The apoptosis pathway : a drugable target in solid tumors

Groot, Derk Jan Alko de

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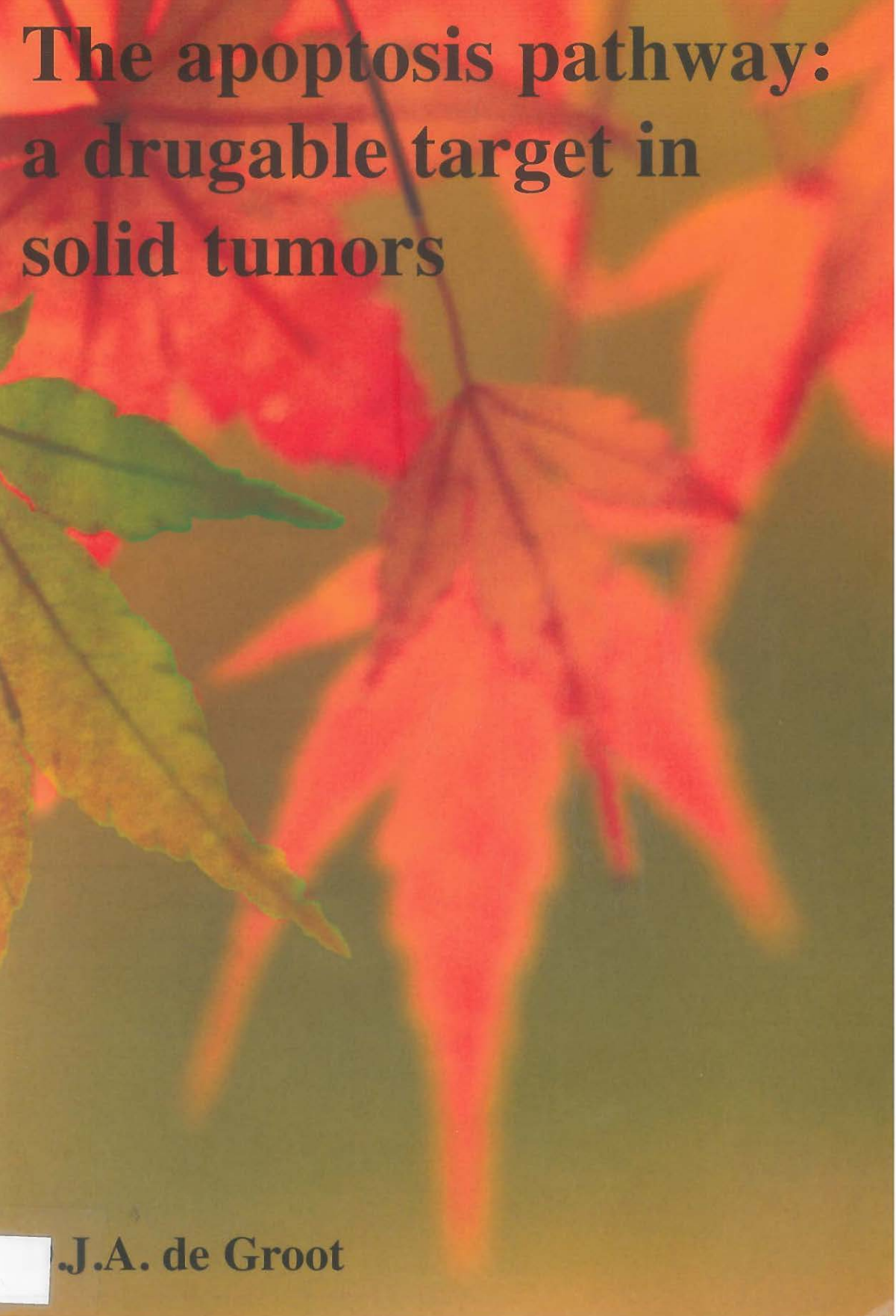

J.A. de Groot

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D.J.A. de Groot
2006

Stellingen

behorende bij het proefschrift
'The apoptosis pathway: a drugable target in solid tumors'

1. NSAIDs zijn interessante medicijnen voor klinische studies in kankerpatiënten om te beoordelen of het effect van chemotherapie ermee verhoogd kan worden.
(Dit proefschrift).

2. Met de opkomst van meer doelgerichte middelen voor het behandelen van solide tumoren kunnen op basis van kennis van de werkingsmechanismen van deze middelen rationele keuzes voor de te testen combinaties gemaakt worden.
(Dit proefschrift).

3. Het multimeriseren van een death ligand levert een potenter apoptose-inducerend ligand op.
(Dit proefschrift).

4. De ABC transpoter MRP1, die door verhoogde efflux, resistentie voor meerdere chemotherapeutica induceert, is tevens de factor die verworven chemotherapie resistente kleincellig longkankercellen gevoelig maakt voor een ontstekingsremmer.
(Dit proefschrift).

5. Verhoging van Fas membraanexpressie en Fas Ligand transport over de celmembraan verhoogt de apoptose inductie door de combinatie cisplatine en indomethacine in ovariumkankercellijnen.
(Dit proefschrift).

6. Anti-oestrogenen verhogen het apoptose-inducerende effect van rhTRAIL.
(Dit proefschrift).

7. XIAP is de enige echte directe caspaseremmer.
(Eckelman et al EMBO reports 2006)

8. Het gebrek aan caspase-8 expressie in neuroblastoomcellen is essentieel voor metastasering.
(Stupack et al Nature 2006)

9. Het aantonen van een bepaald mechanisme in cellijnen van verschillende tumortypes betekent niet dat dit mechanisme een rol speelt in alle betreffende tumortypes.

10. De tijd die een student geneeskunde heeft voor gesuperviseerd patiëntencontact dient meer te zijn dan de tijd nodig voor het verzamelen van handtekeningen.

11. Een tumorbioloog moet een sadist zijn. Eerst worden met veel liefde kankercellen opgekweekt om ze daarna met satanisch genoegen te vergiftigen.

12. Het feit dat iemand je de wind uit de zeilen neemt is niet zo erg, het moet je immers eerder voor de wind zijn gegaan.

13. Hockeyballen zijn gemaakt van uitstekend stressabsorberend materiaal.

14. Noodzaak is de moeder van uitvinding.
(Plato 427 v. Chr. - 347 v. Chr.)

The studies described in this thesis were supported by the MD/PhD program of the University Medical Center Groningen Junior Scientific Masterclass and grant 99-1880 of the Dutch Cancer Society

The printing of this thesis was financially supported by Stichting Werkgroep Interne Oncologie Groningen, Groningen University Institute for Drug Exploration (GUIDE) and Faculteit der Medische Wetenschappen.

De Groot, D..J.A.
The apoptosis pathway: a drugable target in solid tumors
Dissertation University of Groningen. With summary in Dutch.

ISBN: 90-367-2889-4

Drukwerk advies Jaap Reiding
Lay-out: E.P. de Groot
Cover design: B.H. de Groot

Rijks*universiteit* Groningen

The apoptosis pathway: a drugable target in solid tumors

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 20 december 2006
om 16:15 uur

door

Derk Jan Alko de Groot
geboren 26 december 1976
te Groningen

Promotor:	Prof. dr. E.G.E. de Vries
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ISBN: 90-367-2889-4

Contents

1

General introduction

Apoptosis is a key mechanism responsible for controlling the number of cells during embryogenesis, selection in the immune system, development of the central nervous system and tissue homeostasis. Defects in the regulation of the apoptosis machinery may lead to cell survival beyond the intended lifespan of cells or premature death of cells. The result of such deregulation may vary between cancer versus autoimmune and neurodegenerative diseases. Deregulation of apoptosis pathways can also cause decreased chemotherapy sensitivity in tumors and therefore knowledge of apoptosis induction and novel approaches to modulate apoptosis induction in malignancies is needed. Apoptosis can be induced by death ligands activating membrane bound death receptors and hereby activating an intracellular cascade of caspases resulting in apoptosis. Intracellular triggers can also activate caspases and cause apoptosis. COX inhibitors also known as nonsteroidal anti-inflammatory drugs are used to reduce inflammation, but recently it has been found that these agents also contain anti-tumor features. These features are among others apotosis induction and modulation of apoptosis pathways. This thesis focuses on activation and modulation of death receptor apoptosis pathways in human models of solid tumors that are known for developing chemoresistance.

In **chapter 2** the role of COX inhibitors in cancer therapy is reviewed with emphasis on modulation of the different apoptosis pathways based on preclinical models. The rationale behind the combination of COX inhibitors in combination with conventional chemotherapeutic drugs in the treatment of patients with solid tumors is then discussed.

Drug resistance is the major reason for chemotherapy failure in small cell lung cancer treatment. A common drug resistance mechanism of tumor cells is the inability to go into apoptosis. In **chapter 3** the apoptosis inducing potential of the COX inhibitor indomethacin was investigated in a doxorubicin resistant small cell lung cancer cell line panel GLC_4 and GLC_4-Adr. Doxorubicin resistance in GLC_4-Adr could be circumvented by exposure to indomethacin while the parental cell line GLC_4 was relatively resistant to indomethacin-induced apoptosis. This study aimed to evaluate the possibility of circumventing chemotherapy resistance by exposure to COX inhibitors.

In **chapter 4** the mechanism by which indomethacin induces apoptosis in the doxorubicin resistant cell line GLC_4-Adr and not in the parental cell line GLC_4 was further investigated. The differences between these cell lines were evaluated and one of the major differences, overexpression of the ABC transporter MRP1 was investigated by determining indomethacin-induced apoptosis after downregulation of MRP1 with RNA interference. A substrate for MRP1 is glutathione and therefore intracellular gluthathione levels were evaluated to

determine wheter gluthathione levels are of importance for indomethacin-induced apoptosis.

Resistance to cisplatin–based chemotherapy frequently occurs and is the major reason for chemotherapy failure in ovarian cancer treatment. To increase cisplatin sensitivity, apoptosis induction by the cyclooxygenase COX-1/COX-2 inhibitor indomethacin and the COX-2 inhibitor celecoxib was investigated in a p53 mutant ovarian cancer cell line model in **chapter 5**. In addition, the apoptotic ability of these drugs in combination with death ligand agonistic anti-Fas antibodies and cisplatin was investigated.

Resistance to chemotherapy as well as to anti-hormonal therapy is a major problem in the treatment of breast cancer patients. Combinations of chemotherapeutic agents and death receptor ligands have been extensively investigated in breast cancer cell line models. The combination of anti-hormonal agents and death receptor ligands, however, has not been thoroughly evaluated yet. In **chapter 6** the combination of anti-estrogens and the death receptor ligand TNF related apoptosis inducing ligand (TRAIL) was investigated in an intrinsic rhTRAIL resistant and a rhTRAIL sensitive cell line model.

In vivo systemic use of the death receptor ligand rhFas Ligand (rhFasL) or anti-Fas antibodies is not possible due to the massive liver toxicity of these agents. To decrease liver toxicity a modified rhFasL was constructed by conjugating six rhFasL molecules with a linker protein sequences. In **chapter 7** efficacy of this Mega Fas Ligand (MFL) was tested in a panel of different chemotherapy and death ligand resistant cell lines. Apoptosis induction of MFL was investigated as well as MFL in combination with the drug for which resistance was induced. Factors in the Fas apoptosis pathway that determine MFL sensitivity were also evaluated. In **chapter 8** the results of this thesis are summarized and future perspectives are given.

2

Nonsteroidal anti-inflammatory drugs to potentiate chemotherapy effects. From lab to clinic

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In press Crit Rev Oncol Hematol.

1. Abstract

Most solid tumors express the cyclooxygenase-2 (COX-2) protein, a target of NSAIDs. COX-2 overexpression in tumors is considered a predictor of more advanced stage disease and of worse prognosis in a number of studies investigating solid malignancies. Therefore, NSAIDs are evaluated as anticancer drugs. NSAIDs inhibit proliferation, invasiveness of tumors, and angiogenesis and overcome apoptosis resistance in a COX-2 dependent and independent manner. This review will focus on the rationale behind NSAIDs, including selective COX-2 inhibitors, in combination with conventional chemotherapeutic drugs or novel molecular targeted drugs. Studies investigating anti-cancer effects of NSAIDs on cell lines and xenograft models have shown modulation of the Akt, NF-κB, tyrosine kinase and the death receptor-mediated apoptosis pathways. COX-2 expression in tumors is not yet used as biomarker in the clinic. Despite the increased risk on cardiovascular toxicity induced by selective COX-2 inhibitors, several ongoing clinical trials are still investigating the therapeutic benefits of NSAIDs in oncology. The antitumor effects in these trials balanced with the side effects data will define the precise role of selective COX-2 inhibitors in the treatment of cancer patients.

2. Introduction

Solid tumors are one of the leading causes of death in the Western countries with an increasing number of cancer patients every year. Although the prognosis of these patients has improved the last decade, there is still a need for novel treatment modalities. Therefore new targets for anti-cancer treatments are sought. From large retrospective and prospective population-based studies it was learned that regular use of both non-selective non-steroidal anti-inflammatory drugs (NSAIDs), and selective cyclooxygenase-2 (COX-2) inhibitors is associated with an important decreased incidence of colorectal, breast, bladder, prostate as well as lung cancer [1-7].

Preclinical data suggested that the inhibition of COX-2 is responsible for this decrease in cancer incidence. In addition there is increasing evidence that selective and non-selective COX-2 inhibitors have COX-2 independent effects that can account for the anti-tumor effect of these agents. Moreover, data from cell lines and animal models have shown that NSAIDs in combination with chemotherapy enhances efficacy or can even circumvent drug resistance. Similar findings have been described for NSAIDs in combination with novel molecular targeted therapeutics. This review will focus on potential benefits of selective or non-selective COX-2 inhibitors added to conventional or experimental cancer treatments. COX-2 dependent and COX independent mechanisms for this sensitization will be described.

3. Physiological function of cyclooxygenases

3.1. Cyclooxygenases

Cyclooxygenase (COX) is the enzyme that catalyses the conversion from arachidonic acid to prostaglandins (PGs) [8]. There are three isoforms of COX, COX-1, COX-2 and COX-3. The *COX-1* gene was cloned by three separate groups in 1988 [9-11]. In 1991, Xie *et al.* discovered an inducible *COX* gene named *COX-2* [12], while *COX-3* was discovered in 2002, being a splice variant of *COX-1* [13]. COX-1 is involved in maintenance of the gastric mucosa, in regulation of renal blood flow in the afferent vessels of the kidney and in regulation of platelet aggregation. The second isoform, COX-2 is an inducible isoform, which is involved in inflammation and tumorigenesis. The third isoform, COX-3 is involved in anti-inflammatory reactions and the production of anti-inflammatory PGs [13].

COX-2 has two functionalities, i.e. cyclooxygenase activity to oxydate arachidonic acid and peroxidase activity to convert chemicals, from for instance tobacco smoke, into highly reactive mutagens that can bind to DNA [14]. PGs and thromboxane A_2 are the end products of the conversion of arachidonic acid by COX. The process of converting arachidonic acid into PGs and thromboxane is initiated by the conversion of arachidonic acid into prostaglandin H_2 (PGH_2). PGH_2 is converted by tissue specific isomerases into five primary active structurally related PGs. These PGs include PGE_2, PGD, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2) via tissue specific PG synthetases [15-18]. Eight types of prostaglandin receptors have been recognized. All are membrane-bound G-protein coupled receptors encoded by different genes. COX-2 expression is induced by several mitogenic and proinflammatory stimuli including basic fibroblast growth factor [19], transforming growth factor β1 [20], epidermal growth factor [21], vascular endothelial growth factor (VEGF) and Tumor Necrosis Factor alpha (TNFα), lipopolysaccharide, and interleukins 1α and 1β [22]. Transcriptional upregulation of COX-2 can occur by NF-κB, cAMP response element, nuclear factor-interleukin 6, PEA3, nuclear factor of activated T cells 1, Ras/Raf/MAPK, AP-2, and SP-1 [23-27].

3.2. COX knock-out mice models

Knock-out mice have been made to investigate the physiologic function of COX-1 and COX-2. Mice lacking COX-2 developed cardiac fibrosis, nephropathy and peritonitis. The causes of death of mice three weeks after birth were peritonitis or kidney malfunction [28]. Male COX-2 null mice were fertile while females were infertile. In all COX-2 null mice kidney morphology was abnormal. The kidneys were paler and smaller than those of COX-2 wild-type mice with less and poorly

developed glomeruli. In addition, renal tubuli were more dilated and atrophied compared to those in wild-type mice [28].

3.3. NSAIDs

Most research with respect to COX-1 and COX-2 protein function, however, was performed with NSAIDs. There is 60% homology between the amino acid structures of COX-1 and COX-2 [9-11]. The COX-2 cyclooxygenase active site has a slightly larger hydrophobic side-pocket than the active site of COX-1, which can be used to make COX-2 selective inhibitors [29-31]. Selective COX-2 inhibitors were designed to prevend peptic ulcer, gastrointestinal bleeding, and/or perforation of gastroduodenal ulcers which is associated with prolonged use of NSAIDs. The NSAID aspirin inhibits cyclooxygenases by covalent binding to the active site of the enzyme, while all other NSAIDs and COX-2 inhibitors inhibit cyclooxygenase enzymatic activity by competing with the substrate for the active site. Although aspirin binds to COX-1 and COX-2 at a similar site, arachidonic acid can still be converted by COX-2 due to the larger active site of COX-2 [32]. The relative potencies as inhibitors of COX-1 and COX-2 for non-selective NSAIDs as well as selective COX-2 inhibitors are illustrated in Figure 1 [33-35]. Selectivity for either COX-1 or COX-2 can be observed at relatively low concentrations of the COX inhibitor. All COX inhibitors, however, can inhibit both COX-1 and COX-2 at higher concentrations because of the homology of the active sites of these enzymes.

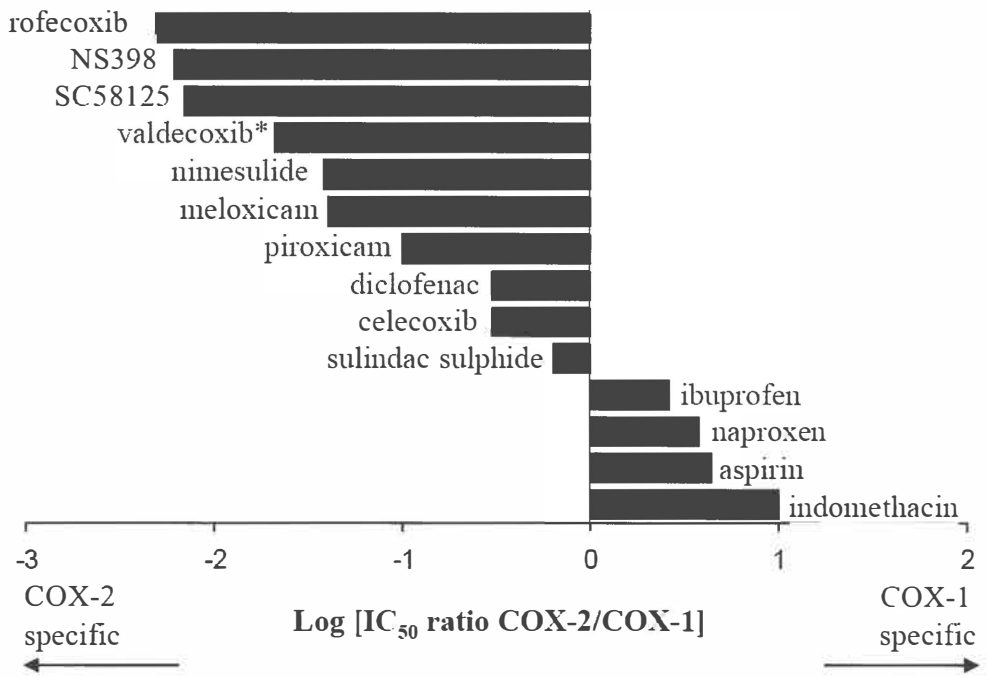

Figure 1. COX-1 and COX-2 selectivity of different drugs as measured by the William Harvey Human Modified Whole Blood Assay [33]. * Measured by using stimulated peripheral human monocytes [34].

The anti-cancer effects of NSAIDs are often observed at concentrations that exceed the COX-2 inhibitory concentration. Therefore these anti-cancer effects are considered to be partially COX-2 independent. For instance, COX-2 independent apoptosis induction of celecoxib has been described in HT29 colon cancer cells. Celecoxib-mediated apoptosis is induced in these cells by 3-phosphoinositide-dependent kinase (PDK1) inhibition. PDK1 can phosphorylate Akt and therefore celecoxib is an indirect inhibitor of the Akt pathway. To prove that PDK1 is the most important target of celecoxib-mediated apoptosis a constitutive active PDK1 mutant was introduced in the HT29 cells. Overexpression of the constitutive active mutant of PDK1 ($PDK1^{A280V}$) inhibited apoptosis as potent as the pancaspase inhibitor zVAD [36].

4. Potential role of COX-2 in cancer development

4.1. COX-2 and carcinogenesis

The role of COX-2 and therefore NSAIDs in cancer development and cancer chemoprevention has been extensively reviewed in the past [37-40]. To investigate the role of COX-2 in cancer development in more detail a number of

COX-2 knock-out models were used. In heterozygous adenomatous polyposis coli *(Apc)* knock-out mice all the animals develop intestinal polyps [41]. The role of COX-2 expression in this polyp formation was investigated by studying double knockout mice with a heterozygous *Apc* knockout and a homozygous COX-2 knockout genotype. These double knockout mice developed 86% less polyps compared to the COX-2 wild-type mice [42]. This effect was dose dependent because the COX-2 heterozygous knock-out mice showed an intermediate reduction of polyp formation. Another mouse model addressing the role of COX expression in carcinogenesis is the multiple intestinal neoplasia (*Min*) mouse model. The *Min* mice also have a chemically induced nonsense mutation in the *Apc* gene and all these mice develop intestinal neoplasia, although the polyp formation is less than in *Apc* knock-out mice. When *Min* mice are crossed with COX-1 or COX-2 null mice on a C57Bl/6 background, polyp formation was decreased by 70-80% [43, 44]. Therefore it can be concluded that COX-1, as well as COX-2, play a key role in intestinal carcinogenesis.

Overexpression of COX-2 in transgenic mice using a mammary gland specific murine mammary tumor virus promoter caused mice to develop mammary cancer after successive rounds of pregnancy. This did not happen in virgin females, probably because proliferating epithelial cells in the pregnant mammary gland are more susceptible to mutagenic events in critical tumor suppressor genes. Mice that received the empty vector containing the MMV promoter did not develop mammary tumors [45].

