SPARSOMYCIN AND ITS ANALOGUES

A PRECLINICAL STUDY ON NOVEL ANTICANCER DRUGS

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A preclinical study on novel anticancer drugs

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SPARSOMYCIN AND ITS ANALOGUES

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een wetenschappelijke proeve op het gebied van de
geneeskunde en tandheelkunde

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Aan Joke en kinderen
Aan mijn ouders.
(Moim rodzicom)

Abbreviations

AUC - area under the plasma concentration versus time curve

ARA-C - cytosine arabinoside

CHO - Chinese hamster ovary cells

CL_{CR} - creatinine clearance

CL_{NR} - non-renal clearance

CL_{R} - renal clearance

C_{ss} - steady state concentration

D - dose

dSm - deshydroxy-sparsomycin

EdSm - ethyl-deshydroxy-sparsomycin

5-FU - 5-fluorouracil

i.d. - internal diameter

i.p. - intraperitoneal

i.v. - intravenous

LD10 - dose lethal to 10% of the animal population

LD50 - dose lethal to 50% of the animal population

MEM - minimal essential medium

MTX - methotrexate

n.s. - not significant

PSI(s) - protein synthesis inhibitor(s)

PSm - n-pentyl-sparsomycin

R_{GF} - renal glomerular filtration

RPE - retinal pigment epythelium

s.c. - subcutaneous

SD - standard deviation

SEM - standard error of the mean

Sm - sparsomycin

T/C - treatment to control ratio of respective median survival times

Contents

CHAPTER 1

OUTLINE OF INVESTIGATIONS AND OBJECTIVES IN THIS THESIS

In 1981 Ottenheijm and co-workers from our university succeeded in the synthesis of an antitumor antibiotic Sparsomycin (Sm). Sm was already discovered in 1962 as a natural product of Streptomyces sparsogenes and Streptomyces cuspidosporus. It appeared that Sm is a potent inhibitor of ribosomal protein synthesis. The drug showed attractive antitumor activity in several rodent tumor models. In 1964 Sm entered a phase I clinical trial. This study was discontinued because of probable drug-induced retinotoxicity observed in two patients.

The chemical synthesis and preparation of new Sm analogues by the group from Nijmegen initiated a new phase in Sm research. Highly purified Sm as well as its analogues have become available in quantities sufficient for preclinical and clinical investigations.

Because its mechanism of action is completely different from that of the cytotoxic drugs in common use, we were very pleased to become involved in the research and to study the innovative possibilities of these compounds especially in combination chemotherapy.

All synthetic derivatives were primarily tested for their inhibitory activity in cell free protein synthesizing systems, and parallelly in the L1210 murine leukemia clonogenic assay. Compounds with the highest inhibitory activity were subjected to preclinical studies. The results of these studies are reported in this thesis.

The introductory chapter gives a detailed history of Sm and reviews its chemistry, biological properties and pharmacology as well as selection procedures for new analogues (Chapter 2).

The analogues were analyzed and their physicochemical properties studied in relation to their in vitro and in vivo activity (Chapter 3). Subsequently, Sm and selected analogues were tested for in vivo antitumor activity in eight murine tumor models (Chapter 4). Because the mechanism of action of Sm is different from that of other cytotoxic drugs, its use in combination chemotherapy was evaluated. It appeared that Sm is a potent modulator of the cytotoxic effect of cisplatin in vitro (Chapter 5) and in vivo (Chapter 6). Later, it was found that cisplatin is not the only compound of which the activity can be modified by Sm. Modification of in vitro cytotoxicity of several other cytotoxic drugs has been described (Chapter 7).

In order to understand the relationships between compounds in terms of activity and toxicity, preclinical pharmacokinetic studies with the three most promising analogues i.e. deshydroxy-Sm, ethyl-deshydroxy-Sm and n-pentyl-Sm were performed in mice. The compounds were compared with each other at equitoxic doses (Chapter 8). These studies were followed by pharma-

cokinetic and toxicological studies of Sm in beagle dogs (Chapter 9). The pharmacokinetics of the three most promising analogues in beagle dogs are presented in Chapters 10, 11 and 12.

Finally, attention was paid to the possible mechanisms underlying Sm-induced retinal toxicity that was described first in 1964. Much effort was spent on reproducing this toxicity in animals (Chapter 13).

The thesis is completed by a general discussion and summary. (Chapter 14).

CHAPTER 2

SPARSOMYCIN

A review of preclinical and clinical studies

2.1 Introduction

Mankind's encounter with protein synthesis inhibitors (PSI) dates from his earliest existence. Many bacterial toxins and plant products act by inhibiting protein synthesis. Through the millenia, man has learned to use these toxins as medicine. PSI agents were used with success to induce vomiting and to treat various protozoal diseases (1,2,3), and leukemia (4,5). Several potential antitumor agents like homoharringtonine (6), bruceantin (7) and emetine (4,8) have been derived from these traditional drugs. Until now all of the compounds studied were very toxic to humans, and not one compound from this group appeared to be active on solid tumors. Most of these compounds possess a very complicated structure, which is difficult for the organic chemist to modify.

Sparsomycin (Sm) is one of the first PSI agents that has been made accessible by chemical synthesis and on which systematic studies on the relationship between structure and activity have been performed. From these studies it appeared that small changes in the structure of Sm result in significant changes in its biological activity and toxicity. For this reason Sm is an attractive candidate for further preclinical evaluation. This chapter reviews the data available on Sm and introduces the reader to preclinical studies on Sm and its analogues as reported in this thesis.

2.2 Discovery and initial studies

Sm was detected initially in beers and was isolated subsequently in 1962 from a Streptomyces sparsogenes fermentation broth (9,10). A few years later, the antibiotic was also isolated from a culture filtrate of Streptomyces cuspidosporus (11,12). Owen et al. found that this newly isolated antibiotic was active in vitro on KB human epidermoid carcinoma cells and on 11 out of 20 rodent tumor models tested in vivo (9). These data prompted further development of the drug and lead to its introduction in a clinical setting just two years after its discovery.

2.3 Phase I clinical trial

In 1964, five patients suffering from far advanced cancer took part in this study (13). The patients were treated with increasing daily doses of the drug. The initial doses (0.0016-0.016 mg/kg/day) were based on toxicological studies in animals. In general, the patients tolerated Sm without abnormal side effects. In four patients a rise in SGOT was noticed after therapy, but two of them had liver metastases. For details see Table 1.

Table 1. Data on five patients treated with Sm (13)

Patient nr and diagnosis	Total dose (mg)	(mg/kg)	Treatment (days)	Level of alkaline phosphatase	Level of SGOT	Observations at necropsy
1. Carcinoma of cecum with liver metastasis	5.5	0.085	10	Increased before therapy; no further change	Increased after therapy	Liver metastasis
2. Carcinoma of the colon with lung and liver metastasis	12.0	0.24	13	No change	No change	Eye changes; no metastasis
3. Carcinoma of the lung	7.5	0.15	15	Increased one week after therapy; remained elevated.	Increased one week after therapy; normal 3 weeks later.	Eye changes; no liver metastasis
4. Carcinoma of the lung	7.7	0.136	10	Increased before therapy; continued to rise.	Increased before therapy; continued to rise	Liver metastasis
5. Osteogenic sarcoma	14.1	0.154	17	Increased before therapy; continued to rise	Normal before therapy; increased one week after therapy.	Fatty changes in liver

2.4 Eye toxicity in humans

Two patients, nr 2 and 3, reported blurred vision, one after 13 days of therapy (total dose 0.24 mg/kg) and one after 15 days of therapy (total dose 0.15 mg/kg) (13). The postmortem investigations were performed 2 and 2.5 months, respectively, after Sm therapy and showed widespread degeneration of the retinal pigment epithelium which was particularly marked in the posterior temporal areas. The impaired vision, the progressive objective changes noted during ocular examination in the form of ring scotomata, and the histologic evidences of retinal pigment epithelium degeneration at necropsy were suggestive for ocular toxicity. The study was discontinued.

It is interesting to discuss these data in retrospect. Nowadays the use of incremental daily doses of cytotoxic drugs in phase I studies has been abandoned because of the high risk of accumulation and toxicity (14). The two patients, both males, that developed retinopathy had very low initial body weights- both 50 kg - suggesting poor general condition. Although patient 5 received a similar total dose of 0.15 mg/kg over 17 days, in contrast to patients 2 and 3, he did not develop retinopathy. The initial body weight of this patient was 92 kg. On the basis of these observations one might speculate that the eye toxicity of Sm may be related to previous treatment, poor general condition, and cachexia. Under normal conditions, the blood retina barrier should be impermeable to the hydrophilic Sm (15). This hypothesis will be discussed in detail in chapter 13.

Additionally, patient 2 was treated with thioridazine concomitantly with Sm. This drug appeared to possess its own retinotoxic potential probably also related to the inhibition of protein synthesis (8). Thus, retinopathy in this patient might be attributed to a synergism between this drug and thioridazine. This deserves further studies.

After discontinuation of this phase I study, intensive research was initiated in order to elucidate the mechanism of eye toxicity (17). Electroretinographic and histopathologic investigations in healthy rats and monkeys treated with Sm did not show any abnormality of the retina. The only finding was that some electroretinographic abnormalities in sick and moribund animals were probably related to their poor general condition. Further clinical studies were not undertaken.

2.5 Elucidation of the chemical structure

The structure of Sm remained unresolved until 1970, when Wiley and MacKellar reported the results of synthetic, spectroscopic, and degradation studies (18). The complete structure of Sm is presented in Fig. 1.

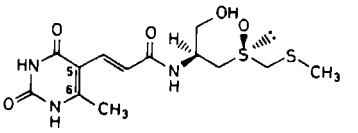


Fig.1 Structure of Sm

The molecular structure of Sm consists of a C(6)-methylated uracil moiety, and a cysteine derived amino acid fragment. The configuration of the chiral carbon atom was shown to be S, correlating with D-cysteine. Some years later, Ottenheijm and co-workers showed that the sulphoxide function of Sm possesses the R configuration (19).

2.6 Biological activity and mechanism of action

Studies initiated in the late sixties on the mechanism of action and biological activity showed that Sm possesses antiviral activity (20), inhibitory activity on Gram-positive and Gram-negative bacteria as well as on a number of fungi (9) and parasites (21).

The different metabolic parameters altered by Sm for E. coli were studied by Slechta et al. (22). Protein synthesis was affected at a drug concentration lower than that affecting the synthesis of DNA and RNA. Cell growth and DNA synthesis were inhibited in parallel in the presence of the drug, whereas RNA accumulated as tRNA. The release of amino acids into the growth medium due to growth inhibition was also reported. These results were confirmed later by others, and the isolation of a Sm-resistant strain of E. coli was reported (23).

Preferential inhibition by Sm of protein synthesis rather than DNA of RNA synthesis has also been reported in growing L cells (24) and in mouse livers (25). Sm does not, however, inhibit protein synthesis in intact reticulocytes (26). In reticulocyte lysates (27), as well as in mouse livers (25), yeast (27), plasma cell tumors (28), and E. coli systems (29), Sm prevented the run-off of polysomes freezing them on the mRNA.

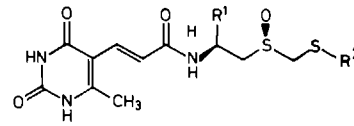
2.7 Synthesis of Sparsomycin and its anlogues

The synthesis of Sm was investigated concurrently by the research groups lead by Ottenheijm and Helquist. Both groups succeeded in synthesizing Sm, but used different approaches. The synthetic endeavours of both research

groups are described in recent review articles (30,31).

After the total synthesis of Sm was accomplished, Ottenheijm's group prepared a large number of structural analogues of the drug (30,31). Most of the derivatives possessed only one, and some only two modifications of the original structure. The analogues were tested for their inhibitory activity in cell free protein synthesizing systems as well as in the L1210 murine leukemia clonogenic assay. The structures of those analogues selected for preclinical investigations are presented in Table 2.

Table 2. Structures of Sm analogues selected for preclinical investigations.



Nr	name	R1	R2
I	Sparsomycin (Sm)	CH_2OH	CH_3
II	n-butyl-Sm	CH_2OH	$(CH_2)_3CH_3$
III	n-pentyl-Sm	CH_2OH	$(CH_2)_4CH_3$
IV	benzyl-Sm	CH_2OH	$CH_2C_6H_5$
V	n-octyl-Sm	CH_2OH	$(CH_2)_7CH_3$
VI	deshydroxy-Sm	CH_3	CH_3
VII	ethyl-deshydroxy-Sm	CH_3	CH_2CH_3
VIII	n-butyl-deshydroxy-Sm	CH_3	$(CH_2)_3CH_3$
IX	n-octyl-deshydroxy-Sm	CH_3	$(CH_2)_7CH_3$

2.8 Activity of the novel Sparsomycin analogues

In the cell free protein synthesis systems, ribosomes from both E. coli and S. cerevisiae were used. The Sm analogues were investigated using the following tests:

1. Polyphenylalanine (PolyPhe) synthesis assay. This test uses synthetic mRNAs, thus it does not require a physiological initiation. Sm preferentially inhibits the polymerization of amino acids encoded by polyuridylic, polyadenylic, and polycytidylic acids (24,29). The inhibition of peptide bond formation observed in this system suggests that Sm does not act as an inhibitor of initiation, but that it may be an inhibitor of

the elongation step in protein synthesis.

2. Puromycin reaction. The antibiotic puromycin, functioning as an analogue of the 3' end of the aminoacyl-tRNA, is used extensively as an acceptor in model assays for peptide bond formation (32). N-blocked aminoacyl-tRNAs bound to the peptidyl transferase P-site on the ribosome react with puromycin, forming N-acetylaminoacyl-puromycin. This reaction is also sensitive to Sm (32,33,34).
3. Fragment reaction. Monro and Marcker (35) tested peptide bond formation using N-acetylaminoacylpentanucleotide and puromycin as donor and acceptor substrates, respectively. The reaction requires only the presence of the large ribosomal subunit and an alcohol to increase the affinity of the ribosome for the 3'-terminal oligonucleotide. This assay, termed "fragment reaction", eliminates the requirements for natural substrates. All the available data indicate that this process takes place at the peptidyl transferase centre, which is in agreement with the normal mechanism of peptide bond formation (35). The reaction is sensitive to Sm (36) and this sensitivity was the definite proof that the action of Sm occurs at the peptidyl transferase centre. Similar inhibitory results were obtained with ribosomes from human tonsils (37), yeast (38), wheat germ (39), and mammalian cells (40).

The investigations on the analogues of Sm in cell free protein synthesizing systems were completed by studies using the L1210 clonogenic assay. L1210 is a murine lymphatic leukemia cell line used frequently to screen antitumor activity.

In Table 3. the inhibitory activity of the selected Sm analogues in cell free systems as well as in the L1210 leukemia clonogenic assay are presented as ED50 or ID50 values representing the 50% effective or inhibitory dose, respectively. For the sake of brevity only data concerning analogues chosen for further investigation are presented. The results of other analogues prepared are reported in references 30 and 31.

2.9 Structure activity relationships

From structure activity relationship studies (of which part of the results are presented in Table 3. and depicted in Fig. 2) the following conclusions can be drawn:

1. The presence of two chiral centres in the molecule accounts for the existence of four stereoisomers, all of which have been synthesized (33). Of these stereoisomers only that compounds with S_CR_S configuration is a strong inhibitor of protein synthesis and of L1210 colony formation.

Table 3. Biological activity of sparsomycin analogues. The results are expressed as ED50 (cell free systems) and ID50 values (L1210). These values represent the median effective or inhibitory dose (in μM).

compound	Cell free systems					L1210
	PolyPhe* synthesis		Puromycin reaction	fragment reaction		clonogenic assay
	E.coli**	S.cerev.***	E.coli	E.coli	S.cerev.	
I Sm	8.5	5.2	0.10	3.2	2.8	0.48
II n-butyl-Sm	1.8	1.0	0.06	5.1	4.8	0.10
III n-pentyl-Sm	1.0	0.17	0.03	2.6	2.7	0.06
IV benzyl-Sm	0.6	6.0	0.13	7.6	2.6	0.11
V n-octyl-Sm	1.6	1.3	0.25	60.0	300.0	0.11
VI deshydroxy-Sm	14.6	50.0	0.25	18.0	39.0	0.07
VII ethyl-des-hydroxy-Sm	12.0	11.1	0.07	10.3	10.4	0.15
VIII n-butyl-des-hydroxy-Sm	3.0	1.9	0.05	12.1	15.0	0.05
IX n-octyl-des-hydroxy-Sm	8.5	1.4	0.46	46.0	60.0	0.03

* polyphenylalanine synthesis test (see text)

** Escherichia coli

*** Sacharomyces cerevisiae

2. The presence and position of the sulphoxide moiety in the cysteinol side chain is of importance. When the positions of the sulphoxide function (S alpha) and the sulphur (S beta) atom are reversed, the biological activity is reduced markedly.
3. The hydrophobic sulphinyl side chain is of great importance for the activity. An increase in the length of this side chain (compounds II-V) markedly enhances the activity of the analogues in all the in vitro assays. An optimum was observed for a side chain with 5-6 carbon atoms attached to the bivalent sulphur atom (42). These results seem to indicate that the hydrophobic group introduced in the molecule interacts with a ribosomal region that forms part of the peptidyl transferase centre. This hydrophobic group is also partially involved in the binding of the aminoacyl-tRNA molecule, but is not implicated in the puromycin binding site. It can be speculated, that physiologically, this binding site could

be used for binding of lipophilic amino acids. The effect of the hydrophobic side of Sm chain on its biological activity will be discussed in detail in chapter 3.

4. The hydroxyl function of Sm was blocked by acetylation (35). The resulting O-acetylated compound shows activity similar to that of Sm itself, indicating that the hydroxyl group is not essential for the activity. Further, some deshydroxy compounds were prepared (compounds VI-IX) in which the hydroxymethyl group is substituted by a methyl group. These drugs showed some surprising physicochemical properties and high biological activity in the in vitro and in vivo assays. The significance of structural modification of Sm's hydroxymethyl function for the activity will be discussed in chapter 3.
5. The C(6)- methyl group on the uracil ring and the trans geometry of the alkene function of Sm are important for the activity. The analogue which lacks the C(6)-methyl group shows low activity, whereas the compound with a cis-alkene function is inactive.

The structure activity relationship studies are summarized in Fig. 2.

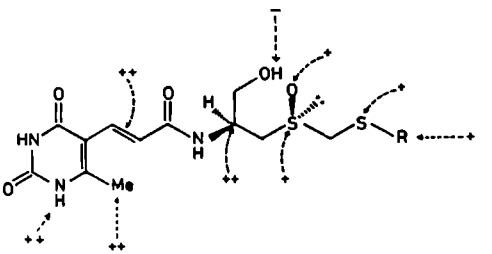


Fig. 2. Importance for activity of different functional groups.
- unimportant, + important, ++ essential.

2.10 A mechanistic rationale for Sparsomycins' activity as an inhibitor of peptidyl transferase

Sm has structural similarities with the 3' end of the acceptor tRNA molecule (Fig. 3). The binding of Sm to a site on the peptidyl transferase centre that partially overlaps the interaction site for the aminoacyladenine moiety of the aminoacyl-tRNA, thereby blocking peptide synthesis, is the most acceptable mechanism to explain Sm's activity. This may disturb also the correct positioning of the 3' end of the large aminoacyl-tRNA molecule. In both cases the formation of the peptide bond will be inhibited. The mechanistic rationale for Sm's interaction with the peptidyl transferase centre is outlined in Fig. 4.

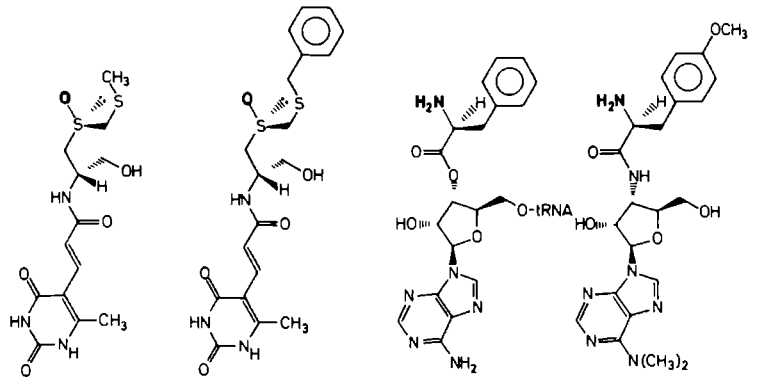


Fig. 3. Sparsomycin (1) Benzylsparsomycin (18) L-Phenylalanyl tRNA Puromycin

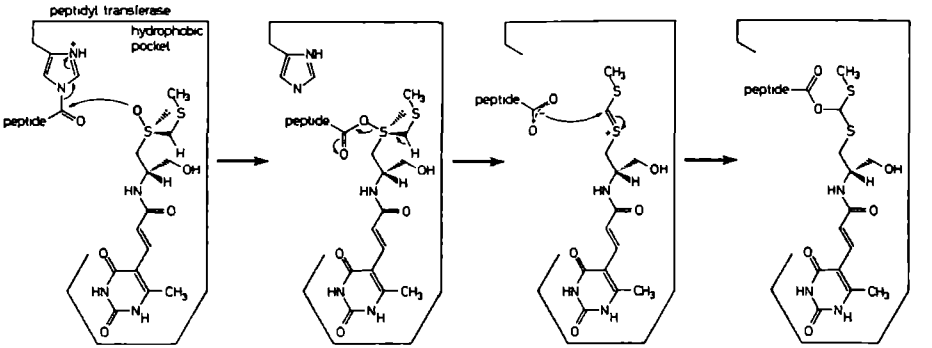


Fig. 4. Mechanism of action of Sm (44,45).

2.11 Cytotoxicity.

Sm, being an inhibitor of protein synthesis, must be intrinsically toxic to cells permeable to the drug. The cytotoxicity is probably related to the deficiency of proteins, especially those with a rapid biological turnover and of vital importance to the cell. It has been reported for E. coli (22) that the antibiotic is mainly bacteriostatic and not bactericidal. Similarly, Chinese hamster fibroblasts incubated for 1 hour with Sm concentrations that inhibit 99% of their protein synthesis recover totally after removal of the drug (46). We obtained similar results with Chinese hamster ovary cells (47) (see chapter 4). CHO cells do not recover their synthetic capabilities after exposure periods exceeding four hours. Fibroblasts are sensitive to Sm only during the S phase of the cell cycle (46). This suggests that the synthesis inhibition of histones may be a critical factor for cell survival.

The permeability of prokaryotic and eukaryotic cells to Sm is an impor-

tant factor influencing cytotoxicity. Intact rabbit reticulocytes are impermeable to Sm (26). If the other bone marrow cells are also impermeable to Sm, than this may explain why Sm is not toxic to bone marrow in vitro (31) and in dogs (48).

2.12 In vitro antitumor activity

The first in vitro antitumor activity testing of Sm was reported in 1962 (8). Sm was found to be exceptionally active on KB human epidermoid carcinoma cells. However, this activity was not confirmed by other investigators (49).

Since 1964 several researchers have tried to prepare more active Sm analogues (49). The in vitro activity of Sm and several of its analogues on L1210 leukemia cells (43,50) and on Ehrlich ascites carcinoma cells (51,52) has been reported. These assays were used to select compounds with a higher antitumor activity than the unmodified drug. For the L1210 leukemia system, a good correlation was found between the in vitro and in vivo antitumor activity of the tested drug (42).

Recently, Sm was tested for cytotoxicity against human and murine tumor cells and human bone marrow (31). Sm, at concentrations that were toxic to 24% of the human tumors and 80% of the murine tumors, did not decrease the viability of human bone marrow cells (see Table 4). However, this "selective" cytotoxicity to the tumor cells but not to the bone marrow was only apparent, as bone marrow toxicity is not a dose limiting factor for this drug.

2.13 Antitumor activity of Sparsomycin in vivo

In 1962, Owen et al. reported a marked antitumor activity of Sm in 11 out of 20 rodent tumor models (8). The data from this publication are, however, difficult to interpret. The activity criteria used are somewhat different than those used nowadays, and it seems that in at least some cases activity was found with doses that were considerably toxic. The present general criterion for minimal activity in solid tumors is tumor volume reduction of $\leq$ 42% of the control without obvious toxicity. This criterion was not fulfilled in any tumor system investigated by Owen et al. (8). Of the 20 tumor models tested, many have disappeared from the screening panel; and only one, the Lewis lung carcinoma, is still in use. Sm was then, as now, not active on this tumor.

Table 4. In vitro effect of Sm on murine tumors, human bone marrow and human tumor cells lines derived from xenografts in nude mice expressed as the number of responses / the number tested (31).

test system	Sparsomycin (µg/ml)*			
	0.03	0.1	0.3	1.0
Murine tumors: P388, L1210, B16, LL, Co38		4/5	5/5	5/5
Human bone marrow (CFU-C)		0/4	2/4	4/4
Human tumor cell lines:				
lung - non-small cell	0/7	0/10	5/8	8/8
- small cell	0/3	1/6	3/6	4/4
large bowel	0/5	1/7	2/6	2/2
melanoma	1/4	1/7	5/7	4/4
pleuraepithelioma	0/2	2/2	2/2	
stomach	0/4	1/5	5/5	1/1
breast	1/2	2/3	2/3	1/1
kidney		1/1	1/1	1/1
thyroid		1/1	1/1	1/1
sarcomas	1/3	1/3	3/3	1/1
testis		0/1	1/1	1/1
Human tumor cells lines (total)	3/30	11/46	30/43	24/24
	10%	24%	70%	100%

* continuous drug exposure. Tumors and bone marrow are considered to be responsive if the colony count is $\leq$30% of the control.

During the course of studies on the antitumor properties of Sm analogues, Sm was reinvestigated (49). Sm was thoroughly tested on four tumors. Cures were not observed in any case. In general, multiple dose schedules were more effective than single doses. Sm was moderately active on P388 leukemia (T/C 156 $\pm$ 8 %) and showed borderline activity on L1210 leukemia (T/C 125 $\pm$ 9 %). There was no activity on B16 melanoma and there was only 50% reduction of tumor volume in Walker 256 carcinosarcoma.

In our studies with synthetic Sm, activity was not observed in P388 and/or in L1210 leukemias. The details on antitumor testing of the Sm analogues are presented in Chapter 4.

2.14 Antitumor activity of Sparsomycin in combination with cisplatin

There is sufficient evidence to indicate that protein synthesis inhibitors may potentiate the activity of other cytotoxic agents (53,54,55). This potentiating effect was also observed for Sm (47) in Chinese hamster ovary cells in vitro (see Chapter 5). Preincubation of the cells with a low Sm concentration that inhibits protein synthesis by only 20%, protected cells from cisplatin toxicity. Higher Sm concentrations, which inhibited protein synthesis by 92%, rendered cells more sensitive to cisplatin. Similar experiments were performed in vivo using L1210 leukemia bearing mice (56, Chapter 6.1). Pretreatment with Sm for 3-6 hours before cisplatin administration increased the number of cures from 0/6 for cisplatin alone to 4/6 for the optimal combinations. It is interesting to note, that Sm up to a certain level protected mice from weight loss induced by cisplatin. These data suggest that Sm may not only potentiate the antitumor activity of cisplatin, but also protect normal cells from its toxicity.

2.15 Toxicity of Sparsomycin

Of the animals studied, mice and rats are the most susceptible to Sm induced organ damage (57). Dogs and monkeys show hardly any gross or microscopic pathology at toxic doses of Sm. Except for eye toxicity no organ pathology in humans has been observed.

Sm induces emaciation and cachexia in mice and rats (57). The animals suffer from diarrhoea (sometimes bloody) and haematuria. Occasionally, mice develop orbital bleeding and eye degeneration (58,59,60). However, this pathology is not related to the retina toxicity seen in humans. It is probable that such haemorrhagic events are due to a deficiency in blood coagulation factors synthesized in the liver.

Microscopic examination of rat organs, frequently disclosed abnormalities of the pancreas and the male reproductive system (57).

Sm induces emesis, bloody diarrhoea, petechies, dehydration, renal insufficiency, and hypothermia in dogs (48,57). Haematological and biochemical studies reveal marked leukocytosis, mainly granulocytosis, elongation of prothrombine time, fibrinogen deficiency, and a decrease in plasma albumin. There is no evidence of bone marow depression. In contrast, mild erythroid and myeloid hyperplasia were observed in monkeys (17). The platelet count remains normal. Occasionally, the number of reticulocytes decreases (48). Emesis, diarrhoea, and anorexia are probably the cause of dehydration and renal insufficiency. There is no evidence that Sm damages the kidneys directly. All hematological and biochemical disturbances are reversible within

two weeks of discontinuing of the treatment (48).

There were no observable pathological changes in the retinas of any animal treated with toxic doses of Sm (17,48).

A marked increase in SGOT and SGPT, sometimes giving the impression of anicteric hepatitis, were observed (48,57). Typically, for dogs as for man, abnormalities in the liver function appear after discontinuation of the treatment (13,48).

Sm particularly affects liver protein synthesis (25,61-63). The drug preferably interacts with membrane bound ribosomes producing export proteins (62). The liver polysomes affected by Sm disaggregate, thus making mRNA translation impossible.

After treatment with Sm, plasma proteins, synthesized in the liver, disappear from plasma according to their biological half-lives. For example, blood coagulation factor VII has a biological half-life of 3.5 hours in humans. In dogs, it disappears nearly totally within 6-7 hours after a single dose of Sm suggesting a similar short biological half-life (48). Plasma albumin, which has a biological half-life of approximately 20 days, decreases by 30% after treatment with Sm for nine days (48).

In some pathological situations, the rate of protein synthesis may increase. This happens with inflammation or cancer cachexia (65) where the liver compensates for the losses to the tumor. In these situations Sm may inhibit this compensatory mechanism and be particularly toxic to the host. The toxicity studies on analogues of Sm fell beyond the scope of this thesis.

2.16 Pharmacology of Sparsomycin

Sm is a nonpolar small hydrophilic molecule (M.W. 361). After i.v. administration to the dog, approximately 10 % of the drug is bound to plasma proteins (Zylicz, unpublished). More than half of the drug dosis is excreted by the dog in the urine (48). Tubular excretion and absorption has been suggested. The drug is eliminated from plasma with a $t_{1/2}\ \beta$ of 0.7 hours (48). There are sufficient data to propose that Sm's pharmacokinetics may be non-linear at higher doses, which could result from saturation of tubular secretion. When administered in daily sequential doses the drug clearly accumulates in plasma. Introduction of a more lipophilic analogue with a more rapid non-renal clearance would thus be an advantage. Details on the pharmacokinetics of Sm are given in Chapter 9. The pharmacokinetics of the most promising Sm analogues in mice and in beagle dogs are presented in Chapters 10, 11 and 12.

2.17 Conclusions

Sm is a cytotoxic drug which acts according to a novel and unexplored principle. Sm itself is toxic and not active in the usual tumor models. However, it can act as a strong potentiator of the cytotoxicity of other anticancer agents and concomitantly may diminish their toxicity on normal cells. This could be an important application for this class of compounds in the future. Small modifications in the chemical structure of Sm may lead to the development of less toxic analogues showing even better effects in drug combination chemotherapy.

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CHAPTER 3

THE INFLUENCE OF PHYSICOCHEMICAL PARAMETERS ON THE BIOLOGICAL ACTIVITY IN VITRO AND IN VIVO OF SPARSOMYCIN: A STRUCTURE-ACTIVITY RELATIONSHIP STUDY

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Abstract. The lipophilicity of 11 sparsomycin analogues was measured by determination of Rm, log k^o and log P values. A linear correlation between these parameters was observed. Water solubility and drug toxicity in mice were found to be linearly correlated with lipophilicity. The deshydroxy-sparsomycin analogues (**9-12**) showed an interesting phenomenon: increase in hydrophobicity was accompanied by a considerable increase in water solubility. All the derivatives studied are more lipophilic than sparsomycin (**1**) itself and all show higher biological activity than **1** in the in vitro and in vivo systems studied. Moreover, these analogues are 5-100 times less toxic than **1**. The sparsomycin analogues **4**, **8** and **9** show the highest antitumor activity against L1210 leukemia in mice, their median T/C values are 386, 330 and 216%, respectively. Sparsomycin (**1**), showing a T/C value of 117%, is only marginally active against this tumor.

Introduction

Sparsomycin (**1**)[1] is a broad spectrum antibiotic active against parasites,[2] viruses,[3,4] fungi,[5a] and bacteria.[6,7] It is a potent inhibitor of protein biosynthesis, and there is ample evidence that its site of interaction is the large ribosomal subunit. The drug competes with the binding of the natural substrate, i.e. L-amino acyl-tRNA, thereby blocking the peptidyl transferase center.[1] Ever since the isolation of sparsomycin from *Streptomyces sparsogenes* in 1962, much attention was focused on its antitumor activity.[1,5a] In earlier reports[8,9] on structure-activity relationships we described the synthesis of several analogues of sparsomycin which are more potent protein synthesis inhibitors than the natural product **1** itself. Subsequently, the influence of lipophilicity on the biological activity in cell-free (i.e. protein synthesis inhibition) and whole-cell systems (i.e. growth inhibition) was investigated systematically.[9] Increase of the lipophilicity of the drug by substituting the SMe group by larger alkylthio groups (see structure of **1**) causes an increase of the biological activity.

Surprisingly, replacement of sparsomycin's hydroxymethyl group at the chiral carbon atom by more lipophilic substituents (alkyl or aralkyl groups) causes a decrease of the protein synthesis inhibitory capacity of these compounds.[10]

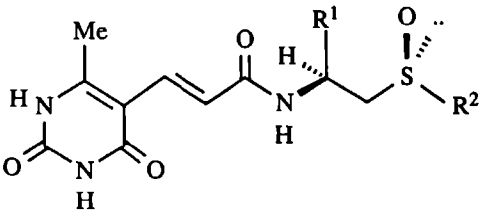
The analogue having the hydroxymethyl group substituted by a methyl group (**9**)[9] is five times more active than sparsomycin against L1210 leukemia in vitro, whereas the activity of the compounds having the hydroxymethyl moiety substituted by larger alkyl or aralkyl groups[10] is equal to or even slightly less than that of **1**. Therefore, we felt the need to study systematically the relationship between physicochemical properties of sparsomycin analogues, i.e. their lipophilicity and water solubility, and their biological activity. A number of lipophilic sparsomycin analogues was selected (see Table I).[9] The following biological activities were studied: the protein synthesis inhibition in a cell-free system, cytotoxicity in vitro, antitumor activity in vitro, toxicity in vivo, and antitumor activity in vivo. The results described in this

report are a firm basis for selection of the best drugs to be investigated in further pharmacological and toxicological studies.

Results and Discussion

The structure of the compounds studied in this report is shown in Table I. Their physicochemical properties, i.e. their lipophilicity and water solubility, are given in Table II. In Table III the biological activity data of all drugs are presented. Regression equations describing the linear correlation between various physicochemical parameters studied and between lipophilicity and toxicity are shown in Table IV.

Table I. Sparsomycin and Analogues



compd	R^1	R^2
1	CH_2OH	CH_2SMe
2	CH_2OH	$CH_2S(CH_2)_2Me$
3	CH_2OH	$CH_2S(CH_2)_3Me$
4	CH_2OH	$CH_2S(CH_2)_4Me$
5	CH_2OH	$CH_2S(CH_2)_7Me$
6	CH_2OH	CH_2SCH_2Ph
7	CH_2OH	$(CH_2)_5Me$
8	CH_2OH	$(CH_2)_6Me$
9	Me	CH_2SMe
10	Me	CH_2SEt
11	Me	$CH_2S(CH_2)_3Me$
12	Me	$CH_2S(CH_2)_7Me$

On the basis of their structure the compounds studied (see Table I) can be devided into three classes, i.e. a series of sparsomycin analogues (**1-2-3-4-5**), two desthio analogues (**7-8**) and a group of deshydroxy-sparsomycin analogues (**9-10-11-12**). Compound **6** was included in order to study the influence of the presence of an aromatic ring in the side chain on the biological activity. One representative of each class, i.e. compounds **4**, **8** and **9** have attracted our attention because these compounds in relation to sparsomycin, combine a high antitumor activity with a low toxicity in vivo.

Compounds **7** and **8** are structurally related to compounds **3** and **4**, respectively. In the former two compounds the bivalent sulfur atom has been replaced by a methylene group. Compounds **9**, **11** and **12** carry identical side chains as compounds **1**, **3** and **5**, respectively. However, the hydroxymethyl group of the latter compounds has been replaced by a methyl group.

Table II. Physicochemical Parameters

compd	Rm	log k^o	log P	water solubility (mg/mL)
1	-0.638	-0.676	-1.713	3.8
2	-0.261	0.117	-0.665	1.5
3	-0.028	0.777	0.143	0.57
4	0.160	1.168	0.600	0.28
5	0.895	3.333	>2.424	0.013
6	-0.042	0.803	0.129	0.76
7	0.235	1.111	0.410	0.89
8	0.560	1.771	1.021	0.22
9	-0.404	-0.209	-0.699	18.0
10	-0.190	0.079	-0.301	8.8
11	0.218	1.290	0.819	0.94
12	1.151	3.533	>2.424	0.015

Physicochemical Properties. The lipophilicity of the sparsomycin compounds can be well characterized by the experimentally simple determination of Rm values (Table II). Excellent linear correlations were found (entries A, B and C of Table IV) between this parameter and the

other lipophilicity parameters measured, i.e. log k^o and log P (Table II). The sparsomycin analogues **1**, **3** and **5** have Rm, log k^o and log P values that are lower than those of the corresponding deshydroxy-sparsomycin derivatives **9**, **11** and **12**, respectively (Table II). The linear correlation calculated between the Rm values of the series **1-5** and **9-12** versus the number of carbon atoms attached to the bivalent sulfur atom is given in entries D and E, respectively, of Table IV. It can be seen that these linear regression lines have equal slopes. The difference in the Rm values of two corresponding structures in these series, e.g. **1** and **9**, is equal to the effect of a single methylene group.

Water solubility of the drugs (Table II) correlates linearly with all lipophilicity parameters used; in entries F and G of Table IV only the correlation with log k^o is shown for the series **1-8** and **9-12**, respectively. Surprisingly, the deshydroxy-sparsomycin derivatives **9**, **11** and **12** are better water soluble than the corresponding sparsomycin analogues **1**, **3** and **5**, respectively, despite their increased lipophilicity. E.g. compound **9** is five times more water soluble than sparsomycin **1** itself. We do not have an explanation for this interesting phenomenon.

The pKa values of sparsomycin **1** (8.97 ± 0.02) and deshydroxy-sparsomycin **9** (9.02 ± 0.02) were measured.[5b] From this we conclude that the hydroxy function of sparsomycin is poorly dissociated since the pKa values are virtually the same.

Biological Activity In Vitro and In Vivo. The biological activity was studied in four in vitro assays and in one in vivo system; the results are given in Table III. The following conclusions can be drawn. Of the hydroxy-containing sparsomycin analogues **1-8**, the *S*-oxo-*S*-*n*-heptyl derivative **8** possesses the highest affinity for the ribosomal peptidyl transferase. The high activity in the phenylalanine polymerization (polyPhe) assay of analogues carrying a hydrophobic group attached to the sulfoxide function (e.g. **4** and **8**) suggests that these compounds interact with a hydrophobic region on the peptidyl transferase. Probably this region functions as the recognition site for the lateral chains of hydrophobic amino acids during protein synthesis.[1,8]

Comparison of the data obtained for compounds **1-5** in the polyPhe assay, in the L1210 clonogenic assay and in the L1210 in vivo system shows that there is an optimum in activity for pentyl-sparsomycin **4**. This optimum in activity is most pronounced in the in vivo L1210 system.

Substitution of the bivalent sulfur atom by a methylene group (compare the data of compound **7** vs. **3** and **8** vs. **4**, respectively) has no serious effect on the in vitro and in vivo biological activity. The in vivo antitumor activity of compounds **7** and **8** is somewhat lower than that of compounds **3** and **4**, respectively. In both cases a surprisingly better T/C ratio was obtained by extension of the side chain with a single methylene group.

In the deshydroxy-sparsomycin series **9-12** an optimum in activity was observed for compound **11** in the protein synthesis inhibition assay and in the growth inhibition assays of T24 and WiDr tumor cells. In this series compound **10** has the lowest activity in the L1210

clonogenic assay.

Table III. Biological Activity

	cell-free	whole-cell			in vivo	
	polyPhe synthesis	growth inhibition			LD_{50}	T/C
compd	*E.coli*	T24	WiDr	L1210		L1210
	$ED_{50}(\mu M)$	$ID_{50}(\mu M)$			(mg/kg)	(%)
1	8.5	0.91	0.92	0.48	0.26	117
2	3.0	0.65	0.89	0.14	NT	NT
3	1.8	0.23	0.31	0.10	2.33	156
4	1.0	0.28	0.40	0.06	4.7	386
5	1.6	0.06	0.22	0.10	29.5	151
6	0.6	0.34	0.51	0.11	5.0	151
7	0.75	0.35	0.39	0.14	1.6	135
8	0.46	0.16	0.28	0.15	6.1	330
9	14.5	0.44	0.58	0.07	2.33	216
10	12.0	0.34	0.48	0.15	8.7	184
11	3.0	0.09	0.16	0.05	16.5	164
12	8.5	0.14	0.28	0.03	NT	NT

NT, not tested

Despite the lower affinity for the ribosome of compounds **9** and **10** in comparison with sparsomycin **1** itself, these analogues are more active than **1** in vivo against L1210 leukemia.

Surprisingly, the antitumor activity of **11** is lower than that of **9**, whereas the hydroxymethyl analogue of **11**, i.e. **3**, has a higher antitumor activity than **1**.

The benzyl derivative **6** is quite active in the in vitro assays and although more active than sparsomycin in vivo, this compound is one of the less active analogues as far as antitumor activity in tumor-bearing mice is concerned.

Replacement of sparsomycin's methyl group at the bivalent sulfur atom by alkyl or aralkyl

groups (i.e. **2-6**) and of its methylthio function by alkyl groups (i.e. **7** and **8**), both leading to increased lipophilicity of the drug, results invariably in an increase of the biological activity in all assays tested.

The activity of the sparsomycin analogues **1-8** against the two human tumor cell lines (T24 bladder and WiDr colon carcinoma) is related to the lipophilicity of the compounds. In general, an increase of the lipophilicity results in an increase of the activity.