4.2. COX-2 and tumor angiogenesis

Neoangiogenesis is a crucial event in tumorigenesis and development of metastases. Tumor growth beyond 2–3 mm in size requires neovascularization and inhibition of angiogenesis at this stage is therefore a potential important target in cancer therapy. One of the first observations indicating that COX proteins may be of importance in angiogenesis was described in a study published in 1997 [46]. Both COX-1 and COX-2 are involved in tumor vascularization. COX inhibitors can directly affect angiogenesis [47]. PGI_2 regulates endothelial sprouting as well as VEGF-induced vascular permeability [48-50]. PGE_2 can induce VEGF production by activation of two different pathways, the ERK2/JNK1 pathway and by translocation of hypoxia-inducible factor from the cytosol to the nucleus [51, 52].

Angiogenesis was blocked in nude mice carrying Colon-26 cells after treatment with diclofenac. There is mounting evidence that PGs participate in angiogenesis, regulating the production of proangiogenic factors such as VEGF [48, 53]. COX-1 mediated induction of endothelial cell tube formation as was

shown by Tsujii et al [54]. The increased PG production can stimulate non-malignant stromal cells to produce VEGF. The VEGF than stimulates local endothelial cells to proliferate into the tumor [51]. Interestingly, stromal cells from COX-2 knock-out mice show a 94% reduction in VEGF levels compared to wild-type fibroblasts. Wild-type fibroblasts exposed to a selective COX-2 inhibitor also had a 92% lower VEGF expression [55].

High PGE_2 levels have been associated with metastatic disease in breast cancer patients due to increased proliferation and angiogenesis, which also suggests autocrine and paracrine signaling [56, 57]. A study in breast cancers in which COX-2 expression was compared to VEGF expression by confocal immunofluorescence analysis showed a positive correlation between COX-2 and VEGF expression [58].

4.3. COX-2 and tumor progression

Progression of tumors can be induced by hormones such as estrogens and androgens, but also by PGs or by downregulation of anti-apoptotic proteins. In breast cancer PGE_2 overexpression in the tumor induces aromatase production in human adipose stromal cells in the breast and concomitantly estrogen production, which stimulates tumor proliferation [59]. In human breast cancers a strong correlation between COX-2 expression and cytochrome P450 enzyme aromatase (CYP 19) was found [60]. Thus, COX-2 may be the cause of progression of estrogen-dependent breast cancer either directly by stimulating tumor-cell proliferation, or indirectly by upregulating aromatase activity [60]. The LS-174 colorectal carcinoma cell line, which does not generate detectable prostaglandins, increases DNA synthesis and growth after addition of PGE_2 to the culture medium [61]. These effects were most likely caused by activation of the PI3K/Akt pathway as PI3K/Akt inhibitors also inhibited the PGE_2-mediated increased tumor growth. This activation of the PI3K/Akt in turn is most likely regulated by PG-coupled G proteins. In colon cancer cell lines COX-2 also provides cells with a more invasive and metastatic phenotype compared to cells with less COX-2 expression as was observed in COX-2 transfected Caco-2 cells [62]. With the same technique, invasion caused by COX-2 overexpression is investigated in the MDA-231 breast cancer cell line. COX-2 overexpression in the MDA-231 breast cancer cell line enhanced cell motility and invasiveness as was investigated with Matrigel invasion experiments thus suggesting a mechanism of COX-2 stimulated metastasis [63].

5. NSAIDs and radiotherapy

Upregulation of prostaglandin synthesis after irradiation is a tumor protective effect. Selective COX-2 inhibitors have also been described to enhance

radiotherapy efficacy primarily by inhibition of angiogenesis [55]. This is a COX-2 dependent effect because neutralization of COX-2 derived PGE_2 has the same effect as celecoxib exposure *in vivo* in Col26 colon cancer cells. In this study, tumor vasculature was measured with contrast magnetic resonance imaging (MRI) [64]. Apart from its involvement in angiogenesis, COX-2 overexpression can also directly affect radiosensitivity of tumor cells as shown in eight oral squamous cell carcinoma cell lines [65]. The level of the COX-2 expression in these oral squamous cell carcinoma cell lines correlated with increased tumor radiation resistance [65]. The COX-2 expressing NCI-H460 and A549 cells were sensitized for radiotherapy by exposure to celecoxib. Downregulation of COX-2 reduced the radiation-enhancing effects of celecoxib in A549 cells. In contrast, the COX-2 nonexpressing MCF-7 and HCT-116 cells were not radiosensitized by celecoxib. HCT-116 cells, transfected with COX-2 expression vector, were also radiosensitized by celecoxib, demonstrating that radiosensitization by celecoxib occurs in a COX-2 dependent manner. Reduced production of PGE_2 after celecoxib treatment was not instrumental, since the addition of PGE_2 had no effect on the radiosensitizing effects of celecoxib. Thus, the question remains via which mechanism COX-2 inhibition by celecoxib affect radiosensitivity [66].

6. NSAIDs and chemotherapeutic agents.

6.1. Combination of NSAIDs and chemotherapy in cell line models

6.1.1. NSAIDs to bypass conventional chemotherapy resistance

One of the first steps to investigate the efficacy of NSAIDs in cancer therapy is to combine them with conventional chemotherapeutic agents. Part of the rationale for combining NSAIDs with chemotherapy involves circumvention of chemotherapy resistance mechanisms.

The Bcl-2 family of pro and anti-apoptotic proteins promotes or inhibits apoptosis at the mitochondrial level. Bcl-2 family members confer a clinically important resistance to chemotherapeutic agents in a number of hematologic and solid malignancies, including acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, chronic lymphoblastic leukemia, multiple myeloma, prostate cancer, malignant brain tumors, and neuroblastoma [67-72]. Downregulation of Bcl-2 increases toxicity for chemotherapeutic agents in a number of tumor cell lines [73, 74]. NSAIDs such as the selective COX-2 inhibitors SC-58125 and NS-398 can downregulate Bcl-2 and subsequently induce apoptosis in colon and prostate cancer cell lines [75]. On the other hand, an increase in proapoptotic Bax protein and a decrease in Bcl-X_L protein can also induce apoptosis as shown in HCT 116 colorectal carcinoma cells [76]. Celecoxib,

aspirin and indomethacin could induce apoptosis by Bak and Bax upregulation, mitochondrial membrane potential loss and activation of caspase-3 [77-79] (Fig 2).

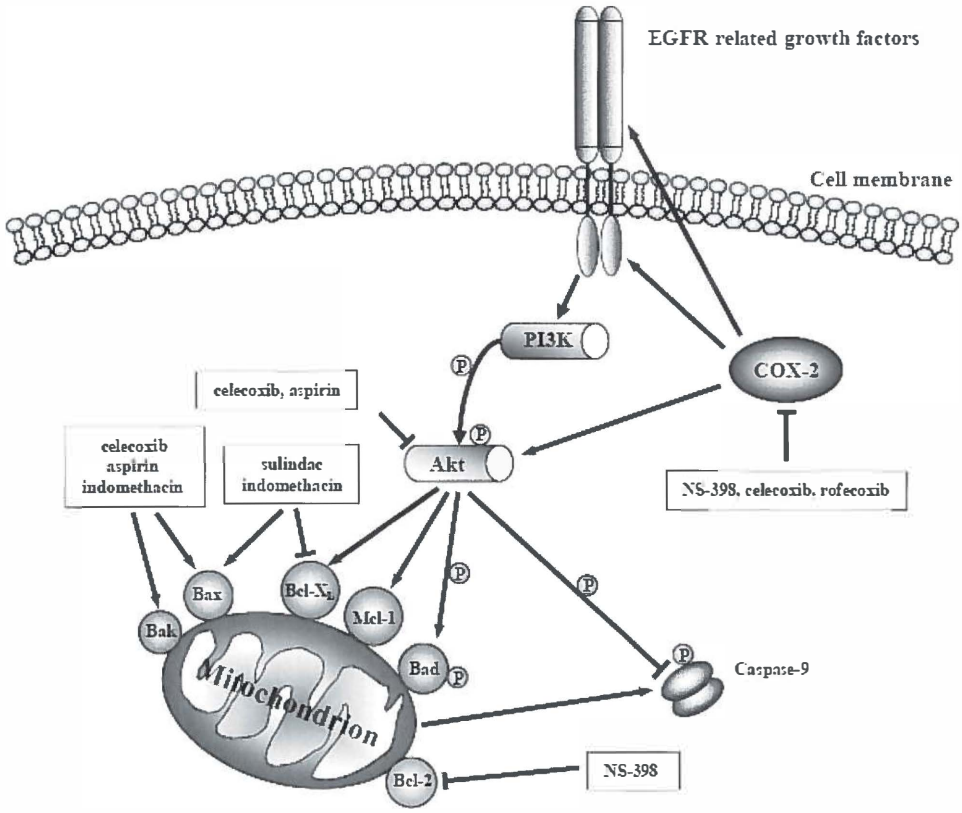

Figure 2. Akt can be upregulated by COX-2, while both Akt and COX-2 are inhibited by NSAIDs. Inhibition of Akt prevents phosphorylation and inactivation of Bad and caspase-9 resulting in decreased apoptosis. Different NSAIDs can modulate expression of Bcl-2 family members. Modulation of Bcl-2 family members involves downregulation of anti-apoptotic members and upregulation of pro-apoptotic members.

There is also an indirect way of targeting the Bcl-2 family of anti-apoptosis proteins namely by inhibiting Akt signaling. Akt/PKB is a serine/threonine protein kinase that functions as a critical regulator of cell survival and proliferation. In recent years, it has been demonstrated that PI3K/Akt signaling components are frequently altered in human cancers. Increased extracellular signaling by growth factors and increased signaling of intracellular components of the Akt pathway such as Ras are frequently observed. The third mechanism of increased Akt

signaling is the decreased activity of Akt regulatory proteins. Akt phosphorylation is associated with phosphorylation and thus inactivation of the pro-apoptotic proteins Bad and caspase-9. Phosphorylated Bad cannot bind to Bcl-2, while phosphorylated caspase-9 cannot be activated resulting in a reduced activity of mitochondrial apoptosis [80, 81]. Inhibition of Akt phosphorylation by celecoxib and aspirin leads to apoptosis in the human A549 non-small cell lung cancer cell line [82]. In an *in vivo* model of spontaneous metastatic breast cancer celecoxib-induced apoptosis correlated with a significant decrease in Akt activation [83]. Celecoxib also suppressed Akt phosphorylation and kinase activity in cultured human C611B cholangiocarcinoma cells, which correlated with Bax translocation to mitochondria, cytochrome c release into the cytosol, followed by activation of caspase-9 and caspase-3. Addition of PGE_2 to these cells blocked the apoptotic actions of celecoxib [84]. In another study with cholangiocarcinoma cell lines celecoxib induced reduction of Akt phosphorylation, whereas the absolute cellular Bcl-2 and Bax levels remained unaltered. Akt protein was inhibited by LY294002 decreasing the viability of these cell lines. These effect were partially COX-2 mediated because after addition of PGE_2 to the growth medium apoptosis induction was decreased [85]. Stable transfection of the human lung adenocarcinoma cell line CL1.0 with the *COX-2* gene results in activation of the PI3K/Akt-dependent pathway, which promotes cell survival by PI3K/Akt dependent up-regulation of the Mcl-1 protein level (Fig 2) [86].

The inhibitors of apoptosis (IAPs) are a family of proteins that function as intrinsic regulators of the caspase cascade. Members of this family include, survivin, c-IAP1, c-IAP2, XIAP, livin, NAIP, ILP-2 and Bruce. These proteins have been identified to regulate the activity of both initiator (caspase-9) and effector caspases (caspase-3 and -7). IAPs have also been implicated in decreased tumor responses in cytotoxic therapy by several experimental studies. In ovarian cancer cell lines it has been shown that the ability of cisplatin to down-regulate XIAP may be an important determinant of chemosensitivity. Antisense downregulation of XIAP expression increased cisplatin sensitivity in cisplatin sensitive cell lines and to a lesser extend in cisplatin resistant cell lines [87-89]. XIAP overexpression in the human myeloid leukemia cell line U-937 suppressed apoptosis in vitro following treatment with 1-[β-D-arabinofuranosyl]cytosine [90]. In this type of chemotherapy resistance NSAIDs can enhance therapy efficacy by modulating IAP expression [91]. In intestinal epithelial cells PG signaling protects normal and transformed intestinal epithelial cells from apoptosis by, rapid induction of cellular inhibitor of apoptosis protein (c-IAP) 2 and delayed induction of another member of the IAP family, livin. Inhibition of COX-2 in cells overexpressing this enzyme decreases c-IAP2 expression and promotes apoptosis,

both of which are reversible by PGE2 addition [91]. COX-2 protein overexpression in non-small cell lung cancer (NSCLC) cell lines stabilizes survivin by decreasing ubiquitination of survivin, thus increasing resistance to apoptosis [92]. This effect was also described after exogenous PGE_2 exposure of COX-2 non-overexpressing tumor cells [92, 93]. Exposure of HT-29 colon carcinoma cells to sulindac decreased survivin mRNA and protein expression [94].

NF-κB is a member of the Rel family of transcription factors usually present in the cytoplasm as hetero or homodimers. NF-κB exerts its anti-apoptotic or apoptotic effects through transcriptional activation or inactivation of specific genes, a process that only occurs in the nucleus. NF-κB activity is tightly controlled through regulation of NF-κB translocation to the nucleus. In the cytoplasm, NF-κB is bound to inhibitor molecules alpha (IκBα) or I kappa B beta (IκBβ). When bound to IκB, NF-κB cannot enter into the nucleus. IκB can be phosphorylated by IκB kinase, which results in proteasomal degradation of IκB and subsequent translocation of NF-κB to the nucleus. Constitutive NF-κB activation, observed in many malignant tumors, protects the cells from apoptotic stimuli, such as anticancer treatments. NF-κB can also confer resistance to cytotoxic therapy for instance by upregulation of anti-apoptotic proteins such as c-IAP1, c-IAP2, XIAP, TRAF1, TRAF2, Bfl-1/A1, Bcl-X_L, or c-FLIP (Fig 3) [95]. Interestingly numerous factors including ionizing radiation and certain chemotherapy agents can induce NF-κB activation. Therefore downregulation of NF-κB activity could be a target for chemosensitization. *In vivo* administration of NF-κB siRNA by adenoviral delivery reduces HCT116 tumor formation in xenograft models in the presence but not the absence of the chemotherapeutic drug irinotecan [96]. Xenografts of HT1080 fibrosarcoma cells transfected with a modified IκBα, which is a NF-κB inhibitory protein, are more sensitive to irinotecan and TNF exposure [97]. Thus NF-κB inhibition can enhance *in vivo* sensitivity to chemotherapeutic agents. Inhibition of NF-κB is one of the COX-2 independent mechanisms by which NSAIDs induce apoptosis [98]. Aspirin inhibits IκB kinase (IKKβ). This prevends the phosphorylation and subsequent degradation of IκB. Due to an increase in IκB/ NF-κB complexes, NF-κB is trapped in the cytoplasm and transcriptionally inactive [99] (Fig 3). A similar effect, i.e. inhibition of IKKβ activity, was reported for sulindac [100]. Unlike aspirin and sulindac, the COX-2 inhibitor SC236 affected neither the phosphorylation, degradation, nor expression of IκB-α. Instead, SC236 worked directly on NF-κB suppressing the nuclear translocation of NF-κB [101]. Celecoxib suppresses constitutively active NF-κB and drug-induced activation of NF-κB by a number of agents, such as TNF, phorbol ester, okadaic acid, lipopolysaccharide (LPS), and IL-1β without cell type specificity [102].

NSAIDs can also decrease chemotherapy resistance by inhibition of members of the ATP binding cassette family of drug transporters. In COR L23R, DLKP and A549 human lung cancer cell lines anthracycline resistance was circumvented by co-exposure to NSAIDs such as meclofenamic acid, diclofenac, naproxen, fenoprofen, phenylbutazone, flufenamic acid, flurbiprofen, ibuprofen and ketoprofen [103]. Celecoxib can decrease, in a COX-2 independent manner, multidrug resistance protein-1 (MRP1) expression in A549 lung cancer cells and therefore increase anthracycline sensitivity [104]. In addition, in HL-60 human leukemia cells incubation with the NSAID meloxicam also resulted in decreased MRP1 expression. This downregulation, however, is COX-2 dependent, because preincubation of these cells with PGE2 neutralized the increase in chemotherapy sensitivity [105]. In NCI H460 lung cancer bearing mice, sulindac increased the anti-cancer effect of doxorubicin [106].

The modulation of NSAIDs on chemotherapy resistance mentioned in the former section was mostly anthracycline resistance. Modulation of platinum containing chemotherapeutic agents occurs through different pathways. For instance the selective COX-2 inhibitor JTE-522 increases cisplatin sensitivity by decreasing Bcl-2 expression in T24 bladder cancer cells [107]. Oxaliplatin and the NSAID etodolac decreased survivin expression and increased death and growth inhibition in RKO colon cancer cells [108]. On the other hand NSAIDs can also inhibit platinum containing chemotherapy efficacy. Nimesulide inhibits the cytotoxic effect of cisplatin in head and neck squamous cell cancer cell lines [109].

6.1.2. NSAIDs combined with novel molecular targeted therapeutics

Recent data on combinations of NSAIDs and novel molecular targeted anti-cancer therapeutics have provided evidence that there are cross-talks between pathways targeted by COX inhibitors and other molecular targeted therapies. Activation of Her-2/Her-3 tyrosine kinase signaling in colorectal cancer cell lines resulted in elevated COX-2 expression levels and subsequently in an increase in PGE_2 production [110, 111]. Upregulation of COX-2 protein mRNA and expression has also been observed in a breast cancer cell. A complex including nuclear Her-2 binds at a specific nucleotide sequence of the COX-2 promoter region and hereby promotes transcription [112]. Exposure of HCA-7 rectal carcinoma cells to the combination of celecoxib and trastuzumab decreased growth *in vitro* as well as in a xenograft model. Therefore targeting COX-2 and HER-2 with a combination of NSAIDs and anti-HER-2 antibody such as trastuzumab or a HER-2 tyrosine kinase inhibitor is a rational combination [113].

The epidermal growth factor receptor (EGFR; erbB1) is a member of the tyrosine kinase receptor family, which includes HER2/neu (erbB2), erbB3, and

erbB4 [114, 115]. The ErbB receptors are present at the cell surface and share a common structure composed of an extracellular ligand-binding domain, transmembrane segment, and an intracellular tyrosine kinase domain [114]. LS174T colorectal carcinoma cells exposed to PGE_2 show rapid induction of Akt signaling. EGFR-specific tyrosine kinase inhibitors can completely abolish Akt activation. This rapid transactivation of the EGFR occurs via an intracellular pathway because inactivation of EGFR ligands with inhibitory antibodies did not inhibit PGE_2-mediated Akt activation (Fig 2) [116]. In Caco-2, LoVo and HT-29 colon cancer cell lines PGE_2 exposure induces phosphorylation and therefore activation of downstream targets of the EGFR pathway such as ERK2. Inactivation of EGFR kinase with selective inhibitors significantly reduces PGE_2-induced ERK2 activation [117]. PGE_2, however, can also directly activate EGFR signaling and thereby stimulate cell proliferation [116, 118]. The mechanism by which this occurs includes PGE_2-mediated metalloproteinase activation resulting in shedding of EGFR ligand from the plasma membrane and thus enhanced EGFR signaling. Another mechanism involves activation of the cAMP/protein kinase A pathway leading to increased expression of amphiregulin, a ligand of EGFR [119].

The TNF family of death receptors and ligands is a family of apoptosis-inducing proteins. Several human death receptors have been identified [120, 121]. Apoptosis is triggered upon binding of specific TNF superfamily ligands, such as TNF, FasL (CD95L/APO-1L) or TNF-related apoptosis inducing ligand (TRAIL), to a receptor TNFR1, TNFR2, Fas or DR4 (TRAIL-R1/APO-2), DR5 (TRAIL-R2/KILLER/TRICK2) respectively. Besides binding to the agonistic receptors TNFR1, TNFR2, Fas, DR4 and DR5, FasL can also bind to the soluble inhibitory decoy receptor, DcR3, while TRAIL can bind to membrane-bound DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), which both lack functional death domains and are therefore unable to induce apoptosis. TRAIL can also bind to a soluble TNF family receptor, osteoprotegerin. However, the physiological significance of this interaction appears to be minimal. Upon trimerization of the death receptors, an intracellular death-inducing signaling complex (DISC) is formed composed of trimerized receptor molecules and recruited TNF receptor associated death domain (TRADD) or Fas-associated death domain (FADD) and procaspase-8 molecules. Following DISC assembly a cascade of effector caspases and substrates are activated. The nucleus is condensed and fragmented, the cytoplasm is decreased and apoptosis induction is complete [122, 123].

Several studies revealed that death receptor apoptosis pathways are involved in chemotherapy-induced apoptosis. NSAIDs are also capable of inducing apoptosis via the TRAIL and Fas signaling pathways as was described by Han *et al.* They showed that FADD is necessary to induce apoptosis with indomethacin in

Jurkat cells [124]. Indomethacin also induces apoptosis in an acquired doxorubicin resistant SCLC cell line via death receptor signaling [125]. In a hepatocellular cell line model, exposure to NS-398 resulted in Fas ligand upregulation and Fas-mediated apoptosis in COX-2 overexpressing cell lines [126]. TRAIL receptor DR5 was upregulated in colon, prostate and NSCLC cell lines after exposure to sulindac sulfide and celecoxib respectively. These NSAIDs induced apoptosis, which was further enhanced in combination with TRAIL [127, 128] (Fig 3).