Acute Toxicity. We observed a striking linear relationship between drug toxicity in mice and lipophilicity; an increase of the lipophilicity results in a decrease of the toxicity. The correlations found for the sparsomycin compounds **1**, **3-8** and for the deshydroxy-sparsomycin compounds **9-11** are depicted in entries H and I, respectively, of Table IV. Removal of the hydroxy group decreases the drug's toxicity considerably; compare the data of compounds **9** vs. **1** and **11** vs. **3**, respectively (Tables II and III). Comparison of the LD_{50} values of compounds **7** vs. **3** and **8** vs. **4**, respectively, learns that substitution of the bivalent sulfur atom by a methylene group has a variable effect on in vivo toxicity.

A rationale for the decreased toxicity of the more lipophilic analogues could be the following. Pentyl-sparsomycin **4** has a total body clearance in Beagle dogs that is twice that of sparsomycin **1**.[11] A more rapid clearance permits on one hand the administration of higher doses of the drug, but on the other hand produces a lower AUC (area under the curve). In other words, the body is exposed less to more lipophilic analogues of sparsomycin, e.g. **4**, resulting in a lower toxicity. The increased volume of distribution and increased protein binding of these lipophilic derivatives may explain further the increase of the LD_{50} value.

The role of the substituent at the chiral carbon atom of sparsomycin on the biological activity in vitro has been investigated earlier.[10] It was concluded that the affinity for the ribosome and the antitumor activity in vitro are hardly affected by replacement of this substituent by larger alkyl or aralkyl groups, or by its *O*-methylation or *O*-acylation. Some of these changes even cause a slight decrease in activity. Only when the hydroxymethyl group is replaced by a methyl group an increase in the in vitro activity in the L1210 clonogenic assay was observed. This study shows that also in vivo deshydroxy-sparsomycin **9** is a better antitumor drug than sparsomycin. Unfortunately, substitution of the methylthio group in **9** by larger alkylthio groups, as in compounds **10** and **11** does not give rise to an increase of the in vivo antitumor activity, as observed in the series **1-2-3-4**.

In conclusion: the therapeutic index of sparsomycin can be considerably improved by minor modification of the molecular structure. The drug with an *n*-pentyl side chain attached to the bivalent sulfur atom, i.e. **4**, shows the highest antitumor activity in mice. *S*-Oxo-*S*-*n*-heptyl-sparsomycin **8** and deshydroxy-sparsomycin **9** also pair high antitumor activity with low toxicity in comparison with the unmodified antibiotic. Compound **9** shows the unique feature of combining an increased lipophilicity with a considerable increase in water solubility.

Table IV. Linear regression equations[1]

			y = ax + b				
entry	y	x	b	a	r	n	compds
A	log k°	log P	-0.63	0.92	0.986	12	**1-12**
B	log k°	Rm	-0.30	0.40	0.989	12	**1-12**
C	Rm	log P	0.07	2.28	0.981	12	**1-12**
D	Rm	Cn	-0.90	0.22	0.998	5	**1-5**
E	Rm	Cn	-0.64	0.22	0.999	4	**9-12**
F	log k°	log Sol	0.51	-1.55	-0.973	8	**1-8**
G	log k°	log Sol	1.28	-1.23	-0.999	4	**9-12**
H	log k°	log LD_{50}	-0.06	0.49	0.937	7	**1,3-8**
I	log k°	log LD_{50}	0.66	0.47	0.858	3	**9-11**

[1]Abbreviations used: a, slope; b, intercept; r, correlation coefficient; n, number of compounds used in linear regression analysis; Cn, number of carbon atoms (see text); Sol, water solubility.

Experimental Section

Drugs. The syntheses of sparsomycin and analogues **2-12** have been described earlier.[8,9,12,13] The compounds used in this study are listed in Table I.

Chromatography. Rf values were determined by high-performance thin-layer chromatography (hptlc) using Merck RP-8 $F_{254\,s}$ plates (eluent 60% methanol/water). Rm values were calculated from these Rf values by employment of the following formula: Rm = log (1/Rf - 1).

Drug concentrations were determined using a semi-automated high-performance liquid chromatography (hplc) system from Kratos Analytical (Ramsey, New York, U.S.A.). This system consisted of two Spectroflow 400 pumps, Multiport Streamswitch with two six-way valves (Rheodyne, USA), a 757 UV-Vis Detector and a DS 610 Multi-range strip chart recorder. The connection of the sampling valves for the back flushing method has been described before.[14] The whole system was operated by means of an hplc solvent programmer (9224, Kipp Analytical, The Netherlands) equipped with a digital time indicator.

The columns used are: a concentration column (5 cm x 3 mm id) filled with LiChrosorb RP-8 (10 μm particle size) and an analytical column (10 cm x 4.6 mm id) filled with CP^{tm} Spher C8 (8 μm particle size); both packing materials were obtained from Chrompack B.V. (Middelburg, The Netherlands).

Solvent A (flow 1.3 mL/min), used for sample concentration and cleaning of the columns consisted of filtered tap water. Solvent B (flow 1.0 mL/min), used to flush back the sample from the concentration column through the analytical column consisted of 0.1 M phosphoric acid buffer (pH 6.7) with different amounts of methanol (Fisons, England, hplc grade) added.

Determination of log k values by HPLC. For each drug the retention time (t_r) was determined on hplc for at least five methanol concentrations (see above). In general, the results for each drug could be described by the following equation:[15] $\log k' = \log k^o + bM$, where k'= the capacity factor at a certain percentage of organic solvent in the eluent, k^o = the capacity factor obtained by extrapolation to 0% of organic solvent in the eluent, b = slope and M = percent of methanol in the eluent. The capacity factor k' is calculated from the difference between the retention time (t_r) of the drug and the retention time (t_o) of the unretained substance: $\log k' = \log (t_r - t_o) - \log t_o$.

Octanol/water partition coefficient. The partition coefficients (P) of the drugs were measured by mixing 1 mL of a solution containing 12 μg/mL of a drug dissolved in demineralized water and 1 mL of 0.1 M phosphoric acid buffer (pH 7.4) saturated with 2 mL of 1-octanol (Merck, Germany). After mixing for 1 min on a Vortex mixer and centrifugation at 1400 g for 10 min, 1 mL of the water phase was analyzed by hplc (see above). Increasing the time of mixing did not change log P. Each test was repeated three times and each sample was determined in duplicate. The coefficient P was calculated from the following formula: $P = (C - C_w)/C_w$, where C = total concentration of drug in both phases and C_w = concentration of drug in the water phase.

Water solubility. An excessive amount of each drug was shaken overnight with 5 mL of demineralized water at room temperature (19.5 ± 0.3 °C). The drug suspension was filtered through a paper filter (Rundfilter, nr.595, Schleicher & Schull, Germany). The last mL of the filtrate was collected and the concentration was determined by hplc (see above). Each experiment was performed twice.

Dissociation constant pKa. The pKa values of **1** and **9** were determined following the method of Hurwitz and Liu.[16] The UV absorption of several samples was measured at 328 nm (DU-7 UV-Vis Spectrofotometer, Beckman, USA). Samples with the same drug concentration dissolved in different borax and phosphate buffers (pH 3.5-12) were prepared and analyzed. The pKa value was calculated from the following formula: $pKa = pH - \log (A-A_a/A_b-A)$, where pH = pH of the buffer, A = absorbance at a specific pH, A_a = absorbance in acidic solution (pH 3.5) and A_b = absorbance in basic solution (pH 12). The pKa value is expressed as a mean of the results of each sample (n = 10) ± SD.

Inhibition of protein synthesis in a cell-free system. The protein synthesis was measured by the polyphenylalanine synthesis (polyPhe) assay. This assay has been described by us in detail elsewhere.[8] Polymerization of phenylalanine using polyuridylic acid as template is generally accepted as a measure for the total polymerization capacity of cellular extracts. Ribosomes from *Escherichia coli* MRE 600 were used. ED_{50} represents the 50% effective dose. The values given are the average of three experiments and have an average error of 20%.

In vitro cytotoxicity. The cytotoxicity against two human tumor cell lines was determined following a new automated technique for large-scale drug cytotoxicity testing, i.e. the PIT method.[17] The 50% inhibitory dose (ID_{50}) was calculated. In vitro antitumor activity was determined in the L1210 murine leukemia clonogenic assay in soft agar.[13] The 50% inhibitory dose (ID_{50}) was calculated.

Toxicity in mice. The toxicity of each drug was determined following the NCI protocol.[18] The tests were done on male CDF_1 mice and each drug, suspended or dissolved in 0.9% NaCl, was injected ip daily x 9. There were 5 dose levels and 10 mice per dose level. The LD_{50} values (mg/kg/inj) were calculated using Weils' tables.[19] The 95% confidence interval was usually within 15-20% of the value shown. These data were reported elsewhere.[20]

Antitumor activity in mice. The antitumor activity of the drugs against L1210 leukemia was determined in male CDF_1 mice inoculated with 10^5 vital L1210 cells ip on day 0. Animals were treated with drugs dissolved or suspended in 0.9% NaCl ip on days 1-9. The median survival time of each treated group (T) was compared with that of the control group (C). The results are expressed as a T/C x 100% ratio at the optimal dose (this dose is approximately equal to the LD_{10} value). These data as well as the results of activity tests against other murine tumors (i.e. P388 leukemia, RC carcinoma and B16 melanoma) were reported elsewhere.[20]

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CHAPTER 4

IN VIVO ANTITUMOR ACTIVITY OF SPARSOMYCIN AND ITS ANALOGUES IN EIGHT MURINE TUMOR MODELS

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SUMMARY

Sparsomycin (Sm) is a known inhibitor of ribosomal protein synthesis with an attractive anticancer potential. Recently, several analogues of Sm which are more active than the parent drug were selected for further study on the basis of in vitro investigations. Six analogues as well as the parent drug were tested for their antitumor activity in eight in vivo murine tumor models: P388 and L1210 leukemias, RC renal cell carcinoma, B16 melanoma, C38 colon carcinoma, LL Lewis lung carcinoma, C22LR osteosarcoma and M5076 sarcoma. Sm itself appeared to have only borderline activity on L1210 leukemia. The analogues that were most active in vitro showed also the highest in vivo activity. The most sensitive tumors were RC, L1210 and P388. Minimal activity was found on B16 and no activity on C22LR, M5076, C38 and LL. The most active compounds are deshydroxy-Sm, ethyl-deshydroxy-Sm and n-pentyl-Sm. There was a considerable loss of activity when L1210 leukemia was implanted s.c. while the drugs were administered i.v. Only one drug, ethyl-deshydroxy-Sm appeared to be active in this assay. No single most effective compound could be found in this study. The overall activity of Sm and its analogues is moderate. The three analogues which show high activity in three ascitic tumors will be further investigated using human tumor xenograft models.

INTRODUCTION

Sparsomycin (Sm, NSC-059729) is a potent inhibitor of ribosomal protein synthesis (1). The natural product was isolated in the early sixties from a fermentation broth of Streptomyces sparsogenes (2). Sm was found to have antitumor activity against KB human epidermoid carcinoma cells in vitro and several rodent tumors in vivo (3). Based on these data, Sm entered a phase I clinical study in 1964. However it was withdrawn after the finding of an unusual retinal toxicity (ring scotomas) in two out of the five treated patients (4). Since then several investigators tried to synthesize more active and less toxic analogues; Sm was reinvestigated during these studies as a reference drug (5). Sm was found to be active against P388 leukemia (T/C $\pm$ 160%) and to have borderline activity against L1210 leukemia (T/C $\pm$ 125%). It was also moderately active on Walker 256 carcinosarcoma in rats, but it had no effect on B16 melanoma and C38 colon carcinoma.

The revival of interest in this drug was stimulated by its present accessibility through a flexible total synthesis (6) and by the preparation

of more active and less toxic analogues (7,8). For the review on the total synthesis of Sm and the development of its structural analogues see references 9 and 10.

Recently we found that pretreatment of in vitro growing CHO cells (11) or L1210 leukemia bearing animals (12) with Sm significantly enhance the cytotoxic effect of cisplatin.

In this present study we report our findings on the dose-effect relationship of several Sm analogues when tested against eight murine tumors in vivo. These analogues were selected from a larger pool on the basis of their activity in cell-free protein synthesizing systems and the L1210 clonogenic assay (7,8). The correlations between the in vitro and in vivo activity of analogues will be discussed.

MATERIALS AND METHODS

Drugs

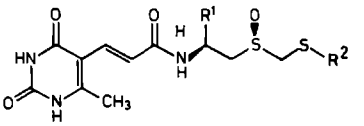
Sm and its analogues were synthesized as previously decribed (6,9). Drugs were acquired in a freeze dried form and dissolved or suspended in buffered physiological saline before use. For those drugs that do not dissolve in water 1% carboxy-methyl-cellulose was added. For i.v. studies, n-pentyl-Sm was formulated in 10% propylene glycol and 0.03 M phosphate buffer (pH = 7). In this formulation n-pentyl-Sm was stable even at 37°C for 12 weeks (unpublished). After dissolution (or after suspension), the drugs were stored for not longer than 10 days in the dark at +4° C. The concentration of the drug in the solution was regularly measured with HPLC (13).

The structure of Sm and its analogues studied are listed in Table 1.

LD50 tests

The tests were performed and the LD50 values calculated according to the method of Weil (14). Four to five dose levels with 10 CD2F1 male mice per dose level were used. Drugs were injected i.p. for 9 days. For three compounds (I,VI and VII) single i.v. dose LD50 values were also determined. This was not possible for n-pentyl-Sm (III) as the maximal solubility of this compound in non toxic formulation was only 0.5 $mg.ml^{-1}$.

Table 1. Sparsomycin and analogues



Nr	name	R1	R2
I	Sparsomycin (Sm)	CH_2OH	CH_3
II	n-butyl-Sm	CH_2OH	$(CH_2)_3CH_3$
III	n-pentyl-Sm	CH_2OH	$(CH_2)_4CH_3$
IV	benzyl-Sm	CH_2OH	$CH_2C_6H_5$
V	n-octyl-Sm	CH_2OH	$(CH_2)_7CH_3$
VI	deshydroxy-Sm	CH_3	CH_3
VII	ethyl-deshydroxy-Sm	CH_3	CH_2CH_3

Tumor models

L1210 and P388 leukemias, and RC renal cell carcinoma tumor lines were kindly supplied by dr. G. Atassi (Inst. Jules Bordet, Lab. for Experimental Chemotherapy, Brussels, Belgium). Dr Atassi also performed control experiments on L1210 leukemia as well as tests on the M5076 sarcoma. The B16 melanoma, C38 colon carcinoma, C22LR osteosarcoma and LL Lewis lung carcinoma tests were performed at the Radiobiological Institute TNO in Rijswijk (P.L.). After being thawed from liquid nitrogen, not more than three passages in vivo were used for the study. The protocol details are presented in the footnotes of tables 3-7.

Animal survival (P388, L1210, RC, B16 and M5076) was recorded daily and the median survival time of each group (6 animals/group) was calculated. The final results for these tumors are presented as the treatment (T) to control (C) ratio of the respective median survival times. From each experiment, performed at 3 to 5 different dose levels (the increments were equal to a factor of 1.3 to 1.5), an optimal T/C was selected (see footnote Table 3). If more experiments were performed, the median optimal dose and the median T/C value at the optimal dose are given, as well as the ranges. Minimal activity criterion for these tumors was T/C $\geq$ 125%. The long term survivors (LTS) were defined as animals survival longer than 60 days without evidence of tumor growth.

C38, C22LR and LL tumors were inoculated s.c., as a fragment or as 1-2 $.10^6$ tumor cells in both flanks (for methodological details see ref.16). After randomization (5 mice per group = 10 tumors) animals were treated i.p. on days 2-10. The tumors were measured with calipers two to three times a week. The mean values were plotted on a semi-log graph (days vs tumor volume), and the volume doubling time was calculated as the time required to increase the tumor volume from 500 to 1000 mm^3. The growth delay factor (GDF) was calculated as follows :

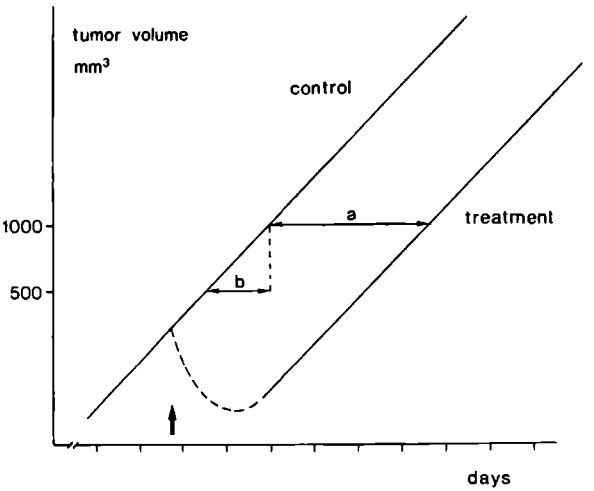


Fig. 1. Growth delay factor (GDF)

$$GDF = \frac{a}{b}$$

where:

a - growth delay between control and treatment group at the level of 1000 mm^3 (days).

b - volume doubling time of the control (days).

The control doubling times (b) were 5.3, 2.3, and 3.3 days for C38, C22LR and LL, respectively.

RESULTS

LD50 values

The LD50 values of the compounds studied after administration as a single dose as well as after nine daily doses are presented in Table 2. Five different Sm analogues that differed from each other by the length of the hydrophobic R2 side chain were investigated. There is a linear relation (r =

0.998) between the number of the carbon atoms in the R2 chain (lipophilicity) and log LD50 (for compounds I,II,III and V): the higher the lipophilicity of the drug, the lower its toxicity. Both deshydroxy analogues (VI and VII) showed substantially higher LD50 values than their hydroxylated counterparts, e.g. compare the LD50 value for compounds I and VI.

Table 2. LD50 values for sparsomycin and its analogues.

Nr	drug	LD_{50} single dose i.v. (95%) ($mg.kg^{-1}$)	LD_{50} daily x 9 i.p. (95%) ($mg.kg^{-1}$ per inj.)
I	Sm	11.3 (8.8-13.5)	0.26 (0.17-0.38)
II	n-butyl-Sm	nd	2.33 (1.8-3.2)
III	n-pentyl-Sm	nd	4.7 (3.7-5.9)
IV	benzyl-Sm	nd	5.0 (4.0-6.3)
V	n-octyl-Sm	nd	29.5 (24.0-37.0)
VI	deshydroxy-Sm	23.2 (18.1-29.8)	2.33 (1.8-3.2)
VII	ethyl-deshydroxy-Sm	82.3 (74 - 91)	8.0 (6.95-9.2)

nd = not done

Antitumor activity

The optimal treatment schedule was determined for n-pentyl-Sm (III) in L1210 leukemia; this data are presented in Table 3. Optimal sche dules were those with a daily drug administration for 7-14 days and a total dose of about 30 $mg.kg^{-1}$. The LTS (2/24) were only observed during the 9 day daily schedule. Mice treated with a 14 day daily schedule showed slow growth of a solid intraperitoneal lymphoma probably due to selection of the tumor cells. On the basis of these data, a 9 day daily treatment schedule was chosen for all the other experiments.

All compounds except Sm (I) appeared to be active on RC carcinoma, L1210 leukemia and to a lesser extent on P388 leukemia (see Tables 4 - 6). An increase of the lipophilicity resulting from elongation of the R2 methyl side chain in Sm (series I, II, III and V) considerably increased the antitumor activity until the optimum was reached with n-pentyl-Sm (III). A further increase of the chain length (n-octyl-Sm, V) did not increase the activity.

All the experiments reported here were performed with male animals.

Table 3. Schedule dependency of n-pentyl-Sm (III) on L1210 leukemia[a]

treatment schedule	dose range ($mg.kg^{-1}$)	optimal dose[b] ($mg.kg^{-1}$)	total dose[c] ($mg.kg^{-1}$)	T/C[d] at optimal dose (%)	LTS/total[e]
day 1	10.0 - 38.3	15.0	15.0	128	0/18
day 1-5	3.2 - 7.0	4.8	24.0	150	0/18
day 1-7	3.2 - 7.0	4.8	33.6	206	0/18
day 1-9	1.5 - 5.0	3.3	29.7	175	2/24
day 1-14	1.4 - 3.2	2.1	29.4	206	0/18
day 1,3,5,7,9	2.2 - 7.5	5.0	25.0	160	0/24
day 1,4,7,10	5.0 - 17.0	11.3	45.2	165	0/24
day 1,5,9	5.0 - 25.5	17.0	51.0	140	0/24

a. 10^5 L1210 leukemia cells were inoculated i.p. on day 0 in CD2F1 male mice. Treatment i.p. was started on day 1.

b. Optimal dose was selected from an experiment with 3-5 dose levels. For selection the following criteria were used: maximal T/C, no early toxic deaths before day 9, no weight loss (T - C) in the first 5 days exceeding 4 g.

c. Total dose required for optimal treatment.

d. T/C is the ratio of the median survival time of the treatment group and that of the control. There were usually 10 animals/experiment as controls.

e. LTS/total - the ratio of the number of long term survivals to total number of animals used in experiment.

Limited data from parallel experiments suggest that female mice were able to survive higher doses of the drugs and had relatively better treatment results (unpublished).

A significant number of LTS were observed for mice bearing i.p. L1210 leukemia and RC carcinoma. The drugs producing the highest number of LTS are n-pentyl-Sm (III) and deshydroxy-Sm (VI). LTS were also seen in animals bearing RC carcinoma treated with n-butyl-Sm (II). However these data wait further confirmation. The three most active compounds (i.e. III, VI and VII) were administered i.v. to the animals bearing L1210 leukemia and RC renal cell carcinoma s.c. In L1210 leukemia (see Table 5 B) compound III and VI were inactive while compound VII showed some activity and a median T/C value of 139% (range 122-156). In RC carcinoma compounds III and VI

showed activity of about 150% (see Table 6 B), only compound VII showed a slightly lower T/C value (138%).

Table 4. Antitumor activity of sparsomycin and its analogues on P388 leukemia[a]

drug	dose range	median optimal dose ($mg.kg^{-1}$)	T/C range at optimal dose (%) ($mg.kg^{-1}$)	median T/C (%)	LTS/total
I	0.063 - 0.5	0.125		88	0/24
II	1.0 - 3.4	2.3	127 - 160	144	0/48
III	2.0 - 7.0	5.0	146 - 175	161	0/48
IV	1.0 - 8.0	2.0		133	0/24
V	10.0 -33.8	22.5		150	0/24
VI	1.0 - 3.4	2.9	136 - 170	153	0/48
VII	3.2 - 8.0	6.2		150	0/24

a. 10^6 P388 leukemia cells were inoculated i.p. in the CD2F1 male mice on day 0. Treatment i.p. on days 1 - 9.
For other explanations see footnotes of Table 3.

Table 5. Antitumor activity of sparsomycin and its analogues on L1210 leukemia

Exp.	drug	dose range ($mg.kg^{-1}$)	median optimal dose ($mg.kg^{-1}$)	T/C range at optimal dose (%)	median T/C (%)	LTS/total
A	I	0.25 - 1.0	0.25		125	0/18
	II	1.0 - 3.4	1.5		145	0/24
	III	1.5 - 8.0	5.4	154 - 175	164	2/42
	IV	1.8 -20.0	6.4	110 - 171	141	0/96
	V	9.0 -30.0	15.0	120 - 152	136	1/78
	VI	0.5 - 4.0	2.0	163 - 248	206	5/84
	VII	3.0 - 12.0	6.1	158 - 185	172	1/42
B	III	-	5.0	-	111	0/20
	VI	-	2.0	-	111	0/20
	VII	6.0 - 7.8	6.9	122 - 156	139	0/40
C	III	4.0 - 5.0	4.5	111 - 125	118	0/20
	VI	-	2.0	111 - 125	118	0/20
	VII	-	6.0	-	125	0/10

A. 10^5 L1210 cells were inoculated i.p. on day 0 in CD2F1 male mice. Treatment i.p. on days 1-9.

B. 10^6 L1210 cells were inoculated s.c. in the right scapular region of CD2F1 male mice. Treatment was i.v. on days 1-7.

C. 10^4 L1210 cells were inoculated intracerebrally on day 0 in CD2F1 male mice. Treatment was i.v. on days 1 - 7.

Table 6. Antitumor activity of sparsomycin and its analogues on RC carcinoma

Exp.	drug	dose range ($mg.kg^{-1}$)	median optimal dose ($mg.kg^{-1}$)	T/C range at optimal dose (%)	median T/C (%)	LTS/total
A	I	0.125 - 1.0	0.125	-	100	0/24
	II	0.7 - 2.3	1.5	-	600	9/24
	III	1.4 - 4.8	3.9	227 - 600	414	8/48
	IV	1.0 - 8.0	3.4	139 - 158	149	0/48
	V	9.0 -33.8	17.5	161 - 179	170	0/48
	VI	0.5 - 2.5	1.6	230 - 304	277	5/54
	VII	3.2 - 8.0	4.1	-	197	0/24
B	III	5.0 - 5.8	5.4	156 - 159	158	0/20
	VI	-	2.0	153 - 154	154	0/20
	VII	-	6.0	134 - 141	138	0/20

A. 10^6 RC renal carcinoma cells were inoculated i.p. on day 0 in CD2F1 male mice. Treatment was i.p. on days 1-9.

B. 10^6 RC renal carcinoma cells were inoculated s.c. on day 0 in CD2F1 male mice. Treatment was i.v. on days 1-7.

For other explanations see footnote of Table 3.

All the drugs were rather ineffective on B16 melanoma (Table 7). The minimal activity criterion of T/C = 125% was reached only by the three most active compounds (i.e. III, VI, and VIII) and was not higher than 131%. No activity was observed on M5076 sarcoma. In three s.c. growing tumors, only n-pentyl-Sm showed some activity on C38 colon carcinoma (see Table 8) but none of the compounds was active on C22LR osteosarcoma and LL Lewis lung carcinoma. The GDF for positive controls at optimal doses on C22LR and LL tumors were all significantly higher than the GDF obtained with Sm analogues.

Table 7. Antitumor activity of sparsomycin analogues on B16 melanoma and M5076 sarcoma.

Exp.	drug	dose range ($mg.kg^{-1}$)	median optimal dose ($mg.kg^{-1}$)	T/C range at optimal dose (%)	median T/C (%)	LTS/total
A	I	0.25 - 1.5	0.5	-	90	0/24
	III	1.4 - 6.0	4.0	-	131	0/20
	IV	1.0 - 8.0	2.0	-	108	0/24
	V	5.0 -20.0	20.0	-	116	0/24
	VI	1.0 - 3.4	2.3	-	131	0/20
B	III	4.0 - 8.0	4.0	-	84	0/12
	VI	2.0 - 4.0	2.0	-	82	0/12
	VII	6.0 -12.0	6.0	-	90	0/12

A. 1:10 tumor brei from a solid B16 melanoma was inoculated i.p. on day 0 in C57BL male mice. Treatment was i.p. on days 1-9.

B. 10^6 M5076 sarcoma cells were inoculated s.c. in the right flanks of B6C3F1 male mice on day 0. Treatment was i.p. on days 1-9.

For other explanations see footnotes of Table 3.

Table 8. Antitumor activity of sparsomycin analogues on C38 colon carcinoma, C22LR osteosarcoma and LL Lewis lung carcinoma.

Exp.	drug	dose range dose ($mg.kg^{-1}$)	median optimal dose ($mg.kg^{-1}$)	GDF[d]	LTS/total
A	III	1.4 - 6.0	6.0	1.87	0/20
	VI	1.0 - 3.4	2.3	0.98	0/20
B	III	2.0 - 4.0	4.0	0.99	0/10
	VI	1.0 - 2.0	2.0	0.79	0/10
	VII	3.0 - 6.0	6.0	1.0	0/10
C	III	2.0 - 4.0	4.0	0.39	0/10
	VI	1.0 - 2.0	2.0	0.92	0/10
	VII	3.0 - 6.0	3.0	0.35	0/10

A. C38 colon carcinoma tumor fragment of about 1 mm^3 were implanted in both flanks of C57BL male mice on day 0. Treatment was i.p. on days 2 - 10.

B. 10^6 C22LR osteosarcoma cells were inoculated in both flanks of male (C57BLxCBA)F1 mice on day 0. Treatment was on days 2-10.

C. 2 x 10^6 cells of LL Lewis lung carcinoma were inoculated in both flanks of C57BL male mice on day 0. Treatment was i.p. on days 2 - 10.

d. GDF - growth delay factor is the ratio of the tumor growth delay between control and treatment groups and the volume doubling time of the control. For other explanations see footnotes of Table 3.

DISCUSSION

The LD50 value for Sm in a multiple schedule (daily x 9) is 0.26 $mg.kg^{-1}$ per inj., this value is 44 times lower than Sm's single LD50. For deshydroxy-Sm (VI) and ethyl-deshydroxy-Sm (VII) this ratio is 1:10, thus close to the number of the doses administered. These differences suggest that in daily schedules Sm may be only administered in small doses because of cummulative toxicity. This may explain the low activity of Sm (I) in all the models tested.

The difference in LD50 values between Sm (I) and deshydroxy-Sm (VI) correlates well with pharmacokinetic studies performed in mice (unpublished). In that study, deshydroxy-Sm (VI) had a total body clearance

that was twice as high as that for Sm. Thus, it seems probable, that removal of the hydroxyl group improves membrane transport as well as excretion of the drug. This is the reason why deshydroxy-Sm (VI) in contrast to Sm (I) does not accumulate in plasma.

The antitumor activity of synthetic Sm (I) on P388 leukemia (T/C = 88%) is much lower than previously reported for the natural product (T/C= 160%)(5). For L1210, the activity is comparable with the older data (5). New Sm analogues, selected according to their in vitro activity in cell free systems and the L1210 clonogenic assay, appeared to be more active that the parent drug. The in vitro activity of the drugs increased with the length of the hydrophobic R2 side chain until the optimum was reached (7,8).

In vivo, increase of drug lipophilicity correlates well with the increase in LD50. But the increase of antitumor activity is much smaller than would be expected from the increased LD50. This is in contrast to much higher in vitro activity of more lipophilic compounds (7,8).

The divergence between the in vitro and in vivo results may be explained by the pharmacokinetic behaviour of the compounds. Two compounds found to be active when tested on i.p. growing carcinomas deshydroxy-Sm (VI) and n-pentyl-Sm (III), were inactive when administered to animals bearing a sensitive tumor (L1210 leukemia) inoculated s.c. Only ethyl-deshydroxy-Sm (VII) was active in this setting. In the RC carcinoma s.c. model all three compounds were moderately active.

Compounds III, VI and VII showed no effect on L1210 leukemia growing intracerebrally.

The good activity of the three compounds (i.e. III, VI and VII) on RC renal cell carcinoma is of interest, because this tumor showed a good responsiveness to drugs that are clinically active but inactive on L1210 leukemia (hexamethyl-melamine, bleomycin and busulphan) (17).

In conclusion, Sm analogues appeared to be moderately active on two lymphocytic murine leukemias (L1210 and P388) and on RC renal cell carcinoma. None of the drugs were active on solid tumors. The n-pentyl-Sm (III) and the deshydroxy compounds (VI and VII) showed the highest antitumor activity in the tumor models tested. Ethyl-deshydroxy-Sm (VII) differed from the other drugs by its outstanding high LD50 and its activity on L1210 leukemia s.c. The three most active compounds will be further investigated using human tumor xenografts in nude mice.

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CHAPTER 5

IN VITRO MODULATION OF CISPLATIN CYTOTOXICITY BY SPARSOMYCIN INHIBITION OF PROTEIN SYNTHESIS

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J. Natl. Cancer Inst. 1987;78:701-705.

In Vitro Modulation of Cisplatin Cytotoxicity by Sparsomycin Inhibition of Protein Synthesis [1,2]

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ABSTRACT—Inhibition of protein synthesis can alter cellular responsiveness to the classical anticancer drugs The in vitro response of Chinese hamster ovary (CHO) cells to cisplatin with or without sparsomycin (Sm) was studied with the use of [^{3}H]leucine and [*methyl*-^{3}H]thymidine incorporation and clonogenic assay Pretreatment of exponentially growing CHO cells with 1 μg Sm/ml for 3 or 5 hours decreased [^{3}H]leucine incorporation by 20% and resulted in significant resistance to cisplatin ($P = 005$) Sm in a concentration of 10 μg/ml reduced [^{3}H]leucine and [*methyl*-^{3}H]-thymidine incorporation after 3 hours by 92 and 84%, respectively, and resulted in potentiation of the cisplatin cytotoxicity ($P = 004$) This effect was the same in the case of nonproliferating cells ($P = 005$), while protection due to Sm (1 μg/ml) was seen only during cell proliferation Simultaneous incubation and postincubation with Sm proved to have much less or no potentiating effect on cisplatin The mechanisms of both protection and potentiation are still not clear, but our data indicate that Sm is a promising drug for further studies on the modulation of the cancer cell response to classical anticancer drugs —JNCI 1987, 78 701-705

A body of information indicates that protein synthesis inhibitors can positively modulate the responsiveness of malignant and normal cells to classical anticancer drugs (*1-4*). Many normal tissues proliferate faster than most of the human tumors (*5, 6*). Preferential inhibition of protein synthesis and subsequent cell cycle arrest in normal, but not in malignant, cells would make the normal tissues insensitive to the S-phase-specific cytotoxic agents, and larger doses of these drugs could be safely administered (*7*) In support of this concept, protection by cycloheximide of rat bone marrow against cytarabine and mechlorethamine (*8*) as well as of rat intestinal mucosa against cytarabine (*9*) has been described.

In contrast to the protective effect on normal cells, protein synthesis inhibitors can directly influence cancer cells, exerting a synergistic effect with classical anticancer drugs. Corbett and Griswold (*10*) reported that anguidine in combination with 5-fluorouracil cured 80% of mice bearing the C38 colon carcinoma. However, a clinical trial in patients with colorectal cancers failed to prove this synergism (*11*). Hromas et al (*7*) reported that anguidine enhanced the in vitro cytotoxic effect of cisplatin on CHO cells. Recently, we have reported on the in vivo potentiation of cisplatin antitumor activity on L1210 leukemia-bearing mice (*12*). To produce an optimal effect, Sm should be administered 3-6 hours prior to cisplatin. In these experiments, subtoxic Sm doses induced 67% of cures (vs. 0% for cisplatin alone) and better toleration of cisplatin.

For further evaluation of this concept, we have studied *the effect of protein synthesis inhibition by Sm on* cisplatin cytotoxicity to CHO cells.

In contrast to anguidine, Sm is readily accessible through total synthesis (*13*), and some more active analogues have recently been developed (*14, 15*). Furthermore, a sensitive assay for detection of Sm has been developed with the use of high-performance liquid chromatography (*16*). Sm has its site of interaction in the large ribosomal subunit, where it prevents peptide transfer by interfering with the peptidyl transferase center (*17, 18*). Many prokaryotic and eukaryotic cells proved to be sensitive to Sm (*19*). The antitumor activity of Sm has been confirmed for P388 leukemia and Walker 256 carcinosarcoma (*20*). Recently, Sm was found to be differentially cytotoxic against many in vitro growing human tumor cell lines, but not against human bone marrow (*21*).

This report presents the results of in vitro experiments evaluating the effect of Sm on CHO cells and the synergism of Sm with cisplatin in the same model. Dependent on concentration, Sm can antagonize or potentiate cisplatin cytotoxicity Mechanisms that may explain these phenomena are briefly discussed.

MATERIALS AND METHODS

Cell line.—CHO cells were grown routinely as monolayer cultures in closed tissue culture flasks (Costar, Cambridge, MA) at 37°C in a humidified atmosphere (Heraeus incubator) of 5% CO_2 in air. We used Eagle

ABBREVIATIONS USED CHO=Chinese hamster ovary, rRNA=ribosomal RNA, Sm=sparsomycin

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minimum essential medium with Earle's salts (GIBCO, Grand Island, NY) supplemented with 25 m*M* HEPES[9] buffer, 10% heat inactivated fetal calf serum (GIBCO), 2 m*M* glutamine (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany), and 50 μg gentamicin/ml (Boehringer Mannheim GmbH). Cells were harvested by treatment with 0.025% trypsin (type III, Sigma Chemical Co., St. Louis, MO) and 0.05% EDTA for 3 minutes at room temperature.

Drugs.—Sm was synthesized as described before (*13*). Cisplatin was purchased from Bristol-Meyers Co., New York, NY.

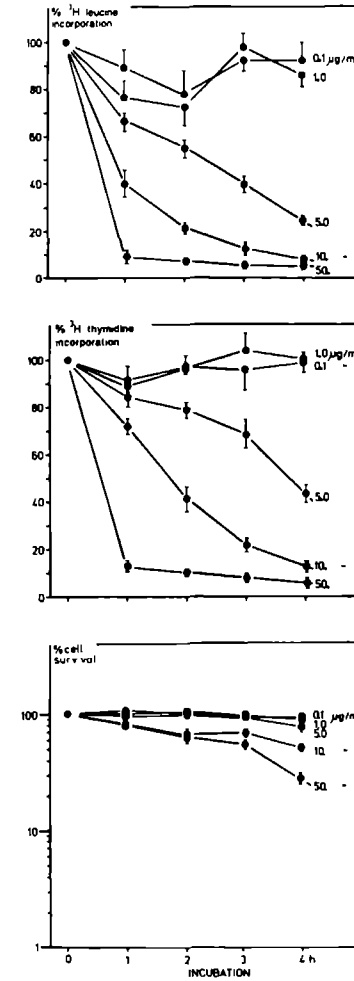
Incorporation studies.—[*methyl*-^{3}H]Thymidine (sp. act., 5 Ci/mmol) and [^{3}H]leucine (sp. act., 130 Ci/mmol) were purchased from Amersham International Ltd., Amersham, England. For incorporation studies, 0.25×10^6 cells immediately after incubation with drugs were suspended in 0.5 ml medium and incubated for 1 hour at 37°C with 1 μCi [*methyl* ^{3}H]thymidine and [^{3}H]leucine. Incorporation of the label was arrested by addition of 250 μ*M* cold thymidine and leucine, respectively (both from E. Merck A.G., Darmstadt, Federal Republic of Germany). Cells were centrifuged at 600×g for 10 minutes at 2°C, and the supernatant was removed. The residue was incubated overnight at 0°C with 2 ml ice-cold 1 *N* $HClO_4$, then harvested on a Millipore microfiber glass filter (type AP, Millipore Corp., Bedford, MA), and counted in a liquid scintillator (Beckman Instruments, Irvine, CA). The studies were performed on duplicate samples.

Survival studies on cells in exponential growth and plateau phase.—To assess cell survival, we used the colony formation technique, with minor modifications (*2, 7*). Exponentially growing cells and plateau phase cells were obtained after 24 and 72 hours of growth, respectively. The modification was that, after incubation with drugs, a constant number of 100 cells was added to each culture dish. Clusters containing more than 30–50 cells were counted as colonies. Cell survival and colony formation after drug treatment were assessed after 5–6 days of incubation and were normalized with respect to those of untreated controls, which had a plating efficiency varying between 45 and 70%. All experiments were repeated at least twice with the use of triplicate samples for each drug concentration. The results are presented as means ± SE.

Flow cytometric studies.—Cells for flow cytometry were harvested, fixed, and stained as described before (*22*). DNA histograms were analyzed with the use of a flow cytofluorograph system 30H (Ortho Instruments, Westwood, MA).

RESULTS

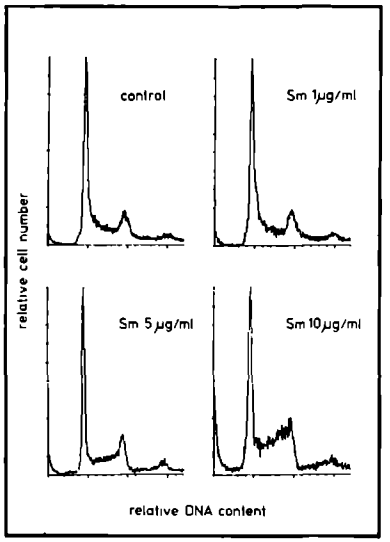
Effect of Sm on CHO cells (text fig. 1).—The effect of Sm on [^{3}H]leucine and [*methyl*-^{3}H]thymidine incorporation by CHO cells was dose dependent and incubation time dependent, with a plateau at the higher dose level (50 μg/ml). Profound changes in protein and DNA syntheses had relatively little impact on the eventual cell survival as deduced from clonogenicity, which proves that most cells recovered after termination of drug incubation. From the above experiments two concentrations (1 and 10 μg/ml) with widely divergent effects were selected for further studies.



TEXT FIGURE 1.—Effect of Sm on exponentially growing CHO cells. *Bars* represent means ± SE. Cells were incubated with Sm in different concentrations for 1, 2, 3, or 4 hr.

DNA histograms (text-fig. 2).—Exponentially growing cells were incubated for 8 hours in the medium containing 0, 1, 5, or 10 μg Sm/ml. The end-point control after 8 hours of incubation was identical to that at the start of the experiment. Incubation with 1 μg Sm/ml did not change the DNA histogram. However, incubation

[9] 4 (2 Hydroxyethyl) 1 piperazine ethanesulfonic acid



TEXT FIGURE 2 —Effect of 8-hr incubation with Sm of exponentially growing CHO cells on the DNA histogram *Note* similarity between 1 μg Sm/ml and control At higher Sm concentrations, there is a clear delay in the cell cycle progression (the G_1 peak is thinner than in the control) and cells accumulate in the late S-phase *Note* also increase of debris before the G_1 peak at 10 μg/ml, probably due to the direct toxicity of Sm to the cells

with 5 and 10 μg Sm/ml resulted in a G_1-S transfer delay and accumulation in the late S-phase.

Preincubation with 1 μg Sm/ml (text-fig. 3).—The 20% decrease in [^{3}H]leucine incorporation after incubation with 1 μg Sm/ml for 5 hours was accompanied by a decrease in [*methyl*-^{3}H]thymidine incorporation inhibition induced by cisplatin. The difference was significant after 5 hours of incubation at all cisplatin concentrations ($P<.05$). The cells were apparently protected against cisplatin cytotoxicity ($P=.005$). Table 1 gives further details.

Preincubation with 10 μg Sm/ml (text-fig. 3).—Pretreatment of exponentially growing CHO cells with Sm at a concentration of 10 μg/ml for 3 hours reduced [^{3}H]leucine to 8% and [*methyl*-^{3}H]thymidine incorporation to 16%. The [^{3}H]leucine incorporation did not decrease further after addition of cisplatin. The survival curve showed strong potentiation of cisplatin cytotoxicity ($P=.004$).

Simultaneous incubation and postincubation with 10 μg Sm/ml —Simultaneous incubation of exponentially growing CHO cells with cisplatin and Sm (10 μg/ml for 3 hr) and postincubation with Sm (10 μg/ml for 3 hr) inhibited [^{3}H]leucine and [*methyl*-^{3}H]thymidine incorporation to the same extent (10 and 18%, respectively) as in the case of preincubation. However, simultaneous incubation of Sm with cisplatin did not have any effect on cell survival ($P=.312$). Postincubation, instead, resulted in a significant but small reduction in cell survival ($P=.028$) However, the shape of the survival curve (parallel to that of the control) suggests an additive rather than a synergistic effect.

Cells in plateau phase.—Preincubation of CHO cells in the plateau phase with Sm at a concentration of 1 μg/ml for 5 hours did not change incorporation of [^{3}H]leucine or [*methyl*-^{3}H]thymidine as well as cell survival. However, preincubation with Sm at a concentration of 10 μg/ml for 3 hours considerably reduced [^{3}H]leucine (to 20%) and [*methyl*-^{3}H]thymidine incorporation (to 50%) and significantly potentiated cisplatin-induced cytotoxicity ($P=.005$).

DISCUSSION

Our data show that Sm is a highly effective inhibitor of protein synthesis, inducing profound but readily reversible biochemical perturbations. From the data available on Sm it is known that its interaction with the ribosome is irreversible (*23*). Upon inhibition of protein synthesis, the cell responds by temporarily enhancing the biosynthesis of rRNA (*24*), providing new ribosomes that should replace those occupied by the drug. In the later stage the rRNA synthesis decreases and the capacity of the cell to restore its biosynthesis decreases as well.

Sm at a low concentration (1 μg/ml) is able to inhibit

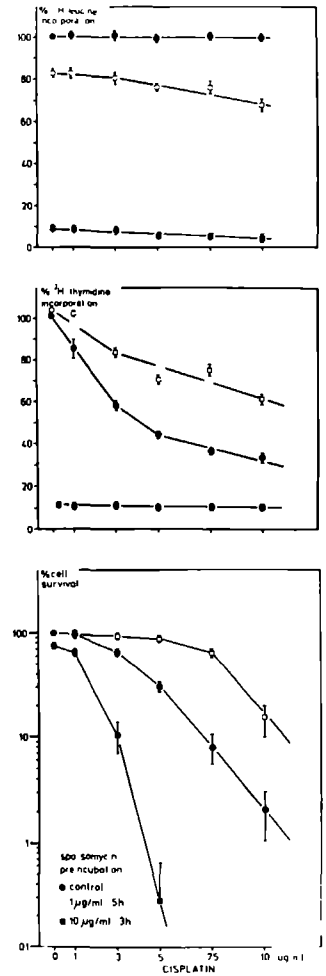
TABLE 1 —*Effect of Sm on cisplatin-induced cytotoxicity Analysis of differences between control and test curves*

Experiment	Sm incubation time, hr	Sm concentration, μg/ml	D_{50} difference[a]	P[b]	AUC ratio[c]
Exponential growth					
Preincubation	3	1	+0.8	.065	143
	5	1	+3 9	.005*	158
	3	10	−2.5	.004*	36
Simultaneous incubation	3	10	0 0	.312	95
Postincubation	3	10	−2.3	028*	58
Plateau-phase cells					
Preincubation	5	1	−0 2	.088	98
	3	10	−4.1	.005*	32

[a] D_{50} is the concentration of the drug required for 50% reduction of cell survival, expressed as the difference between the treatment and control cell populations ($T-C$)

[b] Significance level P as calculated from the Wilcoxon two sample test comparing two survival curves at the level of 5 μg cisplatin/ml. Accepted significance level: $P<.05$ (*)

[c] AUC = area under the curve expressed as treatment-to-control ratio ($T/C \times 100\%$).



TEXT FIGURE 3.—Effect of Sm preincubation on cisplatin cytotoxicity to exponentially growing CHO cells. *Bars* represent means ± SE. Either the cells were incubated with different concentrations of cisplatin alone (control) or the incubation with cisplatin was preceded by incubation with Sm.

only up to 20% of [^{3}H]leucine incorporation without cytotoxic consequences. Sm at a high concentration (50 µg/ml) dramatically decreases [^{3}H]leucine incorporation to 5%. This residual 5% is apparently resistant to Sm. Under these conditions, however, ribosomal protein synthesis is completely inhibited. The cells are rapidly depleted from the enzymes with a high turnover, and cell death follows. The biological effect of Sm at a concentration of 5 or 10 µg/ml is linearly dependent on the incubation time. Up to a certain point, most of the cells survive and are able to proliferate.

Sm at a low and nontoxic concentration (1 µg/ml) actually protects the cells against cisplatin cytotoxicity. At this concentration, Sm does not influence progression through the cell cycle. Kishimoto and Ueberman (*25*) and Verbin and Farber (*26*) suggested that a cell cycle step in the S-G_2 transit phase might be very sensitive to the protein synthesis inhibitors puromycin and cycloheximide. We have considered the possibility that Sm at a concentration of 1 µg/ml can also inhibit this step and would subsequently synchronize cells in an S phase relatively insensitive to cisplatin (*18*). Text figure 2 clearly demonstrates that such an effect does not occur at the level of 1 µg/ml, but only begins to appear at much higher concentrations and after a much longer incubation time. However, even then, the synchronization is not complete and should, therefore, have only a minor impact on cisplatin sensitivity. Thus the observed protective effect is not due to changes in the cell cycle. Another explanation could involve inhibition of the synthesis of an unknown cisplatin agonist. The existence of such a protein agonist has been postulated (*27, 28*), but never definitely proved. Such a mechanism of protective action, however, seems unlikely, because Sm at higher concentrations is expected to inhibit the synthesis of the agonist as well, and this is not in agreement with the potentiation effect that we observed. A further explanation might be that Sm at low concentrations may selectively inhibit degradation of one or more cisplatin antagonists or enhance their synthesis due to increased RNA production. In that case the concentration of the antagonist should increase during preincubation, and more cisplatin molecules should be inactivated. This in turn should result in decreased cisplatin interaction with DNA and does explain the reversal of the inhibition of [*methyl* ^{3}H]thymidine incorporation that we observed. Sm at higher concentrations (i.e., 5 and 10 µg/ml) will reduce protein synthesis in a rather aselective way. This may result in both a reduced turnover of cisplatin antagonist and/or a dramatic reduction in the de novo synthesis. The decreased capacity of the cell to produce antagonist during preincubation will increase its sensitivity to cisplatin.

The absence of cell protection in the case of the plateau phase cells could be due to the different cell densities and different cell number and drug relationships that we used.

Proliferating and nonproliferating cells are equally sensitive to cisplatin (*29*). Sm at higher concentrations potentiates cisplatin activity against both proliferating and nonproliferating cells. This reaction is similar to that described for anguidine by Hromas et al. (*7*). However, Sm acts differently from anguidine. The dominant effect of anguidine at low concentrations was a 'frozen cell cycle' with virtually no cytotoxic expense. Sm at a concentration up to 10 µg/ml, instead, does not induce cell cycle arrest but is already cytotoxic and does not require this mechanism to become able to potentiate cisplatin activity. This shows that cell cycle arrest is not a prerequisite for such an effect, as was suggested earlier (*7*).

Sm at a high concentration (10 µg/ml) inhibits [^{3}H]leucine and [*methyl*-^{3}H]thymidine incorporation to the same extent in preincubation, simultaneous incuba-

tion, and postincubation settings, but the biological effect of this inhibition is different Only cells preincubated with Sm show strong potentiation of cisplatin cytotoxicity This implies that the reaction step inhibited by Sm is localized close to the point of cisplatin addition This could be explained if Sm would inhibit synthesis of cisplatin antagonists like glutathione (*30*) or metallothionein (*31*, *32*) and/or would inhibit the synthesis of the DNA repairing enzymes induced by the action of cisplatin

To address these questions, we are investigating the concentration of cisplatin-DNA adducts as well as their biological half life

A similar concentration dependency of protection and potentiation was described by Pitillo et al (*33*) for the effect of Sm on irradiated *Escherichia coli* bacteria I ow Sm concentrations protected *E coli* against radiation, whereas high Sm concentrations potentiated radiation-induced cytotoxicity

In conclusion, the intensity and duration of the Sm-induced inhibition of protein synthesis may become a useful and selective tool for manipulation of the cellular response to the cytotoxic effect of cisplatin Our in vivo observations on L1210 leukemia bearing mice (*12*) give a new dimension to this statement This may open new therapeutic prospects in the biochemical modification of normal and cancer cells

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CHAPTER 6.1

IN VIVO POTENTIATION OF CISPLATIN ANTITUMOR ACTIVITY BY PRETREATMENT WITH SPRASOMYCIN

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IN VIVO POTENTIATION OF *cis*-DIAMMINEDICHLOROPLATINUM (II) ANTITUMOR ACTIVITY BY PRETREATMENT WITH SPARSOMYCIN

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SUMMARY

The influence of protein synthesis inhibition by sparsomycin (Sm) on in vivo cisplatin activity has been studied on BALBc × DBA_2: F_1 mice bearing L1210 leukemia i.p. Sm alone at the dose range from 0.5 to 3.0 mg/kg did not significantly improve animal survival. Sm potentiated cisplatin activity only when given 3 or 6 h prior to cisplatin ($P < 0.001$). Sm 0.5–1.5 mg/kg 3 h prior to cisplatin resulted in a significant prolongation of animal survival ($P < 0.001$) and 66% cures in each group versus 0% due to cisplatin alone. Sm pretreatment decreased weight loss due to cisplatin suggesting that it probably is able to decrease cisplatin toxicity.

INTRODUCTION

Sm (NCS-059729) is known as an inhibitor of ribosomal protein synthesis [1]. Besides antibacterial, antiviral and antifungal activity the drug also appears to be active against several rodent tumors [2]. Sm is borderline active (T/C = 125%) against L1210 leukemia [3].

In 1980 the total synthesis of Sm was reported [4] and several more active and less toxic analogues have been prepared [5].

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Recently, we have found that pretreatment of exponentially growing Chinese hamster ovary cells (CHO) with Sm in a concentration of 10 μg/ml resulted in significant potentiation of the cisplatin cytotoxicity (unpublished data) This effect was the same in the case of non-proliferating cells. Simultaneous and postincubation treatment with Sm proved to have much less or no potentiating effect on cisplatin. Surprisingly, pretreatment of exponentially growing CHO cells with 1 μg/ml Sm for 3 or 5 h resulted in significant resistance to cisplatin The mechanism of both protection and potentiation is still not elucidated, but our data indicate that Sm might influence the activity of cisplatin antagonists.

This study was focused on 3 topics: confirmation of the Sm/cisplatin synergism in an in vivo model, search for the optimal doses and schedules of both drugs and orientative determination of the drug combination toxicity.

MATERIALS AND METHODS

Animals

Female BALBc × DBA_2 F_1 mice weighing 20—24 g were supplied by TNO Rijswijk, The Netherlands Animals were housed in plastic cages with wire bottoms. Drinking water, food, temperature, air humidity and light conditions were provided according to the NCI standards [10]. The stock of L1210 cells received from TNO Rijswijk, was stored frozen in liquid nitrogen. Cells went through 1 passage before every new experiment. Mice were injected i.p. on day 0 with 10^5 L1210 cells suspended in 0 1 ml buffered saline and then randomized in the test and control groups. The tumor cell suspension was injected with a sterile 27 G needle (1 needle per mouse) to avoid formation of subcutaneous lymphomas. Each test group consisted of 6 mice. Experiments were repeated at least twice to achieve good reproducibility. Data presented in the figures are the means of those experiments.

Drugs

Sm was synthesized as described previously [4] and cisplatin (Platinol) was purchased in a freeze dried form from Bristol Meyers, USA. Cisplatin was dissolved in 0.9% NaCl The drugs were injected in a volume of 0.1 ml/10 g of mouse.

Treatment protocol

Mice were injected on days 1, 5 and 9 i.p. according to the protocol in Fig 1. Animals were weighed on days 1 and 5. Good randomisation was ascertained if the weight of an individual animal varied less than 7.5% from the mean weight of all animals. Animals were weighed individually on days 1 and 5. Weight loss was defined as the difference in mean group body weight on day 5 less that on day 1 ± S.D.). The significance of the difference in weight loss between the groups treated with cisplatin alone and Sm/cisplatin combination was determined using Wilcoxon test for unpaired samples; $P <$

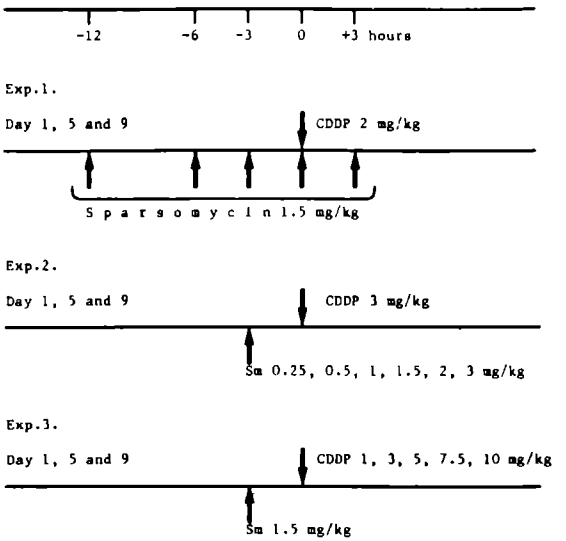

Fig. 1. Treatment protocol.

0.05 was accepted as a significance level. The survival curves of animals treated either with cisplatin alone or with Sm and cisplatin at the same dose, were compared with each other using the Mantel-Haenszel procedure [10].

RESULTS

The median survival time (MST) of the untreated controls ranged from 9 to 10 days (Fig. 2, control a). Sm alone (1.5 mg/kg), given on days 1, 5 and 9 showed no antitumor activity (Fig. 2, control b). Cisplatin alone at the doses used showed optimal activity at the level of 5.0 mg/kg (MST = 31 days, Fig. 3).

Experiment 1

In this experiment, drugs were combined with each other using about half the maximal tolerable doses, i.e. 1.5 mg/kg Sm and 2.0 mg/kg cisplatin. Sm was given 12, 6 and 3 h before, simultaneous to, or 3 h after the cisplatin (Fig. 2). When applied 12 h before, simultaneous to, or 3 h after the cisplatin, little effect of Sm over cisplatin alone was observed (MST = 17—19 days). However, when Sm was given 3 or 6 h before cisplatin the difference between the curves was highly significant ($P < 0.001$) and the MST were 26 and 27 days, respectively. For practical reasons a 3-h pre-treatment with Sm has been selected and this schedule has been kept constant during the following experiments. There were no significant differences in weight loss between the groups treated with cisplatin alone or Sm/cisplatin combination.

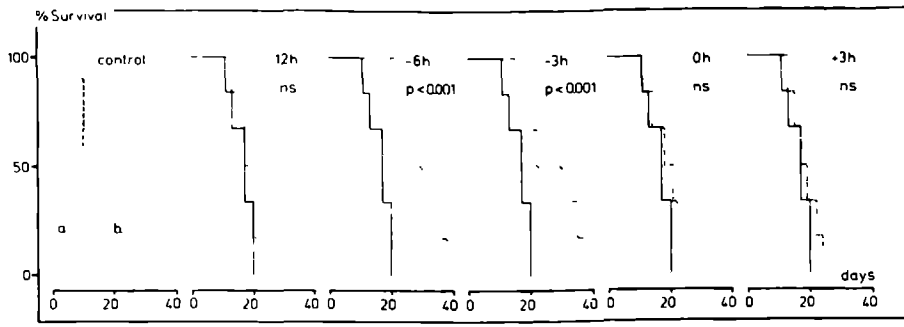

Fig. 2. Survival of animals treated with 2.0 mg/kg cisplatin alone (——) or Sm 1 5 mg/kg prior to the same dose of cisplatin (· · · · ·). The time intervals between Sm and cisplatin injections and significance levels are indicated. Control a (——) represents a group of untreated animals. Control b (· · ·) represents survival of animals treated with Sm 1.5 mg/kg alone on days 1, 5 and 9.

Experiment 2

When Sm in escalating doses was combined with a fixed cisplatin dose (3.0 mg/kg), a significant effect was already obtained with 0.5 mg/kg Sm (Fig. 3, $P < 0.05$). Pretreatment with 0.5—1.5 mg/kg of Sm prior to cisplatin, significantly prolonged animal survival and completely cured 4 out of 6 animals in

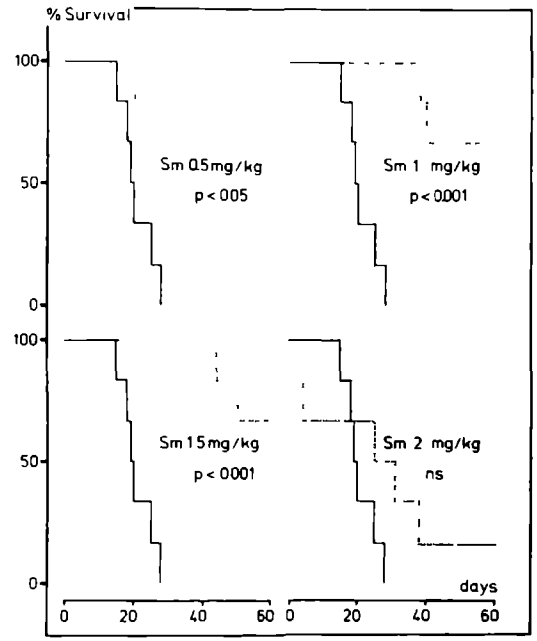

Fig. 3. Survival of animals treated with 3 mg/kg cisplatin alone (——) or Sm in variable doses injected 3 h prior to the same dose of cisplatin (· · ·). The effect of 0.25 mg/kg Sm and cisplatin was not significant and it is omitted in this figure.

each group. The deaths which were observed appeared during the late phase of the experiment and were certainly not due to toxic effect of the drug(s). At the higher dose level (> 2.0 mg/kg), Sm potentiated cisplatin toxicity.

Experiment 3

In the third experiment, Sm (1.5 mg/kg) was combined with escalating doses of cisplatin (Fig. 4). Sm improved animal survival considerably. The differences between the survival curves were significant at 3.0 mg/kg ($P < 0.001$) and 7.5 mg/kg ($P < 0.05$). At the level of 5.0 mg/kg the difference fell just below significance. However, again, in all 3 groups, 4 out of 6 mice were cured. Sm prevented early toxic death in the group treated with 7.5 mg/kg cisplatin. This effect was seen in 2 independent experiments. Interestingly, Sm tended to limit the cisplatin induced weight loss (see Table 1) at the higher cisplatin doses. This effect was significant at the cisplatin dose of 7.5 mg/kg ($P < 0.01$) and 10.0 mg/kg ($P < 0.05$).

DISCUSSION

The in vitro potentiation of cisplatin cytotoxicity recently found by us (unpublished data) has now been suggested in an in vivo model. Just as in

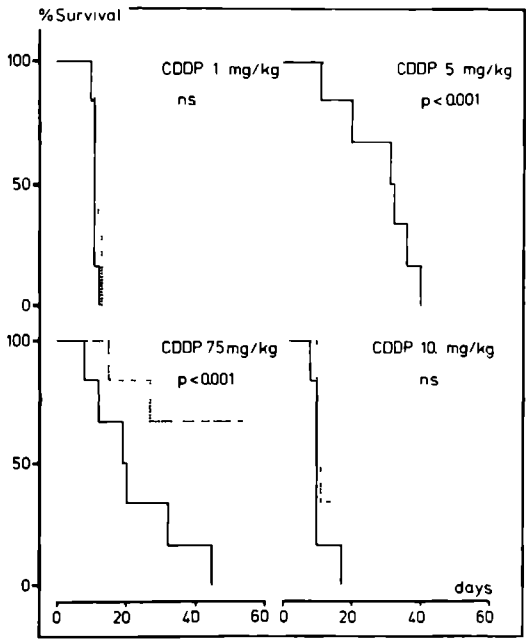

Fig. 4. Survival of animals treated with different cisplatin doses either alone (——) or with Sm pretreatment of 1.5 mg/kg (• • • • • •). The effect of 3.0 mg/kg cisplatin with or without Sm was omitted here and is to be found in Fig. 3.

TABLE 1

MEAN BODY WEIGHT LOSS IN EXPT. 3 ± S.D.

Cisplatin dose (mg/kg)	Sm dose (mg/kg)	Weight loss (g) days 1—5	± S.D.,	P
0	0	+1.3	0.3	
0	0.5	+1.1	0.3	
0	1.0	+0.4	0.4	
0	1.5	+0.1	0.2	
0	2.0	0.9	0.3	
1.0	0	+1.1	0.3	
3.0	0	+1.6	0.9	
5.0	0	-0.6	0.4	
7.5	0	-3.1	0.5	
10.0	0	-3.1	0.5	
1.0	1.5	+1.2	0.2	<0.05
3.0	1.5	+0.2	0.4	
5.0	1.5	−0.1	0.5	<0.01
7.5	1.5	−0.5	0.5	ns[a]
10.0	1.5	−1.8	0.6	

[a]ns, not significant.

vitro the pretreatment with Sm was essential for the enhancement effect. This suggests that the factor(s) involved in this reaction is (are) not present in the tumor cell at the moment of cisplatin administration and its induction is either blocked or delayed by Sm. Alternatively, the factor(s) may be present in the cell before cisplatin administration but have a short half-life. Inhibition of protein synthesis by Sm several hours before cisplatin injection decreases the activity of this hypothetical factor(s) and thereby blocks cellular defenses against cisplatin toxicity.

In the dose range of 0.5—1.5 mg/kg Sm, given in combination with 3.0—7.5 mg/kg cisplatin, Sm does not increase the toxicity of the latter. Instead of this, combination of those drugs appears not only highly effective against the tumor cells but also less toxic than cisplatin alone suggesting that normal and malignant cells may have different susceptibility to the drug combination.

The exact mechanism of this drug synergism is still unknown. Studies evaluating possible involvement of cisplatin antagonists [6—8] as Sm target are in progress.

In conclusion, this study opens a new possibility of chemical modification of the cancer cell on the translational level which may have a profound influence on the therapeutic effect of cytotoxic drugs. However, the data reported here should be interpreted as preliminary. To confirm the hypothesis we are actually studying Sm/cisplatin activity against murine solid tumors.

ACKNOWLEDGMENTS

We are indebted to Mr. J. Reitsma and his co-workers for excellent animal care and assistance during these experiments This work was presented at the Conference 'Chemical Modifiers of Cancer Treatment', American College of Radiology, Clearwater, Florida, 20—24 October, 1985. Supported by a Grant of Queen Wilhelmina Foundation Fund SNUKC-84-1 and Pharmachemie BV, The Netherlands.

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CHAPTER 6.2

IN VIVO POTENTIATION OF CISPLATIN ANTITUMOR ACTIVITY BY PRETREATMENT WITH DESHYDROXY-SPARSOMYCIN AND ETHYL-DESHYDROXY-SPARSOMYCIN

(unpublished)

After completing the series of experiments on potentiating cisplatin activity by pretreatment with sparsomycin (Sm) in L1210 leukemia i.p. (Chapter 6) we performed two similar series of experiments using two promising deshydroxylated analogues: deshydroxy-Sm (dSm) and ethyl-deshydroxy-Sm (EdSm). The tumor model was modified, and L1210 leukemia cells were inoculated s.c. In the same model, EdSm but not dSm showed antitumor activity when administered i.v. (see Chapter 4). We, then performed two experiments in order to further discriminate active and non-active analogues.

CD_2F_1 male mice weighing 25 - 30 g, were inoculated s.c. with 10^6 vital L1210 cells and were treated with drugs i.p. on days 1, 5 and 9. dSm and EdSm were administered 3 hours prior to cisplatin. Animals were weighed on day 1 and 5 of the experiment. Other protocol details are the same as in chapter 4 and 6. The results are expressed as the T/C ratios of the median survival times of the treatment (T) and control (C) groups, respectively.

RESULTS

The weight loss (T-C) was less than 4 g in the groups of animals treated with doses less than 7.5 mg cisplatin/kg. Pretreatment with both dSm and EdSm, potentiated weight loss induced by cisplatin. dSm appeared to be ineffective in potentiation of cisplatin activity (Fig.1). The potentiation up to T/C = 188% seen at the level of 7.5 mg cisplatin/kg was accompanied by the weight loss of 4.0 - 5.8 g. EdSm showed significant potentiation of the antitumor effect of cisplatin at the dose of 10 and 15 mg EdSm/kg preceding 5 mg cisplatin/kg (Fig.2). The maximal T/C value obtained at this level was 313%. One of the six animals from this group survived longer than 60 days and was considered to be cured. The weight loss in animals treated with this combination of drugs was 1.8 to 3.2 g.

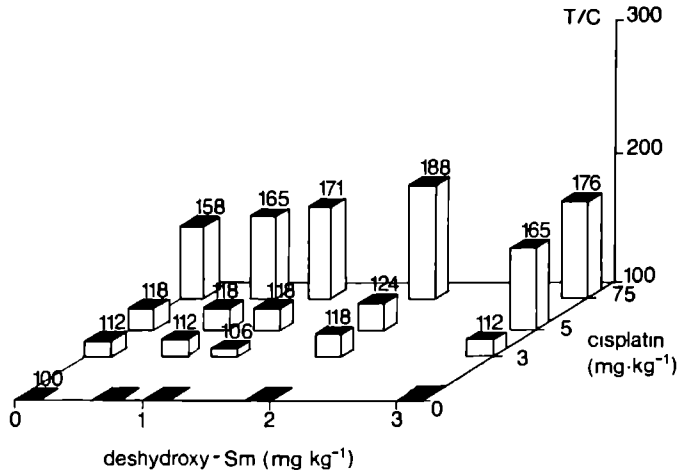

Fig. 1. Potentiation of cisplatin antitumor activity by pretreatment with deshydroxy-Sm (dSm). The results are expressed as the T/C ratio.

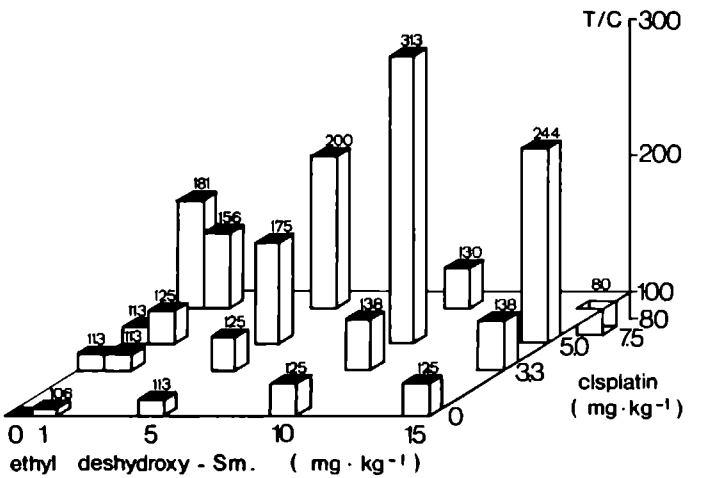

Fig. 2. Potentiation of cisplatin antitumor activity by pretreatment with ethyl-deshydroxy-Sm (EdSm). The results are presented as the T/C ratio.

Cisplatin alone was only moderately active on L1210 leukemia s.c., whereas EdSm showed only minor activity when administered alone. In combination, EdSm appeared to potentiate antitumor activity of cisplatin. The effect was significantly higher than the sum of the effects of each drug alone. dSm appeared to be ineffective in this setting. EdSm potentiated also weight loss induced by cisplatin but in the groups showing maximal therapeutic effects the weight loss was still lower than 4 g. EdSm thus, did not protect animals from the weight loss induced by cisplatin as it was described for Sm in Chapter 6.

The results of this study indicate, that EdSm might be a drug of choice for further preclinical studies on the synergism between sparsomycins and cisplatin.

CHAPTER 7

MODULATION OF IN VITRO CYTOTOXICITY OF SEVEN ANTICANCER DRUGS BY PROTEIN SYNTHESIS INHIBITION USING SPARSOMYCIN

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Submitted to: Int. J. Cancer

SUMMARY

Inhibitors of protein synthesis may modify cell response to cytotoxic drugs. The influence of protein synthesis inhibition using sparsomycin (Sm) on the cytotoxicity of seven classical cytotoxic drugs: 5-FU, ARA-C, MTX, doxorubicin, melphalan, bleomycin and vincristine was studied. Preincubations, simultaneous incubations and postincubations with Sm were investigated in vitro on CHO cells. Preincubation with Sm antagonized the activity of all S phase specific drugs 5-FU, ARA-C, MTX, and vincristin, while postincubation with Sm enhanced their efect. A similar pattern was observed with doxorubicin. Preincubation with Sm potentiated non-S phase specific drugs like bleomycin and cisplatin, but not melphalan. Postincubation with Sm had a potentiating effect on bleomycin but had no effect on melphalan. These results indicate a strong, schedule dependent effect of Sm on various drugs and suggest some potentially useful combinations to test in vivo.

INTRODUCTION

A number of protein synthesis inhibitors (PSIs) have been shown to interact with anticancer drugs (1-9). PSI may either potentiate the activity of the classical cytotoxic agents on tumor cells (3,4,7) or protect normal cells from their toxicity (1,2,8). Both phenomena have been observed in vitro (6,8,10,11), in animal studies (1-4,9) and in the clinical therapy (12-14). There are sufficient data to suppose that PSIs may act differentially on transformed cells while normal cells will be protected (8-11). In patients with acute lymphocytic leukemia, inhibition of protein synthesis by L-asparaginase after the administration of either MTX or ARA-C greatly increases the therapeutic index (12,14). The fact that L-asparaginase is only active on human lymphocytic leukemia has hampered further development of this concept. Many other PSIs tested in the clinical setting were found to be too toxic in phase I studies, and none showed satisfactory single agent activity. However, all were natural products selected on P388 leukemia. None were available as a synthetic product for extensive toxicity and structure activity relationship studies.

Sparsomycin (Sm) is the only PSI that is potentially modifiable through chemical synthesis, and development of less toxic analogues can be expected in future (15,16). Recently, we reported on the synergism between Sm and cisplatin on CHO cells in vitro (17) and L1210 leukemia in vivo (18). Preincubation with Sm appeared essential for the potentiating effect, and

this was dose dependent. Low Sm concentration (1 μg Sm/ml for 5 hours) effectively protected CHO cells from cisplatin toxicity, while higher concentration (10 μg Sm/ml for 3 hours) greatly increased the cytotoxicity of cisplatin (17). Post and simultaneous incubations with Sm had less or even no effect.

Here, we report on the Sm mediated modulation of the in vitro cytotoxicity on CHO cells of seven clinically important anticancer drugs. Comparison between the pre and post incubations with Sm gives valuable indications about the mechanism of these interactions. The results of this study can help in selecting the most interesting drug combination for further in vivo investigations.

MATERIALS AND METHODS

Cell line

CHO cells were grown routinely as monolayer culture in MEM, in closed tissue culture flasks as previously described (17).

Drugs

Sm was synthesized as previously described (16). Melphalan was obtained from Wellcome, Great Britain; ARA-C, MTX and vincristine, from Bergel Manufactoring, Nijmegen, The Netherlands; Doxorubicine, from Farmitalia, Italy; 5-FU from Hoffman-La Roche, Switzerland; and bleomycin from Lundbeck, F.R.G. The drugs were dissolved aseptically before each experiment. Incubation times were as follows: 5-FU and ARA-C, MTX and vincristine, 18 hours; doxorubicine, 1 hour; melphalan, 1 hour, and bleomycine, 3 hours.

Cell survival studies

To assess cell survival the colony formation technique was used as previously described in detail (17). Cells in exponential growth were incubated in a medium containing Sm for 3 or 5 hours either before (preincubation) or after (postincubation) incubation with other cytotoxic drugs. Some experiments were performed with simultaneous incubation. After incubation, the cells were gently trypsinized, counted and known aliquots of the cells were plated in culture dishes. The number of cells plated was adjusted to the expected effect of the treatment to provide 30 - 40 colonies per dish. Cell survival and colony formation after drug treatment were assessed after a 6 day incubation in a drug free medium. A colony has been defined as an aggregate of more than 30-50 cells. The results were normalized with respect

to untreated controls which had a plating efficiency varying between 45 and 70%. The survival of the cells (clonogenicity) is expressed on a dose/log survival plot. The mean and SEM of two independent triplicate observations is provided for each point. The areas under the curves (AUC) were used to calculate the treatment/control ratios (cytotoxic drug with Sm over drug without Sm). Treatment/control (T/C) values higher than 1.50 and lower than 0.50 were defined as significant.

RESULTS

The effect of Sm on CHO cells

When administered as a single drug, Sm at concentrations of 1 or 5 µg/ml for 3 to 5 hours had no influence on cell survival. At 10 µg Sm/ml for 3 hours, cell survival varied from 80 to 100%.

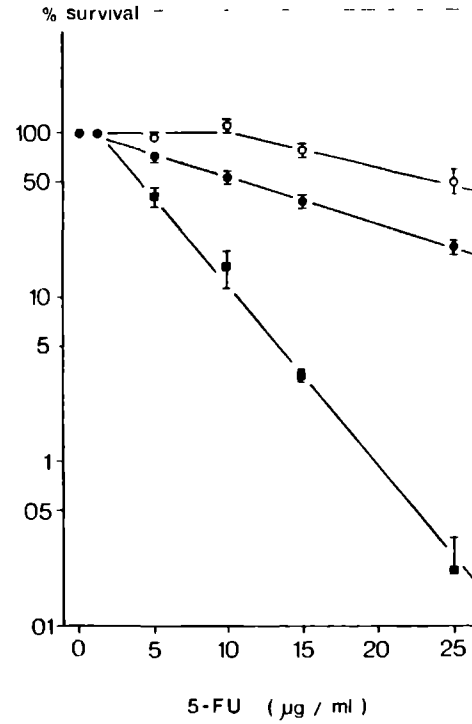
Antimetabolites 5-FU, ARA-C and MTX

Preincubation of exponentially growing CHO cells with Sm (10 µg Sm/ml for 3 hours) antagonized the cytotoxic effect of 5-FU, ARA-C, and MTX (see Fig.1-3). In the case of 5-FU, a similar effect was obtained with 1 µg Sm/ml for 3 hours (see Table 1), suggesting a great sensitivity to protein synthesis inhibition. Simultaneous incubation of Sm and 5-FU during the first 3 hours of the experiment showed neither the inhibitory nor the enhancement effect (Table 1). Postincubation with the same Sm concentration (10 µg Sm/ml for 3 hours) showed a marked increase in the cytotoxic effect of all the three antimetabolites. This enhancement in the cytotoxicity of ARA-C and MTX did not, however, change the pattern of the control curve. When CHO cells were resistant to high doses of ARA-C (see Fig.3), this resistance persisted after postincubation with Sm. Only in the case of 5-FU, did postincubation increase the slope of the exponential part of the curve (see Fig. 1).

Doxorubicin

Preincubation of CHO cells with 1, 5 and 10 µg Sm/ml showed significant antagonism at the two higher Sm concentrations (see Fig. 4). The reaction was probably time dependent as 5 ug Sm/ml for 5 hours had a stronger effect than 10 µg Sm/ml for 3 hours (T/C ratios 2.2 and 1.7, respectively, see Table 1). At low concentrations no effect was seen after 3 nor after 5 hours of incubation. Postincubation with 10 µg Sm/ml showed no significant potentiating effect (T/C ratio 0.8).

Fig.1.
Survival (clonogenicity) of CHO cells treated for 18 hours with different concentrations of 5-FU (●). Preincubation of these cells with 10 µgSm/ml for 3 hours (o) rendered cells insensitive to 5-FU. Postincubation with 10 µg Sm/ml for 3 hours (■) potentiated cytotoxic effect of 5-FU.



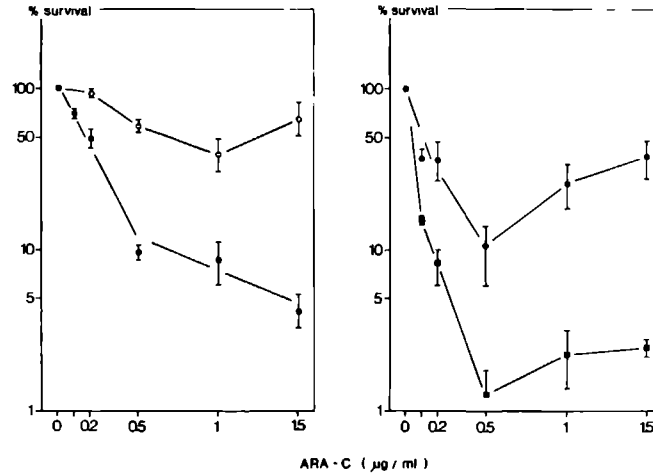


Fig. 2. Cytotoxic effect of ARA-C incubation for 18 hours (●) on CHO cells. Pretreatment of the cells with 10 µg Sm/ml for 3 hours rendered cells resistant to ARA-C (O) (left panel). Postincubation of the cells with 10 µg Sm/ml for 3 hours potentiated cytotoxic effect of ARA-C (■) (right panel).

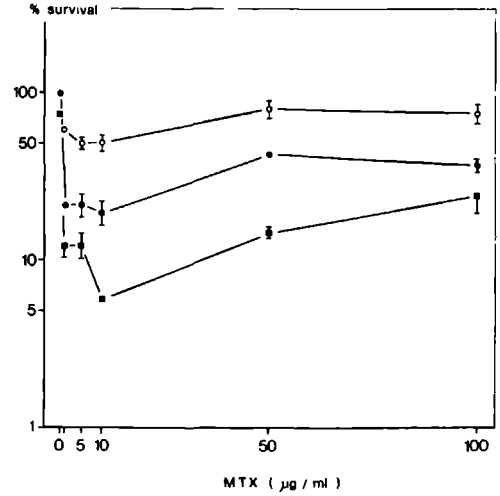


Fig. 3. Cytotoxic effect of MTX on CHO cells after 18 hours of incubation with the drug (●). Preincubation with 10 µg Sm/ml for 3 hours (O) rendered cells resistant to MTX. Postincubation with 10 µg Sm/ml for 3 hours (■) potentiated the cytotoxic effect of MTX.

Fig. 4.
Effect of doxorubicin in different concentrations on survival (clonogenicity) of CHO cells incubated for 1 hour with the drug (●). Preincubation of these cells with 5 µg Sm/ml for 5 hours antagonized the cytotoxic effect (o). Post-incubations with 10 µg Sm/ml for 3 hours slightly potentiated cytotoxic effect of doxorubicin (■).

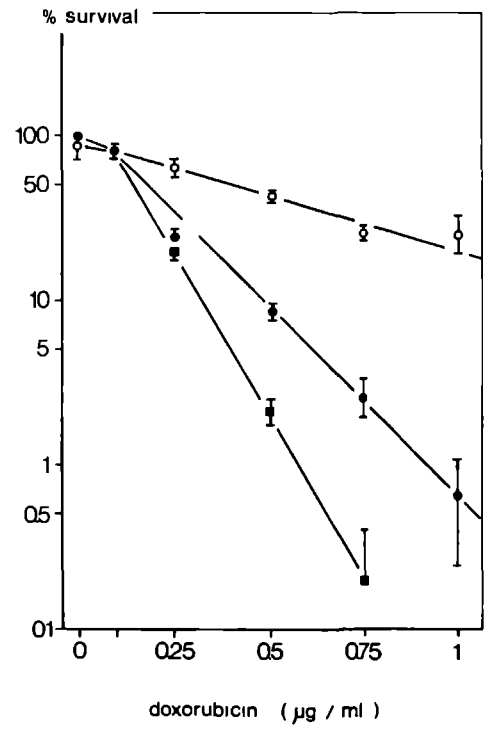


Table 1. Modulation of cytotoxicity of the anticancer drugs by sparsomycin.

Drug	pre-incubation	simultaneous incubation	post-incubation	T/C ratio
		μg Sm/ml(hours)		
5-FU	1.0 (3)			2.31
	10.0 (3)			2.10
		10.0 (3)		1.16
			10.0 (3)	0.36
ARA-C	10.0 (3)			2.70
			10.0 (3)	0.21
MTX	10.0 (3)			1.92
			10.0 (3)	0.26
Doxorubicin	1.0 (3)			1.10
	1.0 (5)			1.02
	5.0 (5)			2.23
	10.0 (3)			1.73
			10.0 (3)	0.75
Melphalan	1.0 (5)			1.91
	10.0 (3)			1.24
			10.0 (3)	0.75
Bleomycin	10.0 (3)			0.34
			10.0 (3)	0.46
Vincristine	10.0 (3)			1.57
			10.0 (3)	0.31
Cisplatin *	1.0 (5)			1.58
	10.0 (3)			0.36
		10.0 (3)		0.95
			10.0 (3)	0.58

* data from the reference 17.

Melphalan

Preincubation with 1 µg Sm/ml for 5 hours showed a strong antagonistic effect (T/C ratio 1.9, see Fig. 6) while preincubation with 10 ug Sm/ml showed a much weaker and insignificant effect (T/C ratio 1.2, data not shown in Fig. 5). Postincubation showed an insignificant effect as well (T/C ratio 0.8).

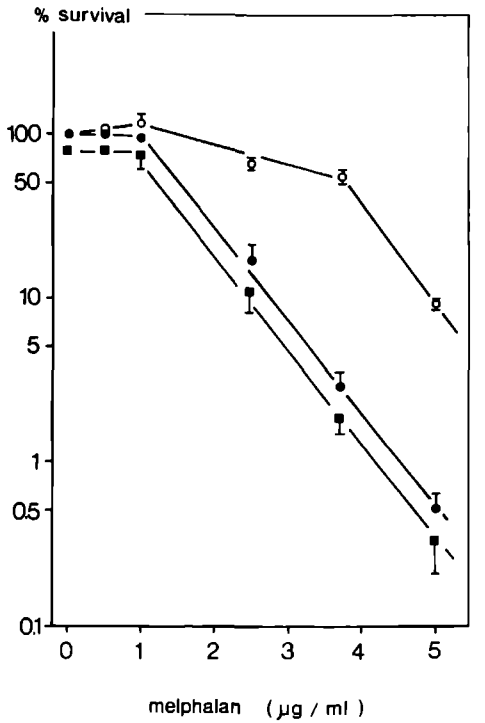


Fig. 5. Cytotoxic effect of melphalan incubated for 1 hour on survival (clonogenicity) of CHO cells (●). Pretreatment with a low Sm concentration, 1µgSm/ml for 5 hours, (o) significantly decreased the cytotoxic effect. Postincubation with 10 µgSm/ml for 3 hours (■) did not change the survival.