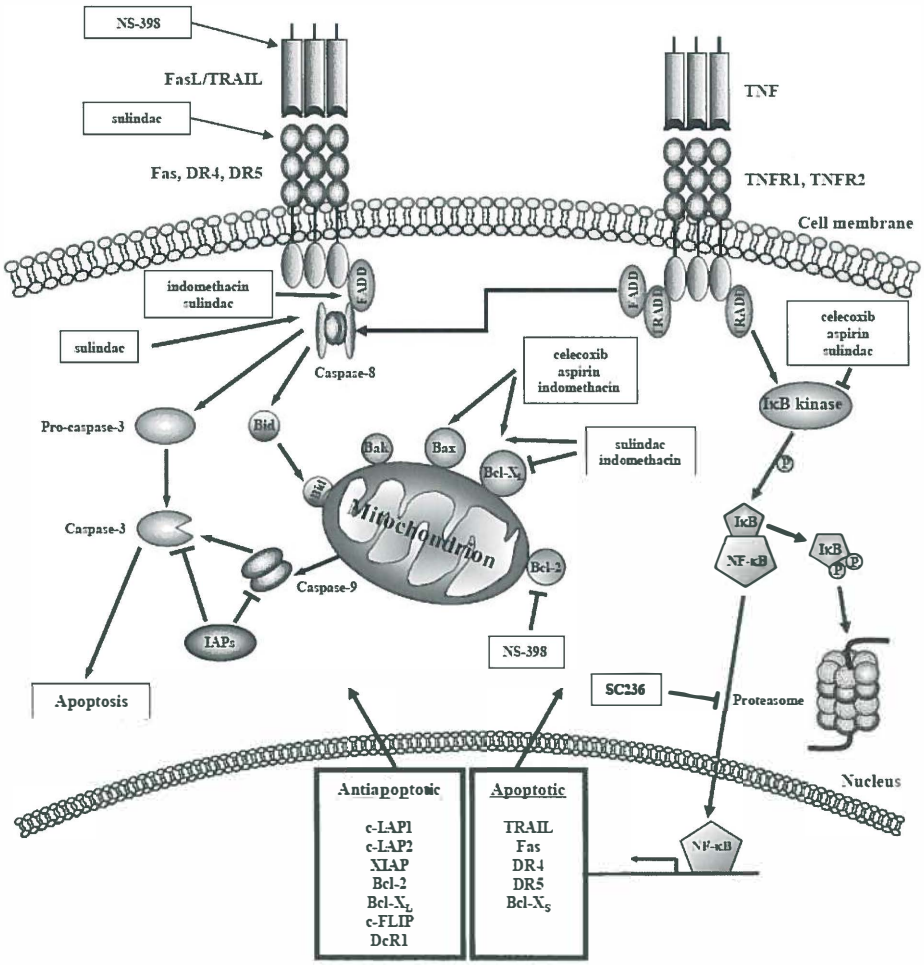

Figure 3. Death receptor-mediated apoptosis is facilitated at different levels by NSAIDs. NS-398 increases FasL expression, sulindac sulfide increases DR5 expression and indomethacin and sulindac sulfide can activate the intracellular part of the death inducing signaling complex. Inhibition of IκB kinases by selective and non-selective COX inhibitors results in decreased NF-κB in the nucleus and subsequently in a decreased transcription of pro- and anti-apoptosis proteins.

COX-2 inhibition can be directly involved in enhancing TRAIL-mediated apoptosis as demonstrated in HT29 colon cancer cells. Both COX-2 inhibition and COX-2 downregulation induced clustering of DR5 at the cell surface and redistribution of the death-inducing signaling complex components (DR5, FADD, and procaspase-8) into cholesterol-rich and ceramide-rich domains [129]. Besides the upregulation of DR5, NSAIDs in particular sulindac might also enhance TRAIL-sensitivity by down-regulating survivin expression [130-132]. Other downstream components of the apoptosis pathway such as anti-apoptotic members of the Bcl-2 family can be affected by NSAIDs as well. For example, the reduction in expression of the anti-apoptotic protein Bcl-X_L in Bax-proficient HCT116 cells by sulindac sulphide augmented TRAIL induced apoptosis. The effect of sulindac sulphide on Bcl-X_L is probably due to a reduction in NFκB activity [133].

6.2. Combination of NSAIDs and chemotherapy in cancer animal models

Teicher *et al.* studied the effect of various different chemotherapeutic agents in combination with NSAIDs on Lewis lung carcinoma bearing mice. Sulindac was an effective modulator of the chemotherapeutic agents cisplatin, cyclophosphamide, melphalan, and carmustine [134]. Indomethacin, doxorubicin and cisplatin only partially reduced the growth of colon tumors inoculated into mice after treatment with the individual drugs. However, a marked synergistic effect was observed when the combinations of indomethacin/bleomycin, indomethacin/doxorubicin or indomethacin/cisplatin were given [135, 136]. Rofecoxib treatment of BALB/c mice inoculated with MC-26 colorectal cancer cells increased survival of the mice and decreased metastatic potential of the cancer cells [137]. In combination with IL-2, indomethacin could eradicate melanoma lung metastases in a nude mice model by stimulating Natural Killer cell formation [138]. In a dog model, the effect of cisplatin could be positively modulated with a NSAID. Dogs with naturally occurring transitional cell carcinomas of the bladder received cisplatin alone or combined with the nonspecific NSAID piroxicam. 10 of 14 dogs in the piroxicam cisplatin group showed complete or partial remission whereas none out of 8 dogs showed remission in the cisplatin group [139].

7. COX-2 expression in human cancer

7.1. COX-2 expression in normal and malignant tissues

COX-2 is constitutively expressed in the human brain, testis, kidney and central nervous system as well as in premalignant and malignant lesions. COX-2 expression can be rapidly upregulated in macrophages, synoviocytes, fibroblasts, osteoblasts, tumor endothelial cells and "activated" endothelial cells [140]. COX-2 expression is described in several tumor types including, colorectal, gastric,

esophageal, hepatocellular, pancreatic, head and neck, non-small-cell lung, ovarian, breast, bladder, cervical, endometrial and skin cancer [141-164]. COX-2 expression is also present in several types of premalignant lesions such as, colorectal adenomas, gastric intestinal metaplasia, Barrett's esophagus, chronic hepatitis, oral leucoplakia, atypical adenomatous hyperplasia of the lung, ductal carcinoma *in situ* of the breast, prostatic intraepithelial neoplasia, bladder dysplasia, cervical dysplasia, actinic keratoses [165-171]. COX-2 expression is not only increased in pre-malignant and malignant lesions, it can e.g. also be increased in the tissue surrounding a malignant lesion [160]. In most studies, COX-2 expression in tumors or premalignant lesions was compared with surrounding tissues and not with other tumor types, which makes a comparison of COX-2 expression between different tumor types difficult. In gastric cancer patients, COX-2 expression is higher in tumors from advanced stage patients and in tumors from patients with lymph node metastases compared to patients with smaller tumors. [172, 173]. However, there are also studies in gastric cancer patients addressing lymphatic invasion, disease stage, or recurrence rate that do not show a correlation between COX-2 expression and more advanced disease [174]. In colorectal cancer a higher COX-2 tumor expression correlated with larger tumor size, deeper invasion and lymph node metastases [175-177]. However, the therapeutic significance of differences in COX-2 expression is questionable, since the anti-cancer effect of NSAIDs may well be COX-2 independent, which needs to be further investigated in clinical studies.

7.2. COX-2 expression and prognosis

In a number of tumors COX-2 overexpression is associated with a worse time to progression or overall survival. In the present review, we have, however, only included prospective studies or studies investigating consecutive cases, as have been described for breast cancer, non-small cell lung, ovarian, esophageal, bladder, gastric cancer as well as malignant mesothelioma [178-195]. In Table 1 survival prognosis of patients with COX-2 overexpressing tumors was compared with patients that did not have a COX-2 overexpressing tumor. In certain tumor types COX-2 overexpression is also associated with other tumor features, which themselves are predictive of worse survival such as Her-2 overexpression in breast cancer [179] and increased VEGF production in the stromal cells surrounding NSCLCs [181].

Table 1. Prognosis of patients with COX-2 overexpressing tumors compared to patients with non-overexpressing tumors (IHC=immunohistochemistry, RT-PCR=reverse transcriptase PCR, WB=Western blot, ISH=in situ hybridization).

Author	Tumor type	Number of patients	Method	Decreased survival
Denkert[178]/Ristimaki[179]	Breast cancer	221/1576	IHC	yes
Wulfing[180]	Breast cancer	200	IHC	no
Yuan[181]	Non-small cell lung cancer	60	IHC, RT-PCR	yes
Kim[182]	Non-small cell lung cancer	84	IHC	yes
Khuri[161]	Non-small cell lung cancer	160	ISH	yes
Ferrandina[183]	Cervical carcinomal	75	IHC	yes
Chen[184]	Cervical adenocarcinoma	53	IHC	no
Erkinheimo[185]	Ovarian serous carcinoma	42	IHC, RT-PCR, WB	yes
Gallo[186]	Head and neck squamous cell carcinoma	52	IHC	yes
Ranelletti[187]	Oropharyngeal squamous cell carcinoma	61	IHC	no
Wulfing[188]/Kim[189]	Bladder cancer	157/37	IHC	yes
Soumaoro[190]	Colorectal cancer	288	IHC	yes
Hull[191]	Colorectal cancer metastases	35	IHC	no
Baldi[190]/Edwards[192]	Mesothelioma	29/48	IHC	yes
Buskens[193]	Esophageal adenocarcinoma	145	IHC	yes
Okano[194]	Gastric cancer	166	IHC	yes

There are also tumor types in which no correlation between COX-2 overexpression and a more malignant phenotype has been observed. These cancers include colorectal, cervical, head and neck. For some other tumor types contradictionary results are reported [180, 184, 187, 191] (Table 1). In these tumor types the importance of COX-2 in carcinogenesis or tumor progression remains questionable.

8. NSAIDs and cancer treatment

8.1. NSAIDs in clinical studies and ongoing trials in cancer patients

A number of clinical studies with NSAIDs in cancer patients are available or ongoing. NDAIDs have been investigated in different types of tumors or tumor stages, and different combinations with chemotherapy. Although the focus of clinical oncological research with NSAIDs was on chemoprevention, the last years the potential therapeutic use of NSAIDs in cancer also obtained attention [196]. The preventive use of selective COX-2 inhibitors was extensively investigated in persons carrying the APC mutation that results in the Familial Adenomatous

Polyposis phenotype. The potential therapeutic use of NSAIDs is currently being investigated in several other tumor types.

First, the clinical studies investigating NSAIDs as monotherapy will be discussed. In the early nineties, indomethacin was already investigated as an anti-cancer agent in a randomized trial conducted in 135 patients with advanced stage cancer (mainly colorectal cancer and liver, pancreatic, and gastric primary cancers) and an expected survival of more than 6 months. At that time less chemotherapy options were available. The patients were randomized to receive placebo, prednisolone, or indomethacin until death. The addition of indomethacin prolonged mean survival with 8.7 months compared to placebo-treated patients [197]. In a pilot study 12 patients, who had biochemical relapse of their prostate cancer after radiotherapy or radical prostatectomy received celecoxib. Prostate Specific Antigen (PSA) doubling times were calculated. Five patients had a decline in their absolute PSA level, three patients had stabilization of the level and of the remaining four patients, three had a marked decrease in their PSA doubling time [198]. In 14 cervical cancer patients tumor material was evaluated at baseline and after 10 days of celecoxib treatment. Celecoxib treatment decreases tumor COX-2 expression and markers of proliferation and neoangiogenesis [199]. In a randomized placebo controlled trial in colorectal cancer patients scheduled for resection of liver metastases 23 patients received rofecoxib and 21 patients received placebo prior to surgery. Only marginally histologically differences were observed in resected metastases of rofecoxib treated patients, without differences in apoptosis index or proliferation index [200]. At the Annual Meeting of the American Association for Cancer Research 2006, phase III results with celecoxib were presented. Fewer polyps were observed in patients with sporadic adenomas taking celecoxib compared to patients taking placebo. This effect was observed in the Adenoma Prevention with Celecoxib (APC) trial and the Prevention of Colorectal Sporadic Adenomatous Polyps (PreSAP) trial, both double blind randomized controlled trials investigating patients that have undergone removal of colorectal polyps. However an increase in cardiovascular events was seen in both studies in the celecoxib group compared to the placebo group [201, 202].

In addition to the anti-tumor activity of NSAIDs as single agents, there is interest in the effects of a combined therapy of chemotherapy with NSAIDs. In a small retrospective study comparing capecitabine in combination with celecoxib compared to capecitabine alone in colorectal cancr patients, the tumor response was increased in the capecitabine/celecoxib group compared to the capecitabine group alone as measured by, proportion of stable disease (62.5% vs 22.8%, $P = 0.001$), and increase in median time to tumor progression (6 vs 3 months, $P = 0.002$). This effect was seen despite the fact that patients on capecitabine/celecoxib

had less favorable disease characteristics (age, performance status, and prior chemotherapies) [203]. In a case report, a small cell lung carcinoma patient was described with multiple brain, lung, liver, and bone metastases recurrence after intensive chemotherapy. The patient showed no signs of remission following cisplatin, etoposide, cyclophosphamide and vincristine chemotherapy. One cycle of vincristine, methotrexate and indomethacin resulted in signs of almost complete remission without any obvious adverse effects. The patient however did not receive methotrexate in the first cycle of chemotherapy [204]. In a phase II trial, twenty-nine patients with stages IB to IIIA NSCLC were treated with preoperative celecoxib daily in combination with 2 cycles of paclitaxel and carboplatin. Twenty-six patients completed preoperative celecoxib treatment and 28 patients underwent complete resection of their tumors. There were no complete pathologic responses, seven patients had minimal residual disease [205]. In a very small study investigating neoadjuvant anti-aromatase therapy (exemestane and letrozole) and celecoxib, 20 patients were randomly assigned to exemestane and celecoxib, exemestane or letrozole. All groups showed a decrease in tumor area. However the differences between the three groups were not significant [206]. In HER-2/neu-overexpressing metastatic breast cancer patients that had progressed while receiving trastuzumab, the addition of celecoxib did not induce objective responses [207]. Rofecoxib in combination with 5-FU and leucovorin did not increase tumor response in colorectal cancer patients. After evaluating the first 10 patients that had entered the study, the study was terminated [208]. Another approach to treat metastatic cancer is targetting endothelial cells in order to inhibit tumor angiogenesis. Twelve patients with pretreated advanced melanoma received treosulfan chemotherapy in combination with rofecoxib. In one patient a partial response occurred and four showed stabilization of their disease [209]. In a prospective multicenter EORTC phase III study 85 patients were randomized to receive either the FOLFIRI regimen or the CAPE/IRI regimen. Both groups were also randomized to receive 2 x 400 mg celecoxib or placebo. This trial was originally designed to include 692 patients, but only 85 patients were randomized due to occurrence of 8 fatal events in this study probably caused by the chemotherapeutic agents and not by celecoxib [210]. Regretfully all the studies mentioned above are small and underpowered and do not allow firm conclusions. Currently a number of phase I-III clinical trials are still investigating the efficacy of selective COX-2 inhibitors as single agent or in combination with chemotherapy in cancer therapy (Table 2).

Table 2. Current open clinical trials with selective COX-2 inhibitors extracted from NCI's Physician Data Query.

Cancer	Phase	Projected accrual	Tumor Stage	Drug
Prostate	II	66	D3	celecoxib
Prostate	II	28	D3	celecoxib
Prostate	II/III	3300	High risk newly diagnosed disease	celecoxib
Prostate	II	70	D3	celecoxib
NSCLC	II	110	IIIA	celecoxib
NSCLC	I	24	IIIB/IV	celecoxib
NSCLC	II	80	II/III	celecoxib
NSCLC	I	6-45	IIB/IV	celecoxib
Pulmonal or pleural malignancies	I	40	Inoperable disease	celecoxib
Esophageal	I	25	Neo-adjuvant	celecoxib
Gastric/Gastroesophageal Junction Carcinoma	II	20	Unresectable, recurrent, or metastatic	celecoxib
Pancreas	I	20	I/II	celecoxib
Breast	II	34	II-IV	celecoxib
Breast/colorectal	III	342	IV/IV	celecoxib
Colorectal	I/II	80	Metastatic colorectal carcinoma	celecoxib
Colorectal	II	Not specified	Metastatic colorectal cancer or local recurrence	celecoxib
Rectal	II	19	II/III	celecoxib
Rectal	I/II	39	III/IV	celecoxib
Glioblastoma multiforme	II	176	Adjuvant treatment	celecoxib
Ewing sarcoma	II	6-36	Metastatic disease	celecoxib
Advanced solid tumors	I	66	Locally advanced or metastatic disease	celecoxib

8.2. NSAIDs to decrease chemotherapy associated side effects in cancer patients

NSAIDs can also decrease chemotherapy associated side effects. In 67 patients with metastatic colorectal cancer, side effects, such as diarrhea and the hand-foot syndrome, were decreased using capecitabine in combination with celecoxib compared to patients using capecitabine alone [211]. A combination of increased efficacy and decreased chemotherapy-induced side-effects can thus be envisioned. Celecoxib increases CPT-11 cytotoxicity in colorectal cancer xenograft mouse models (HT29 cells and Colon-26 cells in nude mice and BALB/c mice), but it can also decrease the severity of CPT-induced late diarrhea. The reduction of diarrhea by selective COX-2 inhibitor suggests an inflammatory component in the pathogenesis of CPT-11-induced late diarrhea. Trifan *et al.* have shown that COX-

2 is induced in the rat colon after CPT-11 treatment and that this is concurrent with an increase in PGE_2 production [212].

8.3. Safety profile of NSAIDs in cancer treatment

Conventional NSAIDs are well-known for their nephrotoxicity. These adverse renal effects occur because of decreased PGs produced by cyclooxygenases. Animal and human data show that COX-2 synthesized prostaglandins are important in the modulation of renal physiology. Therefore, selective COX-2 inhibitors are equal in causing nephrotoxicity as the nonselective NSAIDs [213].

NSAID use increases the risk of gastric and/or duodenal mucosal injury: erosions, ulcers and ulcer complications, especially bleeding [214, 215]. About 15 to 30% of regular NSAID users have one or more ulcers when examined endoscopically, and 3 to 4.5% of NSAID users have clinically significant upper gastrointestinal events, including ulcers and ulcer complications. In the VIGOR study the risk of developing a clinically important upper gastrointestinal event in rheumatoid arthritis patients receiving rofecoxib was compared to patients receiving naproxen. Rofecoxib use was associated with a lower risk of developing clinically important upper gastrointestinal events with a decrease from 3.0 to 1.4% [216, 29].

However, results from recently performed large placebo controlled studies with selective COX-2 inhibitors such as rofecoxib, celecoxib and valdecoxib monotherapy suggest that the use of these agents is not without side effects. The use of rofecoxib was associated with a significant increase in cardiovascular events as was shown in the VIGOR and APPROVe study. The incidence of myocardial infarction increased from 0.1% in the control group to 0.4% in the rofecoxib group. This effect was contributed to the cardioprotective properties of naproxen. In the APPROVe study, the cardiovascular toxicity of rofecoxib was investigated in patients with a history of colorectal adenomas that were treated to reduce the risk of recurrence of neoplastic polyps of the large bowel. Cardiovascular toxicity was lower in the placebo group (0.9%) compared to the rofecoxib group (2.4%). The use of celecoxib was also associated with a significant increase in cardiovascular events (0.7% vs 0.1%) as shown in the APC study in which patients with adenomatous polyps in colon or rectum were treated with celecoxib or placebo [217]. Therefore the potential benefit of selective COX-2 inhibitors in cancer treatment should also be carefully weighed against the increased risk of cardiovascular events. Before these considerations can be made data from solid phase III clinical studies must become available.

9. Conclusion

In cell line models NSAIDs in general are potent antitumor agents. NSAIDs can inhibit angiogenesis, proliferation, invasive growth, and induce apoptosis in a COX-2 dependent or independent manner. There is a great diversity in mechanisms causing the antitumor effect of NSAIDs. The inhibition of PGE_2 production as well as the inhibition of transcriptonal activity of COX-2 are claimed to be the key mechanisms. However, the concentrations needed to induce COX-2 independent antitumor effects using NSAIDs as monotherapy are mostly not clinically achievable. A major issue in the development of new treatment schedules in solid tumors is the safety profile of the selective or non-selective COX-2 inhibitors. The non-selective COX-2 inhibitors are known for their gastrointestinal toxicity and recent evidence has emerged that the selective COX-2 inhibitors increase the risk of cardiovascular events in non-cancer patients. Both risks of these adverse effects must be taken into account when selective or non-selective COX-2 inhibitors are being prescribed to cancer patients. In the preclinical phase of drug development there is accumulating evidence that chemotherapy as well as a number of experimental or novel registered drugs e.g. trastuzumab or rhTRAIL, may have great synergistic efficacy when combined with NSAIDs. Therefore phase II and III clinical studies have to establish the role of NSAIDs in the treatment of cancer patients.

10. References

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3

Indomethacin-induced activation of the death receptor-mediated apoptosis pathway circumvents acquired doxorubicin resistance in SCLC cells

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In press Crit Rev Oncol Hematol.

Abstract

Small cell lung cancers (SCLCs) initially respond to chemotherapy but are often resistant at recurrence. A potentially new method to overcome resistance is to combine classical chemotherapeutic drugs with apoptosis induction via TNF death receptor family members such as Fas. The doxorubicin resistant human SCLC cell line GLC_4-Adr and its parental doxorubicin-sensitive line GLC_4 were used to analyse the potential of the Fas-mediated apoptotic pathway and the mitochondrial apoptotic pathway to modulate doxorubicin resistance in SCLC. Western blotting showed that all proteins necessary for DISC formation and several inhibitors of apoptosis were expressed in both lines. The proapototic proteins Bid and caspase-8, however, were higher expressed in GLC_4-Adr. In addition, GLC_4-Adr expressed more Fas (3.1x) at the cell membrane. Both lines were resistant to anti-Fas antibody, but plus the protein synthesis inhibitor cycloheximide anti-Fas antibody induced 40% apoptosis in GLC_4-Adr. Indomethacin, which targets the mitochondrial apoptotic pathway, induced apoptosis in GLC_4-Adr but not in GLC_4 cells. Surprisingly, in GLC_4-Adr indomethacin induced caspase-8 and caspase-9 activation as well as Bid cleavage, while both caspase-8 and caspase-9 specific inhibitors blocked indomethacin-induced apoptosis. In GLC_4-Adr, doxorubicin plus indomethacin resulted in elevated caspase activity and a 2.7-fold enhanced sensitivity to doxorubicin. In contrast, no effect of indomethacin on doxorubicin sensitivity was observed in GLC_4. Our findings show that indomethacin increases the cytotoxic activity of doxorubicin in a doxorubicin resistant SCLC cell line partly via the death receptor apoptosis pathway, independent of Fas.

Introduction

Lung cancer is the tumour type with the highest incidence in the Western world. Twenty-five percent of lung cancers are of the small cell lung cancer (SCLC) type. These tumours are well known for their initial sensitivity to chemotherapeutic agents and thereafter frequently recur at which time the tumour is drug resistant [1]. A common mechanism for drug resistance shared by chemotherapeutic drugs is the failure of the tumour cells to go into apoptosis. Interestingly, tumour cells have an independent pathway available, which can be used to induce apoptosis, namely, the death receptor ligand signalling pathway [2]. This has raised interest to exploit this pathway to circumvent drug resistance. Fas and Fas ligand (FasL) belong to the TNF family of death receptors and ligands [3-5]. Fas expression is present in many tumours and tumour cell lines including in SCLC [6-10]. After trimerisation of Fas on the cell membrane by extracellular FasL [11], Fas-associated Death Domain (FADD) and caspase-8 bind to the intracellular death domains of Fas and induce a death signal in the cell [12]. This leads to the activation of a cascade of caspases and eventually to cell death. In addition several anti-apoptosis proteins regulate the Fas-mediated death pathway. Important anti-apoptosis proteins are decoy receptor 3 (DcR3), Fas-associated phosphatase-1 (FAP-1), the long and short isoform of FLICE-inhibitory protein ($FLIP_L$ and $FLIP_s$) and the inhibitors of apoptosis family (IAPs) [13-17].

There is an alternative pathway for death receptor-induced apoptosis that involves the mitochondria [18, 19]. This pathway is controlled by pro-apoptotic and anti-apoptotic proteins from the Bcl-2 family. One of the key pro-apoptotic proteins in this pathway is Bid. When caspase-8 is activated in the initial phase of death receptor induced apoptosis, it can cleave Bid. The p15 form of truncated Bid (tBid) translocates to the mitochondria where cytochrome C is released. Cytochrome C activates caspase-9, which activates downstream effector caspases resulting in apoptosis [20].

In several tumour cell lines, including SCLC cell lines, Fas membrane expression is upregulated after exposure to chemotherapeutic agents [21]. This can enhance sensitivity to apoptosis-inducing anti-Fas antibody. Therefore, induction of Fas-mediated apoptosis together with chemotherapy may be an option to overcome drug resistance. At the moment the major problem of FasL or stimulating anti-Fas antibody is the liver toxicity observed in mice [22]. However several attempts are ongoing to circumvent liver toxicity.

Another option to modulate drug resistance is the inhibition of expression of anti-apoptotic members of the Bcl-2 family of apoptosis with non-steroidal anti-inflammatory drugs (NSAIDs). These drugs act by cyclooxygenase (COX) inhibition but can also affect death receptor mediated apoptosis pathways [23] and

induce apoptosis by downregulation of Bcl-2 family members [24-25]. In SCLC cell lines Bcl-2 family members have been described as important factors in chemotherapeutic drug resistance and therefore downregulation of Bcl-2 family members with a NSAID can be an interesting modality to circumvent drug resistance [26]. Human lung adenocarcinoma cells, exposed to NSAIDs showed an effective reduction of the anti-apoptosis Bcl-2 family member Mcl-1 [27].

In this study we investigated the possibility of utilising the Fas-mediated apoptosis route and indomethacin to modulate doxorubicin resistance in an acquired doxorubicin resistant SCLC cell line.

Materials & methods

Cell lines

GLC_4 was derived from a pleural effusion in our laboratory and kept in culture in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS) (both from Life Technologies, Breda, The Netherlands). GLC_4-Adr obtained resistance to doxorubicin, but also to a wide range of other chemotherapeutic agents, by stepwise increasing concentrations of doxorubicin [28-31]. GLC_4-Adr is 190.6 ± 16.2 times more resistant to doxorubicin than its parental cell line. The doxorubicin resistance in GLC_4-Adr is due to a downregulation of the activity of DNA-topoisomerase II (TOPO II) and amplification and overexpression of the *MRP-1* gene. GLC_4-Adr was exposed to 1.2 μM doxorubicin twice weekly. GLC_4-Adr was cultured without doxorubicin for 20 days prior to experiments. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells from exponentially growing cultures were used for all experiments.

Antibodies and reagents

The antibodies used for Western blot analysis were all diluted in Tris buffered saline (TBS) buffer (20 mM Tris-HCl, 137 mM $NaCl_2$ and 0.05% Tween 20) supplemented with 5% skim milk powder (Merck, Darmstadt, Germany). The anti-FasL-, FADD- and XIAP antibody were purchased from Transduction Laboratories (Alphen a/d Rijn, the Netherlands), the Fas-, Bax-, Bcl-2-, Bcl-$X_{S/L}$- and FAP-1 antibody from Santa Cruz (Heerhugowaard, the Netherlands), the caspase-8 antibody from Cell Signalling (Leusden, the Netherlands). The caspase-9, caspase-3- and FLIP antibody were obtained from Pharmingen (Alphen a/d Rijn, the Netherlands). The Mcl-1 antibody was purchased from DAKO (Glostrup, Denmark). The Bid antibody was kindly provided by Dr. J. Borst, the Netherlands Cancer Institute, Amsterdam. The PARP antibody was obtained from Roche (Almere, the Netherlands) and the COX-2 antibody was obtained from Cayman Chemical (Veenendaal, the Netherlands). The anti-mouse secondary antibody was a horseradish peroxidase labelled rabbit anti-mouse, which was diluted 1:1500 in TBS supplemented with 5% milk. The secondary anti-rabbit antibody, a swine anti-rabbit, was diluted 1:1500. Against goat primary antibodies a rabbit anti-goat horseradish peroxidase labelled antibody 1:2000 was used. The fluorescein (FITC) coupled rabbit anti-mouse antibody was diluted 1:20. All secondary antibodies were purchased from DAKO (Glostrup, Denmark). The pro-apoptotic mouse anti-Fas antibody 7C11 was obtained from Immunotech (Versailles, France) and the phycoerythrin (PE) labelled anti-human Fas DX2 and the anti-FasL antibody NOK-1 antibody from Pharmingen, Alphen a/d Rijn, the Netherlands. The mouse

monoclonal CH11 anti-Fas antibody (Upstate Biotechnology, Veenendaal, The Netherlands) was used for confocal laser microscopy. Doxorubicin was obtained from Pharmacia Upjohn (Woerden, The Netherlands). Indomethacin was purchased from ICN Biomedicals (Aurora, Ohio), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), cycloheximide from Sigma Aldrich (Zwijndrecht, The Netherlands), Ponceau S from Sigma-Aldrich and Coomassie blue solution was purchased from Biorad (Veenendaal, The Netherlands). MK-571 was purchased from Merck Sharp (Kirkland, Canada).

SDS gel electrophoresis and Western blot

Proteins for Western blot analysis were extracted by lysing cells with sample buffer containing 0.125 M Tris-HCl, 2% SDS, 10% glycerol and 0.001% bromophenol blue. Samples were boiled for 5 min. Ten μg protein was run on 10% SDS polyacrylamide gels at 200 V and transblotted onto polyvinylidene difluoride membranes (PVDF) (Millipore, Bedford, United Kingdom) with a semi-dry blot system. Equal protein loading was confirmed by Ponceau red staining of membranes and Coomassie blue staining of the gels. Membranes were activated in methanol for 5 min and washed 3 times with H_2O and once with TBS without Tween 20. Membranes were then blocked for 1 h in TBS supplemented with 5% skim milk and probed with the primary antibody for 1 h. Membranes were washed 3 times with TBS and incubated with the horseradish peroxidase bound secondary antibody for 1 h at room temperature. Membranes were washed 3 times with TBS and bands were visualised with chemoluminescence POD or Lumi-light$^+$ (Roche Diagnostics, Basel, Switzerland). All experiments were performed three times.

Confocal laser microscopy

The intracellular localisation of Fas in the cell lines was determined with confocal laser microscopy. Cells were washed once with RPMI 1640 medium containing 10% FCS. Glass slides were coated with 0.1% poly-L-lysine and dried at room temperature. Fifty μl of $4x10^5$ cells/ml were put on glass slides and left to adhere to the slides for 1 h. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS: 6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 mM NaCl; 2.7 mM KCl; pH 7.2) supplemented with 3.3 mM $CaCl_2$ for 15 min. Cells were washed twice with PBS and incubated for 1 h with the CH11 anti-Fas antibody. After incubation with the primary antibody, cells were washed twice and incubated with a FITC coupled rabbit anti-mouse antibody for 30 min and washed twice before they were analysed on a Leica confocal laser microscope.

Flow cytometry

To determine Fas membrane expression cells were harvested from the culture medium by centrifugation at 110 g for 5 min and washed twice with cold PBS supplemented with 2% FCS and 0.1% sodium azide. Cells were then incubated for 1 h with the PE-labelled anti-human Fas DX2 antibody, which was diluted 1:10 in cold PBS supplemented with 2% FCS and 0.1% sodium azide for 1 h on ice in the dark and washed twice with cold PBS. Analysis was performed on a Coulter Elite Flow cytometer (Becton Dickinson, Mount View, CA) with Winlist and Winlist 32 software (Verity Software House, Inc., Topsham, ME). Fas membrane expression was determined as Mean Fluorescence Intensity (MFI). To study also the effect of indomethacin on Fas membrane expression cells were incubated for 24 h with indomethacin. These experiments were performed three times.

Isolation of total cellular RNA, cDNA synthesis and RT-PCR

RNA was isolated from log phase cultures of the cell lines. Cells were harvested by centrifugation at 110 g for 5 min and washed with PBS. RNA was isolated with the guanidine isothiocyanate method. Five µg RNA was treated with 20-100 U DNAse I (Roche Diagnostics Basel, Switzerland) for 30 minutes. Single stranded cDNA was synthesised using M-MLV Reverse Transcriptase (Invitrogen Merelbeke, Belgium) and oligo dT primers according to manufacturers protocol. For RT-PCR 1 µl of cDNA was used as the target in a total volume of 50 µl. Reactions were performed according to standard protocols using the following primers and conditions.

FasL (290 bp, 53°C), upstream 5'-CCTCCAGGCAGGCACAGTTCTTCC-3' and downstream 5'- ATCTGGCTGGTAGACTCTCG-3'; Fas (338 bp, 49°C), upstream 5'-CATGGCTTAGAAGTGGAAAT-3' and downstream 5'-ATTTATTGCCACTGTTTCAGG-3'; FADD (250 bp, 54°C), upstream 5'-AGCTCAAAGTCTCAGCACACC-3' and downstream 5'-TCTGAGTTCCATGACATCGG-3'; Caspase-8 (355 bp, 54°C), upstream 5'-CTGCTTCATCTCTGTATCC-3' and downstream 5'-GCAAAGTGACTGGATGTACC-3'; DcR3 (263 bp, 62°C), upstream 5'-AGCACGCATCGTGTCCACC-3' and downstream 5'-GACGGCACGCTCACACTCC-3'; FAP-1 (276 bp, 54°C), upstream 5'-GGAGTTAGTCTAGAAGGAGC-3' and downstream 5'-ACTGAATCCTAGACCTGAGC-3'; $FLIP_{long}$ (262 bp, 54 °C), upstream 5'-GAACATCCACAGAATAGACC-3' and downstream 5'-GTATCTCTCTTCAGGTATGC-3'; $FLIP_{short}$ (172 bp, 54°C), upstream 5'-

GAACATCCACAGAATAGACC-3' and downstream 5'-TTTCAGATCAGGACAATGGG-3'. All experiments were performed three times.

Mutation screening of Fas

DNA was extracted from the cell lines using a standard laboratory technique. The Fas gene was screened for mutations by denaturing gradient gel electrophoresis of the extracted DNA. The entire coding region, including all splice site junctions, was amplified in 10 amplicons using primers and conditions as described earlier [32]. The amplicons were electrophorised in a 9% polyacrylamide denaturing gradient gel containing 5% glycerol and 20-60% urea-formamide (100% urea-formamide = 7 M urea and 40% deionised formamide). The gels were stained with ethidium bromide and photographed under an UV transilluminator.

Apoptosis assay

Cells ($1.5x10^4$ per well) were cultured in 96-wells plates and optionally pre-incubated with 1 μg/ml cycloheximide for 2 h. Apoptosis was induced by adding the anti-Fas antibody 7C11 (1 μg/ml) for 24 h. To determine whether indomethacin induces apoptosis, cells were incubated with different concentrations of indomethacin. To investigate whether apoptosis induction with indomethacin is Fas-mediated, cells were optionally pre-incubated with 2 μg/ml NOK-1 and incubated with indomethacin for 24 h thereafter. Apoptosis was defined as the appearance of apoptotic bodies and/or chromatin condensation, using a fluorescence microscope. Results were expressed as the percentage of apoptotic cells in a culture by counting at least 200 cells per well. All apoptosis assays were performed three times in twofold.

Inhibition of indomethacin-induced apoptosis

At 1 h prior to indomethacin exposure, cells were incubated with 20 μM broad-spectrum caspase inhibitor zVAD-fmk, caspase-8 inhibitor zIETD-fmk or caspase-9 inhibitor zLEHD-fmk (all from Calbiochem, Breda, The Netherlands). Cells were exposed to the combination of indomethacin and caspase inhibitor for 24 h after which acridine orange was added and the percentage apoptotic cells was calculated. Results are expressed as the percentage of apoptotic cells in a culture by counting at least 200 cells per well. All apoptosis assays were performed three times in twofold.

Caspase-3 activation assay

The cleavage assay was carried out in 6 well plates according to Thornberry *et al* [33]. Activity of caspase-3 was assayed according to the

manufacturer's instructions using the fluorescence peptide substrate Ac-DEVD-AFC (Biomol Tebu-bio, Heerhugowaard, The Netherlands). Fluorescence from free 7-amino-4-trifluoromethyl coumarin (AFC) was monitored in a FL600 Fluorimeter Bio-tek plate reader (Beun de Ronde, Abcoude, The Netherlands) using 380 nm excitation and 508 nm emission wavelengths. Relative caspase-3 activity was calculated by dividing the fluorescence of a sample of treated cells by a sample of untreated cells. Protein from all samples was isolated to confirm apoptosis with PARP cleavage on Western blot. Experiments were performed three times.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

The cell lines were cultured in HAM/F12 and DMEM medium (1:1) (Life Technologies) supplemented with 20% FCS. The effect of doxorubicin and indomethacin on survival was tested MTT assay as described previously [34]. Cells were incubated for four days at 37 °C and 5% CO_2 in a humidified environment with a range of indomethacin concentrations and 10 and 2,000 nM doxorubicin for GLC_4 and GLC_4-Adr respectively. The modulating effect of indomethacin (10 μM and 20 μM) and MK-571 (50 μM) on cell survival by doxorubicin were also tested in the MTT assay using continuous incubation. After a 4-day culture period, MTT (5 mg/ml in PBS) was added and formazan crystal production was measured as described previously. Controls consisted of media without cells (background extinction) and cells incubated with medium instead of chemotherapeutic agents. Experiments were performed three times in quadruplicate.

Statistics

All experiments were performed at least three times on different occasions. Analysis included double sided non-paired t-test. A P-value < 0.05 was considered significant.

Results

Differences between the Fas mediated apoptosis pathway of GLC_4 and GLC_4-Adr

The genes encoding the pro-apoptotic proteins FasL, Fas, FADD, and caspase-8 were all expressed at the mRNA level in GLC_4 and GLC_4-Adr (Fig. 1A).

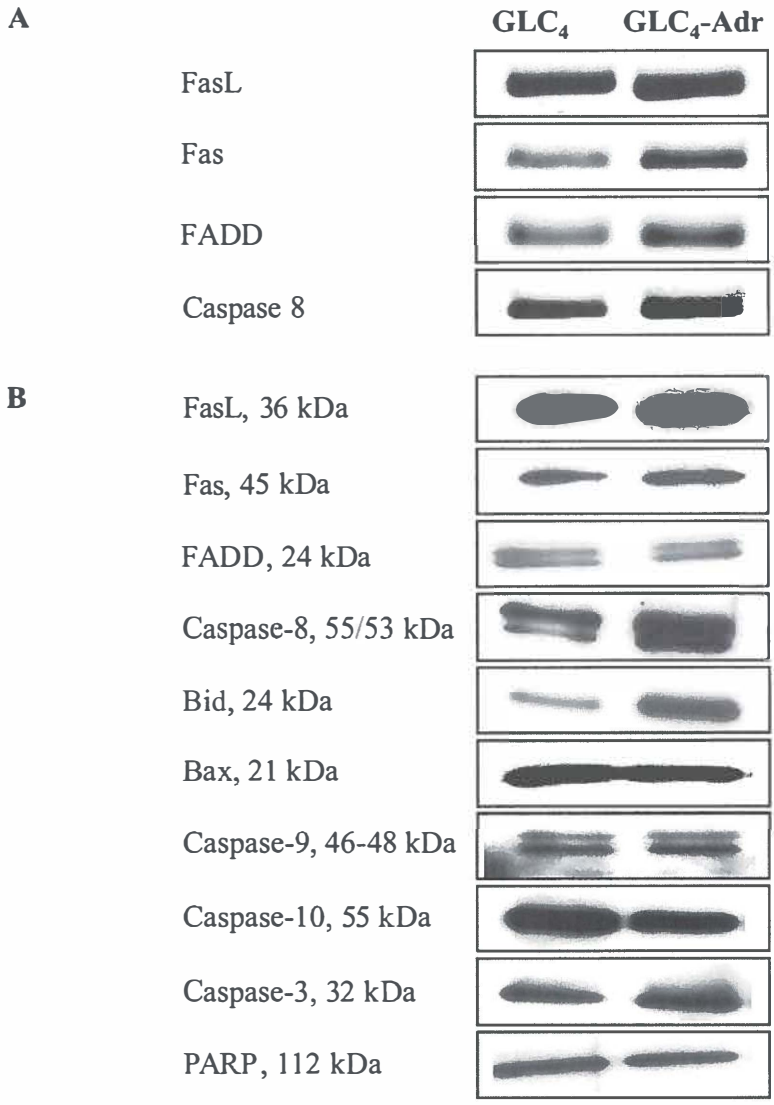

Figure 1. Basic mRNA and protein expression levels of pro-apoptotic proteins in GLC_4 and GLC_4-Adr in the Fas-mediated apoptosis pathway were determined at the mRNA level **(A)** and protein level **(B)**. Representative examples of three independent experiments are shown.

The proteins FasL, Fas, FADD, caspase-8, Bid, caspase-9, caspase-3 and PARP were also present in both cell lines (Fig. 1B). GLC_4 contained less Bid and caspase-8 compared to GLC_4-Adr, while there were no differences in the protein expression of FasL, Fas, FADD, Bax, caspase-9, caspase-10, caspase-3, and PARP between the two lines (Fig. 1B). Mutation analysis of the entire *Fas* gene revealed no aberrant patterns in both cell lines.

Anti-apoptosis genes were present in GLC_4 and GLC_4-Adr. RT-PCR analysis revealed a higher expression of $FLIP_l$, $FLIP_s$ and DcR3 in GLC_4-Adr compared to GLC_4 (Fig. 2A). Western blot analysis showed no differences in expression of the apoptosis inhibitors FAP-1, FLIP, Bcl-2, Bcl-X_L and XIAP between the cell lines. The non-specific anti-XIAP and Bcl-X_L immuno-reactive molecule as indicated (*) served as an internal loading control [35, 36] (Fig. 2B). There were also no differences in COX-2 expression (Fig. 2B).

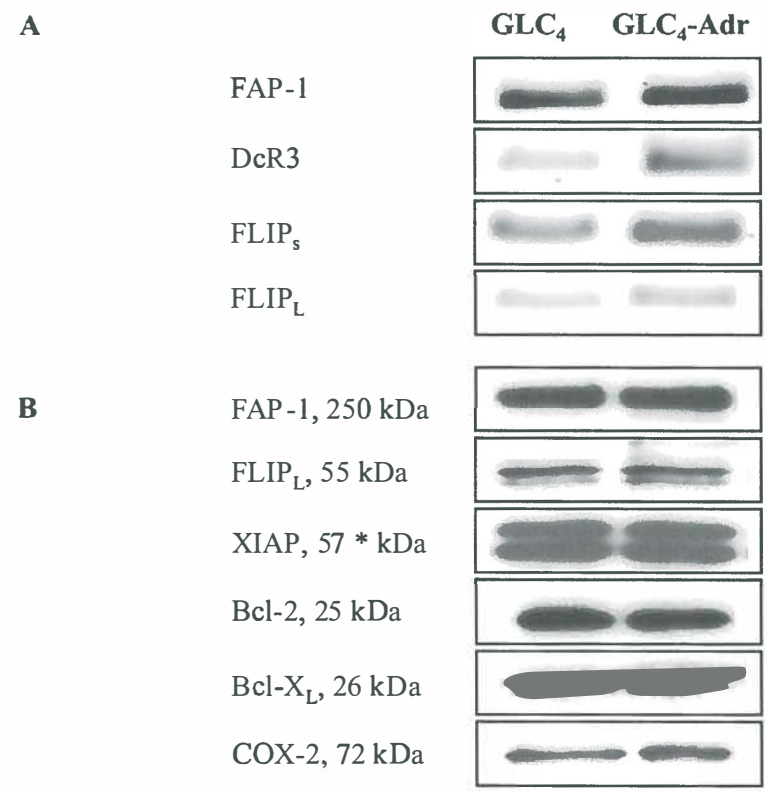

Figure 2. Basic mRNA and protein expression levels of anti-apoptotic proteins in GLC_4 and GLC_4-Adr in the Fas-mediated apoptosis pathway were determined at the mRNA level **(A)** and protein level **(B).** Representative examples of three independent experiments are shown.

Confocal laser microscopy revealed that in both cell lines Fas was present in the cytoplasm and at the cell membrane (Fig. 3). However, as determined by flow cytometry GLC_4-Adr contained 3.1 fold more Fas on the cell membrane than GLC_4. MFI were on average 15.4 in GLC_4 and 47.5 in GLC_4-Adr.

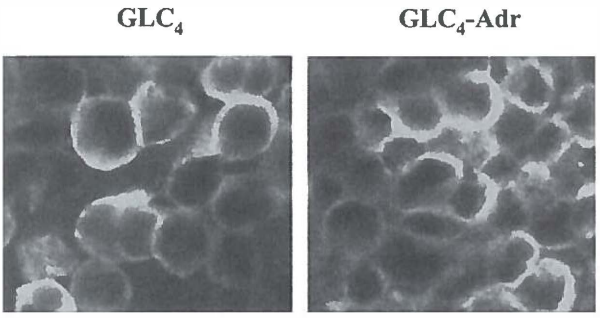

Figure 3. Fas localisation in GLC_4 and GLC_4-Adr determined with the mouse monoclonal CH11 anti-Fas antibody (Upstate Biotechnology) using confocal laser microscopy.

Anti-Fas antibody induces apoptosis

Functionality of the Fas pathway was tested by exposure to anti-Fas antibody (24 h) and a combination of anti-Fas antibody (24 h) and cycloheximide (2 h preincubation). The anti-Fas antibody alone hardly induced apoptosis. Co-treatment with cyclohexamide largely increased apoptosis in GLC_4-Adr (40%) but had almost no effect in GLC_4 (8%) (Fig. 4).

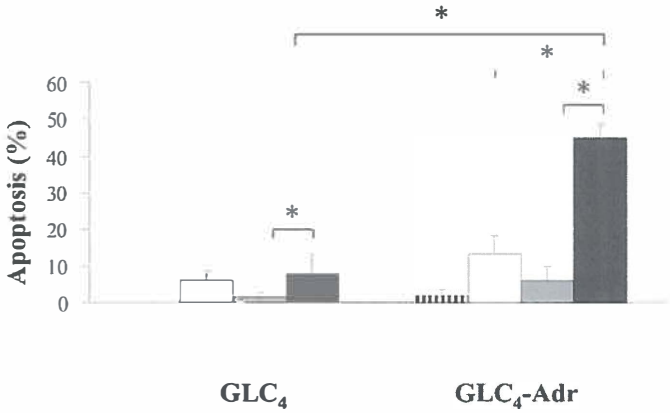

Figure 4. Fas-mediated apoptosis induction. Apoptosis induction in GLC_4 and GLC_4-Adr was determined after exposure to medium (stripes), cycloheximide (white), anti-Fas antibody 1 μg/ml (grey) or both (black) using the apoptosis assay. Data represent the mean ± SD of three independent experiments (* $P<0.05$).