Bleomycin

Both, preincubation and postincubation with 10 ug Sm/ml for 3 hours showed significant potentiation of the cytotoxic effect (T/C ratios 0.3 and 0.5, respectively, see Fig. 6).

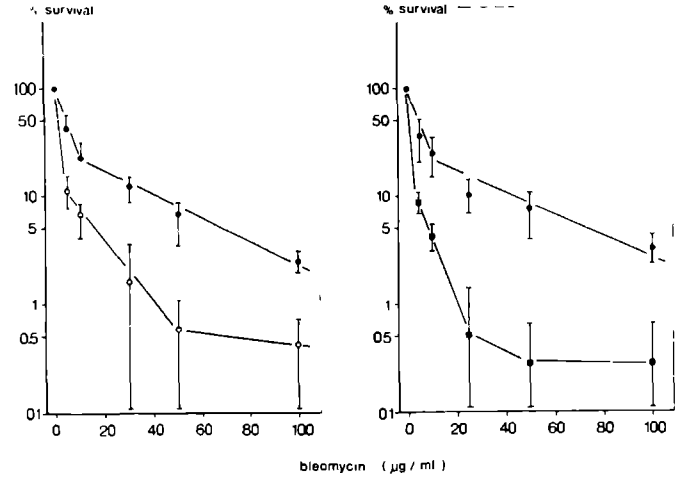
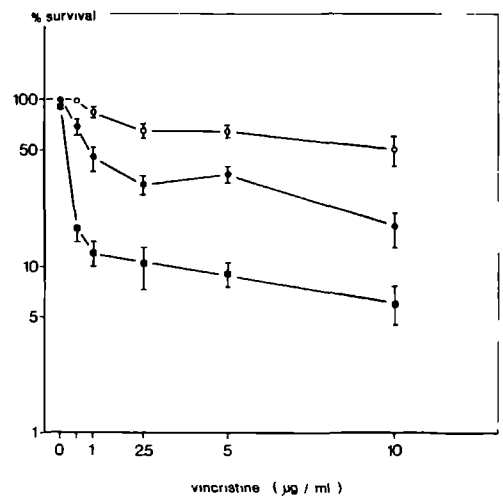


Fig. 6. Cytotoxic effect of bleomycin incubated for 3 hours on CHO cells (●). Preincubation with 10 µgSm/ml for 3 hours (o) potentiated the cytotoxicity of bleomycin. Similar effect was observed in case of postincubation (■).

Vincristine

Preincubation with 10 µg Sm/ml for 3 hours showed an antagonistic effect (T/C ratio 1.6, see Fig. 7), while postincubation with the same concentration showed a significant potentiation effect (T/C ratio 0.3). The enhancement effect due to Sm postincubation did not result in a change of the pattern of the control curve.

Fig. 7.
Cytotoxic effect of Vincristine incubated for 16 hours on CHO cells (●). Preincubation with 10 ug Sm/ml for 3 hours (o) rendered the cells resistant to vincristine. Postincubation with 10 µg Sm/ml for 3 hours potentiated the cytotoxic effect of Vincristine (■).



Little is known about the interaction mechanisms of PSI with other drugs. In general PSI may inhibit synthesis of proteins involved in 1. membrane transport, 2. intracellular activation, 3. intracellular inactivation, 4. interaction with DNA, and 5. DNA repair. The effect will be inversely related to the biological half-lives of the proteins involved. The site of interaction (a specific protein) could be different for each drug or class of drugs.

The antagonistic effect of protein synthesis inhibition by Sm on the cytotoxicity of 5-FU, ARA-C, and MTX is similar to the effect of preincubation with anguidine (6,19). This effect has been called protection, but "antagonism" probably describes this phenomenon better. When MTX was preceded by cycloheximide, much higher doses of MTX were tolerated by the tumor bearing mice, but there was no improvement in the therapeutic effect (20). Similar antagonism has been noted between L-asparaginase administered before MTX or ARA-C in animals bearing L5178Y leukemia (5). These data suggest that protein synthesis inhibition prior to incubation with an antimetabolite either inhibits the active transport or the intracellular activation. L-asparaginase it is known to inhibit formation of MTX-polyglutamates thus decreasing the toxicity and antitumor activity of this drug (21). Similarly, inhibition of ARA-C transport by L-asparaginase has been suggested (5). These observations are also consistent with the work of Capizzi et al. (22). Patients treated with ARA-C or MTX and L-asparaginase for lymphocytic and non-lymphocytic leukemias showed a significantly higher number of complete remissions in comparison to patients treated with ARA-C or MTX but without L-asparaginase (12-14). L-asparaginase was only active when administered after the antimetabolite.

The absence of a modulatory effect seen during the first three hours of simultaneous incubation with both Sm and 5-FU may indicate neutralization of two opposing processes: "preincubation" and "postincubation" effects. To avoid such difficult to interprete situations we abandoned simultaneous incubations in the following experiments. The general interaction mechanism between antimetabolites and PSI, could be a delay in the repair of the damage induced by the drugs. Preincubation with Sm antagonizes the cytotoxicity of doxorubicin, just as it did with the antimetabolites. This reaction is also consistent with that found for anguidine (19). The intracellular accumulation of doxorubicin is not believed to be caused by an active inward transport (23), and it seems improbable that Sm may interfere with this

process. On the contrary, evidence has been presented for an active efflux mechanism across the plasma membrane. If Sm were to inhibit synthesis of this transport protein, the effect would be the opposite of what was observed and it would be similar to what is seen by metabolic inhibitors that increase doxorubicin cellular accumulation and cytotoxicity (24,25). From the above, it may be concluded, that Sm acts on the doxorubicin metabolism rather than membrane transport.

Melphalan showed the same antagonistic effect after preincubation with a low Sm concentration as was seen with cisplatin (17). However, preincubation with higher Sm concentrations did not potentiate the effect of melphalan. This differs from the findings of Hromas et al. (6) who found a significant potentiation of melphalan cytotoxicity on CHO cells when pretreated with anguidine.

The synergism between bleomycin and Sm is very interesting. Both preincubation and postincubation are effective. The picture is somewhat similar to that seen with cisplatin (T/C ratio 0.46 versus 0.58), however, postincubation with Sm showes the stronger modifying effect than in case of cisplatin (17).

The antagonism of Sm and vincristine may be due to a reduction in the cell cycle transverse time and an accumulation of cells in the late S phase (17). As this kind of effect was lethal to only a small number of cells, the rest will be protected from vincristine cytotoxicity by not entering mitosis. Potentiation seen in postincubation experiments may reflect a delay in the recovery after inhibition of the mitotic spindle figure or of DNA synthesis.

In general preincubation with Sm antagonized the cytotoxic effect of all the S phase specific drugs, while postincubation potentiated their effect. Preincubation potentiated the cytotoxicity of all nonS phase specific drugs, while postincubation has less or no effect on these drugs.

In conclusion: Sm in combination with various cytotoxic drugs shows a strong schedule dependent modification in the cytotoxicity. Especially strong potentiating effects were seen with the antimetabolites, and with vincristine and bleomycin. These warrant further investigations in vitro and in vivo.

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CHAPTER 8

PHARMACOKINETICS OF SPARSOMYCIN AND THREE SPARSOMYCIN ANALOGUES IN MICE

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SUMMARY

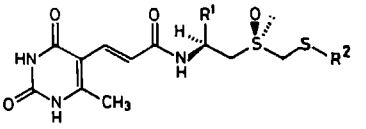
Single dose pharmacokinetics of sparsomycin (Sm) and three Sm analogues: deshydroxy-Sm (dSm), ethyl-deshydroxy-Sm (EdSm) and n-pentyl-Sm (PSm) was studied in BCBA/Rij mice. In previous studies these three analogues showed good antitumor activity in several ascitic murine tumors, while Sm itself did not. EdSm was the only compound that was active on murine L1210 leukemia s.c. when administered i.v. To explain this observation, the pharmacokinetics and the areas under the curves (AUCs) were studied at the LD10 level. The LD10 values ($\mu mol.kg^{-1}$) were: 24 (Sm), 40 (dSm), 167 (EdSm) and approximately 102 for PSm. The dose/AUC relation was constant for all compounds until LD10. The AUC at LD10 of EdSm was 250% higher than that of Sm. dSm showed AUC at LD10 that was 25% lower that that of Sm. PSm showed the AUC at the estimated LD10 dose that was twice as high as that for Sm. However after reduction for protein binding to free drug AUC, this value decreased to 37% of the free drug AUC of Sm. Protein binding of other compounds was negligible. The results of this study suggest that EdSm is the least toxic compound which produces the highest AUC values at the LD10 dose. This may explain its higher antitumor activity in L1210 s.c. model.

INTRODUCTION

Sparsomycin (Sm, NSC 059729) is a well known inhibitor of the ribosomal protein synthesis (1). The drug showed antitumor activity in several rodent tumors (2). The use of and investigations on Sm were limited by poor availability and toxicity of this natural product for more than two decades. Since only recently Sm is available through the total chemical synthesis (3) and a wide range of analogues underwent in vitro structure-activity relationship studies (4-7). The results of these studies stressed the importance of two structural modifications: deshydroxylation in the R1 position (Table 1) and increase of lipophilicity by elongation of the R2 aliphatic side chain. Three analogues deshydroxy-Sm (dSm), ethyl-deshydroxy-Sm (EdSm) and n-pentyl-Sm (PSm) showed high antitumor activity on ascitic P388 and L1210 leukemias and RC renal cell carcinoma (T/C 200-400%) (8). However, when the ascitic tumors (L1210 and RC) were inoculated s.c. a signficant loss of antitumor activity was observed (9). All three compounds showed some activity on RC tumor (T/C 140-150%), but only EdSm was active on L1210 leukemia s.c. (T/C 140%). To understand this loss of activity and select one single most active analogue, we performed a pharmacokinetic study

in mice. A possible relationship between AUC at the LD10 dose and antitumor activity was investigated.

Table 1. Sparsomycin and its analogues



compound	Cn	R1	R2
Sparsomycin (Sm)	1	- CH_2OH	- CH_3
deshydroxy-Sm (dSm)	2	- CH_3	- CH_3
ethyl-deshydroxy-Sm (EdSm)	3	- CH_3	- CH_2CH_3
n-pentyl-Sm (PSm)	5	- CH_2OH	- $(CH_2)_4CH_3$

Cn - number of the carbon atoms determining the lipophilic effect.

MATERIALS AND METHODS

Drugs

Chemical structures of Sm and its three analogues are shown in Table 1. Sm and analogues were synthesized as described before (3,5). Drugs were obtained in a freeze-dried form. Prior to the experiment, drugs were dissolved in 0.9% NaCl, except for n-pentyl-Sm that was dissolved in 15% propyleneglycol and 0.9% NaCl, and kept in dark flasks at 4°C. Before each experiment the drug concentration in solution was analyzed using HPLC (see below).

Animals

Male BCBA/Rij mice, 12-14 weeks old, were purchased from TNO (Rijswijk, The Netherlands). Groups of 15-20 animals needed for one experiment were selected for uniform body weight from the larger populations. The body weight varied less than 3 g within one group. Until the experiments, the animals were housed in standard laboratory conditions: plastic cages, 12/12 hours light-dark schedule, 70%-80% relative air humidity, room temperature of 21 $\pm$ 0.5°C, standard granulated food (Hope Farms, Woerden, The Netherlands) and tap water ad libitum.

Testing the LD10

Four groups of 10 BCBA/Rij male mice were used in each experiment within the dose range indicated by the pilot experiments. The drugs were administered as a single dose i.v. and animal survival was recorded daily for three weeks. The LD10 values were calculated from the dose/survival plots using a microcomputer program for semilog regression and 95% confidence intervals (10). The 95% confidence intervals for LD10 were within 25% - 30%. The single LD10 for PSm could not be determined as the drug was poorly soluble and the amount of propylene-glycol (approximately 50%) needed in the formulation of such a high concentration was toxic to the mice. The single LD10 dose could be estimated from the fractionated experiments by multiplication of the fractionated LD10 by the number of the doses. Such an estimation correctly predicted the single LD10 value for dSm and EdSm.

Pharmacokinetic experiments

Drugs were injected in the tail vein and the time of injection was recorded. The volume of injection was constant: 0.1 ml/10g body weight. Subsequently, the animals were exsanguinated under pentobarbital anaesthesia and blood samples of 0.6-0.8 ml were collected in plastic tubes containing a small amount of powdered heparine. Pentobarbital was always administered 3 minutes prior to exsanguination. Plasma was separated from blood cells by centrifugation within one hour and was kept at $-20^{o}C$ pending analysis. Sm and dSm were studied at three dose levels: 0.5, 1.0 and 2.0 times LD10. EdSM was tested at 0.5 and 1.0 times LD10 because of the very high LD10 for this compound and PSm was tested at the doses of 12 and 24 $\mu mol.kg^{-1}$ (12% and 24% of the estimated LD10 value, respectively) because of poor solubility of this compound.

Analysis

Plasma samples were deproteinized either with 10% trichloroacetic acid (Sm and deshydroxy-Sm) or with 7% perchloric acid (ethyl-deshydroxy-Sm and n-pentyl-Sm). Both acids were from Aldrich (Brussel, Belgium). A semi-automated high-performance liquid chromatography (HPLC) system (Kratos Analytical, Ramsey, NJ, USA) was utilized. This system consisted of two spectroflow 400 pumps, Multiport Streamswitch with two six-way valves (Rheodyne, Cotati, CA, USA), a 757 UV-Vis Detector and a DS 610 Multirange strip chart recorder. The connection of the sampling valves for the back flushing method has been described elsewhere (11). The whole system was operated by means of an HPLC solvent programmer 9224 (Kipp Analytical, Rotterdam, The Nether-

lands). The columns used were: a concentration column (50 x 3 mm id) filled with LiChrosorb RP-8 (10 µm particle size) and an analytical column (100 x 4.6 mm id) filled with CP^{tm} Spher C8 (8 µm particle size); both packing materials were obtained from Chrompack BV (Middelburg, The Netherlands). Solvent A (flow 1.3 ml/min) for sample concentration and cleaning of the columns consisted of distilled water. Solvent B (flow 1.0 ml/min), to carry the sample from the concentration column through the analytical column, consisted of methanol (HPLC grade, Fisons, Loughborough, England) in distilled water. The following methanol concentrations were used: Sm (15%), deshydroxy-Sm (27%), ethyl-deshydroxy-Sm (40%) and n-pentyl-Sm (45%). Drug concentrations were determined from the comparison of the sample peak heights with those of untreated control plasma spiked with known concentrations of the drug. The accuracy of the standard curves was estimated by comparison of the concentration/peak heigth ratios for five different concentrations over a range of 3 logs. The calibration curve was acceptable when the SD of the ratios was lower than 5%. The detection limits for different analogues in plasma varied from 10-30 ng.ml^{-1}.

Protein binding assays

Protein binding of the drugs has been determined with an ultrafiltration method. No aspecific drug binding to the filters (Emit free level TM, Syva, Palo Alto, CA, USA) was found. Heparinized plasma samples of 3 to 5 animals exsanguinated at the same time after injection has been pooled and centrifuged through the filters at 2500 g for 20 minutes. Plasma protein binding was evaluated at each dose level, 10 and 30 minutes after injection. Drug recovery after centrifugation was compared to the total drug concentration. The results are expressed as the percentage of the total drug concentration retained on the filter (bound to plasma).

Fitting of the curves

Curves were fitted using ELSMOS computer program (12) for two compartments model. The AUC was calculated from the fitted concentrations using the trapezoidal rule and starting from the 5th minute of the experiment. Using the exsanguination technique it was impossible to obtain enough data points to determine the alpha phase properly. This also is the reason why the points before 5 minutes were not fitted, and the AUC was calculated from the fifth minute. The $t_{1/2}\,\beta$ and $t_{1/2}\,\gamma$ were calculated from the β and γ elimination rates, respectively, according to:

$$t_{1/2}\,\beta = \frac{0.693}{\beta}$$

The three analogues showed higher LD10 values than the parent drug (see Table 2.) Deletion of the hydroxyl group in R1 position produced higher LD10 for dSm than for hydroxylated Sm (40 versus 24 umol.kg^{-1}). The more lipophilic EdSm showed the highest LD10 167 umol.kg^{-1}. The fractionated LD10 value obtained after administration of PSm for nine daily doses was 11.3 umol.kg^{-1} per inj. The single LD10 was estimated by multiplication of this value by 9 (102 umol.kg^{-1}).

Single dose pharmacokinetics of the four compounds in mice showed two elimination phases. The $t_{1/2}$ was similar for all analogues and was 0.13 $\pm$ 0.05 hour (8 $\pm$ 3 minutes). At LD10 the gamma elimination phase was longest in case of dSm (6-7 hours); in the other analogues $t_{1/2}$ was 0.5 to 1.4 hours (30 - 84 minutes). The terminal half-life of Sm increased almost 5 fold at twice the LD10.

Fig. 2-5

Table 2. Pharmacokinetics of Sm and three Sm analogues in mice.

compound	dose μmol.kg^{-1}	$t_{1/2}$ β (hours)	$t_{1/2}$ γ (hours)	AUC total	AUC free drug	protein binding (%)	dose/ total AUC
				umol.h.l^{-1}			
Sm	12.0	0.11	0.9	6.5	5.9	10.0 $\pm$ 4	1.8
	24.0 *	0.13	1.4	12.9	11.6		1.9
	44.4	0.14	59.3	50.2	45.2		0.9
dSm	20.0	-	-	4.7	3.9	18.0 $\pm$ 6	4.3
	40.0 *	0.2	7.1	9.8	8.0		4.1
	80.0	0.2	6.1	17.9	14.7		4.5
EdSm	83.5	0.08	0.9	13.5	8.4	38.0 $\pm$ 7	6.2
	167.0 *	0.15	1.4	27.3	16.9		6.1
PSm	12.0	0.06	0.5	2.2	0.5	78.0 $\pm$ 10	5.4
	24.0	0.14	0.8	4.6	1.0		5.2
	102.0 **	-	-	19.2	4.2		5.3

* LD10

** estimated LD10

Protein binding was concentration independent for all compounds (see Table 1). Protein binding was proportional to the number of the carbon atoms (Cn) present in R1 and R2 substitutions. Hydroxylated compounds had one carbon atom less, because of the "masking" effect of the hydroxyl group. The correlation coefficient between Cn and protein binding was r = 0.99.

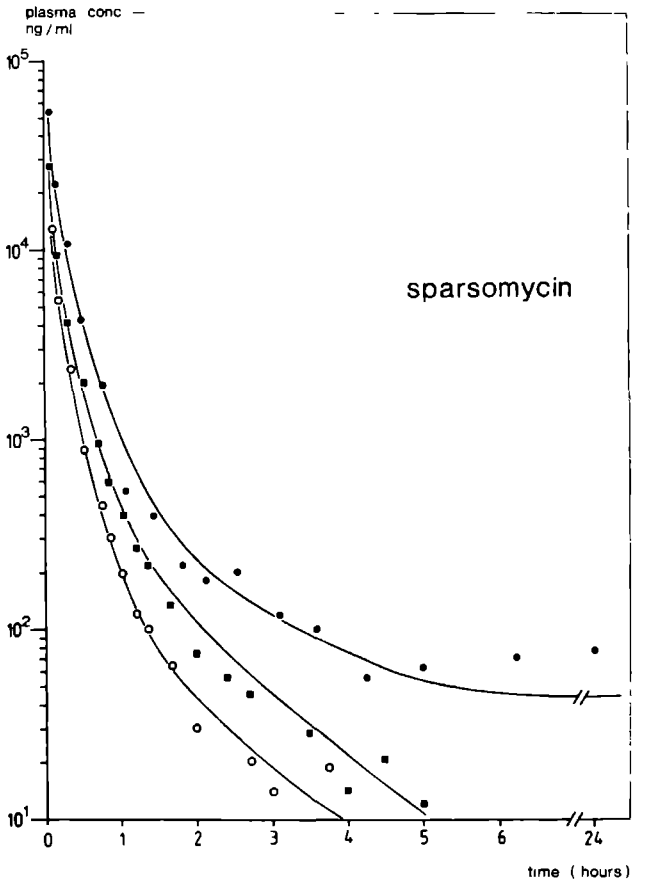


Fig. 2. Pharmacokinetics of Sm administered as a single i.v. bolus to BCBA/Rij mice. (●) 44.4 μmol.kg^{-1};(■)24.0 μmol.kg^{-1} (LD10); (○) 12 μmol.kg^{-1}. Each point represents a sample from a single mouse.

The dose/AUC ratios were constant until the LD10 for three compounds. PSm showed linear dose/AUC relationship until 22% of the estimated LD10 dose. The dose/AUC ratios were smallest for Sm, suggesting lowest total body clearance. Deshydroxylation and increase of lipophilicity (dSm, EdSm and PSm) increased the dose/AUC ratios by a factor 2.3 for dSm, 3.3 for for EdSm and 2.9 for PSm.

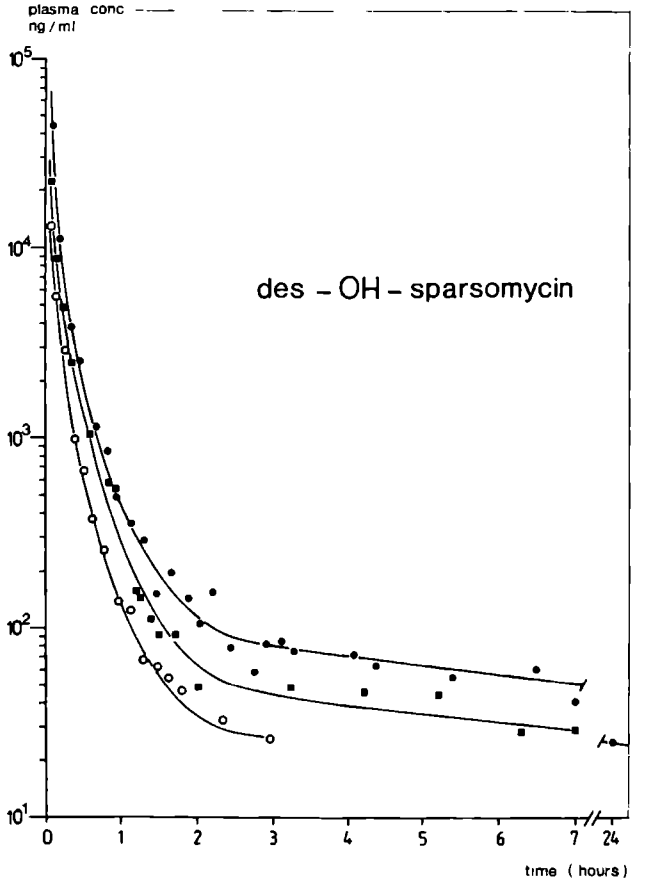


Fig. 3. Pharmacokinetics of deshydroxy-Sm administered as a single i.v. bolus to BCBA/Rij mice. (●) 80.0 μmol.kg^{-1}; (■) 40.0 μmol.kg^{-1} (LD10); (○) 20.0 μmol.kg^{-1}. Each point represents a sample from a single mouse.

At the LD10, EdSm produced the AUC of 27.3 μmol.h.l^{-1} versus 12.9 μmol.h.l^{-1} produced by the equitoxic dose of Sm. The AUC of dSm was 9.8 μmol.h.l^{-1} which was lower than that of Sm. The estimated AUC at LD10 dose of PSm should be 19.2 μmol.h.l^{-1} which is 1.5 times higher than that of Sm. When the total AUCs were reduced to the free drug AUC by subtraction of the protein bound fraction, the relations between compounds changed. Free drug AUC for EdSm remained 1.5 times higher than that of Sm. The AUC of dSm remained approximately 70% of the Sm value while the high total AUC value observed for Psm was only 36% of the free AUC of Sm.

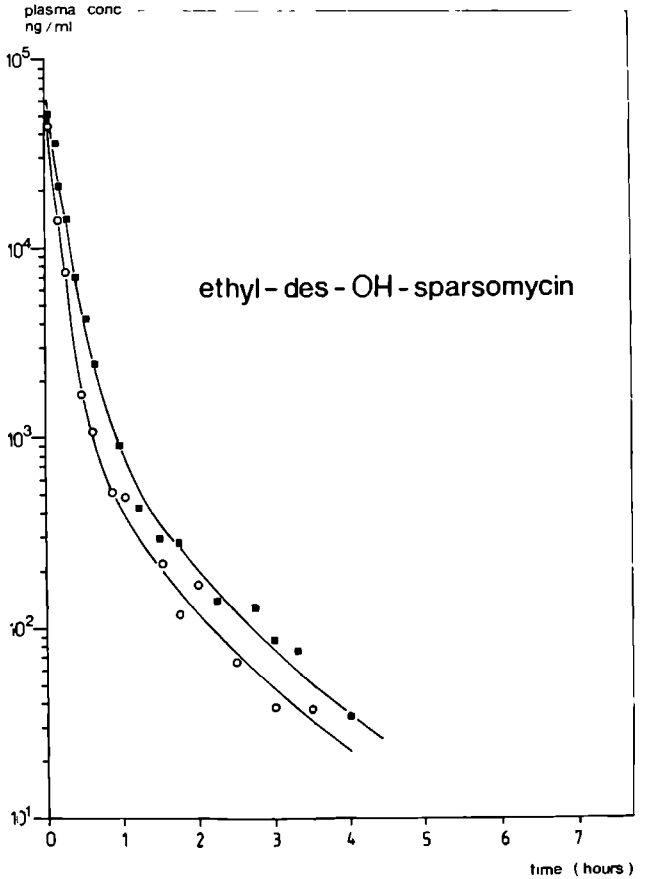


Fig. 4. Pharmacokinetics of ethyl-deshydroxy-Sm administered as a single i.v. bolus to BCBA/Rij mice. (■) 167.0 μmol.kg^{-1} (LD10); (O) 83.5 μmol.kg^{-1}. Each point represents a sample from a single mouse.

DISCUSSION

Previously we have reported that the three analogues dSm, EdSm and PSm showed in mice considerably higher LD50 values and higher antitumor activity, compared to the parent drug (2,7). The highest activity was shown on ascitic tumor models (P388, L1210 and RC). Considerable loss of activity on the same, sensitive tumors inoculated s.c., prompted us to perform pharmacokinetic studies and dose/AUC relation analysis.

EdSm, the only compound that showed activity on L1210 leukemia s.c. showed in this study 2.5 times higher AUC at LD10 in comparison to Sm.

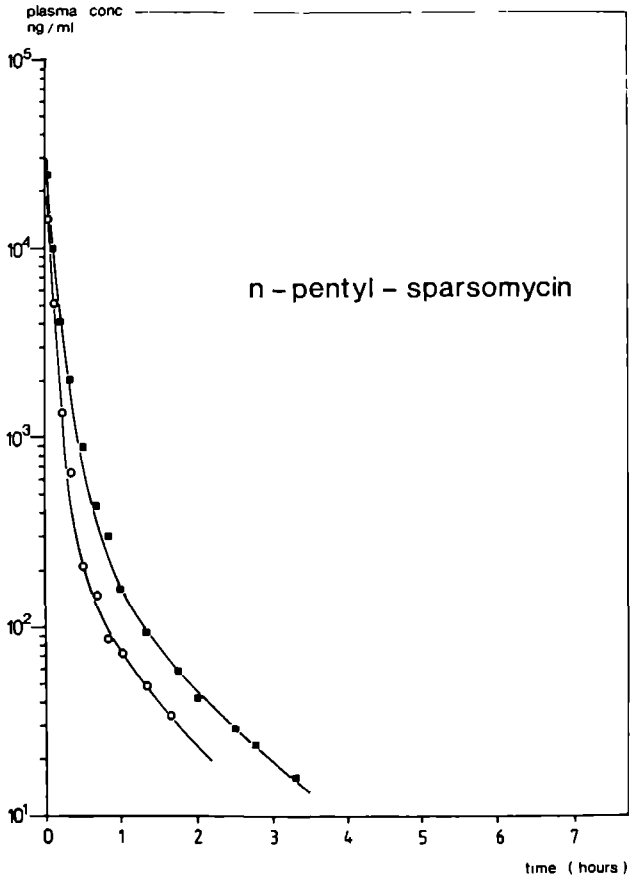


Fig. 5. Pharmacokinetics of n-pentyl-Sm administered as a single i.v. bolus to BCBA/Rij mice. (■) 24.0 μmol.kg^{-1}, (O) 12.0 μmol.kg^{-1}. Each point represents a sample from a single mouse.

Even after reduction for protein binding, the free drug AUC was still 1.5 times higher than that of Sm. dSm showed AUC, both total and free, slightly lower than that of Sm, while in case of PSm very low free drug AUCs could be predicted. These data may suggest, that the free drug AUC may be related to activity on L1210 leukemia s.c. and in this way pharmacokinetic data may contribute to the choice of the most suitable analogue. When the tumor and the drug are administered i.p., the AUC in the ascitic fluid may be very high and high lipophilicity of the drug may ease drug distribution to the tumor cells. This may produce a wrong impression of the antitumor activity of a compound.

The increase of the dose/AUC ratio seen in case of both deshydroxylated analogues suggest that the hydroxyl group of Sm may be an important factor inhibiting metabolism and/or excretion of the drug. Increase of lipophilicity (PSm) increases the dose/AUC ratio too, but the increase is proportionally smaller than the effect of deshydroxylation. EdSm, which is a compromise between deshydroxylation and increased lipophilicity showed a high LD10 and good antitumor activity. Opposite to a certain AUC-activity relation, is the lack of a AUC-toxicity relation. Equitoxic doses of different analogues produced different AUC's. A high AUC at the LD10 dose may thus indicate that the compound expresses a different dose limiting toxicity compared to the other compounds. The most severe toxicity encountered, when the animals were treated with toxic doses of drugs was death due to haemorrhage (unpublished). Orbital, rectal and urogenital haemorrhages were most frequently observed. Appearance of haemorrhage in mice after treatment with Sm was reported in 1968 (14) and similar phenomenon was described by us in beagle dogs (9). The haemorrhagic diathesis seen in dogs was due to exhaustion of blood coagulation factors produced in the liver (9). In mice treated with Sm and its analogues we observed a striking difference in frequency of haemorrhagic events between analogues (un-published). Haemorrhage was clearly a dose-limiting toxicity for hydroxylated analogues. Approximately 30% of the animals dying at LD50 dose of Sm died due to haemorrhage. We did not observe a single case of haemorrhage with both deshydroxylated analogue. Lack of this kind of toxicity may explain higher LD10 and LD50 for these compounds. Higher therapeutic doses may in turn explain higher antitumor activity of these compounds. However, this advantage of EdSm should be interpreted with caution as it may be a species-related phenomenon.

In conclusion: presented data suggest a link between the free drug AUC and antitumor activity of EdSm on L1210 leukemia s.c. This compound too, is a good candidate for further preclinical studies.

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CHAPTER 9

PHARMACOKINETICS AND TOXICOLOGY OF SPARSOMYCIN IN BEAGLE DOGS

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Pharmacokinetics and toxicology of sparsomycin in beagle dogs*

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Summary. Sparsomycin is a cytotoxic drug exhibiting a broad spectrum of in vitro activity against murine tumors and many tumor cell lines. It also appears to be a potent stimulator of the antitumor activity of cisplatin against L1210 leukemia in vivo. However, because of its toxicity the antitumor activity of sparsomycin on murine tumors in vivo has been disappointing. The purpose of our study was to investigate the pharmacokinetics of this drug as well as the possible mechanisms that produce sparsomycin toxicity. Tests on beagle dogs revealed that about 60% of the drug is eliminated by metabolic clearance, while 40% is eliminated by the kidneys. After a single bolus injection of 0.1 mg/kg sparsomycin without narcosis sparsomycin was eliminated with a $t\beta_1$ of 0.6–0.7 h, the AUC being 0.32–0.38 mg·h·l^{-1}, and the volume of distribution (V_d) 0.26 l/kg. In addition to being subject to glomerular filtration, sparsomycin is probably also actively excreted and actively reabsorbed by the renal tubuli. Sparsomycin itself may inhibit its active tubular excretion, thus resulting in a decrease in the drug's renal clearance and its accumulation in the plasma. Sparsomycin appeared to be toxic primarily in the liver, disturbing its function and the synthesis of plasma proteins. Two out of five dogs developed hemorrhagic diathesis due to hypofibrinogenemia and deficiency of other blood coagulation factors. Sparsomycin was not toxic to the bone marrow.

Introduction

Sparsomycin (NSC 059729), which was originally isolated from a bacterial broth of *Streptomyces sparsogenes* [16], is a potent inhibitor of ribosomal protein synthesis [11]. Owen et al. have reported that sparsomycin is active on human KB cells in vitro and on several rodent tumors in vivo [16]. In 1964 the drug was given a phase I clinical trial: five patients received sparsomycin in daily increasing doses for 12–14 days. The trial was prematurely discontinued because of retinal toxicity in two patients [3]. Nowadays this type of scheduling is no longer used because of the high risk of cumulative drug toxicity [2]. Further preclinical research was not performed until 1981. The revival of interest in this drug is justified by the recent development of a flexible procedure for its total synthesis [15] and by the development of some carefully designed new analogs [1, 10].

Synthetic sparsomycin has recently been found to be active in vitro in several murine tumors and in many human tumor cell lines [5]. The results of in vivo screening tests performed in National Cancer Institute (NCI) laboratories over the last 20 years [12] have been disappointing when compared to the data obtained by Owen et al. [16], because the NCI was only able to confirm the antitumor activity of sparsomycin (natural product) for P388 leukemia and Walker 256 carcinosarcoma [12]. However, even though lacking antitumor activity of its own, synthetic sparsomycin has been found to potentiate the activity of cisplatin on CHO cells in vitro [27] and on L1210 leukemia in vivo, inducing 67% cures in optimalized schedules [25]. This synergistic effect, which has also been reported for other protein synthesis inhibitors [6, 7], may provide an important application of sparsomycin in cancer chemotherapy.

The reason for the discrepancy between the in vitro and in vivo antitumor activity of sparsomycin probably results from the toxicity of this drug, which does not allow effective drug levels to be reached in vivo. In a daily ×9 schedule the LD_{10} od sparsomycin in mice is about 40 times smaller than the single LD_{10} dose [12], suggesting that sparsomycin accumulates in vivo.

Experimental data suggest that a reasonable therapeutic index can only be achieved by persistent drug administration [12]. During treatment with sparsomycin the drug sensitive proteins disappear from cells and plasma at a rate proportional to their turnover rate [24]. Thus, proteins with a rapid turnover will disappear first, and if these proteins are critically important to the integrity of the host, they should be the best indicators of drug toxicity. The proteins that are most sensitive to inhibition biosynthesis are those with a high mRNA initiation potential, e.g., ferritin [20] and albumin [21], so that toxicological studies should be focused on liver export proteins.

To achieve the maximal antitumor effect dose limiting toxicities should be clearly defined, and the levels of critical proteins should be monitored.

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The aim of the present investigation was to analyze the pharmacokinetics of sparsomycin together with its effects on some liver derived proteins in order to determine the possible mechanisms of sparsomycin toxicity The results should provide guidelines for the development of better analogs of this drug

Materials and methods

Animals Eleven male beagle dogs (Central Animal Laboratory, University of Nijmegen) weighing between 8 8 and 14 0 kg were used The dogs were fed with standard granulated dog food (Hope Farms, The Netherlands) and had free access to tap water During the experiments, the animals were housed in wire bottomed metabolic cages that enabled urine collection

Drugs Sparsomycin was synthesized as previously described [15] and was aquired in a freeze-dried form The injections were prepared aseptically in the hospital pharmacy (St Radboud Hospital) The drug was dissolved in 0 9% NaCl and kept in dark flasks at 4° C for not longer than 24 h

Dose In a pilot study involving 2 dogs, we had previously determined that nine daily administrations of 0 1 mg/kg sparsomycin was the maximal dose tolerated by the animals A dose of 0 1 mg/kg (2 mg/m^2) daily is close to the maximal dose of 0 05 mg/kg (1 85 mg/m^2) used in humans [3] A 1 4-mg/kg dose was considered to be the maximal single dose that could be tolerated At the time of this experiment, we were not aware of relevant data published by the NCI [13] The doses used for continuous infusions were calculated from the single-dose experiments, so that steady state levels of 50 and 100 ng/ml could be attained The treatment protocols are listed in Table 1

Sparsomycin analysis Sparsomycin concentrations in plasma and urine were determined using high-performance liquid chromatography (HPLC) as previously described [23] using a semi automated HPLC system (Kratos Analytical, USA) For urine analysis, the method was slightly modified, instead of using methanol as the eluent, 10% acetonitrile was applied The detection limits were 10 ng/ml for plasma and 50–80 ng/ml for urine

Pharmacokinetic and toxicological studies The dogs were fasted for 12 h before each experiment Most of the pharmacokinetic studies were performed with the dogs under pentobarbital anesthesia (initial dose, 30 mg/kg) and subjected to intratracheal intubation During each experiment (lasting 5–7 h), the animals received intravenous fluids (0 9% NaCl and 2% mannitol) at a rate necessary to produce the required urine flow (for protocol details, see Tables 1 and 2) Blood samples were taken via a central venous catheter, immediately centrifuged, and frozen at −20° C Urine (7–10 ml) was collected through a urinary catheter At the end of each collection period, the bladder was emptied by gentle massage All urine analyses for the determination of sparsomycin levels, creatinine concentrations, and urinary pH were performed in duplicate Urine samples were kept at 20° C until analysis After the dogs had recovered from anesthesia, they were returned to metabolic cages, where urine collection and blood sampling were continued for 24 h On days 2–8 (expt 1a, dog 632), the sparsomycin concentration in plasma was determined in blood samples taken 5 min before, 5 min after, and 60 min after the drug injection On days 1 and 9, the already described single-dose procedure was repeated Another dog received a single dose of 1 4 mg/kg sparsomycin (expt 7, dog 751), and the drug concentration was determined in bile samples collected through a catheter preoperatively inserted in the choledochal duct Two dogs received continuous infusions of sparsomycin for 3 5 and 4 h 1 received 0 043 mg/kg per hour (expt 8, dog 868), and the other was given 0 025 mg/kg per hour (expt 9, dog 785)

All pharmacokinetic parameters were calculated from plasma and urine concentrations according to well-known procedures [22]

Table 1. Treatment protocol

Expt / dog no	Dose (mg/kg)	Schedule	Anesthesia on day 1	Anesthesia on day 9	Additional hydration	Follow up (days)
1/632	0 1	Q01Dx09	−	−	−	60
3/890	0 1	Q01Dx09	+	+	−	60
4/892	0 1	Q01Dx09	+	+	+	4[a]
5/063	0 1	Q01Dx09	+	−	+	60
6/008	0 1	Q01Dx09	+	−	−	4[a]
Single doses						
2/350	0 1	Q01Dx01	−			5
7/751	1 4	Q01Dx01	+			0[a]
8/868	0 043 mg/kg/h	Cont infusion (3 5 h)	+			5
9/785	0 025 mg/kg/h	Cont infusion (4 0 h)	+			5
10/160	0 1	Q01DX01	−			5
11/172	0 1	Q01DX01	−			5
12/160[b]	0 5	Q01DX01	−			5
13/172[b]	0 5	Q01DX01	−			5

[a] The follow-up was shortened due to death
[b] The experiment was repeated on the same dogs 3 months after the experiment whose results are shown here

Toxicological study Dogs 632, 890, 892, 063 and 008 were subjected to toxicological study following nine daily doses of 0.1 mg/kg sparsomycin. Dogs 892 and 063 additionally received daily hydration (0.5 l glucose saline as a hypodermic clysma) in order to minimize the effects of dehydration. Blood and urine samples were analyzed for changes in biochemical and hematological parameters. Additionally, four single-dose experiments were performed (expts 10–13, dogs 160 and 172) without anesthesia, and a pharmacokinetic study was performed to evaluate exclusively the dynamics of factor-VII changes. Factor VII levels were determined using factor VII-deficient human plasma and dog brain thromboplastine [18]. The 100% value (±SD) was derived from the results obtained for 12 healthy beagles not involved in this study. Thrombotests were performed according to the method of Owren [17]. Student's *t* test was applied to assess the significance of the differences between day-1 and day 9 values, the accepted level of significance was $P < 0.05$.

Eye toxicity In each of the dogs that received a 9-day course, fundoscopy was performed three times, i.e., on the 1st, 9th, and 16th days of the experiment. As dog 892 was killed after the experiment and dog 008 died on day 13, their retinas were subjected to histological evaluation.

Results

The pharmacokinetic results are summarized in Table 2.

Bolus injection of 0.1 mg/kg sparsomycin without anesthesia

Sparsomycin that had been administered to the 2 dogs that were not under pentobarbital anesthesia (expts. 1 and 2) was rapidly eliminated from the circulation as shown by the following values: $t\beta_{1/2}$, 0.60 and 0.68 h, AUC, 0.32 and 0.38 mg h l^{-1}, and volume of distribution (V_d), 0.26 and 0.26 l/kg. The drug was eliminated mainly by nonrenal clearance (CL_{NRSm}), with 40% of the administered dose being consistently recovered in the urine after 24 h.

Bolus injections of 0.1 mg/kg under anesthesia

The dogs that received bolus injections while under pentobarbital anesthesia (expts. 3 and 4) eliminated sparsomycin less rapidly having $t\beta_{1/2}$ values of 0.7 and 0.76 h. In these experiments, the AUCs values increased to 0.99 and 0.78 mg h l^{-1} mainly because of a slow γ-phase with $t\gamma_{1/2}$ values of 7.9 and 9.4 h. The results of expt. 3 are shown in Fig. 1.

The increase in the AUC values was mainly due to reduced CL_{NRSm}, as the renal clearance (CL_{RSm}) remained the same, i.e., about 20 ml/min. As a consequence of the

Table 2 Pharmacokinetic data for sparsomycin administered as an i.v. bolus or by continuous infusion

Experiment	Expt	Dog no	Body weight (kg)	Mean urine flow ± SD (ml/min)	$t\beta_{1/2}$ (h)	AUC (mg h l^{-1})	V_d (l/kg)	CL_{CR} (ml/min)	CL_{RSm} (ml/min)	Percentage of the dose excreted in urine	CL_{Sm} (ml/min)
Bolus injection without anesthesia (0.1 mg/kg) (day 1)	1	632	12.5	0.6±0.5	0.60	0.32	0.26	–	24.6	40.0	62.0
	2	350	13.1	0.7±0.6	0.68	0.38	0.26	–	23.8	39.5	60.2
Bolus injection under anesthesia (0.1 mg/kg) (day 1)	3	890	13.0	0.2±0.05	0.70	0.99	0.26	40.4±17	18.4	84.0	22.0
	4	892	12.5	0.2±0.05	0.76	0.78	0.57	34.3± 3	21.6	81.0	26.7
Bolus injection under anesthesia (0.1 mg/kg) high urine flow (day 1)	5	063	11.8	1.7±0.08	1.17	0.40	0.42	42.3± 7	34.4	70.0	49.2
	6	008	8.8	1.9±0.08	0.83	0.34	0.36	35.7±11	29.3	68.0	43.1
Prolonged drug admin 0.1 mg/kg Q01Dx9 (day 9)	1b[a]	632	10.7	0.2±0.5	0.76	1.87	0.26	–	3.6	38.0	9.5
	3a	890	11.9	0.2±0.04	1.17	1.74	0.27	35.9±16	10.5	100.0	10.5
	4a[b]	892	11.7	0.3±0.08	0.64	0.74	0.32	21.1± 9	21.0	80.0	26.4
Bolus injection under anesthesia (1.4 mg/kg) (day 1)	7	751	11.2	1.0±0.09	1.0	10.80	0.25	–	–	55.0	25.6
Continous infusion, under anesthesia, high urine flow											
0.043 mg/kg/h	8	868	14.0	1.3±0.46	0.60	0.72	–	47.9±9	30.3	62.0	49.0
0.025 mg/kg/h	9	785	13.5	1.0±0.32	0.54	0.35	–	45.3±7	45.0	73.0	61.7

[a] Experiment 1b was performed without anesthesia.
[b] Additional hydration on days 2–8.

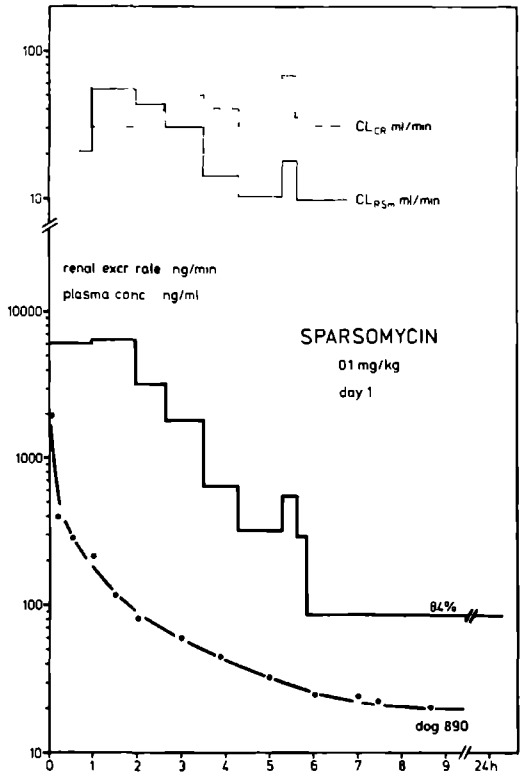

Fig 1 Experiment 3 plasma concentration time curve (●—●) and renal excretion rate time profile (———) of sparsomycin administered as a bolus i v dose (0 1 mg kg) to dog 890 under anesthesia on day 1 of the experiment The percentage of the dose recovered in the urine after 24 h is indicated on the last renal excretion rate bar Changes in the CL_{CR} ( ) and CL_{RS} (———) are shown in the *upper part* of the figure $t\beta$ 0 7 h $t_{1/2}$ 7 9 h

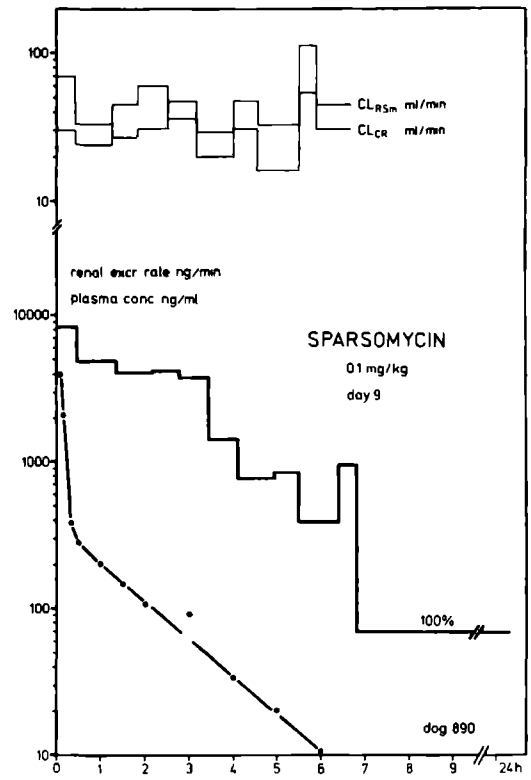

Fig 2 Experiment 3a sparsomycin administered as a bolus i v dose (0 1 mg kg) to dog 890 under anesthesia on day 9 (the last of nine daily doses) $t\beta$ 1 17 h the γ phase was absent For further details see the legend to Fig 1

decreased CL_{RSm}, most of the drug was excreted in the urine (84% and 80% of the administered dose) The volume of distribution (V_d) values in these two experiments were 0 26 and 0 57 l/kg These results were obtained with a relatively low urine flow of 0 2 ml/min

Influence of high urine flow on renal drug clearance

An increase in the urine flow to 1 7 and 1 9 ml/min in expts 5 and 6, respectively, resulted not only in increases in the CL_{RSm} to 34 4 and 29 3 ml/min, respectively, but also an increase of the CL_{RSm} to about 14 ml/min (see Fig 3) The proportions of the drug recovered in the urine were 70% and 68%, respectively The elimination values, $t\beta_{1/2}$, did not drop in these experiments but increased to 1 17 and 0 83 h, respectively An increase in the urine flow resulted in the disappearance of the γ phase and low AUC values of 0 4 and 0 34 mg h l^{-1} Despite the high urine flow, the CL_{RSm} decreased significantly during the 4th h of the experiment but thereafter increased Under vigorous hydration, the V_d values in these experiments increased to 0 42 and 0 36 l/kg, respectively (a 35% increase)

Elimination profile of sparsomycin after daily administration (× 9)

Dogs 632, 890, and 892 were given nine daily i v sparsomycin doses of 0 1 mg/kg In dog 632, a pharmacokinetic study without anesthesia was performed on days 1 (expt 1) and 9 (expt 1a), and on days 2–8, additional blood samples were taken 5 min before the injection, and 5 and 60 min after the injection

Dog 637 became ill during the treatment course Vomiting and anorexia resulted in weight loss (– 1 8 kg) and dehydration On day 9 the dog produced only 110 ml concentrated urine per day (normal level, 500–750 ml/day) From days 1 to 8, the daily measurements of plasma drug levels (expt 1a) revealed that the peak levels (5 min after injection) remained the same i e, 529 ± 53 ng/ml This was also the case for the drug levels 60 min after the injection, i e, 94 ± 16 ng/ml Residual drug levels at 5 min before the injection were detectable only on days 5 (14 ng/ml) and 7 (10 5 ng/ml) On day 9 (expt 1b), the drug elimination profile changed significantly the residual drug level increased to 75 ng/ml, there being a peak value of 605 ng/ml and a 60-min value of 210 ng/ml The β-phase with a $t\beta_{1/2}$ of 0 76 h was followed by a γ-phase with a $t\gamma_{1/2}$

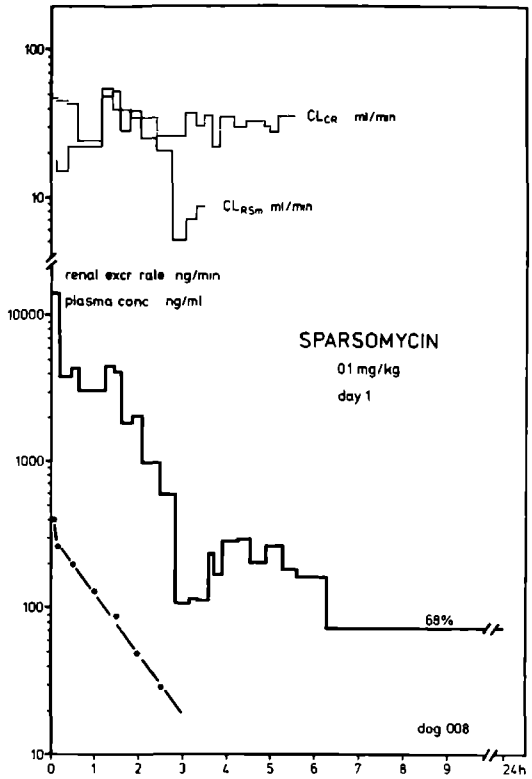

Fig. 3. Experiment 6 sparsomycin administered as a bolus i v dose (0 1 mg kg) to dog 008 under anesthesia with vigourous hydration on day 1 of the experiment tβ , 0 83 h, the γ-phase was absent For further details, see the legend to Fig 1

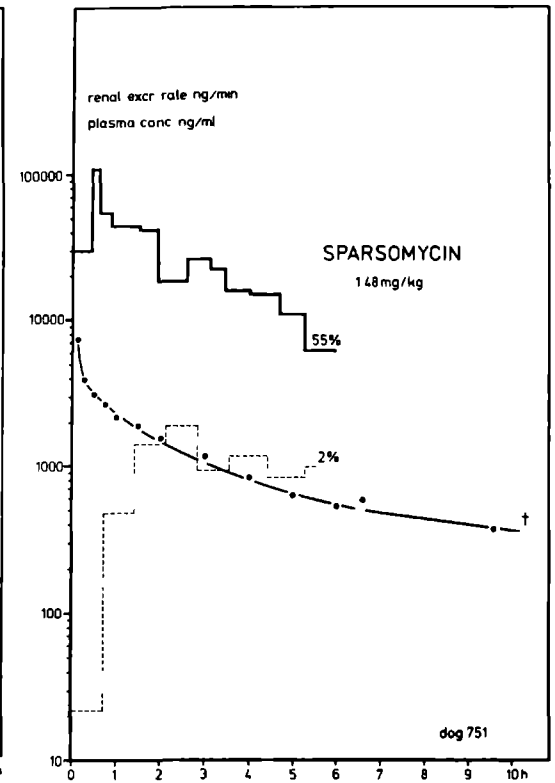

Fig. 4. Experiment 7 sparsomycin administered as a bolus i v dose (1.48 mg/kg) to dog 751 under anesthesia The percentage of the dose recovered in the urine after 6 h is indicated on the last renal excretion rate bar. Sparsomycin concentrations in the bile (— — —) and the percentage of the dose recovered in the bile are indicated tβ , 1 0 h, tγ , 6 7 h

of 20 h The AUC increased to 1 87 mg·h·l^{-1}. Dog 632 recovered promptly within 10 days of the last sparsomycin injection; it started to gain weight and had normal diuresis.

Dogs 890 and 892 (expts. 3 and 4) were subjected to a procedure similar to that used for dog 632, except that the pharmacokinetic studies on days 1 and 9 were performed under anesthesia Dog 892 received additional hydration throughout the experiment (days 2–8, 0 5 l saline-glucose as a daily hypodermoclysis). The elimination profile of sparsomycin in expts. 3 and 4 on day 1 have been described above. On day 9 (expts. 3a and 4a), the drug-elimination profiles were different. Dog 890, which did not receive additional hydration, showed a markedly increased $t\beta_{1/2}$ value of 1.17 and an AUC value of 1.74 mg h·l ^{1} 9 (expt. 3a; Fig 2). Apparently, the slow γ-phase had disappeared, and 100% of the administered dose was recovered in the urine The total body clearance (CL_{sm}) decreased to 10.5 ml/min and was equal to the CL_{RSm}. Dog 892, which was hydrated on days 2–8, showed an unchanged drug-elimination profile in expt 4a, i e , a $t\beta_{1\ 2}$ value of 0 64 h, an AUC of 0.74 mg·h·l^{-1}, a persisting γ-phase. The CL_{sm} remained as high as on day 1, and 80% of the initial dose was recovered in the urine.

The pentobarbital sleeping times after an initial dose of 30 mg/kg pentobarbital were recorded for both dogs during the first and second anesthesia On day 1, dogs 890 and 892 woke up after 1.1 and 1.4 h, respectively, and an additional pentobarbital dose was necessary to keep them asleep. On day 9, the times were 23 and 30 h, respectively, for the same pentobarbital dose. When dog 892 woke up on day 10, it was still stick and hypothermic, and it remained so until day 13, when it was killed for humanitarian reasons. Death was not directly due to sparsomycin toxicity, but rather was attributable to an unexpected delay in pentobarbital metabolism.

High sparsomycin dose: 1.48 mg/kg as an i.v. bolus

In expt. 7, sparsomycin at a dose of 1.48 mg/kg was injected under anesthesia as an i.v bolus (Fig 4) The $t\beta_{1\ 2}$ was 1 0 h, and the AUC was 10 8 mg·h·l^{-1}. The dog died after 10 h, and the amount of the drug recovered in the urine up to the time of death was 55% of the administered dose. Simultaneously measured sparsomycin concentrations in the bile revealed that only 2% of the administered dose had

been eliminated in an unchanged form by this route Autopsy failed to reveal the cause of death

Continuous infusion of sparsomycin

Sparsomycin given as a continuous i v infusion (0 043 mg kg per hour, expt 8) resulted in steady-state plasma concentrations during the first 100 min of the experiment At the same time the Cl_{RSm} increased from 20 0 to 45 0 ml/min, thereby approximating the creatine clearance, CL_{CR} (mean, 47 9 ± 9 0 ml min) The CL_{RSm} remained at a constant level of 40–45 ml min for only 60 min and then started to decline, this resulted in increased sparsomycin levels in the plasma After the discontinuation of sparsomycin infusion, the drug was eliminated with a $t\beta_{1/2}$ of 0 60 h When the sparsomycin plasma concentration dropped to about 60 ng/ml, the CL_{RSm} increased once again to 50 ml/min but had decreased by the end of experiment The CL_{Sm} observed during this experiment was 49 0 ml/min, while 62% of the sparsomycin dose was recovered in the urine

The urine pH dropped from 7 7 at the beginning of the experiment to 6 5 at the end of drug infusion, and had returned to a value of 8 0 at the end of experiment The urine flow was constant during the drug infusion, but increased from 1 0 to 2 5 ml/min immediately after discontinuation of the infusion

The same experiment was repeated using one half of the previous dose (0 025 mg/kg per hour, expt 9), the results are shown in Fig 5 After an initial rise in the CL_{RSm} to 149 ml/min, the clearance deteriorated to values lower than the mean Cl_{CR} (45 3 ± 7 0 ml/min) After an initial steady state, the plasma levels of sparsomycin began to increase during the 2nd h of the experiment Again, after discontinuing drug infusion, sparsomycin was eliminated with a $t\beta_{1/2}$ of 0 54 h The CL_{RSm} was restored to a value of 50 ml/min when the plasma levels dropped below 60 ng/ml The CL_{Sm} was 61 7 ml/min, and 73% of the sparsomycin dose was recovered in urine after 24 h To control variations in the urine flow, a volumetric infusion pump with a constant fluid-infusion rate of 3 0 ml/min was used After an initial equilibration time of 30 min, the urine flow remained constant at 1 1 ± 0 26 ml/min until sparsomycin infusion was terminated, at which time the urine flow increased to 2 3 ml/min The changes in urine pH were similar to those observed in expt 8

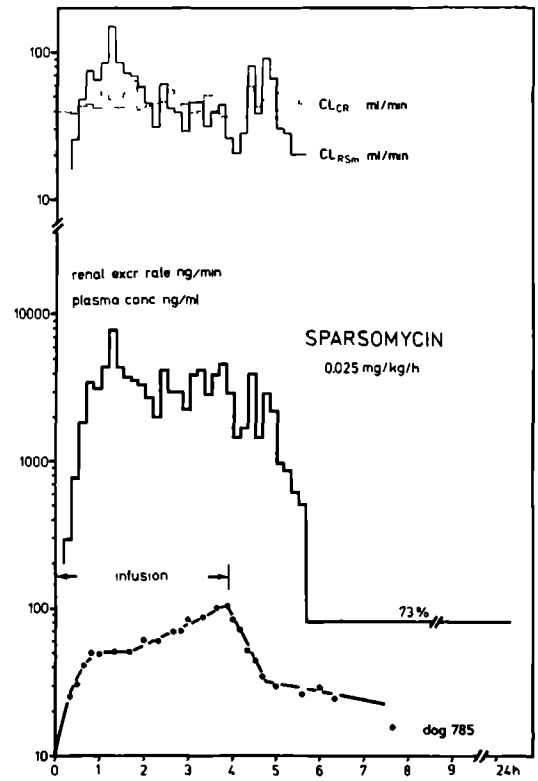

Fig 5 Experiment 9 sparsomycin administered as a continuous infusion (0 025 mg/kg per hour) to dog 785 under anesthesia with vigourous hydration $t\beta$ 0 54 h $t\gamma$, about 8 h For further details, see the legend to Fig 1

Toxicology

As a result of daily treatment with 0 1 mg/kg sparsomycin for 9 days, the 5 animals (dogs 632, 890, 892, 063, and 008) lost body weight (mean loss, −1 8 kg, not significant see Table 3) and became anorexic One dog (008) died because of dehydration, uremia, and hemorrhagic diathesis on day 13, while another (892) was killed on day 13 because it did not recover from the pentobarbital anesthesia given on day 9 The dogs drank water normally but vomited immediately after This phenomenon was most apparent about 1 h after drug injection and resulted in significant hemo concentration and a decrease in urine production (dogs 632, 890, and 008) The dogs that were additionally hydrated (063 and 892) exhibited less hemoconcentration and normal diuresis (about 500 ml/day) Decreased renal creatinine clearance and weight loss were seen in all 5 dogs During therapy, the urinary sodium excretion was significantly reduced to values of < 10 mFq/l per day, while the urine osmolality remained relatively high In the 2 dogs that received additional fluid and sodium, the urinary sodium excretion level increased to > 20 mEq/l per day Despite the considerable decrease in the creatinine clearance (P = 0 02), the plasma creatinine values remained unchanged, and plasma urea levels decreased in all but 1 dog (P = 0 09) Dog 008 developed overt renal insufficiency and died on day 13 In addition to generalized hemorrhagic diathesis, a regenerating proximal tubular necrosis was found at autopsy

Small amounts of glucose were found in one urine sample (dog 632) after 9 days of treatment The loss of urine protein never exceeded 2 g/day (61 collections)

In all dogs, plasma electrolytes remained at normal levels during and after sparsomycin therapy However, the plasma Ca^{2+} level dropped significantly (P = 0 02), probably because of the decrease in plasma albumin

Total plasma protein (TP) decreased from 60 h to 55 g/l but this decrease was masked by hemoconcentration The plasma albumin level decreased from 29 7 ± 1 8 to 21 2 ± 2 7 g/l in 8 days (P = 0 003, Table 3) The mean disappearance half-life time ($t_{1/2}$) was 20 5 days After the

discontinuation of sparsomycin, plasma albumin returned to the pretreatment values within 10 days. The levels of plasma globulins (TP-albumin) did not change during the experiments.

The plasma fibrinogen level decreased dramatically from 1276±98 to 291±265 (P = 0.003). The disappearance $t_{1/2}$ was 3.5 days The 2 dogs with the lowest plasma fibrinogen level on day 9 (dog 892, 160 ml/l; dog 008, < 100 mg/l) showed a considerable tendency towards bleeding in the last 2 days of sparsomycin treatment. In these 2 dogs, gingival bleeding and bloody diarrhea were observed. Both dogs died or were killed on day 13. Prior to death, both dogs had supranormal plasma fibrinogen levels.

The level of plasma factor VII decreased rapidly after the first sparsomycin dose. The mean disappearance $t_{1/2}$ was 5.6±1 5 h (see Fig. 6). The effect of the five-times-higher sparsomycin dose was only slightly stronger, the $t_{1/2}$ being 4.2 h Six to ten hours after drug injection, a plateau of 31±8% of the normal value was observed. At 24 h following a single sparsomycin dose of 0.1 mg/kg, the level of plasma factor VII started to recover (see Fig. 7). The recovery was slower than the disappearance and only reached the 100% level 50 h after the injection. Later on, a small overshoot of plasma factor-VII levels was observed. The recovery after a 0.5-mg/kg dose was delayed for an

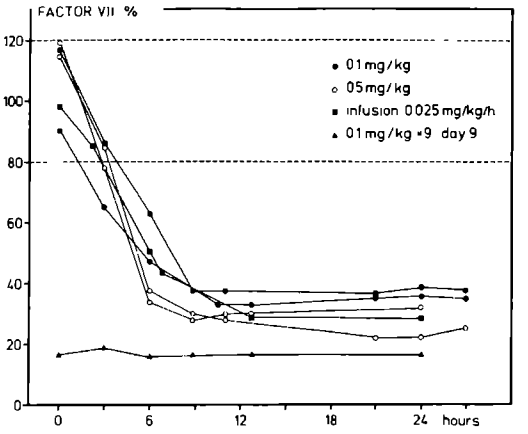

Fig. 6. Changes in plasma factor-VII levels after sparsomycin administration, the sparsomycin doses and schedules are indicated. The SD from the 100% level (mean for 12 normal dogs) is indicated by *dashed lines*

Table 3. Changes in toxicological parameters measured before treatment (day 1) and on day 9 of treatment with sparsomycin (0.1 mg/kg per day)[a]

Parameter	Units	Day 1	Day 9	P	Comments
Body weight	kg	11 8 ± 1 7	10 0 ± 1 8	ns	Recovery within 10 days
Hemoglobin	mmol/l	9 6 ± 1.1	10 9 ± 0 1	ns	Recovery within 2–3 days
Hematocrit	l/l	0 46± 0 04	0 51± 0 05	ns	
WBC	×10⁹/l	10 2 ± 1 7	16 9 ± 12	ns	Two dogs died;
PMN leukocytes	×10⁹/l	6 8 ± 1 0	12.7 ± 10	ns	during the experiment, their
Lymphocytes	×10⁹/l	2 7 ± 0 9	2 0 ± 1 0	ns	WBC counts rese to reach
Thrombocytes	×10⁹/l	159 0 ± 59	110 0 ± 29	ns	66.1 and 49 2 just prior to death
Reticulocytes	%0	9 0 ± 5	0 4 ± 0 8	0 02	Recovery within 3–5 days
Total protein	g/l	60 4 ± 6.9	55 0 ± 11	ns	Recovery within 10 days
Albumin	g/l	29 7 ± 1.8	21 2 ± 3	0 003	
Fibrinogen	mg/l	1276 0 ± 98	291 0 ±265	0 003	Recovery within 2 days
Thrombotest	%	> 100	9 ± 4	0 003	Recovery within 3 days
SGOT	IU/l	31 0 ± 9.7	55.8 ± 28	ns	All liver enzymes returned
SGPT	IU/l	52 4 ± 13 5	65.4 ± 24	ns	to normal levels within 10 days
LDH	IU/l	352 6 ±181	289 0 ±151	ns	One dog had overt hepatitis
Alkaline phosphatase	IU/l	38 6 ± 10 7	64 6 ± 24	ns	
γ-GTP	IU/l	8 0 ± 4 2	17 6 ± 9 4	ns	
Na^+	mmol/l	147 0 ± 2 6	143 0 ± 8	ns	
K^+	mmol/l	3.3 ± 0 4	3 3 ± 0 7	ns	
Ca^{2+}	mmol/l	2 4 ± 0 2	2 2 ± 0 1	0 02	Recovery within 10 days
Glucose	mmol/l	5.2 ± 0.6	4.4 ± 1.1	ns	
Urea	mmol/l	6.5 ± 1 9	3.1 ± 0 7	ns	Recovery within 2–4 days
Creatinine	µmol/l	66 6 ± 18	65 0 ± 10 3	ns	
Creatinine clearance	ml/min	34 4 ± 7	17 4 ± 9 2	0 03	Recovery within 10 days One dog did not recover until 21 days after sparsomycin administration One dog died because of uremia

ns, not significant

WBC, leukocytes, PMN leukocytes, granulocytes (PMN-polymorphonuclear leukocytes)

[a] Mean ± SD for 5 dogs

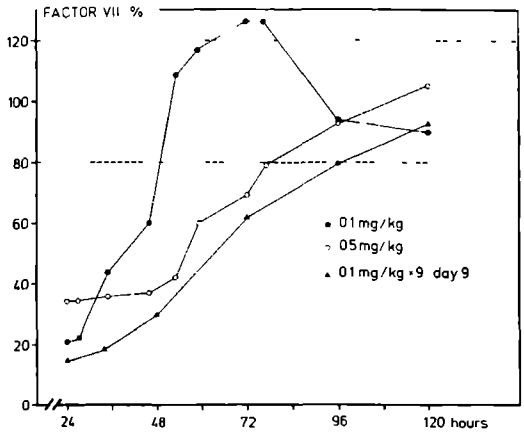

Fig. 7. Recovery of plasma factor VII levels after the final sparsomycin administration

additional 24 h and was slower than in the case of the lower dose the 100% value was not reached until 108 h after drug injection When sparsomycin was administered for 9 days (dog 063), plasma factor-VII levels remained at a plateau for 9 days After the discontinuation of sparsomycin therapy, the plasma factor-VII recovery approximately paralleled that observed after a single 0 5-mg/kg dose

Dog 785 appeared to be deficient in factor VII, having 'plateau' levels of this protein even before drug administration The administration of sparsomycin did not change this plateau level

The thrombotest findings showed control levels (> 100%) for 2 days and later started to decline rapidly On day 9, a mean value of 9%±4% had been reached (P = 0 002) The 2 dogs with hemorrhagic diathesis had values of 5% and 6%

The liver enzymes serum glutamine-oxolate transferase (SGOT), serum glutamine-pyruvate transferase (SGPT), alkaline phosphatase, and γ-glutamyl transpeptidase (γ-GTP) showed elevated levels during and after treatment with sparsomycin There was no apparent difference between anesthesized and nonanesthesized dogs However, the significance of these changes was difficult to evaluate because of considerable scatter One of the dogs (063) developed overt toxic hepatitis after the discontinuation of treatment The derangements in liver enzyme levels in this dog were maximal on day 15, when SGOT increased to 280 IU/l, SGPT to 1,250 IU/l, and γ GTP to 60 IU/l Lactate dehydrogenase (LDH) and alkaline phosphatase were not excessively elevated The derangements disappeared spontaneously, and all liver enzymes in all dogs were at normal levels on day 21 (12 days after last sparsomycin injection)

Eye toxicity

There were no changes in retinal appearance when eyes were checked by performing routine fundoscopy At autopsy (dogs 892 and 008), no morphological changes in the retina were seen

Discussion

In our experiments, we treated dogs with a sparsomycin dose similar to the maximal dose used in phase-I trials in humans [3]

Dogs under anesthesia had much lower CL_{NRSm} values than dogs without anesthesia This might have been due to the specific (enzyme saturation) or nonspecific (decrease in liver blood flow) effects of pentobarbital The increase in CL_{NRSm} values in expts 5 and 6 when vigorous hydration was performed, the expansion of the V_d, and the relative decrease in the amount of the drug recovered in urine all point to the second possibility

In dogs with a low urine flow, a slow γ phase $t\gamma_{1/2}$, about 8 0 h) was seen The CL_{RSm} was very low at this time, suggesting that all of the filtered sparsomycin was (actively?) reabsorbed by the renal tubuli This phenomenon might be an important factor in producing increased drug toxicity However, the γ-phase was not seen in the 2 nonanesthesized dogs An increase in the urinary flow (expts 5 and 6) may result in a decrease in the tubular resorption of the drug and, therefore, a disappearance of the γ-phase On the other hand, dehydration and low urine flows probably result in an increase in urine sparsomycin concentration along with a decrease in the level of the enzyme responsible for drug reabsorption In such a case, all of the filtered sparsomycin is excreted in urine (expt 3a) Additional hydration and normal diuresis would prevent this effect (expt 4a)

Prolongation of the pentobarbital sleeping time indicates that the activity of cytochrome P450 is low Thus sparsomycin might influence the liver metabolism of other drugs

Drug accumulation did not exclusively occur in anesthesized dogs In dog 632 (expt 1a), the blood sparsomycin levels were not elevated until the last day of treatment This observation suggests that, in spite of decreased sparsomycin renal excretion, the liver metabolism or binding capacity was still active or undersaturated until the 9th day of treatment From the rapid deterioration of the general condition of this dog, it was clear that the observed plasma drug accumulation would have resulted in lethal toxicity if the experiment had continued

Continuous sparsomycin infusion provided other interesting data, particularly that the CL_{RSm} could be increased far above the mean CL_{CR} This shows that sparsomycin is also actively excreted by renal tubuli Active excretion can be 'auto-inhibited' by sparsomycin, so that the drug clearance drops This results in acute drug accumulation in the plasma This phenomenon cannot be compensated for by vigorous hydration and increased urine flow Note that a similar phenomenon was also observed for i v bolus injections in expts 5 and 6 (Fig 3) the CL_{RSm} dropped at the end of experiment but climbed again when the sparsomycin plasma levels were no longer measurable It is difficult to propose a reasonable explanation for this phenomenon, as up to now, only a few protein-synthesis inhibitors have been studied clinically However, on the basis of our observations, it can be speculated that sparsomycin either inhibits the synthesis of a short-lived transport enzyme in the renal tubuli or that it saturates the capacity of such an enzyme Another possibility is that pentobarbital interacts with sparsomycin at the level of the renal tubuli

From the results of our continuous infusion studies it can be anticipated that the use of a higher sparsomycin dose would result in nonlinear kinetics with delayed elimination This did occur, and proved fatal in expt 7 (see Fig 4)

The increase in the urine flow and $CL_{R_{SM}}$ after the discontinuation of drug infusion suggests the existence of an unknown vasodepressive activity produced by sparsomycin

In the view of dose scheduling in the phase-I study performed in 1964 [3], it might be argued that the eye toxicity observed in these trials might also have been due to drug accumulation This supposition is based on the following considerations The human blood retina barrier is usually impermeable to water-soluble drugs [4] Moreover, extensive studies on healthy animals have also failed to confirm the retinal toxicity seen in humans [14] Our present findings of an absence of eye toxicity are in agreement with those of previous studies The permeability of the blood-retinal barrier, however, can increase in different diseases [4], and it is also conceivable that it could be changed under different debilitating conditions, e g , after eye irradiation and/or cancer cachexia Both patients who suffered from retinal toxicity had a low initial body weight (50 kg) Thus, it is possible that increased drug plasma levels in combination with increased blood-retinal barrier permeability could be the reasons for the eye toxicity seen in humans

Our study of beagle dogs was extended in order to answer some questions concerning the toxicology of sparsomycin These studies revealed the influence of sparsomycin on liver function Several of the proteins studied showed a decrease in their levels that was proportional to their normal turnover time

Plasma factor VII, which is known to have a short biological half-life of 4–6 h in humans, showed rapid changes after sparsomycin administration to dogs After an initial decay to a 30% level, a plateau was observed in all cases This plateau, however, was an artefact of the method applied, as human plasma free of factor VII was used This phenomenon has been described and quantified by Poller et al [18] in factor VII-deficient dogs Our results, therefore, indicate the total disappearance of factor VII from the plasma of dogs treated with sparsomycin Using our method, however, we were able to determine the length of time between the inhibition and restoration of plasma factor-VII synthesis, this being one aim of the study As in untreated factor VII-deficient dogs [18], the absence of this coagulation factor did not increase the risk of spontaneous hemorrhage

The disappearance of factor VII from plasma was not related to the sparsomycin dose, as 0 5 mg/kg had nearly the same effect on this protein as 0 1 mg/kg dose and continuous infusion This suggests that, over a broad dosage range, the toxicity of sparsomycin may be dependent not on the dosage but on the lenght of the therapy and the disappearance of critical proteins (horizontal' limit) High sparsomycin concentrations may inhibit a wider range of cellular biosynthesis and a wider range of potentially critical proteins (respiratory enzymes') and thereby cause death ('vertical' limit)

Factor VII assays, although useless for predicting the risk of hemorrhage, might provide sensitive dynamic parameters for showing the persistence of protein-synthesis inhibition in liver cells Theoretically, in our experiments, each daily dose was administered just before the recovery of factor VII plasma levels The drug doses should have been more spread out during the second part of experiment, as sparsomycin appeared to accumulate in plasma It is also of interest to note that the recovery of factor VII levels was several times slower than its disappearance, which suggests either low synthetic priority or increased consumption of factor VII

The decrease in plasma fibrinogen levels was well correlated with drug toxicity, and the duration of therapy was clearly limited by this effect The disappearance $t_{1/2}$ value of 3 5 days was very similar to that of human fibrinogen, i e , 3–4 days [9] After the discontinuation of therapy, fibrinogen synthesis recovered promptly, but the exact time of recovery was not estimated in our study Plasma fibrinogen reached supranormal levels 2 days after the last sparsomycin dose This, together with a tremendous increase in the number of young leukocytes, suggests an acute phase reaction, probably as a response to local damage to liver cells The rapid recovery of plasma fibrinogen levels suggests a high priority for its synthesis, which may explain the suppression of albumin [19] and factor-VII synthesis

On day 9, the mean plasma albumin levels had decreased by 8 5 g/l The decrease was slower than that observed for factor VII and/or fibrinogen, and probably did not contribute to the toxic effect of sparsomycin The disappearance t was only a little lower than the supposed biological t of albumin in humans [8]

The liver enzymes SGOT, SGPT, LDH, alkaline phosphatase, and γ GTP exhibited moderately elevated levels during and after the treatment However, it is difficult to differentiate the effect of sparsomycin from the effect of combined pentobarbital/sparsomycin toxicity A spontaneous return to normal levels occurred within 10 days

The decrease in creatinine clearance observed in all dogs might have been due primarily to prerenal factors Dogs treated with sparsomycin vomited frequently and were unable to retain water after drinking Additional subcutaneous hydration was insufficient to restore normal creatinine clearance levels

During treatment with sparsomycin, no bone-marrow toxicity was observed This is in accordance with the findings of earlier toxicological studies [14] and recent in vitro studies [5] During the treatment course, we found decreased numbers of reticulocytes ($P = 0\,01$)

In conclusion, our observations so far allow the following speculations about the pharmacokinetics and toxicity of sparsomycin This drug is mainly eliminated by metabolic clearance (60%), and this may be inhibited or saturated after prolonged drug administration This results in drug accumulation in plasma Slow release of the drug from its binding sites and the enterohepatic circulation of sparsomycin in combination with renal-tubular resorption mechanisms may produce chronic, low plasma sparsomycin levels (below detection limits) and protracted toxicity Acute 'auto inhibition' of tubular excretion results in a decrease in renal drug clearance and, in the case of continuous infusion, increased drug plasma levels The significance of this mechanism for drug toxicity is unknown A decrease in the activity of liver microsomal enzymes (e g , cytochrome P450) after prolonged sparsomycin administration might change the metabolism of other drugs ad-

ministered concomitantly with sparsomycin and thereaby potentiate their toxicity

The dose limiting toxicity for sparsomycin is liver toxicity and hemorrhage The monitoring of plasma fibrinogen levels and thrombotest data should provide valuable indications of sparsomycin toxicity Changes in factor-VII levels might be a useful dynamic parameter for showing the effect of sparsomycin on the host liver The renal toxicity of sparsomycin should not be considered as dose limiting until prerenal factors have been definitively excluded

In future studies a prolonged daily schedule of sparsomycin or its continuous infusion should be used with caution To avoid drug accumulation without losing antitumor activity, the dose schedule should be spread out, and vigorous hydration should always be employed Another possibility would be the introduction of more lipophilic sparsomycin analogs that are more easily eliminated by the liver, and not by the kidneys Such analogs have recently been developed by our group [1, 10], and preliminary studies have indicated that they have favorable in vitro [1] and in vivo antitumor activity [26]

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CHAPTER 10

PRECLINICAL PHARMACOKINETICS OF THE ANTITUMOR ANTIBIOTIC DESHYDROXY-SPARSOMYCIN IN BEAGLE DOGS

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SUMMARY

Sparsomycin (Sm) is a well known inhibitor of protein synthesis with anticancer potential. In order to minimize toxicity of this drug and increase its activity, several analogues were synthesized. Deshydroxy-Sm (dSm) appeared to be a good candidate for further investigations because of its lower toxicity and significantly higher antitumor activity in several ascitic tumors in mice. Pharmacokinetic evaluation in beagle dogs was performed with and without anaesthesia using either single i.v. bolus or continuous infusion administrations. Anaesthesia did not markedly influence the pharmacokinetics. Pharmacokinetic profile of dSm was similar to that of Sm. The drug was eliminated with a terminal $t_{1/2}\ \beta$ of 0.8 $\pm$ 0.08 hours (48 $\pm$ 5 minutes). The mean volume of distribution was 0.4 $\pm$ 0.06 $l.kg^{-1}$. The mean total body clearance was 6.3 $\pm$ 0.8 $ml.min^{-1}.kg^{-1}$. The drug is eliminated mainly by the kidneys (54%). Active tubular secretion and active tubular reabsorption of the drug were observed. The pharmacokinetics was linear. The lethal dose was 0.73 $mg.kg^{-1}$, administered in 3.2 hours continuous infusion. The results of this study provided additional data useful in selection of potentially useful analogues for further preclinical studies.

INTRODUCTION

Sparsomycin (Sm) is a well known inhibitor of ribosomal protein synthesis (1) with a recognized anticancer potential (2,3). The use of this drug was limited until 1981 because of the poor availability from the natural sources, high costs and toxicity.

Since 1981 Sm is available through the total synthesis (4) and several analogues have been synthesized (5,6,7). These analogues were subject of extensive structure-activity relationship studies on in vitro models (5,6,7 and 8). A novel route of synthesis was introduced in 1987 from L-alanine instead of D-cysteine (6). As a result of this a novel Sm-compound : deshydroxy-Sm (dSm) has been synthesized without the hydroxyl group at R1 position (Fig. 1). Preclinical evaluation of dSm revealed unique properties of this compound (9). dSm appeared more lipophilic than Sm because of the exposure of an additional carbon atom normally "masked" by the hydroxyl group (9). The increase in lipophilicity was also accompanied by a fivefold increase of water solubility, probably because of increased aggregation of the molecules. The LD50 values for dSm in mice were significantly higher than that of Sm (23.2 and 11.3 $mg.kg^{-1}$, respectively)(10). dSm appeared to

be active on ascitic P388 and L1210 leukemias (T/C = 153% and 206%, respectively), and RC renal cell carcinoma (T/C = 277%) when administered i.p. daily x 9. There was only slight activity on B16 melanoma (T/C = 131%) and no activity on C38 colon carcinoma. In the same tumor models the parent drug was only borderline active on L1210 leukemia (T/C = 125%) (10,11). Because of promising activity, dSm appeared to be one of the candidates for further preclinical development.

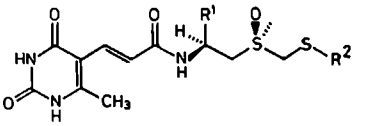

Fig.1. Structure of Sparsomycin (R1 - CH_2OH , R2 - CH_3) and deshydroxy-Sm (R1 - CH_3 , R2 - CH_3).

In a previous report on pharmacokinetics of Sm in beagle dogs we described unusual behaviour of Sm plasma concentrations during continuous infusions (12). However, these data could not be interpreted because of the pentobarbital anaesthesia that influenced drug distribution and excretion (12). In the present study, Ethrane/N_2O anaesthesia was used and it was compared to experiments without anaesthesia. The results of this study help to understand the mechanisms determining the pharmacokinetic behaviour of Sm and dSm.

MATERIALS AND METHODS

Animals

Seven beagle dogs; four males (8.6 - 14.6 kg) and three females (8.2 - 10.4 kg) were obtained from the Central Animal Laboratory of the University of Nijmegen, The Netherlands. The dogs were fed with a standard granulated food (Hope Farms BV, Woerden, The Netherlands) and had free access to tap water. After experiments, the animals were housed in the metabolic cages enabling urine collection for at least 24 hours.

Drugs

dSm was synthesized as described earlier (6) and was obtained in a freeze-dried form. The injections were prepared aseptically. The drug was dissolved in 0.9% NaCl and was kept in dark flasks at 4°C for up to 24 hours.

Pharmacokinetic studies

The dogs were deprived of food for the last 12 hours before each experiment. Exp. 3 - 6 and 8 were performed under Ethrane (Abbot, Campoverde, Italy) and N_2O anaesthesia with thiopenthal (Penthotal, Abbot, Campoverde, Italy) induction (15 mg/kg). Three studies (exp. 1, 2 and 7) were performed without anaesthesia and without additional hydration. During each experiment under anaesthesia that lasted for 5-7 hours the animals received intravenous fluids (0.9% NaCl and 2% mannitol) at the rate of 2.7 $ml.min^{-1}$. The infusion rate was controlled by an infusion pump (IVAC, type 531, San Diego, CA, USA). Continuous infusions of dSm were administered by a 60 ml syringe driver (Infors HT type 5003, Basel, Switzerland) delivering 10 ml per hour. The accuracy of the infusion rate was controlled every hour. The remainder of the solution was regularly checked for definitive drug concentration (see analysis). During experiments, rectal temperature and heart rate (ECG) were continuously monitored. Blood samples were drawn through a central venous catheter. Samples were centrifuged within 1 hour after collection. Plasma was separated and stored at -20°C pending analysis.

During the whole period under anaesthesia urine was collected through a urinary catheter every 10 min. At the end of each collection period the bladder was flushed with 5 ml of saline. Urine samples were made in duplicate for the determination of drug and creatinine concentrations and pH. Urine samples were kept out of light at -20°C pending analysis. After the dogs woke up from anaesthesia, they were returned to a metabolic cage where urine collection and blood sampling were continued for a total of 24 hours.

Drug analysis

dSm concentrations in plasma and urine were determined using a high performance liquid chromatography (HPLC) method previously described (13). The equipment used was a semi-automated HPLC system from Kratos Analytical, Ramsey, NJ, USA. The detection limits for dSm were 10 $ng.ml^{-1}$ in plasma and 50 $ng.ml^{-1}$ in urine.

Fitting of the curves

The curves were fitted using semi-log regression on the curve sections derived graphically. The area under the fitted concentrations versus time curve (AUC) was calculated using the trapezoidal rule. All other pharmacokinetic parameters were calculated according to the following formulae.

$$CL_{dSm} = D \cdot AUC^{-1} \quad (ml.min^{-1}.kg^{-1})$$

$$V_d = CL_{dSm} \cdot t_{1/2} \cdot 0.693^{-1} \quad (l.kg^{-1})$$

$$CL_{RdSm} = CL_{dSm} \cdot \% \text{ of the dose recovered in urine } (ml.min^{-1}.kg^{-1})$$

$$CL_{NRdSm} = CL_{dSm} - CL_{RdSm} \quad (ml.min^{-1}.kg^{-1})$$

where:

$t_{1/2}$ - elimination half-life in the beta phase (hours).