Caspase-8 activation was used as intracellular determinant for activation of the Fas pathway and PARP cleavage as a marker for apoptosis. Surprisingly, no effect of anti-Fas antibody alone on caspase-8 cleavage was found. Intermediate cleavage products of caspase-8 (p41/p43) were detected after exposure to cycloheximide alone or in combination with anti-Fas antibody in both cell lines (Fig. 5). Active caspase-8 (p18 product) was especially observed in GLC_4-Adr but only after co-treatment with cycloheximide and anti-Fas antibody. These results indicate the presence of intracellular inhibitor(s) of the Fas-mediated apoptosis pathway, presumably at the level of caspase-8.

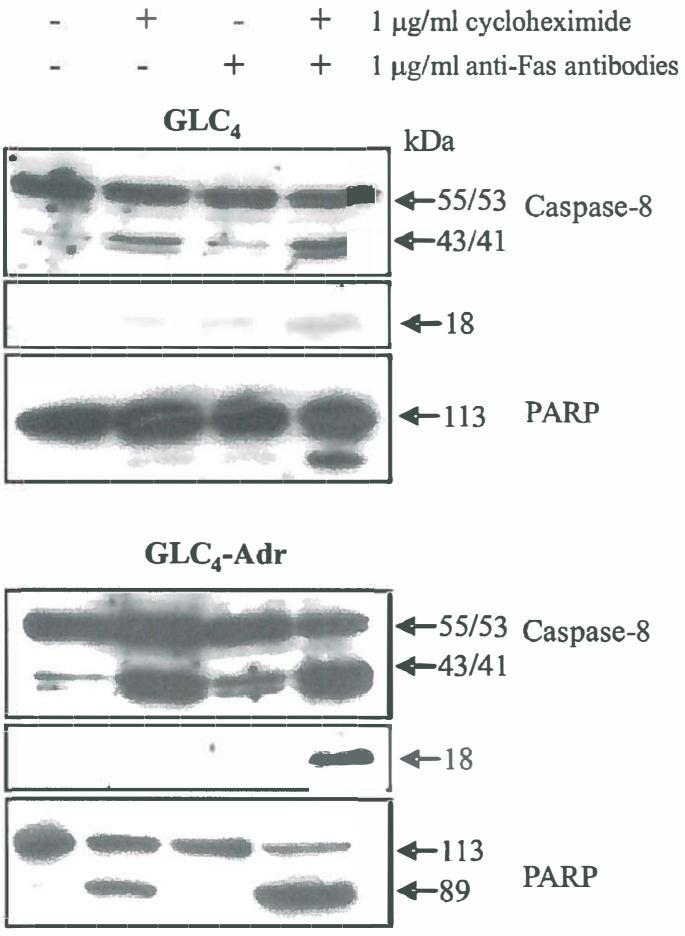

Figure 5. Fas-mediated caspase cleavage. PARP and caspase-8 cleavage were determined after exposing GLC_4 and GLC_4-Adr to anti-Fas antibody for 24h and cycloheximide for 24h and 2h preincubation. Representative example of three independent experiments.

Indomethacin induces apoptosis in GLC_4-Adr but not in GLC_4

Indomethacin alone had hardly any effect on apoptosis induction in GLC_4 but already induced apoptosis (28%) in GLC_4-Adr at 25 μM (Fig. 6).

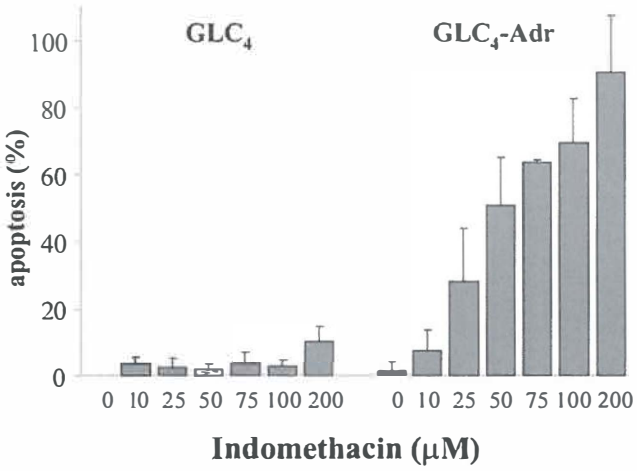

Figure 6. Indomethacin-mediated apoptosis. Apoptosis induction in GLC_4 and GLC_4-Adr was determined after exposure to different concentrations of indomethacin for 48 h.

GLC_4 and GLC_4-Adr were exposed to 0, 25, 50 and 100 μM indomethacin for 16 and 24 h in order to study the apoptosis inducing effect of indomethacin in more detail. Cleavage of caspase-8, Bid and PARP was investigated with Western blotting. Caspase-8, Bid, caspase-9 and PARP activation in GLC_4-Adr occurred 16 h after addition of 50 μM indomethacin. In GLC_4-Adr 100 μM indomethacin induced clearly detectable levels of activated caspase-8 (p18) as well as massive cleavage of full-length caspase-8 and PARP. Indomethacin more effectively induced caspase-8 activation than the combination of anti-Fas antibody and cycloheximide in GLC_4-Adr. However, even at these high indomethacin concentrations, no activation of caspase-8, Bid and PARP was observed in GLC_4 (Fig. 7A).

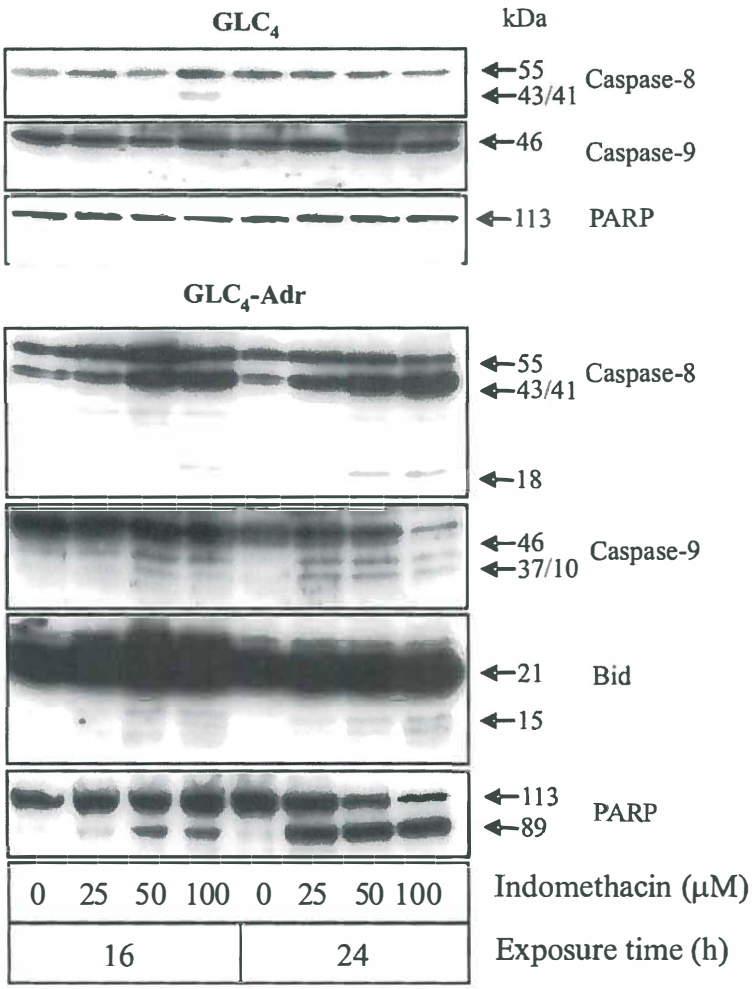

Figure 7 (A). Indomethacin-induced activation of the death receptor apoptosis pathway. Caspase-8, Bid and PARP cleavage were determined after exposing GLC_4 and GLC_4-Adr to 0, 25, 50 and 100 μM indomethacin for 16 and 24 h. Representative example of three experiments are shown.

To further investigate the mechanism by which indomethacin induces apoptosis, GLC_4 and GLC_4-Adr cells were exposed to 25 and 50 μM indomethacin and protein expression levels of anti-apoptotic Bcl-2 family members were analysed. No changes in Bcl-2, Bcl-$X_{S/L}$ or Mcl-1 expression were observed in both cell lines (Fig. 7B).

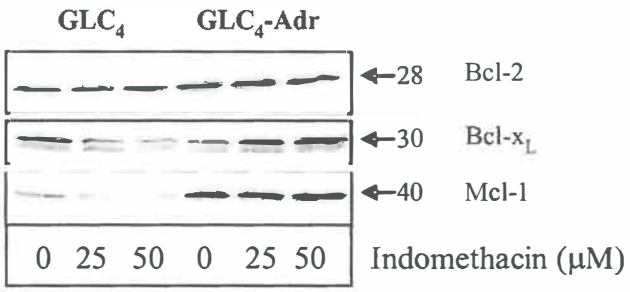

Figure 7 (B). Expression of Bcl-2, Bcl-$X_{S/L}$ and Mcl-1 after 24 h of 25 μM and 50 μM indomethacin exposure.

To determine more quantitatively the effect of various drug combinations on apoptosis in GLC_4-Adr, caspase-3 activation was measured with the DEVD-AFC cleavage assay. Doxorubicin alone showed minimal caspase-3 activation. Doxorubicin in combination with anti-Fas antibody had a slightly additive effect on caspase-3 activation. The combination of doxorubicin with indomethacin was however the most effective combination to induce caspase-3 activation (Fig. 8).

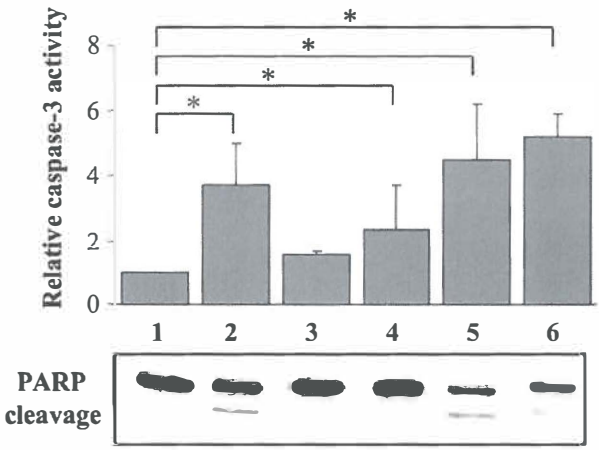

Figure 8. Effects of modulators on caspase-3 activation in GLC_4-Adr after 48 h of exposure to: 1) medium, 2) indomethacin (25 μM), 3) doxorubicin (3 μM), 4) anti-Fas antibody (1 μg/ml), 5) anti-Fas antibody and doxorubicin (3 μM), 6) indomethacin (25 μM) and doxorubicin (3 μM). Exposure to the anti-Fas antibody was only for the last 24h Data represent the mean ± SD of three independent experiments (* $P<0.05$).

Indomethacin induces caspase-8 and caspase-9 activation independently from Fas

The nature of the indomethacin-induced caspase 8 activation was further investigated. Apoptosis induction in GLC_4-Adr could not be prevented by pre-incubation with the anti-FasL NOK-1 antibody (data not shown). This means that indomethacin-induced apoptosis is not caused by autocrine or paracrine Fas/FasL interactions. In addition, indomethacin at a concentration of 50 μM for 24 h did not affect Fas membrane expression (results not shown).

Exposure of GLC_4-Adr cells to indomethacin in combination with caspase inhibitors revealed that indomethacin-induced apoptosis is reduced when cells are exposed to indomethacin in combination with zIETD-fmk, zLEHD-fmk or zVAD-fmk activity. The caspase-8 specific inhibitor zIETD-fmk and the caspase-9 specific inhibitor zLEHD-fmk reduced indomethacin-induced apoptosis by 58% and 44% respectively, the broad-spectrum caspase inhibitor zVAD-fmk by 84% (Fig. 9).

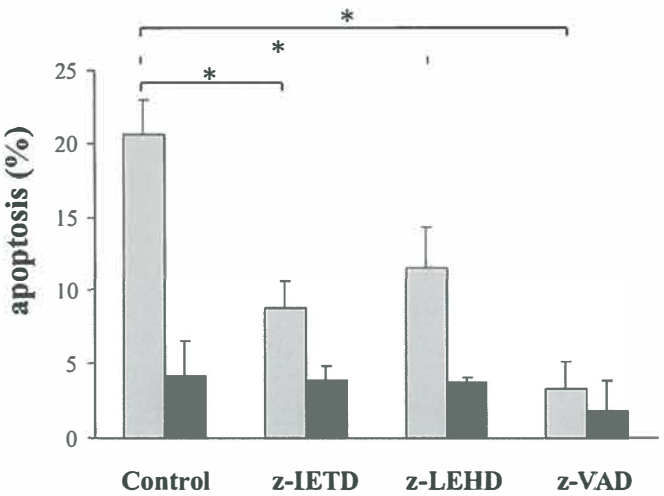

Figure 9. Inhibition of indomethacin-induced apoptosis. Apoptosis induction in GLC_4-Adr after exposure to 50 μM indomethacin (grey) or 0 μM indomethacin (black) in combination with the caspase-8 inhbitor zIETD-fmk, the caspase-9 inhibitor zLEHD or the broad-spectrum caspase inhibitor zVAD-fmk for 24 h.

Modulation of chemotherapy-induced growth inhibition by indomethacin

To investigate growth inhibition after exposure to indomethacin and doxorubicin the MTT assay was used. GLC_4-Adr cells are 190.6 ± 16.2 times more resistant to doxorubicin as compared to GLC_4. Two relatively non-toxic concentrations of indomethacin (10 μM and 20 μM) that induced some caspase activation in GLC_4-Adr, were used in combination with doxorubicin in a MTT assay. A dose of 10 and 20 μM of indomethacin induced respectively 1 and 2 %

growth inhibition in GLC_4, and respectively 15 and 17 % in GLC_4-Adr. In the presence of 20 μM indomethacin there was no effect on doxorubicin sensitivity in GLC_4, while a 2.7-fold increase in doxorubicin sensitivity was observed in GLC_4-Adr (Table 1).

Indomethacin like doxorubicin is also a substrate for the MRP1 drug efflux pump, which is overexpressed in GLC_4-Adr. We observed a similar increase in doxorubicin sensitivity comparing the effect of indomethacin with the effect of MK-571, a well-established inhibitor of MRP1, in the MTT assay (Table 1).

Table 1. 50% inhibiting dose in the MTT assay (μM) of doxorubicin and doxorubicin in combination with indomethacin (10 and 20 μM) in GLC_4 and GLC_4-Adr. Data represent the mean ± SD of three independent experiments (* $P<0.01$).

	GLC_4	GLC_4-Adr
Doxorubicin (μM)	0.014 ± 0.001	2.750 ± 0.050
Doxorubicin + indomethacin (10 μM)	0.011 ± 0.002	1.403 ± 0.203*
Doxorubicin + indomethacin (20 μM)	0.012 ± 0.002	1.003 ± 0.179*
Doxorubicin + MK571 (50 μM)	0.014 ± 0.002	0.743 ± 0.121*

Discussion

This is the first study that illustrates the effective circumvention of doxorubicin resistance by indomethacin-induced activation of the death receptor apoptosis pathway in a doxorubicin resistant SCLC cell line independent of Fas.

Fas-mediated apoptosis could only be induced in GLC_4-Adr but not in GLC_4 in the presence of the protein synthesis inhibitor cycloheximide, demonstrating that the death receptor-mediated apoptosis pathway is functional in the chemotherapy resistant cell line when a cellular block is removed. Interestingly, indomethacin induced apoptosis in GLC_4-Adr but not in GLC_4 in the absence of cycloheximide. No marked intensity differences were observed for pro- and anti-apoptotic proteins involved in the mitochondrial apoptosis pathway. In contrast, several pro-apoptotic proteins important for the death receptor apoptosis pathway were higher expressed in the doxorubicin resistant cell line GLC_4-Adr compared to GLC_4. For instance, the Fas-membrane expression was 3.1 fold higher in GLC_4-Adr compared to the parental cell line. The higher Fas membrane expression may be due to the repetitive incubation with doxorubicin. It, may also serve to facilitate a growth advantage to GLC_4-Adr as was demonstrated in several Fas-positive tumour cell lines [37, 38]. The fact that the difference in Fas membrane expression does not correlate with the Fas expression in total cell lysates may be due to a different distribution of Fas, cytoplasmic and on the cell membrane, as was described in prostate carcinoma cell lines and neuroblastoma cell lines [39, 40]. The expression levels of the pro-apoptotic proteins caspase-8 and Bid were also elevated in GLC_4-Adr compared to GLC_4. Bid has been described to transport and recycle mitochondrial membrane phospholipids [41]. Since doxorubicin has toxic properties towards mitochondrial membranes, increased expression of Bid in GLC_4-Adr may be an additional resistance mechanism. The sensitivity of GLC_4-Adr to Fas-mediated apoptosis, in the presence of cycloheximide, as compared to GLC_4 can therefore be due to the higher Fas-membrane levels as well as elevated expression levels of caspase-8 and Bid or a combination of these factors. Anti-Fas antibody alone induced minimal caspase-3 activation which was only slightly increased by combining it with doxorubicin in GLC_4-Adr. Due to the limited modulatory effects of doxorubicin on Fas-mediated apoptosis an alternative was sought.

The NSAID indomethacin has been identified as an apoptosis inducing agent in different *in vivo* models and among the several mechanisms involved it can induce caspase-3 mediated apoptosis [42-44]. The apoptosis inducing effect of indomethacin in GLC_4-Adr is however not based on Fas/FasL interaction. Indomethacin did not affect Fas membrane expression and apoptosis is not decreased when cells are pre-treated with an inhibiting anti-FasL antibody prior

and during indomethacin exposure. Indomethacin alone induced extensive apoptosis in GLC_4-Adr with activation of caspase-8, caspase-9 and PARP cleavage even at low doses. This did not occur in GLC_4. The apoptosis inducing effect of indomethacin will therefore most likely be due to a Fas receptor independent effect on the death receptor apoptosis pathway. However, we cannot exclude the involvement of other death receptors. Inhibition of either caspase-8 or caspase-9 by zIETD-fmk and zLEHD-fmk, respectively, decreased indomethacin-induced apoptosis. Therefore indomethacin-mediated apoptosis induction in the GLC_4 cell lines depends on a functional mitochondrial apoptosis pathway, which is probably absent in GLC_4 due to the decreased Bid expression. Indomethacin, however, did not decrease expression of Bcl-2, Bcl-$X_{S/L}$ or Mcl-1 in GLC_4 or GLC_4-Adr which is in contrast to results described for lung adenocarcinoma cell lines [27]. The fact that indomethacin can activate caspase-8, Bid and caspase-9 in GLC_4-Adr, makes it a good alternative for agonistic anti-Fas antibody. Indomethacin added to doxorubicin largely increased doxorubicin effects on caspase-3 activation and cytotoxicity in GLC_4-Adr cells. Indomethacin like doxorubicin is a substrate for the MRP1 drug efflux pump which is overexpressed in GLC_4-Adr [30, 45, 46]. Therefore a subsequent increase in cellular doxorubicin concentration by indomethacin may have partly played a role. Other mechanisms by which indomethacin might induce apoptosis are increased glutathione extrusion mediated by MRP1 [47] or increased ATP consumption by MRP1 ATPase activity in analogy to the observed verapamil-induced ATP consumption in P-glycoprotein overexpressing cells [48].

The role of indomethacin in modulation of doxorubicin toxicity however cannot completely be explained by the MRP1 inhibitory effect. MK-571 is a far more effective inhibitor of MRP1 mediated drug efflux than indomethacin [23]. Despite the similar fold of doxorubicin sensitisation with either drug, this suggests that the observed effect of indomethacin on doxorubicin sensitivity is due to an increase in drug accumulation as well as MRP1-dependent or independent caspase activation in GLC_4-Adr cells.

Interestingly the NSAIDs have recently caught much attention in the treatment of tumours in combination with chemotherapy to potentiate their effect [49]. The first clinical report on a combination of celecoxib, a NSAID and selective cyclooxygenase-2 inhibitor, with chemotherapy appeared [50]. It showed an enhanced response to preoperative paclitaxel and carboplatin in early-stage non-small-cell lung cancer. This approach in SCLC may also be of interest not only because of COX-2 inhibition but also because of the effect observed by us on the alternative apoptotic route compared to the route used by chemotherapy. The observed extensive potentiation of doxorubicin induced inhibition of cell survival

at achievable clinical doses indomethacin [49], deserves testing in the clinic. The potential effect of these concentrations of indomethacin on other chemotherapeutic drugs requires further testing in preclinical models.

Overall, it can be concluded that indomethacin increases the cytotoxic activity of doxorubicin in a doxorubicin resistant SCLC cell line partly via the death receptor apoptosis pathway, independent of Fas.