D - dose ($mg.kg^{-1}$)

Vd - volume of distribution in the beta phase ($l.kg^{-1}$).

CL_{dSm} - total body clearance of dSm.

CL_{RdSm} - renal clearance of dSm.

CL_{NRdSm} - non-renal clearance of dSm.

RESULTS

dSm was administered to the dogs either as an i.v. bolus (exp. 1-4) or continuous infusion (exp. 5-8). Plasma concentration versus time curves showed one distribution and one elimination half-life. The $t_{1/2}\alpha$ was approximately 0.03 hours (2 min) but this could not be determined accurately. The $t_{1/2}\beta$ was 0.8 $\pm$ 0.08 hours (48 $\pm$ 5 min, mean $\pm$ SD). The mean Vd in the beta phase was 0.44 $\pm$ 0.06 $l.kg^{-1}$. The CL_{dSm} was dose independent and was 6.3 $\pm$ 0.8 $ml.min^{-1}.kg^{-1}$. The CL_{RdSm} was also dose independent and was 54 $\pm$ 6% of the CL_{dSm}. The CL_{RdSm} was the same as the CL_{CR} value (3.4 $ml.min^{-1}.kg^{-1}$). The relation between the dose and AUC was linear (r=0.99) and the slope was 2.2.

The highest dose tested, 0.73 $mg.kg^{-1}$ administered as a 3.2 hours infusion, appeared lethal. Dog 172 died during the first night after the experiment. This could be the reason of approximately 10-20 % underestimation of the CL_{RdSm} as urine could not be collected for the full 24 hours.

A typical i.v. bolus experiment (exp. 4) is presented in Fig. 2. During this experiment the urine flow decreased from 1.6 $ml.min^{-1}$ at the beginning of the experiment to 0.14 $ml.min^{-1}$ 30 minutes later. Urine flow recovered during the 2nd and 3rd hour of the experiment. Plasma pharmacokinetic profile of dSm during continuous infusions is presented in Fig. 3.

Analysis of plasma concentration versus renal excretion rate function in comparison to renal glomerular filtration (R_{GF}) is shown in Fig. 4. The curve showed that the renal excretion rate exceeded the R_{GF} at concentrations between 200 and 700 $ng.ml^{-1}$. At concentrations lower than 100 $ng.ml^{-1}$ renal excretion rate was lower than the R_{GF}. Similar pictures were observed in all other experiments except exp. 8. At the highest dose tested (0.73 $mg.kg^{-1}$ in 3.2 hours) renal excretion rate was lower than R_{GF} and the

curves were parallel. (Fig. 5). The changes of the renal excretion rate were not related to the variations in urine flow.

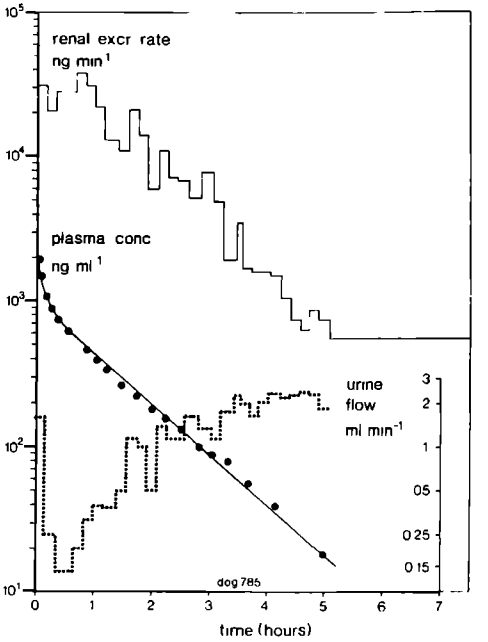

Fig. 2. Plasma (●————●) and renal pharmacokinetics(————) of dSm administered as an i.v. bolus of 0.57 $mg.kg^{-1}$ (exp.4). The $t_{1/2}\beta$ = 0.86 hour (52 minutes). Urine flow (.....) showed a characteristic depression 30 min after the beginning of experiment.

The urine pH in all experiments was usually higher than 8.0 at the beginning of the experiment, decreased to the values of 6.7 - 7.7 in the second and third hour of the experiment, and remained at this level until the end of urine sampling.

To assess the influence of anaesthesia on plasma and renal pharmacokinetics of dSm, similar experiments were done in unanaesthesized dogs (exp. 1 and 2) which did not receive additional hydration. The dogs were rather sick after drug administration and vomited frequently in the first three hours. The dogs did not urinate spontaneously until 7 - 8 hours later, when they passed 50 - 100 ml of highly concentrated urine. In exp. 2 possibly one earlier urine sample was lost and the drug recovery in urine could not be determined. The CL_{dSm} in these experiments were similar to those obtained under anaesthesia.

Table 1. Pharmacokinetic data on dSm in beagle dogs.

Exp. nr.	dog nr.	sex	body weight (kg)	dose ($mg.kg^{-1}$)	type of exp.	$t_{1/2}\beta$ (hours)	C_{ss} ($ng.ml^{-1}$)	AUC ($mg.h.l^{-1}$)	$V_d\beta$ ($l.kg^{-1}$)	CL_{dSM}	CL_{RdSm}	CL_{CR}	CL_{NRdSm}
										$ml.min^{-1}.kg^{-1}$			
1. *	16	F	9.7	0.31	iv bolus	0.6		0.8	0.4	6.7	3.9	-	2.8
2. *	634	F	10.4	0.32	iv bolus	0.7		0.8	0.4	6.9	-	-	-
3.	891	M	14.6	0.42	iv bolus	0.8		1.2	0.5	6.7	3.8	3.5	2.9
4.	785	M	14.0	0.57	iv bolus	0.9		1.4	0.5	6.9	3.9	3.5	3.0
5.	160	M	11.6	0.14	3.2 h. inf.	0.8	118	0.4	0.4	5.6	2.8	3.8	2.8
6.	15	F	8.2	0.22	4 h. inf.	0.8	148	0.6	0.4	6.0	3.3	2.3	2.7
7. *	16	F	9.2	0.23	2 h. inf.	0.8	$\pm$ 300	0.7	0.4	4.8	2.9	-	1.9
8.	172	M	8.6	0.73	3.2 h. inf.	0.9	542	1.7	0.5	7.1	3.1	3.7	4.0
Means:						0.8			0.4	6.3	3.4	3.4	2.9
$\pm$ SD						0.08			0.06	0.8	0.5	0.4	1.2

* experiments without anaesthesia.

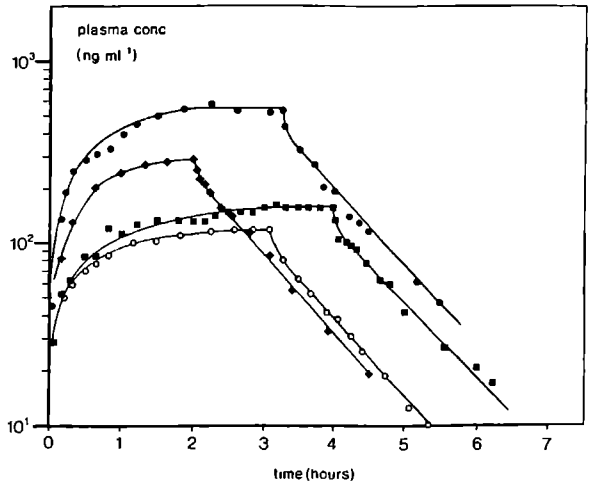

Fig. 3. Plasma pharmacokinetics of dSm administered as continuous infusions. (o) exp. 5, 0.14 $mg.kg^{-1}$ in 3.2 hours. (■) exp. 6, 0.22 $mg.kg^{-1}$ in 4 hours. (◆) exp. 7, 0.23 $mg.kg^{-1}$ in 2 hours without narcosis. (●) exp. 8, 0.73 $mg.kg^{-1}$ in 3.2 hours. In all cases, the $t_{1/2}\beta$ was 0.83 ± 0.02 hours (50 ± 1 minutes).

The CL_{RdSm} and CL_{NRdSm} values in exp. 1 were also within the range found during experiments under anaesthesia. The $t_{1/2}\beta$= 0.62 hours (37 minutes), was only slightly shorter than under anaesthesia and the Vd was smaller.

During continuous infusion experiments (exp. 5-8) the steady state (C_{ss}) was reached after 2 - 3 hours of infusion (Table 1). C_{ss} was reached in all but one experiment. In this experiment without anaesthesia (nr 7) dSm was infused for only 2 hours. Initially it was intended to infuse the drug for 3 - 4 hours, but the dog was restless and vomited frequently so it was decided to terminate the infusion after 2 hours. The C_{ss} reached during continuous infusions were in a linear relation with the dose rate (r = 0.99). After discontinuation of infusion the drug was eliminated with a mean $t_{1/2}\beta$= 0.83 ± 0.02 hours (50 ± 1 min).

In exp. 5, exceptionally, urine was collected 48 longer than usually and also blood samples were obtained regularly in this time. In the second day of experiment plasma concentrations of approximately 20 - 50 $ng.ml^{-1}$ were again detectable and formed a bell-shape curve for about 8 hours. Highest plasma concentrations were seen 32 hours after beginning of experiment. In the same time no drug in urine was detectable.

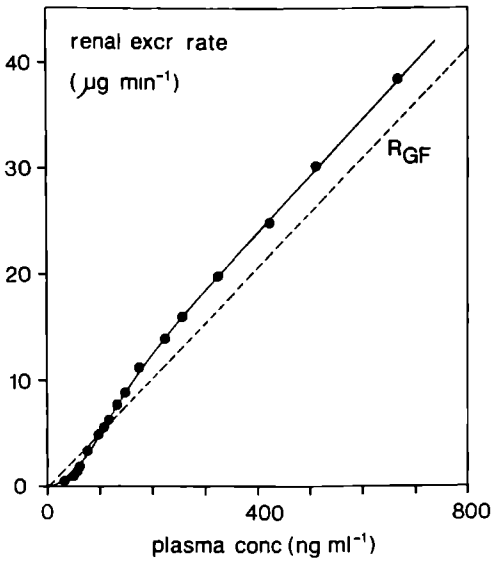

Fig. 4. Tubular titration curve in exp.4. R_{GF} was calculated from the mean CL_{CR}·1.25. The curves were constructed from the renal excr. rate values in 10 minutes urine portions and fitted plasma concentrations. At low dSm plasma concentrations active tubular reabsorption is responsible for renal excretion rates lower than R_{GF}. At higher dSm plasma concentrations active tubular excretion causes renal excretion rates higher than R_{GF}.

DISCUSSION

In pharmacokinetic evaluation of new drugs in dogs, the choice of anaesthesia plays an important role. In a previous study we have reported an unusual behaviour of plasma concentrations of Sm during continuous infusion (12). However, the data could not be interpreted definitely as the pentobarbital anaesthesia used in these studies influenced drug distribution as well as renal and total-body clearances (13). In the present study we used Ethrane/N_2O anaesthesia which proved to have much less influence on drug pharmacokinetics. The data obtained in this study during experiments under anaesthesia are very well comparable to those obtained during three experiments without anaesthesia. To obtain sufficient urine flow and constant CL_{CR}, intensive hydration with saline and 2% mannitol was necessary. Even under these conditions a clear depression of the urine flow was always observed within 20-30 minutes after administration of the drug.

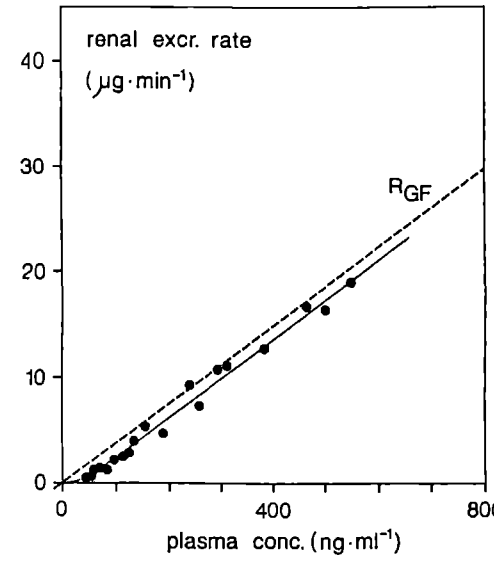

Fig. 5. Tubular titration curves in exp. 8. Renal excretion rate is constantly below the R_{GF} values.

In dogs without anaesthesia and without additional hydration, administration of dSm was accompanied by oliguria that lasted for 7 - 8 hours. These dogs additionally lost fluids by vomiting. The drug was detectable in the gastric juice in only trace amounts (unpublished observation). Depression of the urine flow did not cause the decrease of CL_{CR} and/or CL_{RdSm} except for the extreme cases when the urine flow was approximately 0.1 $ml.min^{-1}$.

Depression of the urine flow may suggest vasoactive properties of dSm. Protein synthesis inhibitors are known to inhibit de-novo synthesis of prostaglandin-synthase which has an extremely short half-life of 5-10 minutes (14). Inhibition of the key enzyme for de-novo synthesis of several kinds of prostaglandins may induce temporary disbalance and higher activity of the species with longer half-lives. When eventually both types of prostaglandines, vasoconstrictive and vasodilatative, will disappear, balance will be restored and the diuresis will reappear because of the intravascular accumulation of fluid and mannitol. Restoration of the urine flow even before termination of drug infusion is very suggestive for such a mechanism.

Analysis of the plasma concentration and renal excretion rate function was performed following the computer models elaborated by Heckman and Van Ginneken (4) and showed the combination of three effects. At first, dSm is

excreted by the R_{GF}. Secondly, the drug is actively secreted by the renal tubuli what is suggested by the renal excretion rate higher than R_{GF}. Thirdly, the drug is actively reabsorbed by the tubuli. At the highest dose tested (exp. 8, 0.73 $mg.kg^{-1}$ in 3.2 hours) tubular secretion was saturated and the renal excretion rate was less than R_{GF}, suggesting that renal tubular secretion may be easier saturated than the renal tubular reabsorption which can result in decrease of CL_{RdSm}.

In the present study, dSm was tested at the doses comparable to those of Sm studied before (12). The pharmacokinetics of dSm and Sm are essentially the same. The phenomenon of exponentially increasing Sm plasma concentrations during continuous infusion is probably related to the type of anaesthesia. At the same dose, dSm plasma concentrations did not show any abnormal behaviour. Sm as well as dSm are cleared from the plasma by an additional non-renal clearance (a.o. tissue binding?) and this clearance may be saturated by pentobarbital used in previous study, but not by other anaesthetics. This might be an explanation for the increase in plasma concentrations seen during continuous infusion of Sm. In that case, rapid decrease of CL_{RSm} suggested during these studies is only apparent and is an effect of unchanged CL_{RSm} divided by the increasing Sm plasma concentrations. The suggestion of tissue binding may be supported by the finding that unchanged dSm may be detected in plasma 32 hours after the beginning of experiment, suggesting that some of the drug may be bound to the tissues and released thereafter. dSm may also circulate in the enterohepatic loop.

In conclusion, dSm and Sm showed similar pharmacokinetics. The dose/AUC relation is linear until the lethal dose. A saturable tubular clearance is proposed. dSm may be a good candidate for further preclinical development.

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CHAPTER 11

DOSE-DEPENDENT PHARMACOKINETICS OF ETHYL-DESHYDROXY-SPARSOMYCIN IN BEAGLE DOGS

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SUMMARY

Sparsomycin (Sm) is a known inhibitor of protein synthesis. Some analogues of this drug proved to be less toxic and more active in several murine tumor models. Ethyl-deshydroxy-Sm (EdSm) was found to be one of the promising compounds of this class. The preclinical pharmacokinetics of EdSm was investigated in beagle dogs under anaesthesia. A dose range of 0.16 - 0.5 mg EdSm.kg^{-1} was investigated. EdSm was administered either as an i.v. bolus or 4 - 5 hours i.v. infusion. The $t_{1/2}\ \beta$ in the i.v. bolus experiments was 0.3 hours and $t_{1/2}\ \gamma$ varied from 2.0 to 4.2 hours. EdSm administered as an infusion produced higher AUC values than the same dose administered as an i.v. bolus. This was due to decrease of the renal clearance occuring 3 - 5 hours after the start of infusion. EdSm was actively secreted as well as actively reabsorbed by the renal tubuli. Both processes were saturable. The dose/AUC relation was non-linear. Two dogs died after the experiments (0.4 and 0.5 mg EdSm.kg^{-1}) because of inhibition of respiratory center and heart arrhytmia. The lethal doses were lower than the lethal doses seen for other analogues. The drug was considered more toxic than the other analogues due to non-linearity of its pharmacokinetics.

INTRODUCTION

Sparsomycin (Sm, NSC-059729) is a well known inhibitor of protein synthesis (1) with anticancer potential (2). The drug administered daily for 13 - 17 days appeared to be too toxic to be used in humans (3), however recently prepared analogues of this drug are very promising as they are less toxic and more active in vitro and in vivo than the parent drug (4,5,6,7). Some modifications of the chemical structure (see Fig.1) are of particular importance. Increase of drug lipophilicity by elongation of the R2 side chain appeared to increase in vitro effectivity of the drug (8). Removal of hydroxyl group from the R1 position made the compound better water soluble and significantly increased the LD50 and antitumor activity in mice (8). One compound, ethyl-deshydroxy-Sm (EdSm), appeared to be outstanding because of good water solubility, high LD50 and good in vivo antitumor activity. This compound showed activity on ascitic P388 and L1210 leukemias (T/C=150 and 172%, respectively), and on RC renal cell carcinoma (T/C=197 %). When the sensitive tumor (L1210) was inoculated s.c. EdSm was the only active Sm analogue (T/C=139%) (8). EdSm also showed significant potentiation of cis-platin activity in the same L1210 s.c. model (unpublished). Similar poten-

tiation activity was found previously in L1210 i.p. model with Sm (9). Furthermore, EdSm's dose-limiting toxicity was neurotoxicity, while lethal toxicity of Sm consisted of haemorrhage (unpublished). The last type of toxicity was seen in all hydroxylated compounds, but not in deshydroxylated Sms. Thus, EdSm appeared to be one of the promising analogues to be studied in phase I in man. Here we report the preclinical pharmacokinetics of EdSm in beagle dogs. This model has been used for the studies on Sm toxicity and pharmacokinetics previously (10).

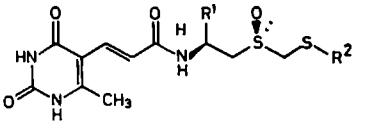

Fig. 1. Structure of sparsomycin (Sm)

Sm: R1 - CH_2OH R2 - CH_3

EdSm: R1 - CH_3 R2 - CH_2-CH_3

MATERIALS AND METHODS

Animals

Five beagle dogs, two males (weight 10 - 13.5 kg) and three females (weight 9 - 10.5 kg) were obtained from the Central Animal Laboratory of the University of Nijmegen. The dogs were used several times in the pharmacokinetic experiments with at least a 3 month rest period between tests. Before the dogs were accepted for these experiments, their general condition, body weight and kidney and liver functions were evaluated. The dogs were fed a standard granulated dog food (Hope Farms, Woerden, The Netherlands) and had free access to the tap water. The pharmacokinetic experiments were performed under anaesthesia (see below), after which the animals were housed in metabolic cages enabling urine collection for a total of 24 hours.

Drug and formulation

EdSm was synthesized following a modified method (10) and was obtained in a freeze dried form. The drug was dissolved in 0.9 % NaCl in a concentration of 1.0 $mg.ml^{-1}$. The solutions were prepared aseptically by the hospital pharmacy. The dissolved drug was stored in dark flasks at $4^{o}C$ for at most 24 hours.

Pharmacokinetic experiments

The dogs fasted for the last 12 hours before each experiment. Experiments were performed under Ethrane (Enflurane, Abbot, Campoverde, Italy) and N_2O anaesthesia with thiopenthal (Penthotal, Abbot, Campoverde, Italy) induction (15 $mg.kg^{-1}$). This anaesthesia was found superior to previously used pentobarbital anaesthesia (10) as it produced more stable circumstances and did not depress renal and non-renal clearances (13). Each experiment lasted for 5-7 hours. The animals received intravenous fluid (0.9% NaCl and 2% mannitol) at a rate of 2.7 $ml.min^{-1}$. The infusion rate was controlled by a pump (IVAC, type 531, San Diego, CA, USA). Continuous infusions of EdSm were administered using a 60 ml syringe driver (Infors HT, type 5003, Basel, Switzerland) at a rate of 10 $ml.h^{-1}$ per hour. The accuracy of infusion rate was controlled every hour. Definite drug concentration was determined in the remainder of the solution (see analysis). During these experiments, the rectal temperature and heart rate (ECG) were continuously surveyed. Blood samples (5 ml) were drawn in heparinized vacuum tubes through a central venous catheter. The samples were centrifuged within one hour of collection. Plasma was separated and stored at -20°C pending analysis.

During whole period under anaesthesia urine was collected through a urinary catheter every 10 min; except in exp. 5 where urine samples were collected every 20 minutes. At the end of each collection period, the bladder was flushed with 5 ml 0.9% NaCl. Urine samples were made in duplicate for determination of the drug and the creatinine concentrations. Urine samples were kept in the dark at -20°C pending analysis.

Drug analysis

EdSm concentrations in plasma and urine were determined using the reversed phase HPLC method as described previously (11). Plasma samples were deproteinized with 10% trichloroacetic acid (Aldrich, Brussel, Belgium). A semiautomated HPLC system from Kratos Analytical (Ramsey, NJ, USA) was used. The system consists of two spectroflow 400 pumps, Multiport Streamswitch with two six way valves (Rheodyne, Cotati, CA, USA), a 757 UV-Vis Detector and DS 610 Multirange strip chart recorder. The system was operated by means of an HPLC solvent programmer (type 9224, Kipp Analytical, Rotterdam, The Netherlands). The connection of the sampling valves for the reverse flushing method has been described elsewhere (11). The columns used were a concentration column (50 mm x 3 mm id) filled with LiChrosorb RP-8 (10 µm particle size) and an analytical column (100 mm x 4.6 mm id) filled with CP^{tm} Spher C8 (8 µm particle size); both packing materials were from Chrom-

pack BV, Middelburg, The Netherlands. Solvent A (flow 1.3 $ml.min^{-1}$) for sample concentration consisted of distilled water. Solvent B (flow 1.0 $ml.min^{-1}$), to flush the sample from the concentration column through the analytical column, consisted of 40% methanol (Fisons, Loughborough, England, HPLC grade) in distilled water. Drug concentrations were determined by the comparison of the sample peak heights with standards made from untreated control plasma spiked with known concentrations of the drug and incubated for 1 hour at 37°C. The accuracy of the calibration curves were estimated by comparing the concentration to peak height ratios for five different concentrations. The calibration curve was acceptable when the SD of the ratios was lower than 5%. The detection limits for EdSm in plasma was 10 $ng.ml^{-1}$ and in urine 50 $ng.ml^{-1}$.

Protein binding assays

The protein binding of EdSm was determined using an ultrafiltration method. Prior to these studies, no aspecific drug binding to the filters (EMIT free level TM, Syva, Palo Alto, CA, USA) was found. Heparinized plasma samples were centrifuged through the filters at 2500 g for 20 min. Drug recovery after ultrafiltration was subtracted from the total drug concentration obtained from the deproteinized sample. The amount of drug retained on the filter is expressed as the percentage of the total drug concentration.

Fitting of the curves

Curves were fitted by semi-log regression of the graphically identified elimination phases and by the usual stripping method. The AUC was calculated using the trapezoidal rule for the fitted concentrations. Other pharmacokinetic parameters were calculated from the following equations:

$$CL_{EdSm} = D \cdot AUC^{-1} \ (ml.min^{-1}.kg^{-1})$$

$$CL_{REdSm} = CL_{EdSm} \cdot \% \text{ of the dose recovered in urine } (ml.min^{-1}.kg^{-1})$$

$$CL_{NREdSm} = CL_{EdSm} - CL_{REdSm} \ (ml.min^{-1}.kg^{-1})$$

where:

D - dose $(mg.kg^{-1})$

$t_{1/2}$ - elimination half-life

CL_{EdSm} - total body clearance of EdSm

CL_{REdSm} - renal clearance of EdSm

CL_{NREdSm} - non-renal clearance of EdSm

AUC - area under the concentration versus time curve.

The tubular titration curves were constructed and interpreted according to the model elaborated by Heckman and Van Ginneken (12).

i.v. bolus experiments

EdSm administered as an i.v. bolus at the dose levels of 0.24 - 0.5 mg.kg^{-1} showed one distribution alpha phase and two elimination phases. $t_{1/2}\alpha$ was approximately 0.03 hours (2 minutes) but it was difficult to determine it accurately. The $t_{1/2}\beta$ was 0.3 - 0.6 hours (20 - 36 minutes), and $t_{1/2}\gamma$ was 2 - 4.2 hours (see Table 1 and Fig. 2 and 3). In exp. 2 no gamma phase was detectable, however the pattern of the renal excretion rate profile suggested its existence. This is the reason of the longer $t_{1/2}\beta$ = 0.6 hours (36 minutes) found in this experiment. Dog nr 891 (exp. 3) died directly after termination of anaesthesia, probably due to depression of the respiratory center. Early death might be the reason for 10 - 20 % underestimation of the mean CL_{REdSm} and similar overestimation of CL_{NREdSm}.

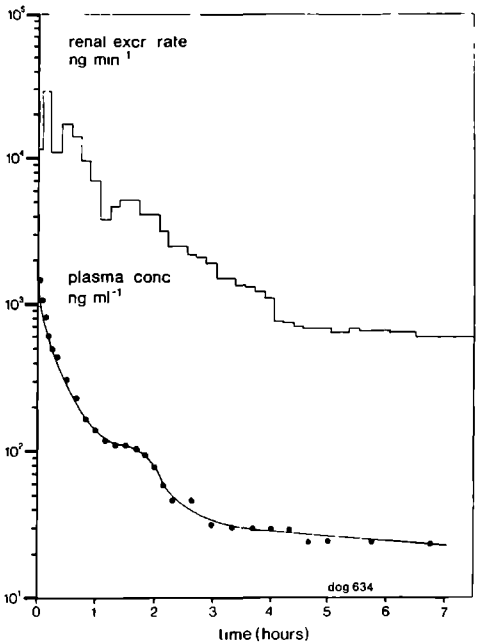

Fig. 2. Plasma and renal pharmacokinetics of ethyl-deshydroxy-Sm in exp.1 administered as an i.v. bolus, 0.24 mg.kg^{-1}. 59 % of the dose was excreted in the urine after 24 hours. The $t_{1/2}\beta$ was 0.3 hours and $t_{1/2}\gamma$ was 4.2 hours.

Table 1. Pharmacokinetic parameters of ethyl-deshydroxy-Sm in beagle dogs.

Exp. nr.	dog nr.	sex	body weight (kg)	dose ($mg.kg^{-1}$)	type of exp.	$t_{1/2\ \beta}$ (h)	$t_{1/2\ \gamma}$ (h)	AUC ($mg.h.l^{-1}$)	CL_{EdSm}	C_{REdSm}	CL_{CR}	CL_{NREdSm}
									$ml.min^{-1}.kg^{-1}$			
1.	634	F	10.5	0.24	iv bolus	0.3	4.2	0.5	7.4	4.3	4.1	3.0
2.	633	F	9.5	0.25	iv bolus	0.6	-	0.6	7.0	3.4	4.1	3.6
3.	891	M	13.5	0.5	iv bolus	0.3	2.0	1.7	5.0	2.5	1.6	2.5
4.	16	M	10.5	0.16	4 h. inf.	-	1.3	0.4	6.4	4.5	2.6	1.9
5.	633	F	9.6	0.19	4 h. inf.	-	0.8	0.5	6.6	4.5	3.8	2.1
6.	633	F	9.9	0.22	5 h. inf	-	0.6	0.6	5.9	4.1	3.4	1.8
7.	15	F	8.9	0.4	4 h. inf.	-	1.5	1.9	3.5	1.0	1.6	2.5

In exp. 1 (Fig. 2) drug was first eliminated with a $t_{1/2}\beta$ of 0.3 hours. In the second hour of the experiment EdSm plasma concentrations showed slight temporary increase of plasma levels that could not be explained by depression of the CL_{REdSm}. In this experiment initially, renal excretion rate was lower than R_{GF} (Fig.4). When the plasma concentrations decreased to approximately 100 $ng.ml^{-1}$, EdSm was eliminated only by the R_{GF}. This coincided with the temporary increase of plasma concentrations (Fig. 2) which were probably of non-renal origin. At the end of experiment, again, renal excretion rate became lower than the R_{GF} which produced a slow $t_{1/2}\gamma$ of 4.2 hours.

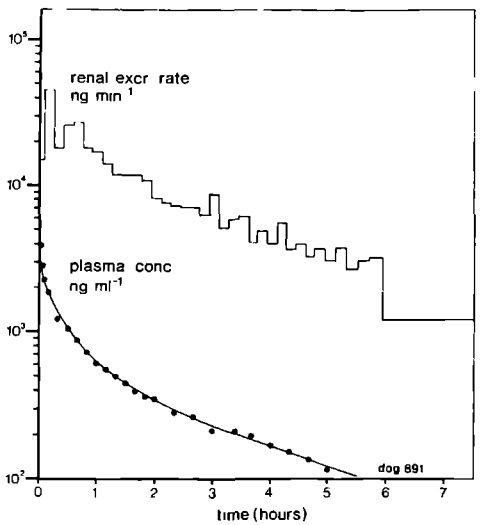

Fig. 3. Plasma and renal pharmacokinetics of EdSm in exp.3 administered as an i.v. bolus, 0.5 mg . kg^{-1}. The $t_{1/2}\beta$ was 0.3 hour and $t_{1/2}\gamma$ was 2.0 hours. 69% of the dose was excreted in urine until the dog died at t=10 hours.

At higher dose, drug was eliminated exclusively by the R_{GF} and no tubular secretion or reabsorption were observed (Fig. 5).

Continuous infusions

EdSm was infused at different doses (0.16 - 0.4 $mg.kg^{-1}$) for 4 to 5 hours. In all experiments a shortstanding steady state was observed within 1 hour after the start of infusion. This primary steady state lasted for only 20 - 80 minutes. After this time plasma concentrations started to increase and if drug was infused long enough, new, secondary steady state was produced.

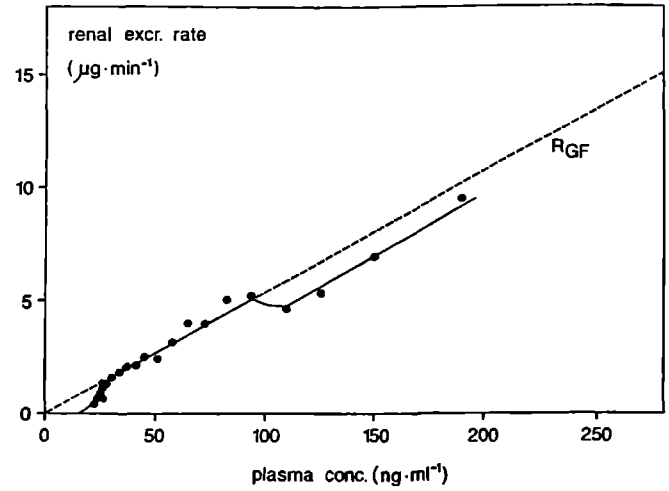

Fig. 4. Tubular titration curve in exp. 1 , i.v. bolus injection. This curve was constructed from the renal excretion rates of individual 10 minutes urine samples and fitted plasma concentrations. The R_{GF} was calculated from the CL_{CR} . 1.25. Renal excretion rates lower than R_{GF} suggest active tubular reabsorption.

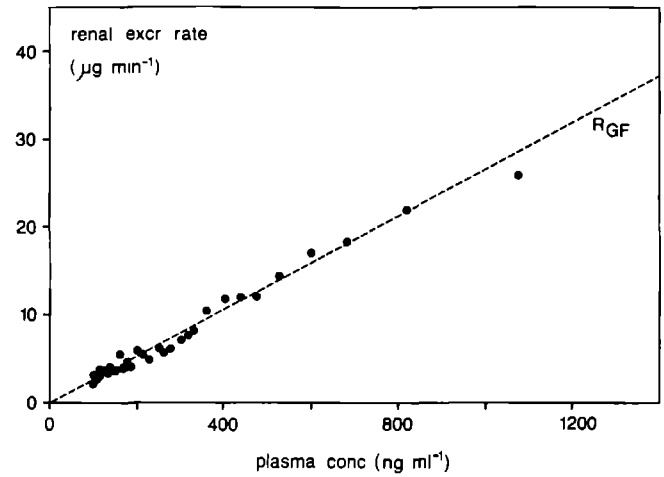

Fig. 5. Tubular titration curve in exp. 2 , i.v. bolus injection of 0.5 mg EdSm.kg^{-1}. EdSm was cleared exclusively by the R_{GF}. Both tubular secretion and reabsorption mechanisms are saturated almost immediately after drug administration.

After discontinuation of the infusions, plasma concentrations decayed rapidly with a $t_{1/2\gamma}$ ranging from 0.6 to 1.5 hours. In exp. 5 plasma EdSm concentrations decayed rapidly for about 30 min and afterwards showed temporary slowing of drug elimination that was restored again after another 30 min. The terminal $t_{1/2\gamma}$ was 0.6 hour (36 minutes).

Analysis of the tubular titration curves in exp.4 (Fig. 6) showed that at the low dose EdSm initially was actively reabsorbed by the renal tubuli, but when the infusion was continued, the drug was also actively secreted by the tubuli (Fig. 7). This process appears saturable at the higher plasma concentrations and the drug continues to be eliminated only by the R_{GF}. This saturation was accompanied by the formation of the secondary steady states. When the drug infusion was discontinued, drug was eliminated initially only by the R_{GF} but later, active tubular reabsorption started to play a role. This was the cause of prolonged terminal $t_{1/2\gamma}$ observed in these cases. In exp.6 (Fig.8) the pattern was essentially the same (data not shown). After discontinuation of drug infusion, desaturation of the renal reabsorption mechanisms was observed what resulted in temporary increase of plasma concentrations. It is probable, that later, renal tubular secretion mechanisms recovered too and the drug was eliminated with a shorter terminal $t_{1/2\gamma}$ of 0.6 hours (36 minutes).

Dog nr 15, (exp. 4) which received 0.4 $mg.kg^{-1}$ in 4 hours, died several hours after experiment due to cardiac arrhytmia and circulatory insufficiency. In this experiment too, a 10-20 % underestimation of the CL_{REdSm} can be expected as the urine could not be collected for a period of 24 hours.

During the experiments a constant pattern of urine flow fluctuations was observed. After a very high urine flow (2-5 $ml.min^{-1}$) observed in the first 10 - 15 minutes of experiment, a urine flow depression was followed. The urine flow values frequently decreased to 0.3 - 0.5 $ml.min^{-1}$ but restored to the average value of 0.75 $ml.min^{-1}$ in the second and third hour. After termination of infusion, a small increase of urine flow was frequently observed. The pH values were initially 8.0-8.4 and decreased to the values of 7.0 - 7.6 later in the experiment.

Dose / AUC relationship

Analysis of the dose/AUC relationship showed that i.v. bolus and continuous infusions followed a different pattern (Fig. 9). Continuous infusions, especially that of 5 hours (exp. 5, Fig. 8) showed steeper increase of the dose/AUC relation.

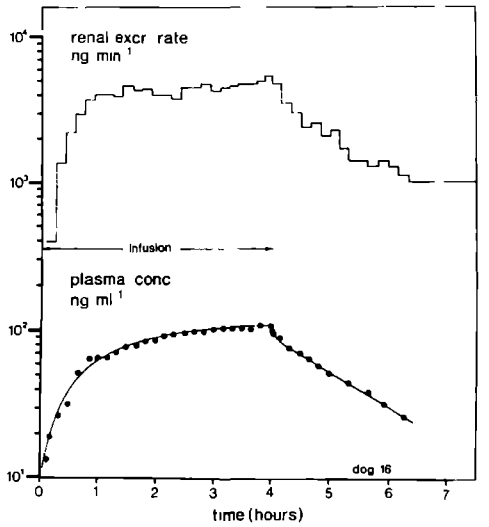

Fig. 6. Plasma and renal pharmacokinetics of EdSm in exp.4 administered as continuous infusion, 0.16 mg.kg^{-1} in 4 hours. 70% of the dose was recovered in urine after 24 hours. The terminal elimination $t_{1/2\,\gamma}$ was 1.3 hours.

At higher doses, the mean CL_{REdSm} tended to be lower than the mean CL_{CR} (Table 1), while the CL_{NREdSm} remained constant (2.5 $\pm$ 0.7 ml.min^{-1}.kg^{-1}). The mean CL_{REdSm} decreased in a dose-dependent manner (r = - 0.96) with an exception of exp. 3 where immediate saturation of the tubular reabsorption resulted in relatively high CL_{REdSm}.

Protein binding

To determine protein binding, ten blood samples from different experiments with EdSm plasma concentrations varying from 54 to 680 ng.ml^{-1} were assayed. Protein binding appeared to be constant in this concentration range and was 40.5 $\pm$ 2.8 %.

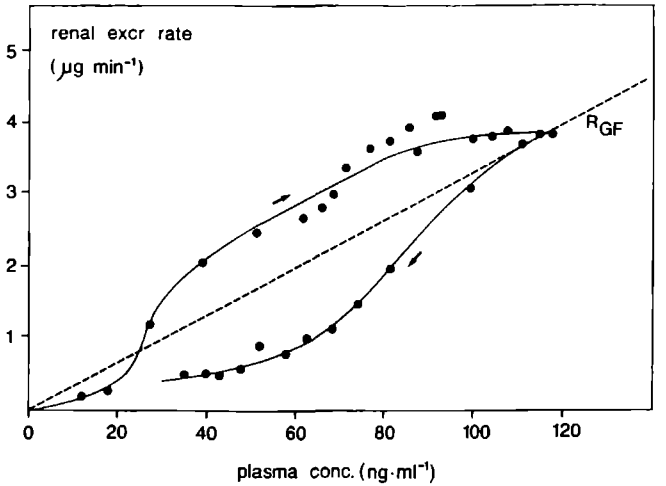

Fig. 7. Tubular titration curve obtained in exp. 6 after administration of EdSm as continuous infusion. Drug is extensively secreted by the renal tubuli which can be concluded from the renal excr. rates higher than R_{GF}. Tubular secretion was saturated at the end of infusion and this was the reason for increase of plasma concentrations (see Fig. 6). After discontinuation of infusion EdSm was excreted initially only by the R_{GF}. Lateron renal reabsorption mechanisms became desaturated and this was the cause of temprorary slowing of drug elimination from plasma.

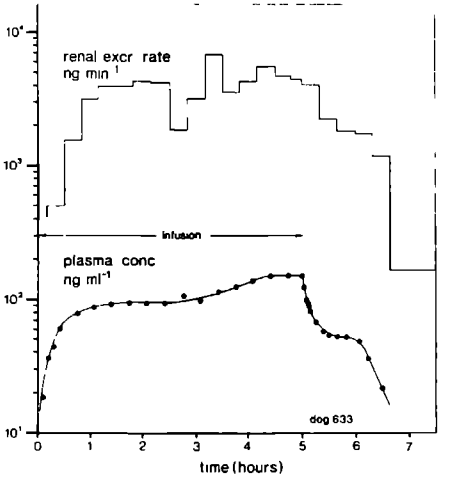

Fig. 8. Plasma and renal pharmacokinetics of EdSm in exp. 6 administered as continuous infusion, 0.22 $mg.kg^{-1}$ in 5 hours. 70% of the dose was recovered in urine after 24 hours. The primary steady state was established after 1.5 hours and in the 4th and 5th hours a new steady state was established. Terminal $t_{1/2}\gamma$ was 0.6 hours.

DISCUSSION

EdSm showed non-linear pharmacokinetics. At lower doses 50 - 70% of the drug dose was excreted in urine (CL_{REdSm}). During experiments a great variability of CL_{REdSm} was observed which was the result of changes in tubular reabsorption and secretion. In low dose continuous infusion drug was eliminated predominantly by the R_{GF} and tubular secretion. When this process was saturated the CL_{REdSm} decreased because of domination of tubular reabsorption. This was the reason of the dose-dependent decrease of CL_{REdSm}. However, when the tubular reabsorption mechanisms became saturated too, CL_{REdSm} decreased to the value of R_{GF}. This was seen clearly in case of exp. 3. Changes in CL_{REdSm} were the reason of non-linearity of the dose/AUC ratio. The dose/AUC ratio for infusions was steeper than that for i.v. bolus. Because of the non-linear pharmacokinetics in cont. infusions and i.v.

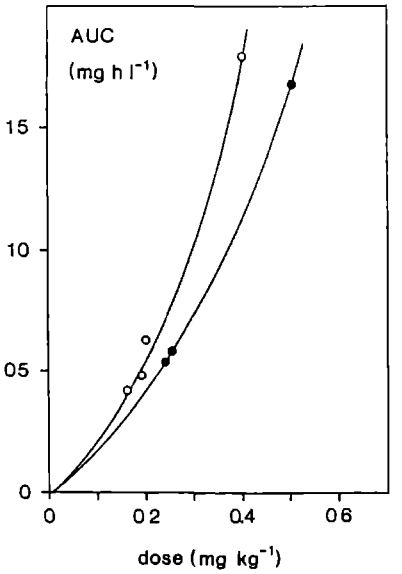

Fig. 9. Relation between the dose and AUC was dependent by the mode of administration; i.v. bolus (●) or continuous infusion (O). The figure shows a dose-dependent increase of AUC due to accumulation of EdSm in plasma.

bolus EdSm appeared to be more toxic in dogs than the two other Sm analogues, which showed a linear pharmacokinetics (deshydroxy-Sm) or non-linear pharmacokinetics with a much less steep increase of the dose/AUC relation (n-pentyl-Sm, unpublished).

These experiments showed clearly, that the changes of renal tubular secretion and reabsorption were due to saturation of the carrier proteins and not inhibition of their synthesis and decay of activity. In the latter case the changes would be time but not dose dependent.

From the above experiments it may be concluded that EdSm is predominantly excreted by the CL_{EdSm} and saturation and desaturation of tubular secretion and resorption mechanisms determines its complicated pharmacokinetics. EdSm which had the highest LD50 in mice among all Sm analogues and showed good antitumor activity, showed non-linear pharmacokinetics in the dog with a steep increase of the dose/AUC relation. Two dogs died because of drug toxicity at doses which were lower than non-toxic doses of the other analogues. The mechanism of this non-linear pharmacokinetics might be related to the inhibition of the synthesis and/or saturation of enzymes involved in the tubular secretion of the drug.

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CHAPTER 12

PHARMACOKINETICS OF THE ANTITUMOR ANTIBIOTIC n-PENTYL-SPARSOMYCIN IN BEAGLE DOGS

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Submitted to: Invest. New Drugs

SUMMARY

N-pentyl-sparsomycin (PSm) is a lipophilic analogue of sparsomycin (Sm), which is a well known inhibitor of protein synthesis. This compound was selected for preclinical pharmacokinetic studies because of its high in vitro and in vivo antitumor activity. In this study in which the drug was evaluated in beagle dogs under anaesthesia, the drug concentrations in plasma, urine and bile samples were determined using high performance liquid chromatography (HPLC). Plasma protein binding was approximately 54%. The mean $t_{1/2\,\beta}$ was 0.2 hours (12 minutes) and $t_{1/2\,\gamma}$ was 0.75 $\pm$ 0.1 hours (45 $\pm$6 minutes). During continuous infusions up to 5.25 hours, the steady state was reached in 3 out of 6 experiments, suggesting that in some cases the real $t_{1/2\,\gamma}$ was longer than measured. PSm was actively reabsorbed from the renal tubuli. This process was saturable at the higher doses. Tubular reabsorption played only a minor role in pharmacokinetics as most of the drug (67%) was eliminated by the non-renal clearance. The non-renal clearance was saturable at higher doses of PSm and was the reason for non-linearity of pharmacokinetics.

INTRODUCTION

Sparsomycin (Sm, NSC-059729), an antibiotic isolated in 1962 from Streptomyces sparsogenes (1) is a well known ribosomal protein synthesis inhibitor (2). The drug showed a potent anticancer activity in KB cells in vitro and in some rodent tumors in vivo (2,3). In 1964 Sm was studied in a phase I clinical trial but its testing was discontinued because of unusual retinal toxicity (ring scotomas) seen in two out of the five patients (4). Due to the drug's poor availability from the natural sources and the expense, further research and use of this drug was delayed for two decades.

Following the chemical synthesis of Sm in 1981 (5) and structure activity relationship (SAR) investigations (6,7), in vivo studies on the analogues were performed. Sm analogues are unique among protein synthesis inhibitors as several of them showed lower toxicity and higher activity in vivo than the parent drug (8).

During the SAR studies it was found, that analogues with a hydrophobe R2 side chain (see Fig.1) shows higher affinity to the ribosomes and higher activity in cell free systems which synthesize proteins (9). Based on these findings, a hydrophobic pocket in the larger ribosomal subunit has been proposed (7). This place on the ribosomal acceptor site is probably desti-

nated for binding with the more lipophilic aminoacids. Additional studies showed that the analogue with five carbon atoms in the R2 aliphatic side chain, n-pentyl-Sm (PSm), possessed the best in vitro activity (9). PSm showed high LD50 in mice (4.7 $mg.kg^{-1}$) when administered daily for nine days. The comparable value for Sm is 0.26 $mg.kg^{-1}$ (8). PSm was active on P388 and L1210 leukemias in vivo (median T/C = 160% and 164%, respectively), RC renal cell carcinoma (T/C = 414%) and B16 melanoma (T/C = 131%)(8). There was only minimal activity on C38 colon carcinoma (8). Based on these data, PSm has been considered as a candidate for clinical trial.

The biologic effect of a protein synthesis inhibitor depends on the disappearance of the native proteins. The higher their turnover rate, the more rapid the biological effect. If PSm is excreted and/or metabolized by enzymes with a short life span, then inhibition of protein synthesis using this drug also inhibits its own elimination which would result in drug accumulation in plasma and in increased toxicity. If this effect were to occur, it would be most clearly seen during continuous infusion.

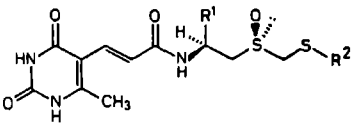

Fig. 1. Structure of Sm : R1 $-CH_2OH$, R2 $-CH_3$
and PSm : R1 $-CH_2OH$, R2 $-(CH_2)_4CH_3$

The purpose of this study is to describe the plasma pharmacokinetics of PSm and to assess whether accumulation in plasma occurs, which would increase toxicity during prolonged administration.

MATERIALS AND METHODS

Animals

Four beagle dogs, one male (13-16 kg) and three females (8.7-10 kg), were obtained from the Central Animal Laboratory of the University of Nijmegen. The dogs were used several times in the pharmacokinetic experiments with at least a 3 month rest period between tests. Before the dogs were accepted for these experiments, their general condition, body weight, and kidney and liver functions were evaluated. The dogs were fed a standard granulated food (Hope Pharms BV, Woerden, The Netherlands) and had free access to the tap

water. The pharmacokinetic experiments were performed under anaesthesia, after which the animals were housed in metabolic cages enabling urine collection for a total of 24 hours.

Drug and formulation

PSm was synthesized following a modified method, originally described by Ottenheijm et al. (5, HCJ Ottenheijm, personal communication) and was acquired in a freeze dried form. The injections were prepared aseptically by the hospital pharmacy. The drug was dissolved in 10% propylene glycol and 0.03 M phosphate buffer (pH = 7) in a concentration of 0.5 mg/ml and filtered through a 0.2 um filter (FP 030/2 Schleicher & Schnell, Dassel, F.R.G.). The dissolved drug was stored in dark flasks at $4^{o}C$ for up to 24 hours.

Stability of the drug in this formulation was tested at 4^{o}, 20^{o}, 40^{o} and $60^{o}C$ for 12 weeks. Only at the highest temperature the drug concentration decreased by 13.5 % in 12 weeks. At the other temperatures PSm was stable.

Pharmacokinetic experiments

The dogs were deprived of food for the last 12 hours before each experiment. Experiments were performed under Ethrane (Enflurane, Abbot, Campoverde, Italy) and N_2O anaesthesia with thiopental (Penthotal, Abbot, Campoverde, Italy) induction (15 mg/kg).

Each experiment lasted for 5-7 hours. The animals received intravenous fluid (0.9% NaCl and 2% mannitol) at a rate of 2.7 ml/min. The fluid infusion rate was controlled by an infusion pump (IVAC, type 531, San Diego, CA, USA). Continuous infusions of PSm were administered using a 60 ml syringe driver (Infors HT, type 5003, Basel, Switzerland), delivering 10 ml per hour. The accuracy of infusion rate was controlled every hour. The remainder of the solution was routinely checked to determine the definitive drug concentration (see analysis). Rectal temperature and heart rate (ECG) were continuously surveyed. Blood samples (5 ml) were drawn in heparinized vacuum tubes through a central venous catheter. These samples were centrifuged within one hour of collection. Plasma was separated and frozen at $-20^{o}C$ pending analysis.

During the whole experiment urine was collected through a urinary catheter every 10 min. At the end of each collection period, urinary bladder was flushed with 5 ml of 0.9% NaCl. Urine samples were made in duplicate for determination of the drug and the creatinine concentrations. Urine samples were kept in the dark at $-20^{o}C$ pending analysis.

After recovery from anaesthesia, the dogs were returned to a metabolic cage where urine collection and blood sampling were continued for 24 hours.

Dog 785 (exp. nr 3) was subjected to perioperative bile drainage. Bile samples, about 2 ml every 20 min, were collected for determination of drug concentration. After this experiment, the dog was sacrificed by an overdosis of barbiturates.

Drug analysis

PSm concentrations in plasma, urine and bile were determined using the reversed phase HPLC method as previously described (10). Plasma samples were deproteinized with 7% perchloric acid (Aldrich, Brussels, Belgium). A semiautomated HPLC system from Kratos Analytical, (Ramsey, NJ, USA) was utilized. This system consists of two Spectroflow 400 pumps, a Multiport Streamswitch with two six way valves (Rheodyne, Cotati, CA, USA), a 757 UV-Vis Detector and DS 610 Multirange strip chart recorder. The connection of the sampling valves for the reverse flushing method has been described elsewhere (10). The system was operated by means of an HPLC solvent programmer (type 9224, Kipp Analytical, Rotterdam, The Netherlands). The columns used were a concentration column (50 x 3 mm id) filled with LiChrosorb RP-8 (10 µm particle size) and an analytical column (100 x 4.6 mm id) filled with CP^{tm} Spher C8 (8 µm particle size); both packing materials were from Chrompack BV (Middelburg, The Netherlands). Solvent A (flow 1.3 ml/min) for sample concentration and cleaning of the columns consisted of distilled water. Solvent B (flow 1.0 ml/min), to carry the sample from the concentration column through the analytical column, consisted of 45% methanol (HPLC grade, Fisons, Loughborough, England) in water. Drug concentrations were determined by the comparison of the sample peak heights with standards made from untreated control plasma spiked with known concentrations of the drug and incubated for 1 hour at 37°C. The accuracy of the calibration curves were estimated by comparing the concentration to peak heigth ratios for five different concentrations. The calibration curve was acceptable when the SD of the ratios was less than 5%. The detection limits for PSm were: in plasma 5 ng.ml^{-1} and in urine 20 ng.ml^{-1}.

Protein binding assays

The protein binding of PSm was determined using an ultrafiltration method. Prior to these studies, no aspecific drug binding to the filters (EMIT free level TM, Syva, Palo Alto, CA, USA) was found. Heparinized plasma samples were centrifuged through the filters at 2500 g for 20 min. Drug recovery

after ultrafiltration was subtracted from the total drug concentration obtained from a deproteinized sample and expressed as the percentage of the total drug concentration.

Fitting of the curves and expression of the results

Curves were fitted by semi-log regression of the graphically identified elimination phases and by the usual stripping method. The area under the fitted concentration versus time curve (AUC, $mg.h.l^{-1}$) was calculated using the trapezoidal rule. Other pharmacokinetic parameters were calculated from the following equations:

$$CL_{PSm} = D \cdot AUC^{-1} \quad (ml \cdot min^{-1}.kg^{-1})$$

$$V_d \text{ beta} = CL_{PSm} \cdot t_{1/2} \cdot 0.693^{-1} \quad (l \cdot kg^{-1})$$

$$CL_{RPSm} = CL_{PSm} \cdot \% \text{ of the dose recovered in urine } (ml \cdot min^{-1}.kg^{-1})$$

$$CL_{NRPSm} = CL_{PSm} - CL_{RPSm} \ (ml \cdot min^{-1}.kg^{-1})$$

where:

D - dose ($mg.kg^{-1}$)

$t_{1/2}$ - elimination half-life

V_d - volume of distribution (in the beta and gamma phases)

CL_{PSm} - total body clearance of PSm

CL_{RPSm} - renal clearance of PSm

CL_{NRPSm} - non renal clearance of PSm

AUC - area under the concentration versus time curve

Tubular titration curves were obtained by plotting fitted plasma concentrations against renal excretion rate. R_{GF} was obtained from the mean $CL_{CR} \cdot 1.25$. Curves were interpreted according to the method of Heckman and Van Ginneken (11).

RESULTS

PSm was administered either as an i.v. bolus (exp. 1, 0.07 mg $PSm.kg^{-1}$ and exp. 2, 0.53 mg $PSm.kg^{-1}$) or as a continuous infusion for 2 - 5.25 hours at the doses ranging from 0.08 to 1.1 mg $PSm.kg^{-1}$ (exp. 3-8). In total eight experiments were carried out on four dogs. The pharmacokinetic data are shown in Table 1 where the means $\pm$ SD are presented.

After administration of i.v. bolus (exp. 1 and 2) the curves showed one distribution and two elimination phases. The alpha distribution phase had an estimated $t_{1/2\,\alpha}$ of approximately 0.03 hours (2 minutes) but could not be determined accurately. The drug was eliminated from plasma with the $t_{1/2}\,\beta$ of

Table. 1. Pharmacokinetic parameters of n-pentyl-Sm in beagle dogs.

Exp. nr.	dog nr.	sex	body weight (kg)	dose ($mg.kg^{-1}$)	type of exp.	$t_{1/2}\beta$ (h)	$t_{1/2}\gamma$ (h)	C_{ss} ($ng.ml^{-1}$)	AUC ($mg.h.l^{-1}$)	V_d β ($l.kg^{-1}$)	V_d γ ($l.kg^{-1}$)	CL_{PSm}	C_{RPSm}	CL_{CR}	CL_{NRPSm}
												$ml.min^{-1}.kg^{-1}$			
1.	785	M	12.6	0.07	iv bolus	0.2	0.74		0.11	0.18	0.65	10.1	3.8	3.7	6.3
2.	634	F	10.0	0.53	iv bolus	0.2	0.76		0.89	0.18	0.65	9.8	3.0	3.5	6.8
3.	634	F	9.8	0.08	4 h inf.	-	- *	38.5	0.15	-	-	9.0	5.2	3.1	3.8
4.	16	F	9.2	0.21	4 h inf.	-	0.78	-	0.39	-	0.63	9.0	2.0	2.5	7.0
5.	15	F	8.4	0.21	3.5 h inf.	-	0.62	110.0	0.43	-	0.44	8.2	3.0	2.9	5.2
6.	785	M	16.0	0.24	3.5 h inf.	-	0.83	-	0.57	-	0.50	7.0	2.6	4.5	4.4
7.	785	M	16.0	0.24	2 h inf.	-	0.90	-	0.46	-	0.68	8.8	3.3	3.4	5.5
8.	16	F	9.9	1.1	5.25 h inf.	-	0.60	580.0	3.04	-	0.28	6.0	2.5	2.9	3.5
Means						0.2	0.75			0.18	0.55	8.5	3.2	3.3	5.3
± SD						0.0	0.1			0.0	0.15	1.4	1.0	0.6	1.3

* The part of the curve after discontinuation of the infusion was not evaluable.

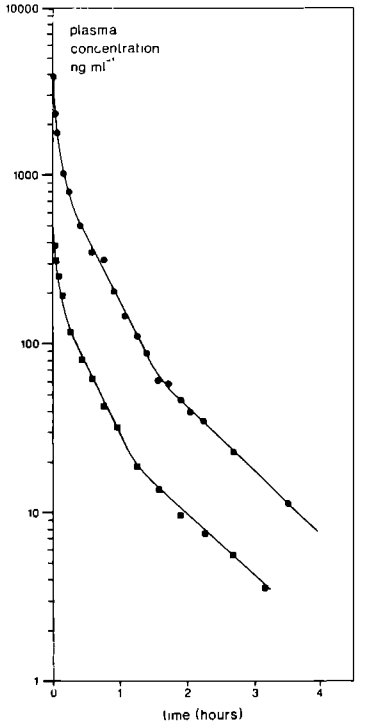

Fig. 2. Plasma pharmacokinetics after bolus injections of 0.53 mg PSm.kg^{-1}, exp. 2 (●), and 0.07 mg PSm.kg^{-1}, exp. 8 (■). The $t_{1/2}\beta$ was 0.2 hours (12 min) and $t_{1/2}\gamma$ was 0.75 hours (45 min).

0.2 hours (12 minutes) and $t_{1/2}\gamma$ of 0.75 (45 minutes) (Fig.2). When the drug was administered as continuous infusion (Fig.3) only terminal $t_{1/2}\gamma$ of 0.75 $\pm$ 0.1 hours (46 $\pm$ 6 minutes) could be indentified, equal to the $t_{1/2}\gamma$ seen in i.v. bolus experiments. In experiments 4, 6, and 7, steady state was not reached during 2 to 4 hours of infusion. In experiments 3, 5, and 8 the steady state was reached within 1-2 hours, and in these experiments, shorter terminal half-lives were observed then in exp. 4, 6 and 7 (0.6 versus 0.8 hours).

PSm was actively reabsorbed by the renal tubuli (Fig. 4-7) as the renal excretion rate was lower than the calculated R_{GF}. At the lowest dose tested (exp.1 Fig.4) tubular reabsorption was dominant and only 3.75% of the dose was recovered in urine after 24 hours (CL_{RPSm} = 0.4 ml.min.kg^{-1}). At the higher dose administered as a single i.v. bolus (exp.2, 0.53 mg.kg^{-1}) PSm was eliminated exclusively by the R_{GF} (Fig.5). When the low PSm dose was

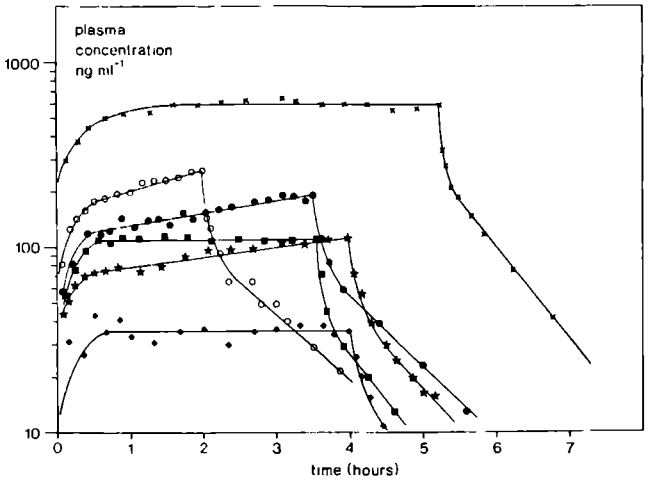

Fig. 3. Plasma pharmacokinetics of PSm determined during and after continuous infusions. exp.3. (◆), 0.08 mg.kg^{-1} in 4 hours. exp.4. (★) 0.21 mg.kg^{-1} in 4 hours. exp.5. (■), 0.21 mg.kg^{-1} in 3.5 hours, exp.6. (●), 0.24 mg.kg^{-1} in 3.5 hours, exp.7. (O), 0.24 mg.kg^{-1} in 2 hours, exp.8 (x) 1.1 mg.kg^{-1} in 5.25 hours. The terminal $t_{1/2\gamma}$ was 0.75 $\pm$ 0.1 hours (45 $\pm$ 6 minutes).

administered as continuous infusion for 4 hours, beyond tubular reabsorption also tubular secretion was seen (exp. 3, Fig. 6). At the higher doses, tubular secretion was saturated and tubular reabsorption was dominant (exp. 6. Fig. 7). At the end of the infusion tubular reabsorption was saturated and the drug was excreted exclusively by the R_{GF}. This pattern was seen also during exp. 8 (1.1 mg.kg^{-1} in 5.25 hours infusion, data not shown).

In the dose range between 0.07 and 0.53 mg PSm.kg^{-1} the relation between dose and AUC was linear with correlation coefficient r = 0.97 and the mean dose/AUC ratio was 0.53 $\pm$ 0.07. At the highest dose tested, 1.1 mg PSm.kg there was a 30% decrease of the CL_{PSm} and the dose/AUC ratio decreased to 0.36. This apparent non-linearity at the dose of 1.1 mg PSm.kg^{-1} (exp. 8) was due to decrease of CL_{NRPSm}. The CL_{RPSm} was also lower than the mean of all experiments, but this was due to decrease of the renal function and low mean CL_{CR}.

The volume of distribution of the beta phase (i.v. bolus experiments) was 0.18 l.kg^{-1} and for the gamma phase 0.55 $\pm$0.15 l.kg^{-1}.

To determine the protein binding, nine blood samples from different

experiments with PSm concentrations varying from 63 to 774 $ng.ml^{-1}$ were assayed. A constant protein binding of 54 $\pm$ 4.3% was found over this concentration range.

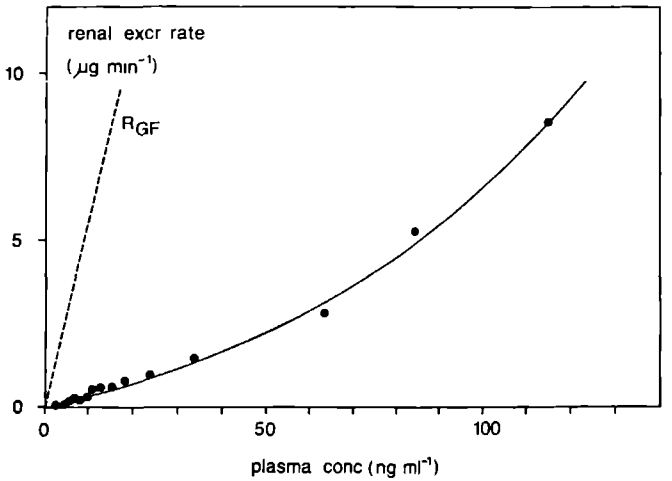

Fig. 4. Tubular titration curve in exp. 1. The slope of R_{GF} curve was obtained from the mean CL_{CR} . 1.25. The renal excretion rate in this experiment was far below the R_{GF} value due to extensive tubular reabsorption.

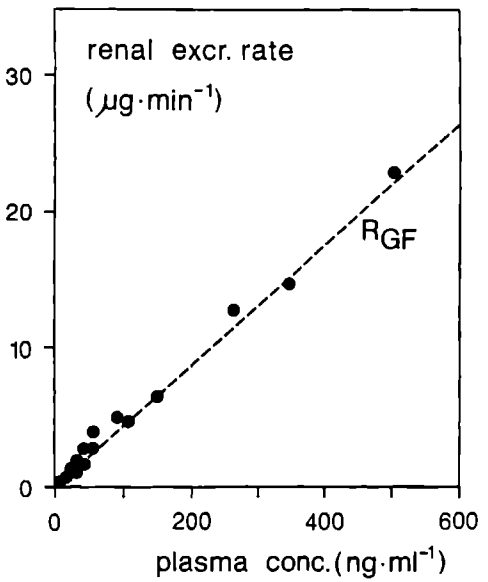

Fig. 5. Tubular titration curve in exp. 2 after administration of a high dose of PSm (0.53 $mg.kg^{-1}$) as an i.v. bolus. Due to immediate saturation of the carrier PSm is eliminated exclusively by the R_{GF}.

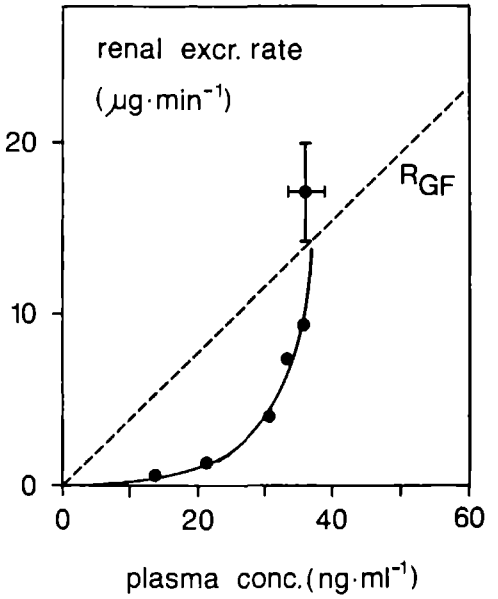

Fig. 6. Tubular titration curve in exp. 3. During continuous infusion, initially drug was reabsorbed by the tubuli. When the plasma concentrations reached steady state, renal excretion rate was higher than the R_{GF} suggesting tubular excretion. The bars show the SD of the plasma concentrations at the steady state (horizontal) and the renal excretion rate at the steady state (vertical). After discontinuation of infusion again, tubular reabsorption was observed.

In exp. 7 no metabolites of PSm in 1:10 diluted bile were observed. Only trace amounts of unchanged PSm in the bile were noted.

DISCUSSION

PSm, an analogue of Sm, was selected from a dozen other analogues because of its good antitumor activity (8) and its optimal lipophilicity as determined by in vitro and in vivo studies (9). The drug is hardly water soluble, and while it is instable in several organic solvents it is stable in propylene glycol (D. Blok personal communication).

Pharmacokinetic evaluation revealed that the drug is reabsorbed by the renal tubuli. In one experiment only at the lowest dose administered as continuous infusion (exp. 3) it was found that PSm might also be actively excreted by the renal tubuli.

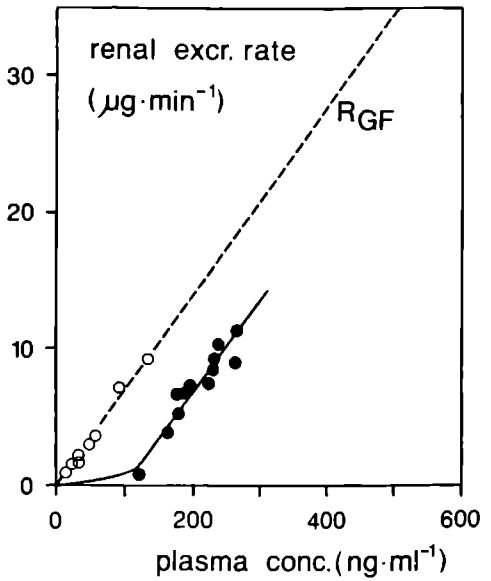

Fig. 7. Tubular titration curve during exp. 7 (continuous infusion). Initially (●) only tubular reabsorption was observed. After discontinuation of infusion drug was eliminated exclusively by the R_{GF} (o), probably due to saturation of the tubular reabsorption.

The dominant tubular reabsorption was saturable either at the end of the infusion or immediately after administration of high PSm dose as an i.v. bolus. The CL_{NRPSm} may also be saturated at the higher doses and this is probably the reason why the pharmacokinetics is nonlinear.

In a previous study (12), Sm was continuously administered by infusion to beagle dogs under anaesthesia. It then induced a considerable reduction in the renal clearance with a subsequent exponential increase in the Sm plasma concentration. In two experiments performed with different doses, such changes appeared at the same time suggesting the decay of a transport enzyme. The pattern of PSm and kidney interaction is quite different from that observed with Sm. The changes are dose but not time dependent, which suggests saturation and not decay of the enzyme activity. Inhibition of the de-novo synthesis of the carrier enzymes would induce time dependent events. It is difficult to explain why the plasma concentration versus time curve in 3 out of 6 experiments steadily increases and does not show a definable steady state even during long ($5 \times t_{1/2}^{\gamma}$) infusions. It could be possible that in some cases the real terminal half-life is greater than that calcu-

lated from the data.

In conclusion: the more lipophilic analogue of Sm, PSm shows a different pharmacokinetic behaviour than is seen with Sm. The CL_{PSm} is two times higher than that for Sm (11), which would lower the chance of accumulation and toxicity. The dose-dependent pharmacokinetics of PSm is related to saturation of non renal mechanisms of drug elimination as well as disturbance of the renal function.

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CHAPTER 13

STUDIES ON POSSIBLE MECHANISMS OF SPARSOMYCIN-INDUCED RETINOTOXICITY IN RATS

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SUMMARY

Sparsomycin (Sm) is a potent inhibitor of protein synthesis with an anti-cancer potential. Two years after its discovery in 1962 a phase I clinical trial revealed serious drug-induced retinotoxicity in two out of five patients. The mechanism of this toxicity still remains unresolved. The recent development of several promising analogues of Sm prompted us to re-investigate the possible toxic action of Sm on ocular targets. The effect of a maximal tolerable treatment with Sm on ocular rhodopsin and opsin levels were studied in normal albino rats with or without permeabilization of the blood-retina barrier with sodium iodate, and in Royal College of Surgeons (RCS) rats. The RCS rats are known to have deficient degradation of visual pigment in retinal pigment epithelium (RPE) and develop a defective blood-retina barrier.

Treatment with subtoxic doses of Sm daily for 10 days causes accumulation of the drug in the plasma. However, Sm does not significantly reduce ocular rhodopsin content and no changes in ocular morphology were observed. A single injection of sodium iodate was found to reduce rhodopsin but not opsin levels. Sm administered after sodium iodate lowered opsin levels by 27% suggesting only partial inhibition of biosynthesis even under these extreme conditions. In tumor bearing cachectic animals, Sm administration did not affect rhodopsin levels. Rhodopsin accumulation in RCS rats was only affected to a minor extent by treatment with Sm. These results strongly indicate that Sm might only become retinotoxic upon extensive degradation of the blood-retina barrier as in combination with strong metabolic poisons.

INTRODUCTION

Sparsomycin (Sm, NSC-059729) was discovered in 1962 as a metabolite of Streptomyces sparsogenes (1). The antibiotic was found to be active on KB human epidermoid carcinoma cells in vitro and several rodent tumors in vivo (1). The drug proceeded rapidly to a phase I study that started already in 1964 (2). Five patients were treated with Sm that was administered daily in increasing doses. At day 13 and 15 of the treatment, respectively, two patients developed blurred vision and ring scotomas, later confirmed by histopathological examination as widespread degeneration of the RPE especially accentuated in the posterior temporal areas. The trial was discontinued and the drug was assumed retinotoxic.

On assignment of the National Cancer Institute (Bethesda, MD) additional

toxicological studies in rats and monkeys were performed (3). Sm was administered in subtoxic doses daily for up to 28 days. Retinotoxicity was assessed by electroretinography and eye histology. Abnormal electroretinograms were only observed in moribund rats, probably due to their poor general condition. No changes were seen in monkeys. Histopathological changes were not detected in any animal. After these studies were completed in 1967, most of the investigations on Sm were discontinued because of poor supply of the drug.

In the late sixties it was discovered that Sm is a potent inhibitor of cellular protein biosynthesis (4). After its chemical structure was elucidated in 1976 (5), synthetic Sm became available in 1981 (6). Thereupon a wide program of structure-activity relationship studies produced three derivatives which were good candidates for a phase I study (7). These developments prompted us to investigate the alleged retinotoxic potential of Sm on a biochemical level. Since the photoreceptor cell maintains a high rate of biosynthesis of visual pigment protein (opsin), which is compensated by circadian phagocytotic action of the adjacent RPE (8), we investigated the effect of Sm treatment on the ocular rhodopsin and opsin levels in rat. In addition, possible synergistic effects of cachexia, and a metabolic poison - sodium iodate-inducing breakdown of the retina-blood barrier, were studied.

MATERIALS AND METHODS

Drugs

Sm was synthesized and purified as described before (6) and acquired in a freeze-dried form. The drug was dissolved in phosphate buffered saline (PBS, pH = 7.4) and kept at $4^{o}C$ in dark flasks for maximally 10 days. Sodium iodate ($NaJO_3$) was from Merck, FRG, and was used as a 5% solution in PBS.

Animals

Male albino Wistar rats, 200-250 g, were supplied by the Central Animal Laboratory of the University of Nijmegen. The rats were adapted to a 12/12 hour light/dark schedule for at least one week prior to experiment. Animals were always sacrificed after 24 hour of dark adaptation to ensure maximal regeneration of rod visual pigment (opsin to rhodopsin). Drugs were administered i.p. during the light phase except for the final doses 30 min prior to exsanguination (exp. 1) which were carried out in darkness.

Pink-eyed RCS rats (males and females) were supplied by the same facility, at the age of 19 days and body weight of 30-40 g. Young animals were weaned up to a week before the experiments started and then adapted to

standard rat granulated food (RMH, Hope Farms, Woerden, The Netherlands) and tap water. At day 20 the animals were placed in a dark cabinet and remained there for the rest of the study so that all visual pigment remains in the rhodopsin state. All procedures were carried out in dim red light (λ > 610 nm).

Treatment protocol

Exp. 1. Sm administration at maximal tolerable dose daily for 10 days. Fourty healthy Wistar rats were randomized to control (n=10) or treatment groups (n=5). Five control animals were sacrificed on day 0, and single controls were sacrificed simultaneously with the treatment groups. Animals were exsanguinated under pentobarbital anaesthesia by a cardiac puncture 30 minutes after respectively 1,2,3,4,6 and 10 daily doses of 0.5 mg Sm/kg i.p. This dose was found the maximally tolerable one in a previous pilot study. After exsanguination the eyes were extirpated for rhodopsin analysis. Plasma was isolated from the collected blood by centrifugation and stored at -20^{o}C pending analysis. Plasma Sm concentrations were determined by quantitative high performance liquid chromatography (9). For histologic examination two randomly selected eyes, from two different animals, were fixed in Bouin solution.

Exp.2. As exp.1 but following pretreatment with sodium iodate 0.05 mg/kg (10) on day 0. Animals were treated on days 1-5 with 0.5 mg Sm/kg i.p. and were sacrificed 24 hours after the last injection. Eyes were analysed for rhodopsin and opsin content.

Exp.3. Effect of cancer cachexia. 15 Wistar rats were inoculated s.c. in the right flank with 10^{6} viable Walker 256 carcinosarcoma cells. The tumors became palpable in 7 days and the animals were then randomized in three groups (n=5). Tumor bearing rats without further treatment served as control. The other groups were treated with daily doses of either 0.15 mg Sm/kg i.p. or with 0.30 mg Sm/kg i.p. for 5 days. Additionally, 5 healthy rats served as "normal" control at day 0. All eyes were analysed for rhodopsin content.

Exp.4. Sm administration to RCS rats. The animals were supplied in nests of 5-6 animals, at 2-3 nests per time and the experiments had to be performed in several phases. The animals were housed in darkness, and were treated on postnatal days 20-24 with a daily dose of 0.5 mg Sm/kg i.p. They were sacrificed either on day 25 or 31 and the eyes were assayed for rhodopsin.

Rhodopsin determination

Eyes were extirpated in darkness under pentobarbital anaesthesia with or without previous exsanguination. The animals were sacrificed afterwards by cervical dislocation. Extirpated eyes were placed in light-tight aluminium containers and quick-frozen at -80°C. For rhodopsin assay the eyes were thawn in darkness in plastic Eppendorf tubes containing 0.25 ml of PIPES buffer (pH 6.5) and cut in fine pieces with sharp scissors. The PIPES buffer contained 20 µM PIPES (Sigma, St. Louis, USA), 120 mM NaCl, 5 mM KCl, 1 mM $MgCL_2$, 1 mM $CaCl_2$, 0.5 mM EDTA, 1 mM DTE, titrated to pH 6.5 by 1 M NaOH. Thereafter the scissors were rinsed three times with 0.25 ml PIPES buffer containing 1% Ammonyx LO (Fluka, Buchs, Switzerland). Each rinse was added to the corresponding tissue homogenate. Rhodopsin was then extracted from the tissue homogenate by gentle rotation for 1 hour at 4°C, whereupon samples were centrifuged at 15000 g at 4°C for 30 minutes. The clear supernatant was assayed for rhodopsin by differential spectroscopy under standard conditions as described elsewhere in details (11) employing a PU 8800 spectrophotometer. The rhodopsin concentration was calculated from the difference in A_{500} before and after illumination, using a molar absorbance coefficient of 40300. In exp. 2 the illuminated samples were frozen again at - 80° for opsin determinations using ELISA.

Opsin assay

The enzyme-linked immunosorbent assay (ELISA) for quantitative determination of opsin using anti-bovine rhodopsin antisera (12) as applied to mice (13) was adapted for rats. The rat opsin standards required to construct ELISA calibration curves were prepared by illumination of pooled rhodopsin extracts of six rat eyes (Fisher x Copenhagen: F1) of which the rhodopsin content was determined by spectroscopy. Polystyrene 96 well flat-bottom microtiter plates were obtained from Greiner (Frickenhausen, FRG). The plates were coated with rat opsin by incubation of 1:6400 diluted opsin extract for 16 hours at 4°C (final detergent concentration 0.00065%). After coating, the wells were washed three times with PBS (0.02 M phosphate buffered saline, pH 7.4). The inhibition assay was carried out in the pre-incubation mode essentially according to Schalken et al. (13) using serially diluted antiserum CERNJS843 elicited against purified bovine rhodopsin (12,14). Calibration curves were constructed by semi-log plots of the resulting absorbance at 492 nm versus the amount of opsin added (0.001 - 5 pmol opsin per well). The opsin contents of the rat eye extracts were assayed in the same way using triplicate samples at various dilutions, and

calculated from the resulting absorption at 492 nm using the linear part of the sigmoidal calibration curve (15 - 85% of maximal absorption).

Statistics

The duplicate standard deviation was used as a quality control. The mean rhodopsin content of both eyes of an animal was used to calculate the mean of the group. Because the duplicate SD value was very small (3.2% for rhodopsin), occasionally one eye value, due to submission of the second eye to histopathologic examination, were accepted as means of two eyes (exp.1 only). The means of Sm plasma concentrations after different number of administrations were compared to the values obtained after the 1st administration. The data were analysed using the Kruskall-Wallis test for parallel independent groups. The same test was used to compare rhodopsin and opsin concentrations. The accepted level of significance was $p<0.05$.

RESULTS

General technical aspects

The overall SD for duplicate samples for rhodopsin in controls was 0.06 nmol/eye (2-4% of the measured values). For opsin this value was 0.09 nmol/eye (3-5% of the measured values).

Exp. 1. Dose and plasma concentrations

The dose of Sm administered to healthy Wistar rats (0.5 mg Sm/kg i.p. daily, during 10 days) was the maximal tolerable dose and it was derived from a pilot study on 20 rats. At day 10 in exp.1 the animals had lost 21 $\pm$ 8% of body weight and were moribund. Plasma Sm concentrations were measured 30 min after the daily drug injection and were significantly elevated in all cases in relation to day 1 values (see Fig. 1). Although no definite pattern could be discerned, the levels seem to plateau after 4 days at 600 - 800 ng Sm/ml.

Effect of Sm administration of rhodopsin levels

The ocular rhodopsin content showed a small (7%) but not significant drop (see Fig. 2) already after a single dose, 30 min after Sm administration. No real progression of this effect during further treatment, nor any correlation with the plasma Sm concentrations was observed. Instead, after 10 days of treatment, the ocular rhodopsin levels had recovered to the control values. To eliminate this possible acute effect of Sm in subsequent experiments the animals were sacrificed 24 hours after the last injection, under

the assumption that this time interval should allow full recovery from any acute effect. Histologic examinations of the eyes did not show any morphological abnormalities at any time.

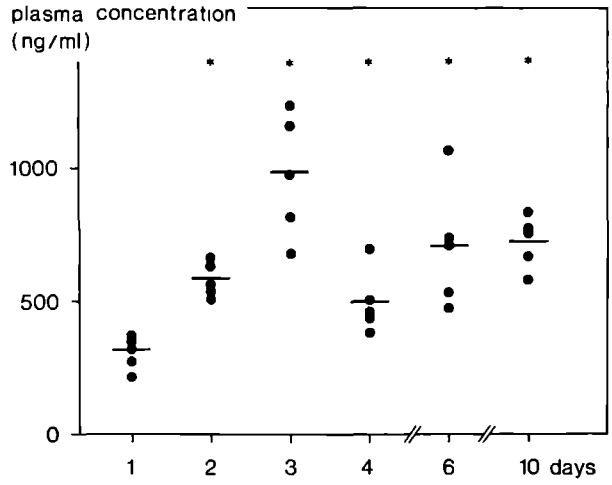

Fig. 1. Sm plasma concentrations in Wistar rats 30 min after respectively 1,2,3,4,6 and 10 daily Sm doses of 0.5 mg/kg. * $p<0.05$

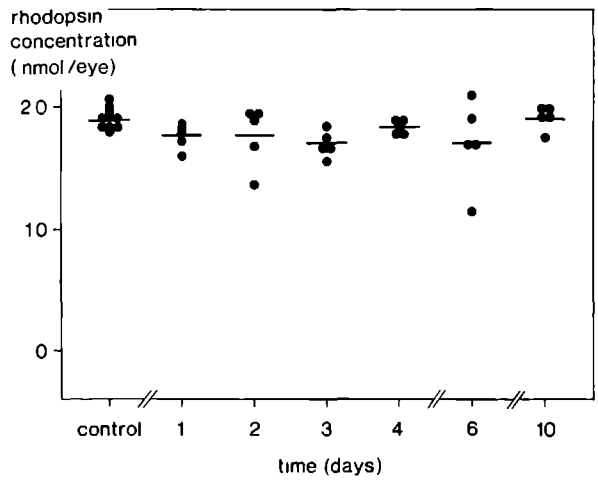

Fig. 2. Mean rhodopsin concentrations in eyes of Wistar rats treated with 0.5 mg Sm/kg for respectively 1,2,3,4,6 and 10 days. The differences between the means were not significant.

The controls sacrificed at the same time as treated animals produced constant and normal rhodopsin levels, indicative for adequate dark adaptation and constant experimental conditions.

Exp. 2. Combined treatment with sodium iodate and Sm

In this experiment (Fig. 3) rats were pretreated with sodium iodate in order to destabilize the RPE and permeabilize the blood-retina barrier. Control animals only treated with sodium iodate showed a tenfold decrease in rhodopsin content which dropped from 1.76 $\pm$ 0.3 nmol/eye in untreated controls to 0.17 $\pm$ 0.02 nmol/eye for animals treated with sodium iodate ($p<0.03$). Treatment with Sm did not significantly affect the rhodopsin levels in normal (1.53 $\pm$ 0.16 nmol/eye) or iodate pretreated animals (0.14 $\pm$ 0.1 nmol/eye). To differentiate this phenomenon somewhat further, the ocular extracts were also assayed for total visual pigment protein (opsin) using the ELISA method on illuminated samples. Combination of sodium iodate with Sm led to a decrease of opsin levels from 1.93 $\pm$0.25 nmol/eye in untreated controls and 1.62 $\pm$ 0.36 nmol/eye in animals treated with sodium iodate alone to 1.2 $\pm$ 0.3 nmol/eye in animals treated with sodium iodate and Sm ($p<0.003$ and n.s., respectively).

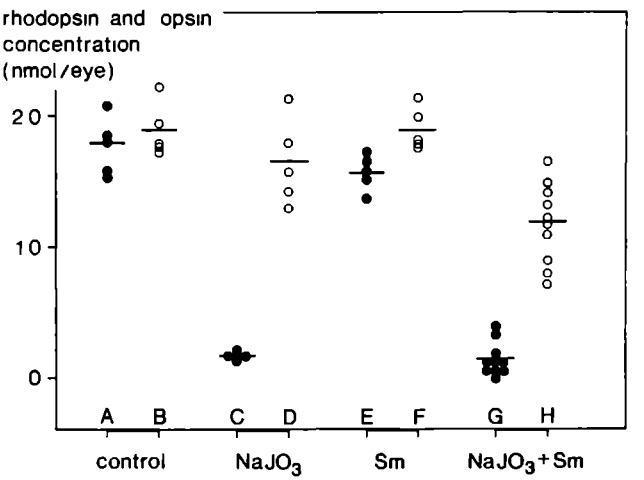

Fig. 3. Mean rhodopsin (●) and opsin (o) concentrations in both eyes of Wistar rats treated for 5 days with 0.5 mg Sm/kg with or without sodium iodate ($NaJO_3$) pretreatment. Control values as well as effects of sodium iodate and Sm alone are also shown. Significance: A-C $p<0.03$, A-E n.s., A-G $p<0.003$, B-D n.s., B-F n.s., B-H $p<0.03$, D-H n.s., F-H $p<0.03$.