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4

Indomethacin induces apoptosis in a MRP1-dependent mechanism in MRP1 overexpressing, doxorubicin resistant SCLC cells

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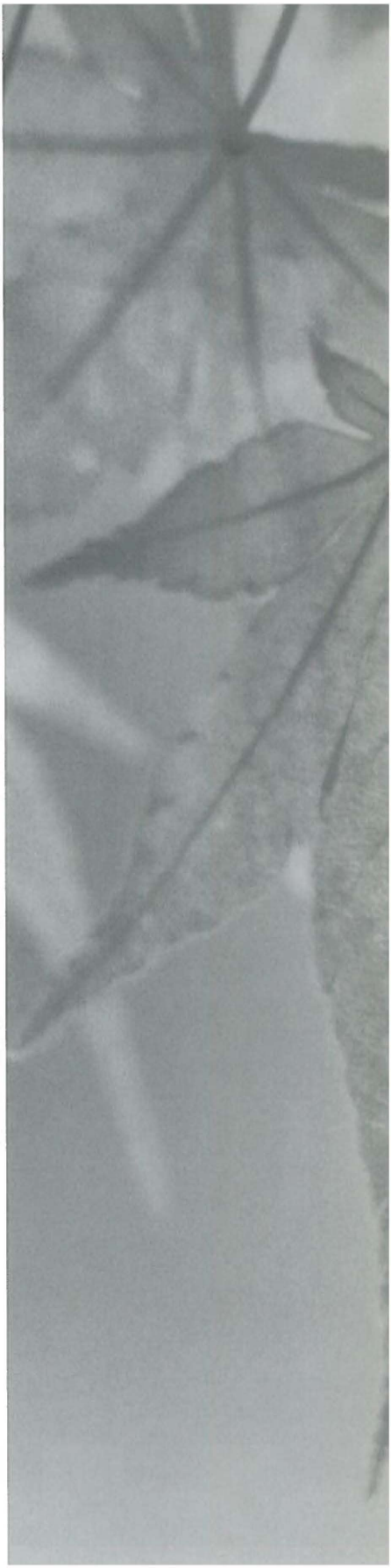
Submitted



Abstract

Small cell lung cancers (SCLCs) initially respond to chemotherapy but are often resistant at recurrence. The non-steroidal anti-inflammatory drug indomethacin is an inhibitor of multidrug resistance protein 1 (MRP1) function. The doxorubicin resistant MRP1 overexpressing human SCLC cell line GLC_4-Adr was highly sensitive for indomethacin compared to the parental doxorubicin-sensitive line GLC_4. **Purpose:** To analyze the relation between hypersensitivity to indomethacin and MRP1 overexpression. **Experimental design:** Effect of MRP1 downregulation using siRNA on indomethacin-induced cell survival and apoptosis was analyzed in GLC_4-Adr and GLC_4. In addition the effect of indomethacin on glutathione levels and mitochondrial membrane potential was investigated. **Results:** Si-MRP1 reduced 2-fold MRP1 mRNA and diminished efflux pump function of MRP1, reflected in a 1.8-fold higher accumulation of MRP1 substrate carboxyfluorescein, in si-MRP1 versus si-Luciferase transfected GLC_4-Adr cells. MRP1 down-regulation decreased 2-fold initial high apoptosis levels in GLC_4-Adr after indomethacin treatment for 24 h, and increased cell survival (IC_{50}) from 22.8 ± 2.6 to 30.4 ± 5.1 μM following continuous indomethacin exposure. MRP1 down-regulation had no effect on apoptosis in GLC_4 or on glutathione levels in both lines. Although indomethacin (20 μM) for 2 h decreased glutathione levels by 31.5 % in GLC_4-Adr, complete depletion of cellular glutathione by L-buthionine (S,R)-sulfoximine only resulted in a small increase in indomethacin-induced apoptosis in GLC_4-Adr demonstrating that a reduced cellular glutathione level is not the primary cause of indomethacin-induced apoptosis. Indomethacin exposure decreased mitochondrial membrane potential in GLC_4-Adr cells, suggesting activation of the mitochondrial apoptosis pathway **Conclusions:** Indomethacin induces apoptosis in a doxorubicin resistant SCLC cell line through an MRP1 dependent mechanism, which may have implications for the treatment of patients with MRP1 overexpressing tumors.

Introduction

Lung cancer is the tumor type with the highest incidence in males in the Western world, while it is rising in females. Small cell lung cancer (SCLC) represents about 25% of all lung cancers. SCLC tumors are well known for their initial sensitivity to chemotherapeutic agents but thereafter they frequently recur at which time the tumor is drug resistant [1]. A common mechanism of drug resistance is the overexpression of drug transporters and the failure to induce apoptosis in tumor cells. The major family of drug transporters is the ATP binding cassette (ABC) family of transport proteins. MRP1, a member of this family of drug transporters, can act as an efflux pump for a number of chemically unrelated agents. Glutathione (GSH) can be conjugated to these agents by glutathione S-transferase and MRP1 transports GSH, GSH conjugates and unconjugated cytotoxic drugs to the extra-cellular compartment [2-6]. GSH is required for several cellular functions such as protein and DNA synthesis, cell cycle regulation, protection against oxidative damage and detoxification of toxins [7].

The non-steroidal anti-inflammatory drug indomethacin is a well-known inhibitor of MRP1 function. It inhibits glutathione S-transferase but also functions as a direct substrate for MRP1 [8]. In addition indomethacin can increase GSH efflux at low concentrations [9]. We previously reported that indomethacin exposure of the MRP1 overexpressing doxorubicin resistant SCLC cell line GLC_4-Adr resulted in caspase-8 and caspase-9 dependent apoptosis induction, which suggests involvement of the extrinsic and intrinsic apoptosis pathway. In contrast, indomethacin exposure of the parental cell line GLC_4 did not induce apoptosis [10]. The use of this isogenic model implies that factors rendering GLC_4-Adr resistant to doxorubicin, are very likely to be causative in indomethacin sensitivity. Insight in this mechanism might open a simple, relatively non-toxic way to exploit MRP1 overexpression for apoptosis induction in chemo-resistant cells. In this study, we therefore investigated whether indomethacin induced apoptosis is related to MRP1 overexpression. In addition the mechanism of indomethacin induced apoptosis with respect to MRP1 function and GSH levels was studied.

Material and Methods

Cell lines

The GLC_4 cell line was derived from a pleural effusion in our laboratory and kept in culture in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS) (both from Life Technologies, Breda, The Netherlands). The GLC_4-Adr sub-line obtained resistance to doxorubicin, but also to a wide range of other chemotherapeutic agents, by stepwise increasing concentrations of doxorubicin [2, 11-13]. GLC_4-Adr is 190.6 ± 16.2 fold more resistant to doxorubicin than GLC_4. The doxorubicin resistance in GLC_4-Adr is due to a down-regulation of the activity of DNA-topoisomerase II (TOPO II) and amplification and a 79 fold overexpression of the *MRP1* gene. GLC_4-Adr was maintained in 1.2 μM doxorubicin twice weekly. GLC_4-Adr was cultured without doxorubicin for 20 days prior to experiments. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. Cells from exponentially growing cultures were used for all experiments. Indomethacin (50 μM) induces 50.6 ± 14.6% apoptosis in GLC_4-Adr and 2.1 ± 1.5% apoptosis in GLC_4 following treatment for 48 hrs.

Chemicals, media and reagents

HAM/F12 and DMEM medium (minimal essential medium (MEM, supplemented with Earle's salts and L-glutamine), Oligofectamine, RPMI 1640, Phosphate Buffered Saline (PBS), Hoechst 33258, MitoTracker Red CM-H_2XRos and Trizol were purchased from Invitrogen Life Technologies (Breda, The Netherlands). Carboxyfluorescein diacetate (CFDA), thiazolyl blue tetrazolium bromide (MTT), L-buthionine (S,R)-sulfoximine (BSO), glutathione reductase, nicotinamide adenine dinucleotide phosphate (NADPH), trichloric acid (TCA), GSH and propidium iodide (PI) were obtained from Sigma-Aldrich BV (Zwijndrecht, The Netherlands), DNase-I from Roche Diagnostics (Mannheim, Germany), doxorubicin-HCl from Pharmachemie BV (Haarlem, the Netherlands), ethylenedinitrilo tetraacetic acid disodiumsalt dihydrate (EDTA) from Merck, (Darmstadt, Germany). MK571 was purchased from Omnilabo (Breda, the Netherlands), the qPCR core kit from Eurogentec (Seraing, Belgium), the RNeasy kit from Qiagen (Venlo, the Netherlands) and Vitrogen from Nutacon (Leimuiden, the Netherlands).

Flow cytometric detection of MRP1 activity

To determine MRP1 activity, cells were incubated with 0.1 μM CFDA as described previously [14] with slight modifications. CFDA is intracellular converted to carboxyfluorescein (CF), which is a fluorescent MRP1 substrate. To establish the effect of indomethacin on the activity of MRP1, 1 x 10^6 cells were

incubated in 0.5 ml RPMI 1640 medium (37°C, 5% CO_2, 1 h) with CFDA. MK571 (50 μM) served as a positive control for inhibition of MRP1 activity. Cells were pelleted for 15 seconds at 12,000 g and re-suspended in 350 μL ice-cold RPMI medium with 0.1 μg/mL PI to distinguish dead cells from living cells. Fluorescence of CF was analyzed with a FACS Caliber flow cytometer (Becton Dickinson Medical Systems, USA). Per sample, 10,000 events were measured. The Winlist 5.1 program (Verity Software House Inc., Topsham, USA) was used to calculate mean fluorescence intensity (MFI) values. The efflux-blocking factor (BF) was defined as the ratio between MFI of substrate plus modulator and MFI of substrate. All measurements were corrected for the negative control (without indomethacin). Experiments were performed in triplicate.

RNA interference

To explore the role of MRP1 in indomethacin induced apoptosis, siRNAs directed against MRP1 (si-MRP1) and Luciferase (si-Luciferase, negative control) were purchased from Eurogentec (Maastricht, The Netherlands). The sense sequence for si-MRP1 was 5'-GGA GUG GAA CCC CUC UCU G-3' and the anti-sense sequence was 5'-CAG AGA GGG GUU CCA CUC C-3'. For si-Luciferase, the sense sequence was 5'-CUU ACG CUG AGU ACU UCG A-3' and the anti-sense sequence was 5'-UCG AAG UAC UCA GCG UAA G-3'.

GLC_4 and GLC_4-Adr cells were seeded in six-wells plates at a concentration of 3 x 10^5/well. The next day, transfection was performed using 200 nM oligonucleotides with Oligofectamine and RPMI 1640 in the absence of FCS according to the manufacturer's instructions. After 4 h, medium containing FCS was added bringing the FCS concentration to 10%. MRP1 function assays with flow cytometer and RNA extraction were carried out 48 h after siRNA transfection.

RNA isolation and quantitative RT-PCR

Cells treated with siRNA for 48 h were washed with ice cold PBS and re-suspended in 1 mL of Trizol. After 5 min incubation at room temperature, the lysate was stored at -80°C until use. RNA was isolated according to standard manufacture protocols followed by a DNase-I treatment. For RNA purification, the RNeasy kit was used following standard procedures. RNA (800 ng) was subjected to a complementary DNA (cDNA) synthesis reaction.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, USA) as described previously [15]. In brief, a qPCR core kit was used and the PCR mixture contained 900 nM of sense and anti-sense primers, 200 nM of fluorogenic probe (labeled by a 5' FAM reporter and a 3'

TAMRA quencher). Each sample was analyzed in duplicate. Primer sequences for MRP1 were for the sense strand 5'-GGT GGG CCG AGT GGA ATT-3', for the anti-sense strand 5'-TTG ATG TGC CTG AGA ACG AAG T-3' and for the probe strand 5' FAM-CTG CCT GCG CTA CCG AGA GGA CCT-TAMRA 3'. Primer sequences for the housekeeping gene GAPDH were for the sense strand 5'- GGT GGT CTC CTC TGA CTT CAA CA-3', for the anti-sense strand 5'-GTG GTC GTT GAG GGC AAT G-3' and for the probe strand 5' FAM-ACA CCC ACT CCT CCA CCT TTG ACG C-TAMRA 3'. Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (C_T value) correlate inversely with mRNA levels.

MTT survival assay

The cell lines were cultured in HAM/F12 and DMEM medium (1:1) supplemented with 20% FCS. The effect of doxorubicin and indomethacin on survival was tested in an MTT assay as described previously [16]. Cells were incubated for 4 days at 37°C and 5% CO_2 in a humidified environment with a range of indomethacin concentrations and. The effect of si-MRP1 down-regulation on cell survival was tested in the MTT assay using continuous incubation with indomethacin. After a 4-day culture period, MTT (5 mg/mL in PBS) was added and formazan crystal production was measured as described previously. Controls consisted of media without cells (background extinction) and cells incubated with medium instead of the cytotoxic agent. Experiments were performed three times in quadruplicate.

Apoptosis assay

The effect of GSH on indomethacin mediated apoptosis induction was tested in cells (1.5×10^4 per well) cultured in 96-wells plates and pre-incubated with 250 µM BSO for 24 h. Apoptosis was induced by adding different concentrations of indomethacin for 24 h.

In addition GLC_4-Adr cells were transfected with si-MRP1 and si-Luciferase. After 48 h these cells were plated in 96-wells plates and exposed to different concentrations of indomethacin for 24 h. Apoptosis was defined as the appearance of apoptotic bodies and/or chromatin condensation, using a fluorescence microscope. Results were expressed as the percentage of apoptotic cells in a culture by counting at least 200 cells per well. All apoptosis assays were performed in duplicate and repeated three times.

GSH assay

Free GSH was measured by a modified method as described by Allen *et al* [17]. Cells were plated in 25 cm^2 flasks and exposed to BSO for 24 h, indomethacin for 2 h or MK571 for 2 h for free GSH measurements after siRNA transfection. Cells were isolated from six wells plates and washed in PBS. Protein was precipitated with 5% TCA and the precipitate was spun down at 4,500 g. The supernatant was diluted to 1 mg/ml protein. All GSH assays were performed three times.

Mitochondrial membrane potential

To determine whether there is an absolute decrease in fluorescence, 3 x 10^5 cells were seeded in six wells plates and exposed to different concentrations of indomethacin for 24 h. 300 nM MitoTracker red® was added for 45 min at 37ºC and cells were analyzed with an Elite flow cytometer (Becton Dickinson, Mount View, USA). Experiments were performed in triplicate. A representative example was shown.

Statistical analysis

The paired Student's t-test, the independent samples t-test or the one-sample t-test was used to calculate statistical differences. Differences were considered significant when $P < 0.05$.

Results

The effect of MRP1 down-regulation on indomethacin-induced apoptosis and cell kill

Compared to the parental cell line GLC_4, the doxorubicin resistant subline GLC_4-Adr, which is highly sensitive to indomethacin, strongly overexpresses MRP1 [13]. The role of MRP1 in indomethacin sensitivity was investigated using a MRP1 siRNA approach. Efficient downregulation was demonstrated, since the MRP1 mRNA expression level, as determined with quantitative RT-PCR, was reduced to 55.2 ± 18.3% in si-MRP1 transfected GLC_4-Adr cells and 44.9 ± 1.1% in si-MRP1 transfected GLC_4 compared to their respective si-Luciferase transfected controls. The absolute MRP1 mRNA expression level in GLC_4-Adr however was 79 times higher compared to GLC_4.

The CF accumulation following 1 h CFDA exposure was used to determine the MRP1 function in siRNA transfected GLC_4-Adr cells. CF accumulation was 84 ± 19% higher in the si-MRP1 transfected cells compared to the si-Luciferase transfected cells indicating that the MRP1 function in GLC_4-Adr was indeed decreased as a result of MRP1 siRNA.

In GLC_4-Adr indomethacin-induced apoptosis was clearly reduced by si-MRP1 transfection. (Fig. 1). There was e.g. a 2.0 ± 0.05 fold reduction in apoptosis at 75 μM indomethacin. At higher indomethacin concentrations similar reductions were observed. In the GLC_4 cell line however, indomethacin concentrations up to 150 μM still did not induce apoptosis under these conditions (data not shown).

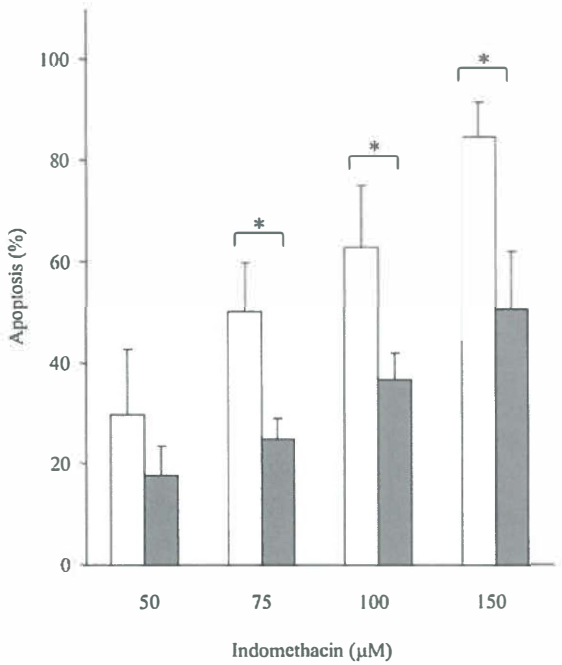


Figure 1. Indomethacin induced apoptosis in GLC_4-Adr 48 h after si-Luciferase transfection (white) or si-MRP1 transfection (grey). Cells were exposed to indomethacin for 24 h. No apoptosis was observed in GLC_4 (data not shown). Data represent the mean ± SD of three independent experiments (* $P < 0.05$).

Apart from a reduction in apoptosis also an increased survival following indomethacin exposure was observed (Fig. 2). The IC_{50} for indomethacin was 22.8 ± 2.6 μM in si-Luciferase transfected GLC_4-Adr and 30.4 ± 5.1 μM in si-MRP1 transfected GLC_4-Adr ($P < 0.05$). No differential effect on cell survival was observed between GLC_4 cells transfected with si-Luciferase or si-MRP1 for indomethacin concentrations up to 100 μM.

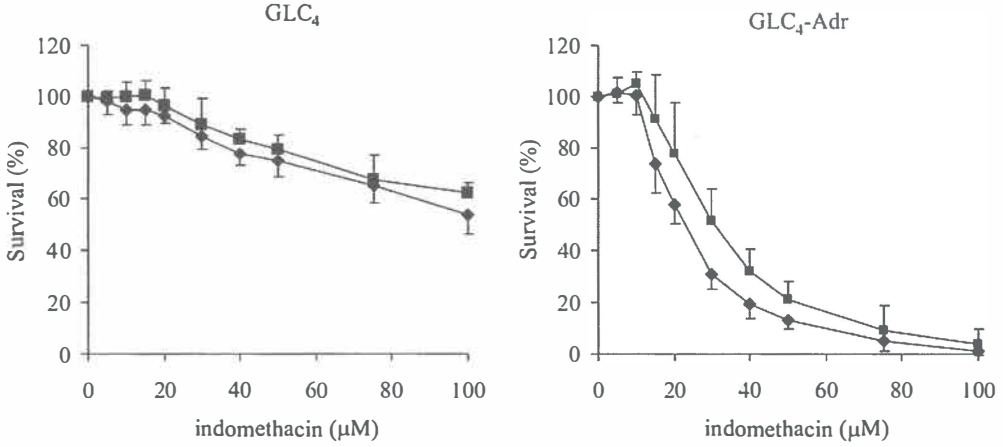


Figure 2. Indomethacin induced growth inhibition. Survival of GLC_4 and GLC_4-Adr cells 48 h after si-Luciferase or si-MRP1 transfection were compared. Data represent the mean ± SD of three independent experiments (* $P < 0.05$).

Effect of indomethacin on GSH levels

To investigate whether the protective effect of MRP1 siRNA to indomethacin induced apoptosis is not only related to a reduction in MRP1 activity but also to an effect on cellular GSH levels, we measured intracellular GSH concentrations. GLC_4 cells have a slightly lower although not significant GSH content of 9.9 ± 2.2 μg/mg of total cellular protein compared to GLC_4-Adr cells with 11.1 ± 6.1 μg/mg ($P = 0.75$).

Interfering with MRP1 function did not affect cellular GSH levels in both cell lines. Firstly, down-regulation of MRP1 with si-MRP1 transfection did not alter intracellular GSH content in GLC_4 nor in GLC_4-Adr. Secondly, when cells were exposed to 50 μM of the specific MRP inhibitor MK571 for 2 h, GSH levels were not affected and no apoptosis was induced (data not shown). Indomethacin exposure alone however lowered GSH levels with 31.5 ± 19.8% in GLC_4-Adr cells ($P < 0.05$) and 20.6 ± 37.7% in GLC_4 cells ($P = 0.28$) (Fig. 3).

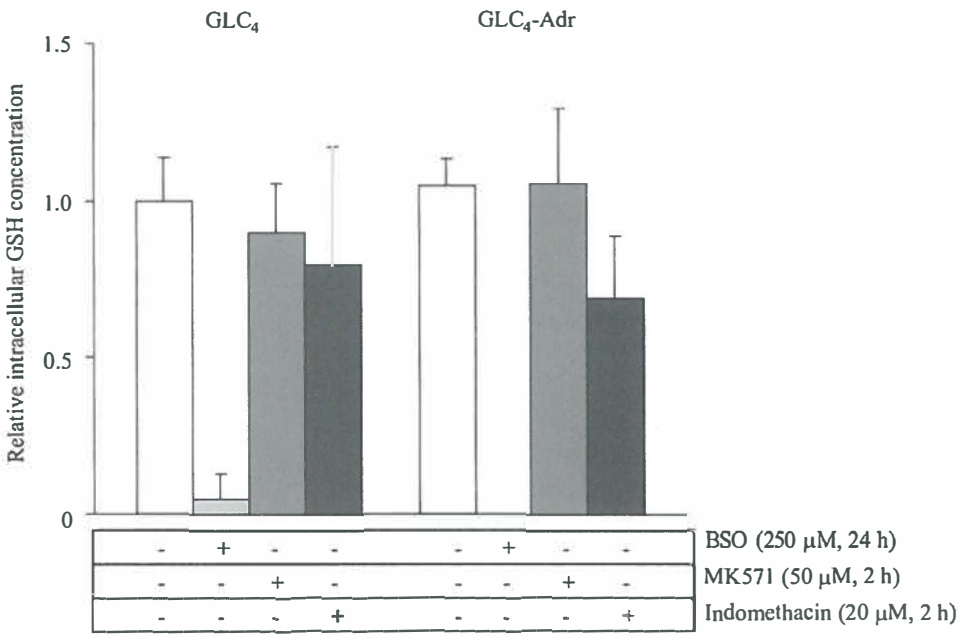


Figure 3. Relative GSH levels in GLC_4 and GLC_4-Adr cells. GSH intracellular concentration was determined after 24 h BSO, 2 h MK571 or 2 h indomethacin exposure. GSH levels were calculated as the relative concentration compared to untreated cells. Data represent the mean ± SD of three independent experiments (* $P < 0.05$).

To confirm the relation between GSH levels and indomethacin-induced apoptosis, cellular GSH levels were reduced with BSO in both cell lines. As shown in Fig. 3, GSH levels were completely diminished after BSO treatment in GLC_4-Adr cells. This resulted in an increase in apoptosis levels in GLC_4-Adr cells treated with indomethacin in combination with BSO compared to cells treated with indomethacin solely, for example from 14.1 % to 35.8 % at 25 µM indomethacin (Fig. 4).

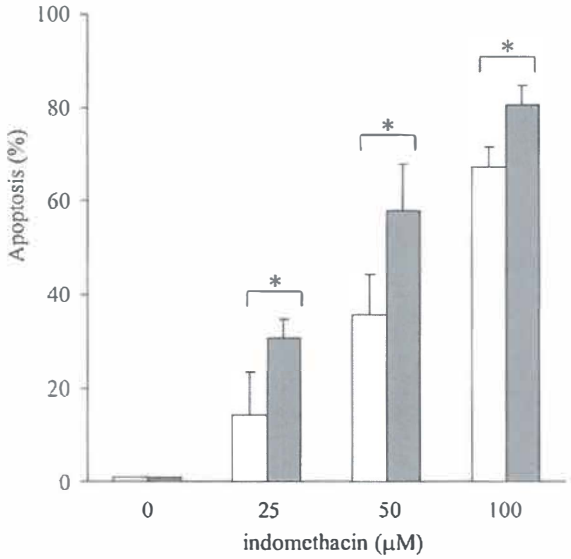


Figure 4. Indomethacin induced apoptosis in GLC_4-Adr after 24 BSO exposure (grey) or control (white). Cells were exposed to indomethacin for 24 h. No apoptosis was observed in GLC_4 (data not shown). Data represent the mean ± SD of three independent experiments (* $P < 0.05$).