As expected, the total opsin content of normal animals is slightly, but not significantly higher than the rhodopsin content (1.93 ± 0.25 versus 1.76 nmol/eye). Remarkably, the total opsin content is slightly but not significantly lowered upon treatment with sodium iodate (1.62 ± 0.36 nmol/eye) in contrast to the rhodopsin levels (0.17 ± 0.02 nmol/eye). Sm alone did change neither the rhodopsin (1.53 ± 0.16 nmol/eye) nor the opsin content (1.95 ± 0.24 nmol/eye). However, the opsin/rhodopsin ratios change from 1.1 ± 0.13 for the controls to 1.26 ± 0.08 for animals treated with Sm alone ($p<0.04$).

Exp. 3. Effect of cancer cachexia

In this experiment (Fig. 4) Walker 256 carcinosarcoma bearing rats were treated for 5 consecutive days with Sm at a daily dose of 0.15 or 0.3 mg/kg. The dose of Sm was reduced because of the increased toxicity of the drug in these tumor-bearing animals. Two animals did not survive the treatment with 0.15 mg Sm/kg, while one animal did not survive the 0.3 mg Sm/kg dosage. All animals, including controls were emaciated and lost 12 ± 9 % of their body weight at randomization (tumor included). Tumor growth by itself did not influence the rhodopsin levels (1.79 ± 0.2 versus 1.75 ± 0.07 nmol/eye in the untreated controls). Treatment with Sm produced the normal pattern: slight decrease in rhodopsin content (0.15 mg/kg - 1.65 ± 0.1 nmol/eye; 0.30 mg/kg - 1.7 ± 0.2 nmol/eye). The differences were not significant.

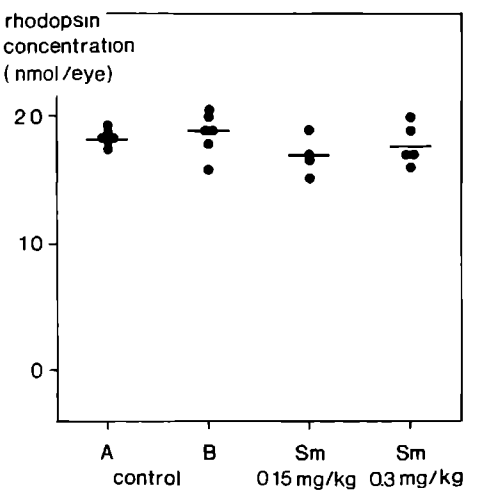

Fig. 4. Mean rhodopsin concentrations in eyes of Wistar rats bearing Walker 256 carcinosarcoma s.c. and treated for 5 days with Sm either 0.15 mg/kg or 0.3 mg/kg. Control values of tumor free (A) and untreated tumor bearing animals (B) are also included.

Exp. 4. Influence of Sm treatment on RCS rats

In this experiment 20 days old RCS rats were treated with four doses of 0.5 mg Sm/kg/day (Fig. 5). Animals were sacrificed on days 25 and 31. Rhodopsin concentrations in untreated rats increased linearly from 1.5 ± 0.2 nmol/eye on day 21 to 2.8 ± 0.1 nmol/eye on day 31. The Sm treatment produced only a small (7.7%) decrease of rhodopsin concentration on day 25 (1.93 ± 0.2 nmol/eye versus 2.09 ± 0.14 nmol/eye in the controls), consistent with the previous results. The differences were not significant.

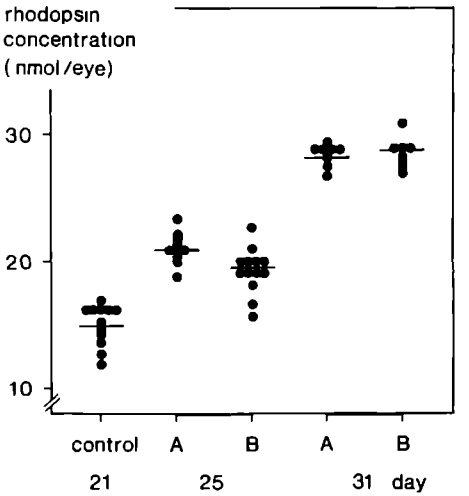

Fig. 5. Mean rhodopsin concentrations in eyes of RCS rats treated on days 20-24 with 0.5 mg Sm/kg (B). Untreated controls on day 21 as well as on day 25 and 31 (A) are presented for comparison.

DISCUSSION

Before entering clinical study with one of the new Sm analogues, several important questions should be addressed.

1. Are the retinotoxic effects observed really due to Sm ?
2. If yes, can retinotoxicity be reproducibly induced by Sm in animal models and is the retinotoxicity of Sm the consequence of inhibition of protein biosynthesis?
3. Does Sm or its analogues penetrate the blood-retina barrier ?

Ad.1. Other factors than Sm may be responsible for retinotoxicity.

The "natural" Sm originally used in the phase I study might not be pure. Contaminants may have been responsible for enhancement of its toxicity and

retinotoxic potential. The "natural" Sm showed a LD50 in mice of 4.32 mg/kg (National Cancer Institute, 1964) compared to 11.3 mg/kg for synthetic, highly purified Sm (7). However, the only contamination of a Sm sample isolated from natural sources and generously supplied by the National Cancer Institute, Bethesda, MD, was a considerable percentage of isosparsomycin as revealed by HPLC analysis (unpublished). Isosparsomycin is a stereoisomer of Sm formed under the influence of light, and might have accumulated during the 20 years of storage. Isomerization of Sm to isosparsomycin would increase rather than decrease the LD50 as isosparsomycin is not active as inhibitor of protein biosynthesis (15).

It can, however, not be excluded that the retinotoxicity is due to isosparsomycin by a mechanism different from inhibition of protein biosynthesis. Isosparsomycin bears some structural similarities to 11-cis-retinal (Fig.6), the chromophore of the rod and cone visual pigments: rhodopsin, iodopsin etc. Therefore, possible competitive inhibition of regeneration of opsin by Sm or isosparsomycin was investigated in vitro. Even at a 1000-fold molar excess to opsin both compounds only slightly inhibited the regeneration of opsin with 11-cis-retinal to rhodopsin (De Grip, personal communication).

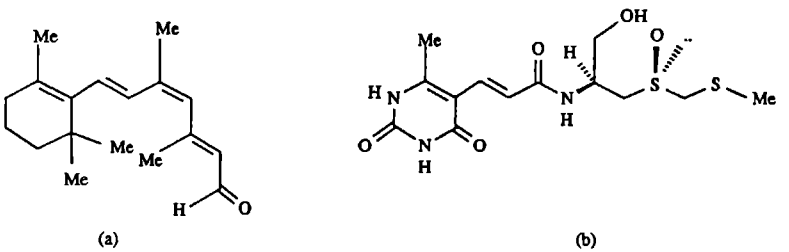

Fig. 6. Structure of (a) 11-cis-retinal and (b) sparsomycin

Another possibility is that synergistic toxicity of Sm occurs with other drugs administered to the patients in the phase I clinical trial. One of the patients that developed retinal damage received, concomitantly with Sm, thioridazine (2). This drug has a recognized retinotoxic potential, similar to that produced by chloroquin (16). Thioridazine is a lipophilic drug accumulating in RPE and is responsible for disruption of membranes. Both chloroquin and thioridazine inhibit the appearance of newly synthesized proteins in the rod outer segment in vitro (16). However, some authors suggest that chloropromazine (similar to thioridazine) may be toxic to the

retina by inducing fotosensitization (17). The possibility of synergistic action between Sm and thioridazine can not be excluded and should be investigated in the future.

Ad.2. Sm itself has little effect on rhodopsin levels.

Finally it should be considered that upon penetration into the RPE and the retina, Sm may inhibit biosynthesis of opsin or of other short living protein(s) involved in degradation of rhodopsin or in retinol transport and/or metabolism. As a result of this, levels of (rhod)opsin in the eye should change upon administration of Sm, and these levels should be indicative for the ocular toxicity of Sm. Complete inhibition of rhodopsin biosynthesis in bovine retinas in vitro by puromycin has been demonstrated by O'Brien (18). The present study was designed to investigate whether Sm could provoke similar effects in vivo. Hydrophilic drugs, like Sm (octanol/water partition coefficient log P = - 1.7 (19) is unlikely to be able to penetrate an undamaged blood-retina barrier, and it is improbable that it would reach sufficient intraocular concentrations to inhibit opsin biosynthesis for a prolonged time (20). Hence it is not surprising that administration of Sm daily for 10 days at the maximally tolerable dose did not significantly change the ocular rhodopsin content.

In all experiments reported here Sm produced a small but not significant decrease in rhodopsin content. Interestingly this decrease was acute and also appeared already 30 minutes after the first injection in dark adapted animals. Hence this probably is not an effect on biosynthesis of opsin or regeneration of rhodospin, but should reflect preturbation of the shedding and phagocytose of the outer segment tops.

Ad.3. Blood-retina barrier.

Another factor that may increase intraocular Sm concentrations is accumulation in plasma. Data on plasma Sm concentrations in humans are not available but we have found indications for accumulation in mice (7), beagle dogs (21) and now in rats (see Fig. 2). We considered the possibility that combination of blood-retina barrier leakiness and increased plasma Sm concentrations may be responsible for the Sm-induced retinal toxicity. However, our results do not indicate any correlation between an increase in plasma concentrations and ocular rhodopsin levels. To test this hypothesis further, we needed to study the effect of a defective blood-retina barrier.

The blood-retina barrier may be disrupted by different pathological conditions (22-24). Artificially, the blood-retina barrier may be disrupted

by application of strongly oxidizing metabolic poisons like sodium iodate (25). Our experiments with this compound showed that the artificial increase of blood-retina barrier leakiness is accompanied by serious changes in retinal metabolism. A dramatic loss of rhodopsin is observed, in agreement with earlier findings (26), but the opsin levels are barely affected. This indicates that due to the toxic effects of sodium iodate on the RPE (25) the regeneration of rhodopsin from opsin is blocked, probably by interference somwehere in the visual cycle (reformation of 11-cis-retinal by RPE). The fact that the opsin levels are almost maintained may be due to (A) maintenance of opsin biosynthesis (photoreceptors) and degradation (RPE) at nearly similar rates or (B) complete block of both processes. If the alternative (B) would be correct, treatment with Sm could not produce a reduction in opsin level as we observed. Hence, we conclude that in the early stage of iodate poisoning, opsin biosynthesis and degradation still proceed and that under those conditions (defective blood-retina barrier, severe metabolic stress) Sm is able to exert an inhibitory action on protein biosynthesis, thereby much less affecting phagocytosis and degradation by RPE. It is difficult to assess from the present data whether this inhibition by Sm is complete or only partial. If biological turnover of opsin in mammals is 8-10 days (8), complete inhibition of biosynthesis for 5 days should decrease the opsin level by 50-60% while we observed a decrease of only 27%. Since the catabolic potential of the RPE may also be affected by sodium iodate (25) this means that rod protein biosynthesis is inhibited under these conditions by Sm, somewhere between 50 and 100%. Alternatively, development of the blood-retina barrier leakiness may be delayed by 2-3 days. Flage et al. (25) reported the first gross morphological changes in the rabbit retina 4 days after sodium iodate treatment.

The RCS rat differs from the normal animal by a genetic deficiency in outer segment phagocytosis in RPE (27). In these animals the continuous biosynthesis of opsin by the rod cell results in a linear increase of rhodopsin concentrations up to the fifth week of life, providing rats are kept in the dark (28). After this time the retinas become dystrophic and animals turn blind. Caldwell and Laughlin (29) showed that the blood-retina barrier of RCS rats is leaky from about the 3rd week of life. This leakiness is probably due to the relaxation of the tight junctions. In this system, treatment with Sm had no significant effect on rhodopsin levels. Hence, defective blood-retina barrier is not per se sufficient to produce a retinotoxic action of Sm.

Two patients that developed retinopathy after treatment with Sm weighed

both only 50 kg and suffered from a far advanced stage of cancer. It is thus possible that both of them were cachectic and in poor general condition. Another patient treated for 17 days with a dose similar to that administered to the above two patients did not develop retinopathy but his initial body weight was 92 kg. Thus it may be speculated that poor general condition may be a risk factor for Sm-induced retinotoxicity. However, experiments with tumor bearing rats failed to show cachexia as a factor responsible for Sm-induced retinotoxicity. The constant rhodopsin values seen in these animals suggest a high priority for opsin biosynthesis and activation even during cachexia.

Conclusion

Our experiments failed to identify a possible mechanism for the alleged retinal toxicity of Sm. Increase of blood-retina barrier permeability by sodium iodate possibly allows Sm to penetrate the retina and inhibit opsin biosynthesis, but these circumstances were extreme in that a strong metabolic poison was required, which was not likely to resemble any condition in vivo. Vision in these rats was already severely affected due to rhodopsin deficiency afore Sm was administered.

Our general conclusion is that the hydrophilic Sm is unlikely to be able to diffuse through the undamaged blood-retina barrier but even a defective barrier is not sufficient to promote Sm retinotoxicity. Only in synergism with secondary factors like strong metabolic poisons, or pathological conditions like retinitis pigmentosa or diabetes mellitus Sm may show retinotoxic action. Whereas, general cancer cachexia seems to have no synergistic effect. While we fail to observe strong retinotoxic action of Sm under conditions which do not deviate from normal, we still feel that Sm analogues which are more lipophilic than the parent drug and show higher antitumor activity produce a higher risk of retinotoxicity and should be investigated for their potential ocular toxicity in the same animal models.

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CHAPTER 14

GENERAL DISCUSSION AND PROSPECTS

The preclinical investigations carried out and reported in this thesis were done in order to select the best analogue for clinical studies. The results indicate that some Sparsomycin (Sm) analogues are less toxic and more active than the parent drug. The three analogues with the most promising activity are deshydroxy-Sm, ethyl-deshydroxy-Sm and n-pentyl-Sm. They showed high activity on three ascitic murine tumor models but were not active on four solid tumors. When the ascitic tumor sensitive to Sm analogues (L1210 leukemia) was inoculated s.c. and the drugs were administered i.v., only ethyl-deshydroxy-Sm showed some activity. On another tumor (RC renal cell carcinoma) s.c. all three analogues were equally active. No activity was found on four human tumor xenografts (gastric, mammary, colon and ovary cancers) in nude mice (Fiebig, personal communication).

However, Sm showed interesting anticancer potential when combined with other cytotoxic agents. Used together with cisplatin, Sm showed significant potentiation of cisplatin antitumor activity in vitro and in vivo without increasing its toxicity. Ethyl-deshydroxy-Sm in combination with cisplatin is the only analogue that shows high activity on L1210 leukemia growing s.c. This striking synergy opens new possibilities in cancer chemotherapy.

Cisplatin is not the only drug that can be potentiated by Sm. Several other compounds, i.e. 5-fluorouracil, methotrexate, cytosine arabinoside, vincristine and bleomycin, also show promising synergy with Sm in vitro. In all experiments performed by us on Sm and by others on different PSI, the sequence of drug administration was critical for the cytotoxic effect. These observations should play a key role in further clinical and in studies concerning the mechanism of action.

Sm and its analogues do not induce bone marrow toxicity. This increases their attractiveness in combination with other anticancer drugs.

Our data indicate that Sm-induced inhibition of protein synthesis may act as a specific modifier of the cytotoxicity of different anticancer agents. Studies should be performed in order to elucidate the mechanisms of the synergy and its potential to kill malignant cells selectively without increasing toxicity to the normal cells.

Further research on Sm analogues should confirm potentiation of cisplatin activity on different murine tumors as well as in human tumor xenografts in nude mice. Potentiation of activity of other anticancer drugs should also be studied in the same way.

The data obtained until now seems to indicate that ethyl-deshydroxy-Sm will be probably the drug of choice for further preclinical studies. The relatively higher toxicity of ethyl-deshydroxy-Sm found in beagle dogs

should not interfere with the choice of this compound for clinical studies if the compound will prove to be active on other tumors. Non-linear pharmacokinetics found in dog, would be dangerous if the drug would be administered daily for a longer time. Synergy between Sm and cisplatin was found when Sm and cisplatin were applied every 4 days. Moreover, the doses necessary to induce optimal effect were lower than the LD10 for Sm.

The drug of choice should be subjected to detailed toxicological evaluation in animals before its use in humans. These studies should be performed with this compound alone and in combinations with other cytostatics. If this drug will not increase toxicity of the second anticancer drug, then the phase I study in man should be also performed with combinations of drugs, for example with cisplatin and increasing dose of ethyl-deshydroxy-Sm.

During toxicological evaluation special attention should be paid to the potential retinotoxicity of the compound. Although extensive investigations were performed on rats in order to study the possible mechanisms of Sm retinotoxicity, no effect of Sm on the retina was found. It is probable, that Sm does not penetrate the blood-retina barrier because it is too hydrophilic. Future studies should test whether more lipophilic analogues, which seem to be more active, do penetrate the blood-retina barrier and induce retinotoxicity.

In conclusion: The use of Sm analogues is an exciting new perspective in cancer chemotherapy. These protein synthesis inhibitors seems to be especially promising in combination with other cytotoxic compounds and not as single drug. Ethyl-deshydroxy-Sm is probably the drug of choice for further preclinical and clinical studies. Future investigations directed to the mechanisms of action and proper scheduling of the drugs are necessary prior to the clinical application of this approach.

Sparsomycin (Sm) is a well known inhibitor of protein synthesis with an anticancer potential. The first clinical trial with this drug derived originally from natural sources, in 1964, was unsuccessful. In 1981 Ottenheijm and co-workers succeeded in the chemical synthesis of Sm. Since this time their main goal was development of less toxic and more active analogues. More than fifty compounds were prepared and studied in vitro for their inhibitory activity. These studies, as well as history, chemistry and biological activity of selected Sm analogues, are reviewed in Chapter 2.

Newly synthesized analogues were tested for their inhibitory activity in vitro on cell free E. coli ribosomes and in L1210 leukemia clonogenic assay. The relation between structural modifications, physicochemical properties, and biological activity is described in Chapter 3.

Two types of structural modification appeared to be important for activity. First, the introduction of a more lipophilic side chain replacing the methyl group caused higher affinity for the ribosomal binding site. Compounds with 5-6 carbon atoms in the aliphatic side chain showed an optimal affinity. These compounds too, showed the highest activity on L1210 leukemia in vitro. Second, removal of the hydroxyl group proved to be critical for the activity in vivo as well as for the physicochemical properties of these deshydroxy compounds. Deshydroxylated analogues were slightly more lipophilic than their hydroxylated counterparts but were significantly better water soluble and showed a favourable LD50 in mice. These compounds too, had distinctive high antitumor activity. The above mentioned two modifications i.e. introduction of a more lipophilic side chain and removal of the hydroxyl group, were combined in the compound ethyl-deshydroxy-Sm. This compound showed a high LD50 and good antitumor activity in several murine tumor models.

Chapter 4 presents a detailed antitumor activity study in eight murine tumor models. Compounds with high in vitro activity showed the increased LD50 in mice. This increase was directly proportional to the lipophilicity of the drug. Three Sm analogues, i.e. n-pentyl-Sm, deshydroxy-Sm and ethyl-deshydroxy-Sm, were found to be the most active analogues. However, activity was manifested only in rapidly growing ascitic tumors, i.e. P388 and L1210 leukemias and RC renal cell carcinoma (T/C = 150 - 600%). When the sensitive tumors (L1210 or RC) were inoculated s.c. and the drug was administered i.v., there was loss of activity for all compounds. On L1210 leukemia s.c. only ethyl-deshydroxy-Sm showed some activity (T/C = 139%). On RC tumor s.c. all three analogues were equally moderately active (T/C= 138 -

150%). There was only slight activity on B16 melanoma and no activity on four solid tumors: C22LR osteosarcoma, C38 colon carcinoma, LL - Lewis lung carcinoma and M5076 sarcoma.

Stimulated by data from the literature on modification of cytotoxicity of some anticancer drugs by protein synthesis inhibitors, the in vitro modulation of cisplatin cytotoxicity by inhibition of protein synthesis with Sm was studied (Chapter 5). These investigations were done with CHO cells. Low Sm concentrations, inhibiting protein synthesis by 20%, protected cells from cisplatin-induced cytotoxicity. Higher Sm concentrations enhanced cisplatin cytotoxicity. Both effects were highly significant. These results were independent of the inhibition of the cell cycle. Data obtained from these studies formed an important cornerstone for further investigations on the synergistic activity of Sm analogues with other cytotoxic drugs.

The same principle of synergism was investigated in L1210 bearing mice (Chapter 6.1). Sm administered 3-6 hours prior to the administration of cisplatin, potentiated significantly the antitumor activity of the latter drug. In the optimal combination, 66% of the animals were cured from leukemia, while cisplatin alone could not produce any cure. In this study, host protection against cisplatin-induced toxicity was also observed. Mice pretreated with Sm lost significantly less weight due to cisplatin. Additionally, the only drug that was active on L1210 leukemia s.c., ethyl-deshydroxy-Sm, was found to have a significant potentiating effect on cisplatin activity (Chapter 6.2). Cisplatin alone as well as ethyl-deshydroxy-Sm alone did not show any significant activity. Combination of the two drugs resulted in a T/C of 313% and a single cure. Deshydroxy-Sm administered in combination with cisplatin in the same tumor model showed only weak and insignificant potentiation of the cisplatin effect.

In a subsequent in vitro study, Sm was found not only to influence the activity of cisplatin (Chapter 7). The combination of Sm with seven classical anticancer agents was tested on CHO cells. Preincubations with Sm antagonized the activity of the following S-phase specific agents: methotrexate, 5-fluorouracil, cytosine arabinoside and vincristine. Postincubation with Sm instead, potentiated their effect. A similar, but less significant effect, was observed with doxorubicin. In contrast, preincubation with Sm enhanced the activity of non-S-phase specific compounds, namely cisplatin and bleomycin, but not of melphalan. Postincubations were ineffective or less effective. The results of this study indicate some potentially useful combinations of Sm with other cytotoxic drugs to be investigated in vivo.

Somewhat disappointing results of antitumor activity testing with Sm analogues prompted us to study the relationship between pharmacokinetics, activity and toxicity in mice (Chapter 8). In this study the area under the curve (AUC) at the LD10 was determined for Sm and its three promising analogues. Increase of the lipophilicity of the drug increased its total body clearance. Lipophilic compounds were also more extensively bound to plasma proteins. Deshydroxy-Sm (VI) was twice as rapidly cleared from the plasma as Sm, but its AUC at the LD10 was lower than that of Sm. Ethyl-deshydroxy-Sm was the only drug that showed a 2.5 fold increase of the AUC at the LD10 in comparison to Sm. Significant increase of the LD10 for this compound is probably related to the fact that ethyl-deshydroxy-Sm has a different dose-limiting toxicity in mice than hydroxylated compounds. Ethyl-deshydroxy-Sm induced no haemorrhages in mice, which were observed after treatment with toxic doses of all hydroxylated analogues.

The pharmacokinetic studies were later extended to beagle dogs (Chapter 9). In these investigations, pharmacokinetic studies were correlated with toxicity. Beside the determination of Sm pharmacokinetics for a single dose, it was found that Sm tends to accumulate in plasma when administered in sequential daily doses. This may explain the toxicity seen after several days of treatment. All the plasma proteins which were studied upon treatment with Sm disappeared from the plasma proportionally to their biological half-life. Thus, if Sm acts only through inhibition of protein synthesis, than the toxicity should be due to disappearance of critical proteins. In beagles, disappearance of plasma fibrinogen was found critical for development of haemorrhagic diathesis seen in 2 out of 5 dogs treated with Sm for 9 days.

Pharmacokinetic studies in beagle dogs were performed also with three promising analogues. In Chapter 10 the plasma and renal pharmacokinetics of deshydroxy-Sm are presented. Chapter 11 describes the pharmacokinetics of ethyl-deshydroxy-Sm and Chapter 12 describes the pharmacokinetics of n-pentyl-Sm. All three compounds, as well as the parent drug Sm, behaved somewhat differently in dogs than in mice. In mice, linear pharmacokinetics up to the LD10 were seen in all cases. In dogs, non-linear pharmacokinetics were found for ethyl-deshydroxy-Sm and n-pentyl-Sm. Ethyl-deshydroxy-Sm was found to be relatively more toxic in comparison to the other analogues, while n-pentyl-Sm produced the highest non-lethal AUCs. Non-linearity of ethyl-deshydroxy-Sm pharmacokinetics was related to extensive, active re-absorption of the drug from the renal tubuli. The same process was observed in case of n-pentyl-Sm but due to its high lipophilicity this compound was,

extensively eliminated by non-renal clearance and active tubular reabsorption played only a relatively minor role.

In the last chapter (Chapter 13) the results of several experiments on Sm-induced eye toxicity in rats are presented. No indication of Sm penetration through the blood-retina barrier was found in healthy animals and therefore it can be concluded that the risk of retinotoxicity should be very low. However, when the blood-retina barrier is disrupted, for example by sodium iodate, Sm partially inhibits opsin biosynthesis. The histopathological appearance of Sm-induced retinotoxicity could not be reproduced in these studies.

SPARSOMYCINE EN ZIJN ANALOGA

een preklinisch onderzoek naar nieuwe geneesmiddelen tegen kanker

Sparsomycine (Sm) is een bekende eiwitsyntheseremmer met mogelijke anti-kanker activiteit. Het eerste klinische onderzoek met Sm, verkregen door isolatie uit bacteriestammen, bij mensen in 1964 is mislukt. In 1981 slaagde Ottenheijm en medewerkers erin Sm synthetisch te maken. Sindsdien heeft deze groep in samenwerking met onze afdeling zich gericht op het ontwikkelen van minder toxische en meer actieve analoga. Meer dan 50 derivaten zijn gesynthetiseerd en in vitro bestudeerd. De resultaten hiervan, evenals de geschiedenis, chemie en biologische activiteit zijn beschreven in **Hoofdstuk 2.**

Nieuw gesynthetiseerde analoga zijn getest op hun remmende activiteit van de eiwit synthese in een celvrij systeem (dat gebruik maakt van ribosomen van E. coli) en op L1210 leukemie cellen in de clonogene assay. Het onderzoek naar de relatie tussen structuurmodificaties, fysicochemische eigenschappen en biologische activiteit is weergegeven in **Hoofdstuk 3.** Twee modificaties blijken voor de activiteit met name belangrijk te zijn. Op de eerste plaats, het invoeren van alkyl groepen op het bivalente zwawel atoom waardoor een hogere lipofiliteit ontstaat en daardoor een hogere affiniteit tot de ribosomale bindingsplaats optreed. Een optimale affiniteit wordt bereikt met stoffen die 5 tot 6 koolstofatomen in de alifatische zijketen hebben. Deze stoffen laten de hoogste activiteit op L1210 leukemie cellen in vitro zien. Op de tweede plaats blijkt dat verwijdering van de hydroxyl groep van belang is voor de biologische activiteit en de fysicochemische eigenschappen. Gedeshydroxyleerde analoga zijn iets meer lipofiel dan hun gehydroxyleerde equivalenten, maar ze zijn significant beter wateroplosbaar en vertonen een gunstige LD50 bij muizen. Gedeshydroxyleerde verbindingen onderscheiden zich door een hoge antitumoractiviteit. Deze twee modificaties: hogere lipofiliteit en deshydroxylering zijn aangebracht in de stof ethyl-deshydroxy-Sm. Deze stof vertoont een hoge LD50 en een goede antitumor activiteit bij de verschillende muizentumoren.

Hoofdstuk 4 geeft een gedetailleerd overzicht van de onderzoeksresultaten betreffende antitumoractiviteit van Sm-analoga bij 8 muizentumoren. Sm-derivaten, die in vitro een hoge remmende aktiviteit vertonen, laten een toename van de LD50 bij muizen zien. Deze toename is evenredig met de lipofiliciteit. Drie analoga: n-pentyl-Sm, deshydroxy-Sm en ethyl-

deshydroxy-Sm blijken de meest actieve analoga te zijn. Deze aktiviteit manifesteert zich alleen bij de snel groeiende ascitesvormende tumoren te weten P388 en L1210 leukemieën en RC renal cellcarcinoma (T/C = 150 - 600%). Wanneer de gevoelige tumoren (L1210 en RC) s.c. geïnoculeerd worden en de stoffen i.v. toegediend worden, blijkt er een verlies van activiteit voor alle derivaten op te treden. Voor L1210 leukemie s.c. vertoont alleen ethyl-deshydroxy-Sm enige activiteit (T/C = 139%). Voor de RC tumor s.c. zijn alle drie analoga even actief (T/C = 138 - 150%). Er wordt een geringe activiteit voor B16 melanoom en geen activiteit voor de volgende 4 solide tumoren C22LR osteosarcoom, LL Lewis longcarcinoom, M5076 sarcoom en C38 coloncarcinoom waargenomen.

Op grond van gegevens uit de literatuur over de modificatie van de cytotoxiciteit van sommige antikankermiddelen door remming van de eiwitsynthese, werd Sm in combinatie met andere cytostatica getest (**Hoofdstuk** 5). Dit onderzoek is verricht op CHO cellen. Het bleek dat lage Sm-concentraties, die 20% van de eiwitsynthese remmen, de cellen beschermen tegen de cytotoxiciteit van cisplatinum. Hogere Sm-concentraties daarentegen, potentiëren de activiteit van cisplatinum. Beide effecten zijn statistisch significant. Deze effecten zijn onafhankelijk van de remming van de celcyclus. De resultaten verkregen tijdens dit onderzoek, vormen de hoeksteen voor verdere onderzoeken betreffende het synergisme tussen Sm en analoga en andere cytotoxische middelen.

Een soortgelijk synergisme is onderzocht in muizen met L1210 leukemie (**Hoofdstuk** 6.1). Indien Sm 3 - 6 uur voor cisplatinum wordt toegediend, wordt de antitumoractiviteit van laatstgenoemde verbinding, significant gepotentieerd. In een optimale combinatie kon 66% van de dieren van leukemie worden genezen, terwijl cisplatinum alleen geen genezing tot stand kon brengen. In dit onderzoek werd ook bescherming tegen cisplatinum-geïnduceerde toxiciteit geconstateerd. Muizen voorbehandeld met Sm, blijken significant minder gewicht te verliezen als gevolg van cisplatinum-behandeling.

Ethyl-deshydroxy-Sm, het enige middel dat actief is tegen L1210 leukemie cellen s.c., blijkt ook een significant versterkend effect op de cisplatinum activiteit te hebben (**Hoofdstuk** 6.2). Zowel cisplatinum alleen als ethyl-deshydroxy-Sm alleen, toonden geen significante activiteit. Een combinatie van beide middelen resulteerde in een T/C van 313 % en in een langdurige overleving bij een van de zes muizen. In hetzelfde tumormodel vertoont deshydroxy-Sm in combinatie met cisplatinum, een zwak en niet-significant effect.

In het in vitro onderzoek dat volgde, blijkt Sm ook de activiteit van andere cytostatica te beinvloeden (Hoofdstuk 7). Een combinatie van Sm met een van 7 klassieke antikankermiddelen is op CHO cellen getest. Voorbehandeling met Sm antagoniseert de activiteit van de volgende S-fase specifieke middelen: methotrexaat, 5-fluorouracil, cytosine arabinoside en vincristine. Nabehandeling met Sm daarentegen, versterkt hun effect. Eenzelfde, hoewel minder significant effect, wordt gezien met doxorubicine. Voorbehandeling met Sm verhoogt de activiteit van de niet S-phase specifieke stoffen: cisplatinum en bleomycin, maar niet de activiteit van melfalan. Deze resultaten geven aan dat het zinvol is om bepaalde combinaties van Sm met andere cytotoxische middelen in vivo nader te bestuderen.

De tegenvallende resultaten van de antitumor activiteit van Sm-analoga heeft ertoe geleid, de relatie tussen farmacokinetiek, activiteit en toxiciteit in muizen verder te onderzoeken (Hoofdstuk 8). Daartoe werd de oppervlakte-onder-de-curve (AUC) bij LD10 bepaald voor Sm en 3 veel belovende analoga. Een hogere lipofiliteit van Sm-derivaten veroorzaakt een hogere totale lichaamsklaring en meer binding aan plasma-eiwitten. Deshydroxy-Sm wordt tweemaal zo snel uit het plasma verwijderd dan Sm, hoewel zijn AUC bij LD10 lager is dan die van Sm. Ethyl-deshydroxy-Sm is het enige analogon welke een verhoging van de AUC bij LD10 te zien geeft t.o.v. Sm. De significante toename van de LD10 en LD50, waargenomen voor deze stof, is waarschijnlijk gerelateerd aan het feit dat ethyl-deshydroxy-Sm een andere dosisbeperkende toxiciteit vertoont dan de gehydroxyleerde analoga. Ethyl-deshydroxy-Sm induceert geen bloedingen bij muizen, wat wel het geval is met de gehydroxyleerde derivaten.

De farmacokinetische onderzoeken zijn later uitgebreid naar beagle honden (Hoofdstuk 9). In dit onderzoek wordt farmacokinetiek gecorreleerd aan toxiciteit. Naast een eenmalige dosis, werd Sm ook meerdere dagen achtereen toegediend. Er werden bij de langdurige toedieningsvorm aanwijzingen gevonden voor cumulatie van Sm in het plasma. Dit zou de toxiciteit kunnen verklaren die na enkele dagen optreedt. Het bleek dat alle bestudeerde plasma-eiwitten tijdens de behandeling met Sm, uit het plasma verdwijnen met een snelheid evenredig aan hun biologische halfwaarde-tijd. Hieruit kan worden geconcludeerd dat, indien Sm uitsluitend werkt als eiwit-synthese remmer, de toxiciteit moet worden toegeschreven aan het verdwijnen van bepaalde kritieke eiwitten. Bij beagle honden blijkt het verdwijnen van het fibrinogeen verantwoordelijk te zijn voor het optreden van de haemorhagische diathese, welke bij 2 van de 5 behandelde honden na 9 dagen Sm-behandeling is waargenomen.

Farmacokinetische onderzoeken in beagle honden zijn ook uitgevoerd met de drie meest belovende analoga. **Hoofdstuk 10** gaat in op de farmacokinetiek van deshydroxy-Sm in plasma en urine. **Hoofdstuk 11** en **12** beschrijven de farmacokinetiek van respectievelijk ethyl-deshydroxy-Sm en n-pentyl-Sm. Alle drie genoemde derivaten, evenals de moederverbinding Sm, geven een verschillend gedrag te zien in honden en in muizen. Muizen vertonen voor alle derivaten een lineaire farmacokinetiek tot aan de LD10. Honden vertonen voor ethyl-deshydroxy-Sm en n-pentyl-Sm een niet lineaire kinetiek. Ethyl-deshydroxy-Sm is relatief de meest toxische stof van de bestudeerde analoga, terwijl n-pentyl-Sm de hoogste niet-lethale AUC geeft. Het feit dat de farmacokinetiek van ethyl-deshydroxy-Sm niet lineair verloopt, wordt verklaard door een sterkere, actieve reabsorptie in de renale tubuli. Dit geldt ook voor n-pentyl-Sm, maar deze stof is veel lipofieler, waardoor eliminatie via andere wegen dan de nieren veel belangrijker wordt en dus ook de actieve tubulaire reabsorptie relatief veel minder belangrijk is.

In het laatste hoofdstuk (**Hoofdstuk 13**) wordt aandacht besteed aan de Sm-geinduceerde oogtoxiciteit bij ratten. Er zijn geen aanwijzingen gevonden dat Sm in gezonde dieren de bloed-retina barriere zou kunnen passeren. Het risico van retinotoxiciteit zal daarom uitermate klein zijn. Is echter de bloed-retina barriere beschadigd (bv. door natriumiodaat, een sterk oxiderend toxisch middel), dan is Sm in staat de biosynthese van opsine gedeeltelijk te remmen. De bij de Sm-geinduceerde retinotoxiciteit optredende histopathologische verschijnselen konden in dit onderzoek niet worden gereproduceerd.

Velen hebben bijgedragen aan het totstandkomen van dit proefschrift, en het is onmogelijk om hier allen bij naam te noemen. Allereerst wil ik de leden van de sparsomycine werkgroep bedanken, die vier jaar lang, iedere maand bijeen kwamen om de meest recente resultaten te bespreken en het toekomstige beleid uit te stippelen. Zij waren mij tot steun en stimuleerden mij met hun kritiek en enthousiasme. Met name wil ik noemen Wim de Grip, die mij wegwijs maakte in de wereld van de retina-biochemie, Leon van den Broek, die voor mij nieuwe sparsomycine-analoga synthetiseerde, Dick de Vos met zijn Pharmachemie-medewerkers die een oogje in het zeil hielden met betrekking tot de gang van zaken en mij met beide benen op de grond lieten belanden wanneer mijn fantasie met mij op de loop dreigde te gaan.

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Histologische preparaten zijn met grote deskundigheid beoordeeld door Prof. Dr. U. van Haelst van de Afdeling Pathologie, St. Radboud Ziekenhuis.

Veel bloed en urine monsters zijn verwerkt door het laboratorium van de Afdeling Hematologie en het Klinisch Chemisch Laboratorium, beide van het St. Radboud Ziekenhuis. De heer H. Dickhaut en zijn medewerkster van het magazijn voor verpleegkundige artikelen, zorgden ervoor dat ik voor ieder karwei, altijd het geschikte gereedschap had. Ik wil tevens de Maurits en Anna de Kock Stichting danken voor het financieren van een biologische werkbank voor het steriel voorbereiden van cel kweken. De dames van de bereiding van de afdeling Klinische Farmacie zorgden voor de vlotte en probleemloze voorbereiding van sparsomycine-oplossingen. De heer E. de Graaf en Mw S. Bakker van de Medische Bibliotheek hielpen bij het verzamelen van de literatuur. Mw Patsy Metzger-Anderson corrigeerde mijn "Poolse" Engels en maakte er Amerikaans Engels van.

Tom Vree en zijn medewerkers van het Laboratorium Onderzoek en Ontwikkeling van de Klinische Farmacie, net zoals de hele staf van deze afdeling, stond altijd klaar om mij te helpen bij het voorbereiden van manuscripten en oplossen van grote en kleine analytische en farmacologische problemen. Met name wil ik hier noemen Eveline Wuis, Emiel Termond, Ita Baars, Elly Kimenai, Marijn Oosterbaan en Rita Dirks. Marijcke Schoots van de Siepkamp verzorgde met grote nauwkeurigheid de lay-out van dit proefschrift.

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Mw. ing. Kasia Adams-Debicka heeft veel tekeningen voor dit proefschrift gemaakt.

En nu, "last but not least" wil ik mijn vrouw Joke en kinderen: Staś, Margot, Bennie, Hanneke en Wanda bedanken voor alles wat jullie in die afgelopen vier jaar voor mij betekend hebben. Mijn vrije tijd die aan het totstandkomen van dit boekje besteed is, is jullie ontnomen. Ik hoop dat jullie daar nu begrip voor kunnen opbrengen.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 13 juni 1955 te Wroclaw (Polen). In 1973 behaalde hij het einddiploma van het Algemeen-vormend Lyceum nr. 3 te Gdańsk (Polen). Daarna studeerde hij geneeskunde aan de Medische Academie te Gdańsk, waar hij in 1979 het artsexamen cum laude aflegde. Aansluitend is hij begonnen met een 7 maanden durend onderzoek naar toxische effecten van industrieel afval op de luchtwegen (afdeling Fysiopathologie, Medische Academie te Gdańsk, hoofd: Prof. Dr. J. Stolarczyk). Begin 1980 emigreerde hij samen met zijn gezin naar Nederland. Daar heeft hij stages gelopen in de huisartsenpraktijk te Wezep bij Dr. D. Post en Drs. H.A.M.H. Verberk en op de afdeling interne geneeskunde van het Ziekenhuis de Weezenlanden te Zwolle (hoofd destijds: Dr. A.F. Casparie). Daarna werkte hij enkele maanden bij de bloedbank Zwolle (hoofd: Mw. Drs. A.G. Russchen - Dekker). Op 1 februari 1981 is hij met de opleiding interne geneeskunde begonnen, aanvankelijk in het Sophia Ziekenhuis te Zwolle (opleider: Dr. T. Tjabbes) en vanaf 1 october 1982 in het St. Radboud Ziekenhuis te Nijmegen (opleider: Prof. Dr. A. van 't Laar). Inschrijving in het specialistenregister volgde op 13 november 1987. Vanaf 1 februari 1984 tot op heden, was hij werkzaam op de afdeling medische oncologie (hoofd: Prof. Dr. D.J.Th. Wagener) en verrichtte hij een onderzoek (subsidie Stichting Koningin Wilhelmina Fonds, SNUKC-84-1) waarvan dit proefschrift het resultaat is. In de loop van 1988 zal hij een opleiding tot hematoloog beginnen op de afdeling Bloedziekten van het St. Radboud Ziekenhuis (hoofd: Prof. Dr. C. Haanen). Hij is getrouwd met Joke Loor en zij hebben vijf kinderen: Staś, Margot, Bennie, Hanneke en Wanda.

Stellingen behorende bij het proefschrift:

SPARSOMYCIN AND ITS ANALOGUES

A preclinical study on novel anticancer drugs

Z. Zylicz

1. De antitumoractiviteit van sparsomycine en zijn analoga kan niet alleen verklaard worden door remming van de eiwitsynthese. Enkele structuuranaloga vertonen in vitro en in vivo een grotere antitumoractiviteit dan sparsomycine terwijl het slechte remmers van de eiwitsynthese zijn.

(dit proefschrift)

2. Bij de beoordeling van de toxiciteit van een reeks van analoga, is het zinvol om bij de bepaling van de lethale dosis (LD10) ook de oppervlakte onder de concentratie-curve (bij de LD10) te betrekken.

(dit proefschrift)

3. Het is niet de loutere aanwezigheid van een polaire groep, die de wateroplosbaarheid van een molecuul waarborgt.

(dit proefschrift)

4. De kracht van sparsomycine-analoga ligt niet in de eigen antitumoractiviteit, maar in de modulatie van de activiteit van andere cytotoxische middelen.

(dit proefschrift)

5. De waarheid dient als een mantel te worden aangereikt, en niet als een natte doek die om de oren wordt geslagen.

(M. Frisch)

6. The dissatisfied dead cannot noise abroad the negligence they have experienced.

(A. Hinton)

7. Het gebruik van superabsorberende papieren luiers bij de verzorging van zuigelingen, vertraagt de ontwikkeling van de fysiologische reflex "nat-vies-huilen" en het optreden van zindelijkheid.

(eigen waarneming gemaakt tijdens het promotie-onderzoek, doch buiten de gewone werkuren)

8. You can never judge another man until you will walk in his moccasins.

(oud Indiaans gezegde)