However, at higher indomethacin concentrations the relative increase in apoptosis was less pronounced. Indomethacin in combination with BSO did not induce apoptosis in GLC_4 at these concentrations (results not shown). These results suggest that at lower indomethacin concentrations GSH levels are important determinants for MRP-dependent indomethacin-induced apoptosis.

Indomethacin-induced loss of mitochondrial membrane potential

Previously, we demonstrated that indomethacin-induced apoptosis in GLC_4-Adr was caused by caspase-8 as well as caspase-9 activation [10]. The role of mitochondria in this indomethacin-induced apoptosis in GLC_4-Adr was, however, not established. Since GSH depletion can induce oxidative stress resulting in activation of the mitochondrial apoptosis pathway, we determined whether indomethacin-mediated apoptosis induction coincided with loss of mitochondrial membrane potential in the cells. Flow cytometry revealed that after indomethacin exposure for 24 h two GLC_4-Adr cell populations exist, a MitoTracker red® high and a MitoTracker red® low population (Fig. 5). The presence of the MitoTracker red® low population (41.8% at 100 μM of indomethacin) at 24 h suggests that loss of mitochondrial membrane potential in

GLC_4-Adr occurs in conjunction with apoptosis induction as at these indomethacin concentrations up to 67.3 % of apoptosis is induced.

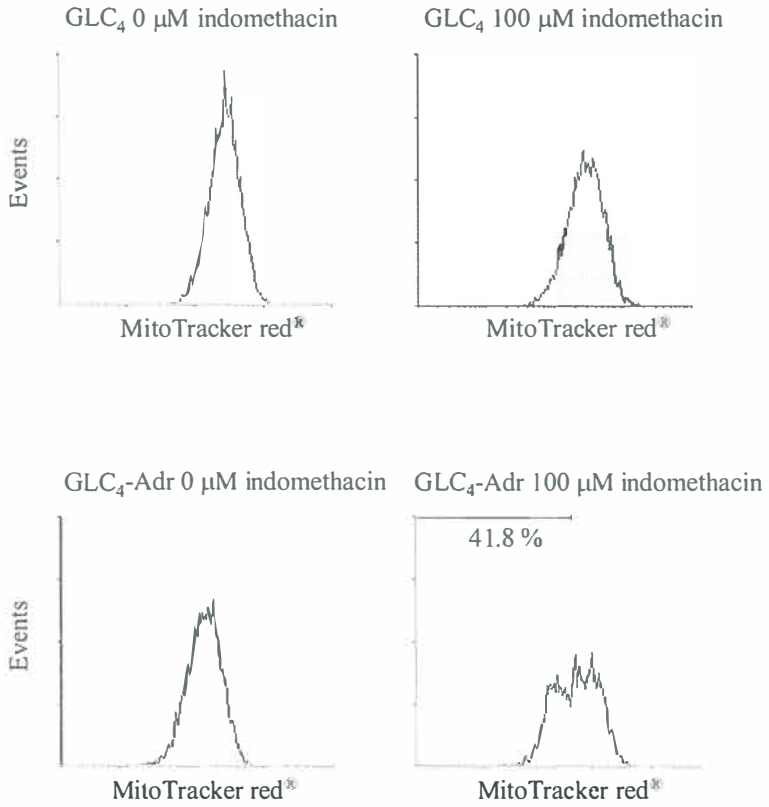


Figure 5. Flow cytometric analysis of intact mitochondria in GLC_4 and GLC_4-Adr cells after 24 h exposure to indomethacin and stained with MitoTracker red®. Cells not exposed to indomethacin were compared to cells exposed to 100 μM indomethacin. A representative example is shown.

Discussion

This is the first study demonstrating that indomethacin can induce apoptosis in an MRP1 overexpressing SCLC cell line partly through an MRP1 dependent mechanism. Efficient down-regulation of MRP1, as shown with the elevated cellular level of the MRP1 substrate CF, resulted in less apoptosis and concomitantly in an increase in cell survival following indomethacin exposure in GLC_4-Adr. Cellular GSH levels in GLC_4-Adr cells were not affected by MRP1 down-regulation but were decreased by indomethacin exposure. In addition, GSH depletion by BSO made GLC_4-Adr cells more sensitive to indomethacin, although this explains only part of the apoptosis inducing effect of indomethacin. Mitochondrial membrane potential was lost simultaneously with apoptosis induction indicating that the mitochondrial apoptosis pathway is at least partly responsible for indomethacin-mediated apoptosis in GLC_4-Adr, which confirms our previous finding that indomethacin-induced caspase-9 activation is important.

Indomethacin is a well-known glutathione S-transferase and a cyclooxygenase inhibitor, but can also have an effect on MRP1 function [18, 19]. Indomethacin not only increases chemotherapy sensitivity through inhibition of MRP1 function, but also stimulates GSH efflux in MRP1 overexpressing cell lines as was demonstrated in polarized Madin–Darby canine kidney (MDCKII) cells [9]. Drug-induced GSH efflux through MRP1 has also been described for verapamil in baby hamster kidney-21 cells transfected with human MRP1 [20]. The GSH levels in GLC_4 and GLC_4-Adr are similar but GSH turnover is much higher in GLC_4-Adr due to its increased MRP1 expression [12]. In the present study, we demonstrated that GSH levels in GLC_4-Adr did not rise by 2 h exposure to indomethacin and even slightly decreased in GLC_4-Adr suggesting that indomethacin-induced apoptosis in GLC_4-Adr is partly caused by indomethacin-induced GSH efflux.

NSAIDs can be metabolized intracellular and the metabolites can form reactive oxygen species. This last mechanism has been implicated in NSAID idiosyncratic toxicity such as bone marrow toxicity or hepatitis [21]. These reactive oxygen species are inactivated by mitochondrial GSH. In our model, cellular GSH levels slightly decreased after indomethacin exposure. Moreover, the reduction of GSH levels by BSO, a drug that specifically inhibits GSH production, increases indomethacin mediated apoptosis in GLC_4-Adr but not in GLC_4. A decrease in GSH levels alone can be an early activator of apoptosis, which has recently been demonstrated in the B cell lymphoma cell line PW after BSO exposure [22]. GSH levels were also depleted in NCI-H889 SCLC cells by the proteasome inhibitor MG132, which also resulted in apoptosis in a caspase 8-dependent manner in these GSH-depleted cells [23]. However, mitochondria of GSH depleted cells also become more sensitive to oxidative stress [24]. Oxidative stress then reduces

mitochondrial membrane potential and induces the release of several essential players of mitochondrial apoptosis pathway, such as cytochrome C and apoptosis-inducing factor in the cytosol as well as activation of caspases and apoptotic protease-activating factor-1 [24, 25]. Previously, we showed that by inhibition of caspase-9 apoptosis induction in GLC_4-Adr cells is decreased by 44% suggesting that the mitochondrial apoptosis pathway is important in indomethacin mediated apoptosis [10]. In the present study, we demonstrated that indomethacin actually causes loss of mitochondrial membrane potential and subsequently activates the intrinsic apoptosis pathway in GLC_4-Adr cells. Taken together our results indicate that indomethacin-induced apoptosis in GLC_4-Adr cells is dependent of MRP1 expression and also depends on activation of the mitochondrial apoptosis pathway. Activation of this pathway may further be enhanced by caspase-8 activation and Bid cleavage, which we reported earlier for indomethacin-treated GLC_4-Adr cells [10].

Until now only few studies used siRNA to down-regulate a member of the ABC family of transporters. A down-regulation of MRP1 and concomitant loss of MRP1 function slightly decreased indomethacin sensitivity indicating that MRP1 activity facilitates indomethacin mediated apoptosis. In addition GSH depletion by BSO increases indomethacin mediated apoptosis. These two findings indicate that MRP1 function and GSH metabolism are important in indomethacin induced apoptosis. The exploitation of a chemotherapy resistance factor such as MRP1 to induce apoptosis is a novel and potentially interesting finding.

In the future identification of MRP1 overexpressing tumors and exposure of these tumors to combination therapies including indomethacin may provide a novel approach in the treatment of MRP1-overexpressing cancers.

Acknowledgments

We thank M. van der Toorn (Department of Allergy) for his help with the GSH assays.

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5

Indomethacin and celecoxib differently affect the Fas apoptosis pathway in human ovarian cancer cell lines

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Submitted

Abstract

Drug resistance is the major reason for chemotherapy failure in ovarian cancer treatment. A common drug resistance mechanism of tumor cells is the inability to go into apoptosis. Fas Ligand (FasL), an inducer of death receptor Fas trimerization, and non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and celecoxib, are known to induce apoptosis in various tumor types. The stable transfected human ovarian cancer cell lines A2780/cmv, transfected with an empty control plasmid, and A2780/m248, transfected with p53 mutated at codon 248, differ in Fas surface levels and were used in the present study. Our aim was to investigate apoptosis induction by the cyclooxygenase (COX)-1/COX-2 inhibitor indomethacin and the COX-2 inhibitor celecoxib. In addition, apoptotic ability of these drugs in combination with agonistic anti-Fas antibody or cisplatin in the ovarian cancer cell lines was determined. Celecoxib induced higher levels of apoptosis in A2780/cmv and A2780/m248 as compared to indomethacin. Celecoxib was also a stronger inducer of caspase-8 and caspase-9 activation, while caspase-8 and caspase-9 specific inhibitors blocked celecoxib and indomethacin-induced apoptosis. However, only indomethacin induced apoptosis could be blocked up to 50% by antagonistic anti-FasL (NOK-1) and anti-Fas (ZB4) antibodies in both cell lines. Confocal laser microscopy revealed that treatment with celecoxib in contrast to indomethacin resulted in a marked increase in granular cytoplasmic FasL colocalizing with Fas. Both indomethacin and celecoxib increased the sensitivity of A2780/m248, the cell line with the highest Fas membrane level, to agonistic anti-Fas antibody without increasing Fas membrane expression. In contrast, cisplatin potentiated indomethacin-induced apoptosis but not celecoxib-induced apoptosis in A2780/cmv and A2780/m248. Our results indicate that indomethacin and celecoxib differently affect the Fas apoptosis pathway in a p53-independent manner. These drugs may therefore have different applications in anti-cancer treatment combinations.

Introduction

Ovarian cancer is the most important cause of death in women with gynecologic malignancies. Despite intensive multimodality treatment, including surgical resection followed by platinum- and paclitaxel containing chemotherapy most patients do relapse and eventually die of their disease. This indicates that drug resistance is a major reason for failure of chemotherapy. Chemotherapeutic drugs show a common reason for drug resistance, namely the failure of tumor cells to go into apoptosis following chemotherapy. This has raised interest in exploring other routes that result in apoptosis. Interestingly, the death receptor ligands induce apoptosis via a distinct pathway.

Trimerization of death receptor Fas by Fas Ligand (FasL) or agonistic anti-Fas antibody binding can activate the apoptosis pathway [1, 2]. The key signaling proteins, FADD, and procaspase-8 form a complex with trimerized Fas, resulting in active caspase-8. This initiates a cascade of caspases activation, which eventually leads to cell death. *In vitro*, chemotherapeutic drugs, such as cisplatin, can increase Fas membrane expression resulting in an enhanced sensitivity for activation of the Fas-mediated apoptosis pathway by FasL or agonistic anti-Fas antibodies. Since ovarian tumors frequently become resistant to chemotherapy, induction of the Fas-mediated apoptosis pathway is a potential option to overcome drug resistance. Intravenous administration of especially anti-Fas antibodies is toxic in mice due to severe liver damage, while soluble FasL appears to be much less toxic.

Over the last years, non-steroidal anti-inflammatory drugs (NSAIDs) have gained major interest for their capacity to induce apoptosis [3-7]. NSAIDs are a group of drugs, which are able to bind and inhibit cyclooxygenase (COX). COX catalyses a key step in the formation of prostaglandins. There are three COX isoforms, COX-1, COX-2 and the recently identified COX-3 [8]. COX-1 is responsible for housekeeping prostaglandin biosynthesis and is expressed in most tissues in the body [9]. COX-2 expression is induced by a wide spectrum of growth factors and pro-inflammatory cytokines, tumor promoters and mitogens. Population-based studies showed that long-term NSAID intake reduces the risk of developing colorectal cancer [10]. Abundant COX-2 expression has also been detected in a variety of tumor types, including ovarian tumors [11]. Antitumor effects of NSAIDs *in vitro* involve the inhibition of tumor cell growth, anti-angiogenesis, and induction of apoptosis. However, several studies have illustrated that NSAIDs can also induce apoptosis in cells not containing COX. Several biochemical mechanisms underlying NSAIDs-induced apoptosis are described, such as inactivation of the NF-κB signaling pathway, activation of the PPAR subtypes α and γ, inhibition of the activation of PPARδ and inhibition of Bcl-X_L and Mcl-1 expression. Sensitization by NSAIDs of Fas-mediated apoptosis has

been demonstrated in two studies [12, 13]. Apart from the antitumor activity of NSAIDs alone, *in vitro* as well as preclinical *in vivo* studies demonstrated a synergistic apoptotic effect by combining chemotherapy with NSAIDs. Currently, several ongoing clinical trials are testing the antitumor effect of NSAIDs in combination with chemotherapy.

The aim of the present study was to investigate apoptosis induction by the COX-1/COX-2 inhibitor indomethacin and the COX-2 inhibitor celecoxib in ovarian cancer cells. P53 mutations frequently occur in ovarian cancers and are related to chemotherapy resistance [14]. We, therefore, used the wild-type p53 expressing human ovarian cancer cell line A2780/cmv, stable transfected with an empty control plasmid, and A2780/m248, stable transfected with p53 mutated at codon 248 to determine whether NSAIDs induced apoptosis is p53-dependent. Both cell lines are resistant to anti-Fas induced apoptosis but A2780/m248 has at least 5-fold higher Fas membrane levels as compared to A2780/cmv. In addition, apoptotic ability of NSAIDs in combination with agonistic anti-Fas antibody or cisplatin in the ovarian cancer cell lines was studied.

Materials & Methods

Cell cultures

The human ovarian epithelial cancer cell lines A2780/cmv (stable transfected with empty control plasmid and expressing low Fas membrane levels) and A2780/m248 (stable transfected with p53 mutated at codon 248 and expressing 6-fold higher Fas membrane levels than A2780/cmv) were cultured in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 1 mg/ml geneticin (Invitrogen) and 10% fetal calf serum (Invitrogen) at 37 °C in a 5% CO_2. The generation of the transfectants was described earlier [15].

Cytotoxicity assay

The microculture tetrazolium assay (MTT) was used to determine the cytotoxicity of indomethacin (ICN Biomedicals, Aurora, Ohio), or celecoxib (kindly provided by Pharmacia, Woerden, the Netherlands) in the cell lines A2780/cmv and A2780/m248. In the combination assays, indomethacin or celecoxib were cocultured with cisplatin (Bristol-Myers Co., Weesp, the Netherlands). For the MTT assay, A2780/cmv cells (1,250 cells/well) or A2780/m248 cells (2,500 cells/well) were seeded in quadruplicate into 96-well culture plates in a total volume of 200 µl and incubated 72 h with or without indomethacin or celecoxib. The assay was performed as described earlier [16]. Cell survival was defined as the growth of treated cells compared to untreated cells. Inhibiting concentration (IC)50 values were defined as the concentration giving 50% cell kill.

Apoptosis assay

For apoptosis measurements, cells were seeded in 96-well culture plates and incubated with or without indomethacin or celecoxib at various concentrations and time periods. To determine whether apoptosis was caspase-dependent, cells were pre-incubation for 1 h with 20 µM zLEHD-fmk or zIETD-fmk (Calbiochem Breda, the Netherlands), then indomethacin or celecoxib was added and cells were incubated for 24 h at 37°C unless indicated otherwise. To analyze whether observed apoptosis was Fas-mediated, antagonistic (blocking Fas-mediated apoptosis) anti-FasL antibody NOK-1 (1 µg/ml, BD Biosciences, Alphen a/d Rijn, the Netherlands) or antagonistic anti-Fas antibody ZB4 (1 µg/ml, Immunotech, Marseille, France) was added 2 h prior to indomethacin or celecoxib treatment. Binding of NOK-1 and ZB4 to FasL and Fas, respectively, blocks the Fas-FasL interaction. To determine the effect of NSAIDs on anti-Fas sensitivity, cells were 2 h or 24 h pre-incubated with indomethacin or celecoxib, followed by 24 h exposure to the apoptosis inducing (agonistic) anti-Fas antibody (7C11, 0.1 µg/ml, Beckman

Coulter, Mijdrecht, the Netherlands). To determine the effect of NSAIDs on cisplatin sensitivity, cells were 2 h or 24 h pre-incubated with indomethacin or celecoxib, followed by 24 h exposure to cisplatin (10 μM). Apoptotic cells were distinguished from vital cells by acridine orange fluorescent staining of nuclei in unfixed cells. Results are expressed as the percentage of apoptotic cells in a culture by counting at least 200 cells per well.

Western blot analysis

Cell pellets were dissolved in phosphate buffered saline (PBS; 6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 mM NaCl and 2.7 mM KCl, pH 7.2). Of one aliquot the protein concentration was determined using the method of Bradford and one aliquot was lysed in 2x sample buffer (0.05 M Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue, 10% 2-β-mercaptoethanol) and heated at 100 °C for 5 min. Ten μg protein was resolved by SDS-PAGE using the BioRad Mini-protean II system (Veenendaal, The Netherlands). Samples were semidry electroblotted on PDVF membranes, activated with methanol, and blocked with skim milk in PBS/Tween for at least 1 h. After washing with PBS/Tween, the blots were incubated with the primary antibody in skim milk in PBS/Tween for 1 h at room temperature. Mouse-anti-FLIP NF6 was kindly provided by Dr. M. Peter (Chicago, IL). Rabbit-anti-caspase-3, rabbit-anti-caspase-9, and rabbit-anti-Bid were purchased from Pharmingen (Becton Dickinson, Erebodegem-Aalst, Belgium), and rabbit-anti-Bax (N20) from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-caspase-8 was purchased from Cell Signaling Technology (Leusden, the Netherlands). Mouse-anti-actin was obtained from ICN Biomedicals (Zoetermeer, the Netherlands). The secondary antibodies were labeled with horseradish peroxidase (all from DAKO, Glostrup, Denmark) and chemilumunescence was detected using the BM-chemiluminescence kit or the Lumi-Light Plus Western blotting kit (Roche Diagnostics, Almere, The Netherlands).

Flow cytometry

Cell pellets were washed with PBS and resolved in 50 μl PBS / 2% FCS / 0.01 mg/ml sodium-azide). Approximately 0.5 x 10^6 cells were incubated at 4°C for 30 min with R-phycoerythrin-conjugated Fas monoclonal antibody DX-2 (BD PharMingen, San Diego, CA). After repeated washes, cells were resuspended in 200 μl PBS and analyzed (10,000 cells) with a Coulter Elite Flow cytometer (Becton Dickinson, Mount View, CA) to detect Fas expression with gate set on intact cells by forward/side scatter analysis.

Confocal laser microscopy

To determine the effect of NSAIDs on cellular FasL localization, cells were seeded in 8-wells Lab Tek chamber slides and 24 h incubated with different indomethacin or celecoxib concentrations. After the incubation period, cells were fixed in 4% paraformaldehyde solution for 15 min, washed with PBS and washed twice with glycine (0.5 M in PBS). Immunofluorescent staining of Fas was performed according to Vekemans et al. [17]. Cells were permeabilized in 0.5% Triton X-100 at room temperature for 5 min. Thereafter, cells were stained for 1 h with anti-FasL antibody (Q-20, Santa Cruz Biotechnology, Santa Cruz, CA), 1:15 times diluted in PBS, 1% BSA, 0.2% Triton X-100. After two wash steps with PBS, cells were incubated for 1 h with FITC labeled rabbit anti-mouse antibody (Dako, Glostrup, Denmark), 1:20 times diluted in PBS + 1% BSA, and washed once in PBS. Nuclei were stained by adding 30 µl Hoechst (150 µg/ml in 1.4-diazabicyclo-[2,2,2]-octane (dabco) solution (2% dabco, 0.1 M Tris-HCl, 30% glycerol, pH 8.0). After adding the cover slip, cells were sailed using nail polish. Specific binding of the antibodies was visualized by the confocal laser microscope (Leica, Rijswijk, the Netherlands).

Statistical analysis

The paired Student's t-test was used to calculate significances. P-values < 0.05 were considered significant.

Results

Indomethacin and celecoxib induce cell growth inhibition and apoptosis in ovarian cancer cell lines

To analyze the effect of indomethacin and celexocib on the growth of the A2780/cmv and A2780/m248, cytotoxicity assays were performed. Indomethacin treatment for 96 h reduced cell growth in both cell lines (Fig. 1A).

A2780/m248 is more sensitive to indomethacin (IC_{50} = 21.0 ± 1.3 μM) than A2780/cmv (IC_{50} = 32.6 ± 5.9 μM) ($P < 0.01$). Celecoxib treatment for 96 h also inhibits the cell growth, but to the same extent in both cell lines (A2780/cmv IC_{50} = 32.5 ± 10.2 μM, A2780/m248 IC_{50} = 36.0 ± 19.5 μM) (Fig. 1B).

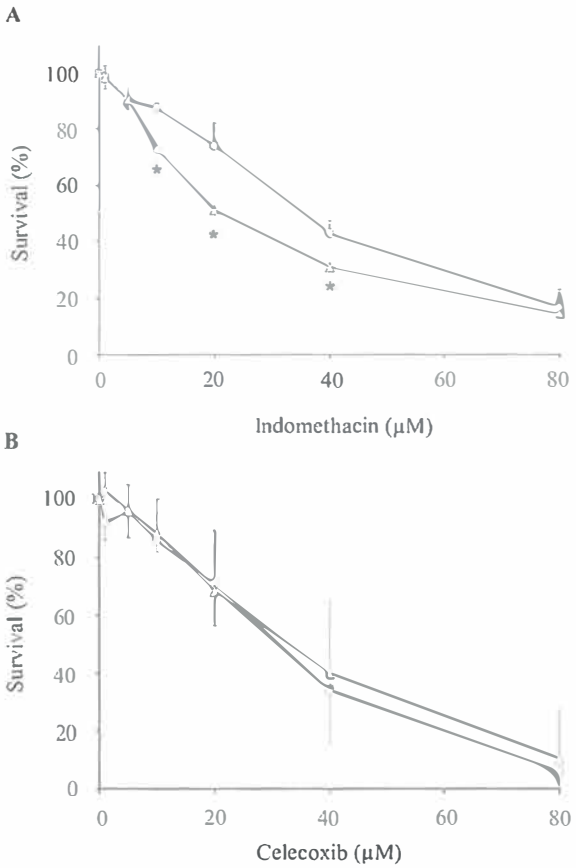


Figure 1. Survival curve of A2780/cmv (○) and A2780/m248 (Δ) treated with indomethacin **(A)** or celecoxib **(B)**. The figures represent the mean survival from three experiments each performed in quadruplicate ± SD. The asterisks indicate significant differences between A2780/cmv and A2780/m248 ($P < 0.05$).

Treatment with up to 500 μM indomethacin for 24 h resulted in 20-30% apoptotic cells in A2780/cmv and A2780/m248. Prolonged indomethacin treatment for 48 h increased the percentage of apoptotic cells in a concentration-dependent manner to more than 80% in A2780/cmv and A2780/m248 (Fig. 2A). In contrast, treatment for 24 h with celecoxib concentrations above 50 μM resulted in a very steep increase in apoptosis up till 90% at 100 μM celecoxib in A2780/cmv and A2780/m248 (Fig. 2B). Prolonged treatment for 48 h with celecoxib concentrations above 50 μM resulted in higher levels of apoptosis (Fig. 2B).

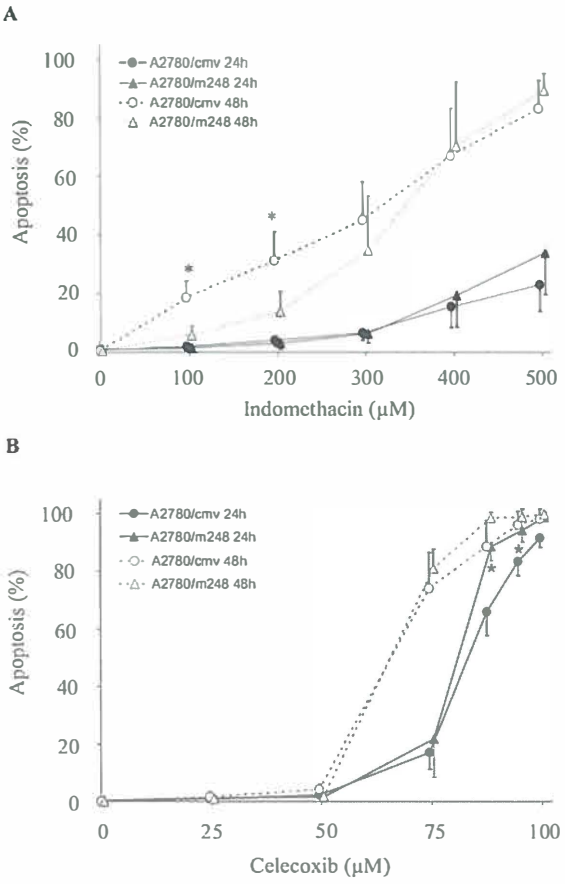


Figure 2. Apoptosis induced in A2780/cmv and A2780/m248 by indomethacin **(A)** or celecoxib **(B)** after 24 h (black line) or 48 h (dotted line) treatment. The figures represent the average apoptosis from three experiments each performed in duplicate ± SD. The asterisks indicate significant differences between apoptosis induction in A2780/cmv and A2780/m248 at 24 h or 48 h ($P < 0.05$).

Indomethacin and celecoxib induced apoptosis is caspase dependent

Western blot analysis showed that caspase-8 was activated following treatment with indomethacin or celecoxib as indicated by the intermediate product p43 after 6 hr of incubation (Fig. 3). Prolonged exposure to indomethacin or celecoxib for 24 hr also resulted in active caspase-8 product p18 (results not shown). In addition, FLIP cleavage was observed in A2780/cmv and A2780/m248. In contrast, Bid cleavage and caspase-9 activation were almost only observed after treatment with celecoxib, which also induced the highest levels of apoptosis as demonstrated by PARP cleavage (Fig. 3).

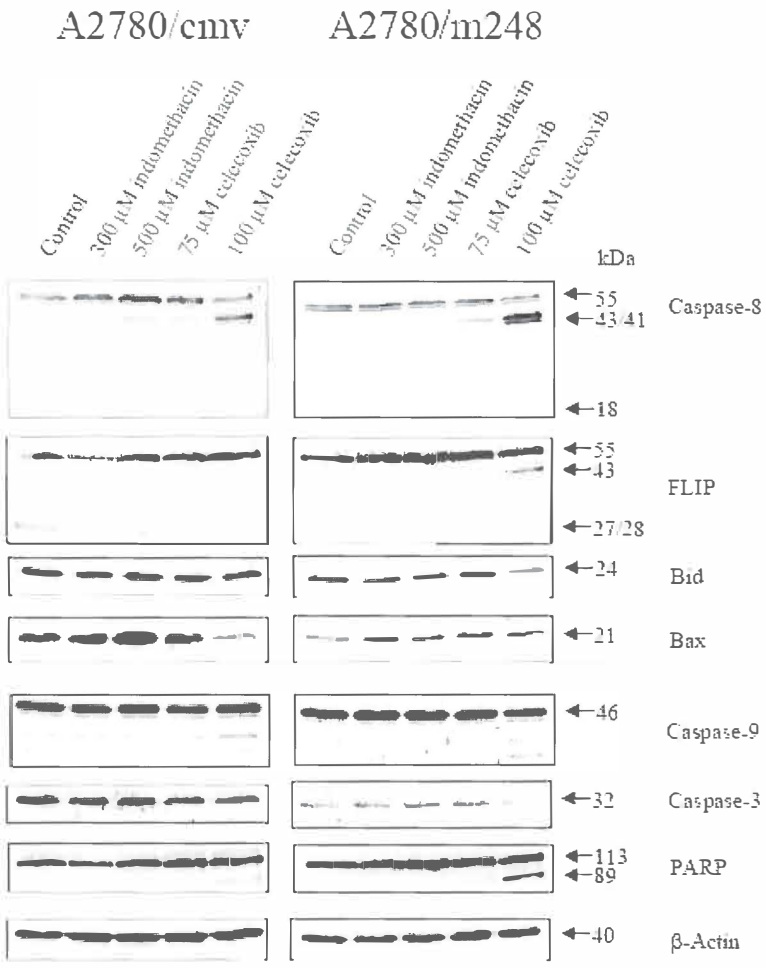


Figure 3. Western blot determination of intracellular caspase activation in A2780/cmv and A2780/m248 after treatment with indomethacin or celecoxib for 6 hr.

To determine whether the mechanism of apoptosis induction by indomethacin and celecoxib is caspase-dependent, we treated cells with the specific caspase-8 inhibitor zIETD-fmk and the caspase-9 inhibitor zLEHD-fmk. Both inhibitors reduced the level of NSAIDs-induced apoptosis, while the largest effect was observed with the caspase-8 inhibitor (Fig. 4).

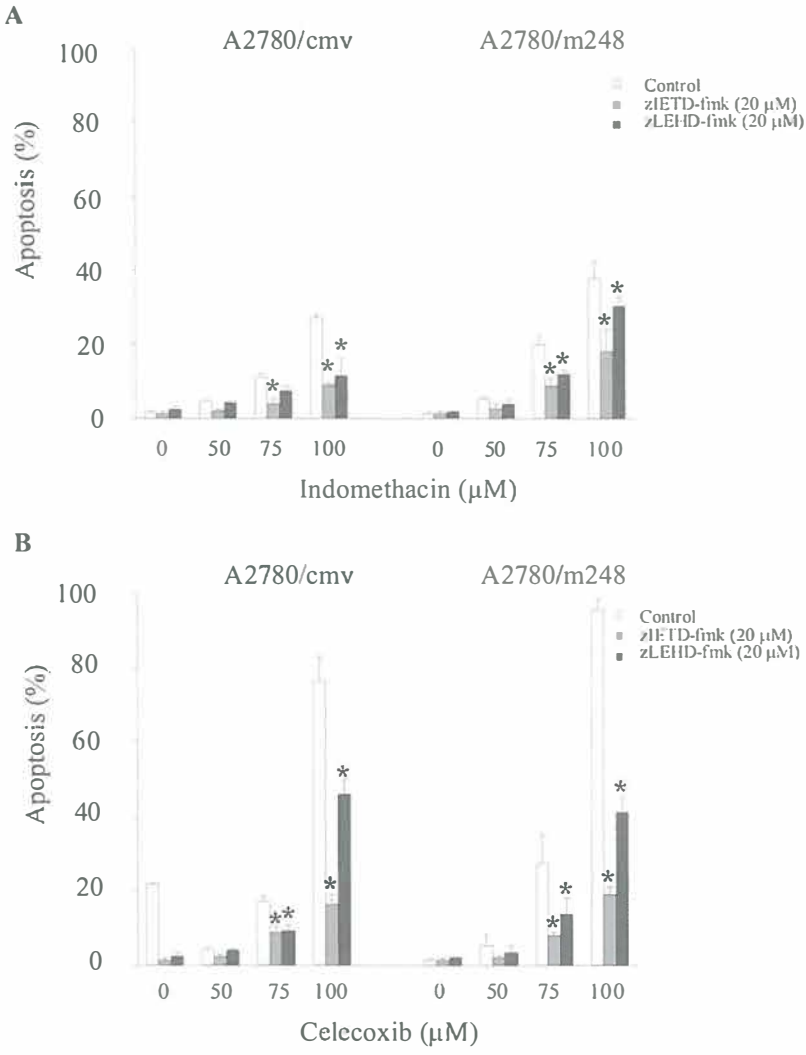


Figure 4. Inhibition of indomethacin- **(A)** and celecoxib-induced **(B)** apoptosis in A2780/cmv and A2780/248 by caspase-8 (zIETD-fmk) and caspase-9 (zLEHD-fmk) specific inhibitors. The figure represents the mean apoptosis from three experiments each performed in duplicate ± SD. The asterisks indicate significant differences between control and zIETD-fmk or zLEHD-fmk co-treated cells ($P < 0.05$).

Indomethacin induced apoptosis is dependent on Fas/FasL interaction

To determine whether the observed apoptosis induction by NSAIDs is Fas-mediated, NSAIDs were combined with the neutralizing anti-FasL antibody NOK-1 or the neutralizing anti-Fas antibody ZB4. The addition of NOK-1 as well as ZB4 resulted in a reduction of indomethacin induced apoptosis in A2780/cmv and A2780/m248 after 24 h of treatment (50 to 55 % reduction at 500 μM indomethacin) as well as after 48 h of treatment (31 to 46% reduction at 300 μM and 7 to 16% reduction at 500 μM indomethacin). Neither the addition of NOK-1 nor ZB4 had any effect on apoptosis induced by the COX-2 inhibitor celecoxib.

To analyze whether this effect was due to an induction of the Fas membrane expression by NSAIDs, their effect on the Fas membrane expression was determined. Flow cytometry analysis showed a large difference in Fas membrane expression between A2780/cmv and A2780/m248. Treatment with NSAIDs did not enhance Fas membrane expression in A2780/cmv and A2780/m248 (Fig. 5A).

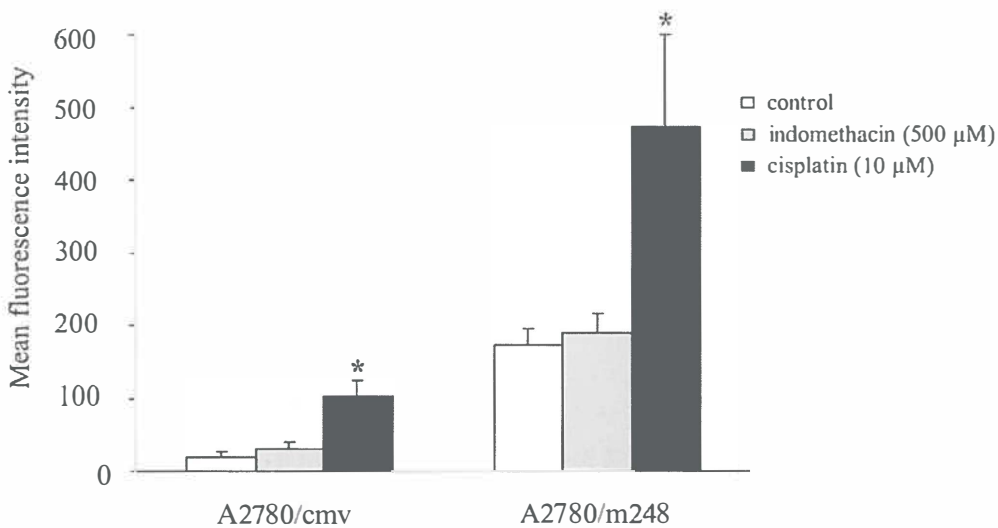


Figure 5A. Fas membrane expression in A2780/cmv and A2780/m248 after treatment with indomethacin or cisplatin for 24 hr. The figures represent the average apoptosis from three experiments each performed in duplicate ± SD. The asterisks indicate significant differences in Fas membrane expression between control, indomethacin or cisplatin treated cells ($P < 0.05$).

Confocal laser microscopy revealed granular FasL staining in the cytoplasm of A2780/cmv and A2780/m248 cells. FasL staining was frequently

present at cell membranes where cell cell contact occurred. Indomethacin treatment did not enhance or relocate FasL staining. Surprisingly, celecoxib treatment resulted in a marked increase of FasL staining in the cytoplasm (Fig. 5B). No increase in FasL expression was observed at the cellular protein level with Western blotting (results not shown). Fas expression remained relatively unchanged following treatment with indomethacin or celecoxib. However, FasL co-localizes with Fas in intense stained granules when celecoxib was used but not with indomethacin (Fig. 5B).

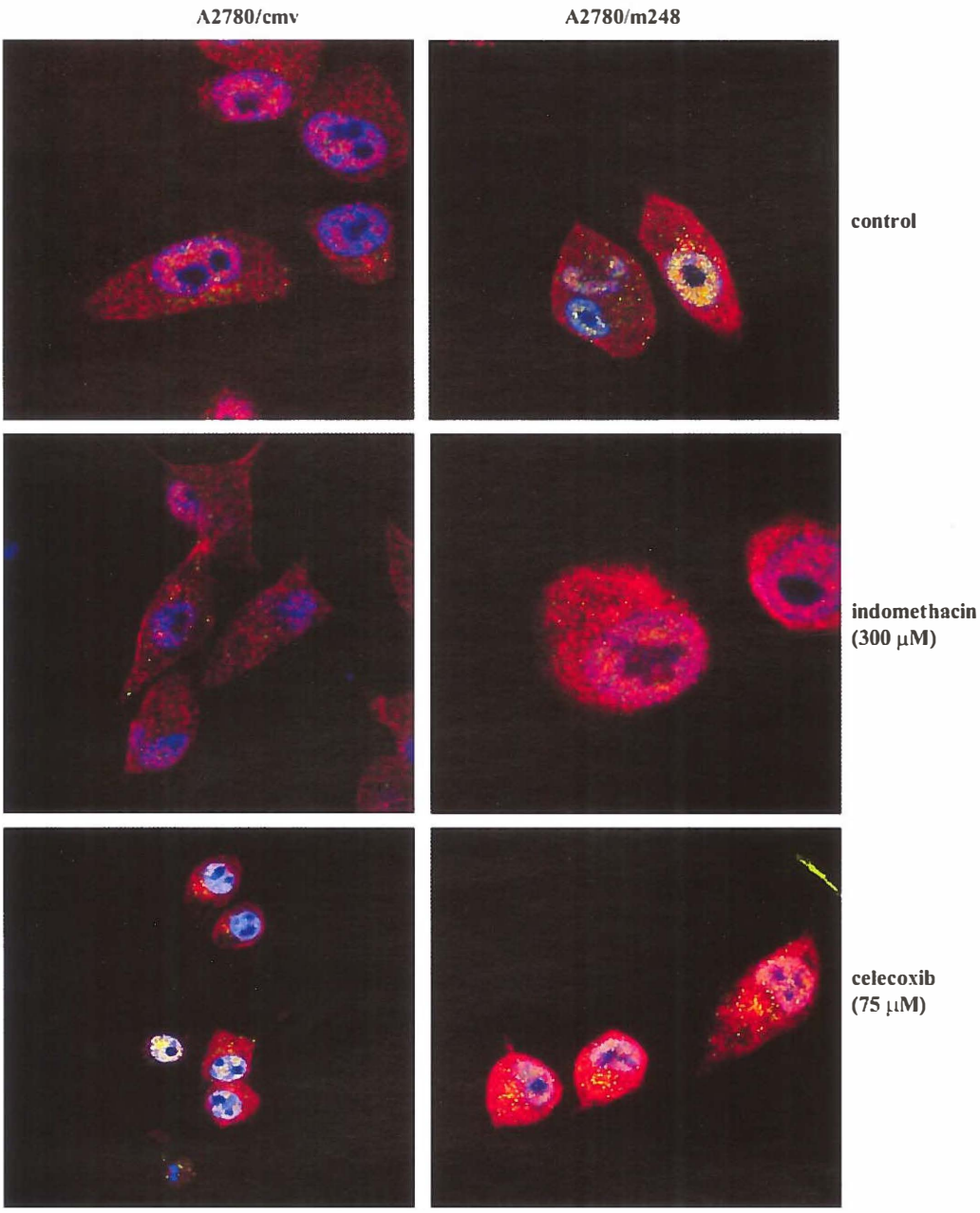
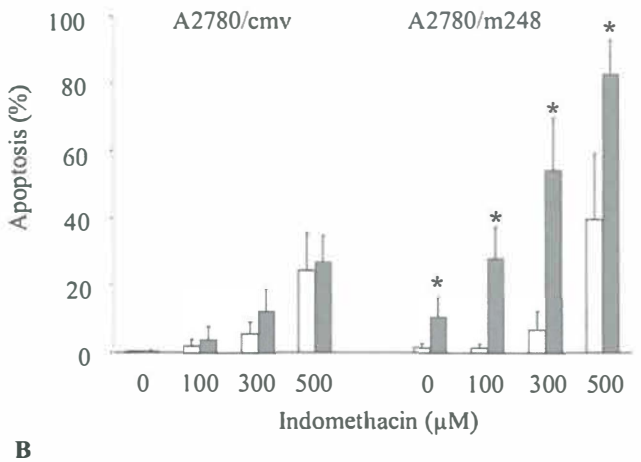


Figure 5B. Cellular localization of FasL and Fas after treatment with indomethacin and celecoxib. Cells were treated with 300 μM indomethacin or 75 μM celecoxib for 8 hr. FasL and Fas were detected with confocal laser microscopy as described in "Materials and Methods".

Indomethacin and celecoxib increase the sensitivity to anti-Fas induced apoptosis

Since NSAIDs induced caspase-8 activation and FLIP downregulation, we determined whether NSAIDs sensitize cells to Fas-mediated apoptosis. A2780/cmv and A2780/m248 were pre-incubated with indomethacin or celecoxib prior to anti-Fas antibody addition. A2780/cmv and A2780/m248 are insensitive to anti-Fas antibody (7C11) induced apoptosis. Pre-treatment with indomethacin (100 - 500 μM) for 2 h resulted in a significant increase in anti-Fas sensitivity in A2780/m248, but not in A2780/cmv (Fig. 6A). Also pretreatment of A2780/m248 with celecoxib (up to 87.5 μM) resulted in enhanced anti-Fas sensitivity (Fig. 6B). Increasing the pre-incubation time for indomethacin and celecoxib from 2 h to 24 h did not result in a stronger increase of anti-Fas sensitivity (results not shown).

A



B

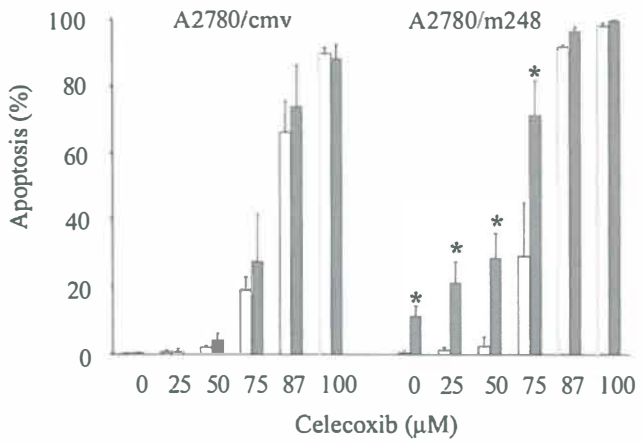


Figure 6. Percentage apoptosis in A2780/cmv and A2780/248 cells induced by anti-Fas antibodies in combination indomethacin or celecoxib. **(A)** Indomethacin incubation for 24 h (white bars) or indomethacin was added 2 h prior to anti-Fas antibody addition (grey bars). **(B)** Celecoxib incubation for 24 h (white bars) or celecoxib was added 2 h prior to anti-Fas antibody addition (grey bars). The figures represent the average apoptosis from three experiments ± SD. The asterisks indicate significant differences between control and anti-Fas co-treated cells ($P < 0.05$).

Indomethacin increases the sensitivity to cisplatin induced apoptosis

To determine the effect of NSAIDs on cisplatin induced apoptosis, A2780/cmv and A2780/m248 were pre-incubated with indomethacin or celecoxib for 2 h prior to cisplatin treatment (24 h). A2780/cmv and A2780/m248 are resistant to cisplatin induced apoptosis. Pre-incubation with indomethacin (100 – 500 μM) for 2 h followed by cisplatin (10 μM ~ IC_{50}) treatment resulted in a strong induction of apoptosis in A2780/cmv and A2780/m248 compared to apoptosis induction by indomethacin treatment alone ($P < 0.05$) (Fig. 7A). This effect was also observed, although less pronounced after 24 h indomethacin pre-incubation (data not shown). Pre-treatment with celecoxib for 2 h (Fig. 7B) or 24 h (results not shown) did not increase cisplatin induced apoptosis.

Cisplatin treatment not only stimulated indomethacin-induced apoptosis but also strongly enhanced Fas membrane expression in A2780/cmv and A2780/m248 (Fig. 5A). The anti-FasL antibody NOK-1 was added to the combination of indomethacin and cisplatin to investigate the involvement of the Fas FasL interaction in apoptosis induction. In both cell lines NOK-1 treatment inhibited apoptosis resulting in a 1.8-fold reduction in apoptosis in A2780/cmv and a 1.7-fold reduction in A2780/m248 following treatment with 500 μM indomethacin in combination with 10 μM cisplatin. Surprisingly, NOK-1 added to the combination of celecoxib and cisplatin also inhibited apoptosis. A 2.7-fold reduction in apoptosis was observed in A2780/cmv and a 2.8-fold reduction in A2780/m248 following treatment with 87.5 μM celecoxib in combination with 10 μM cisplatin.

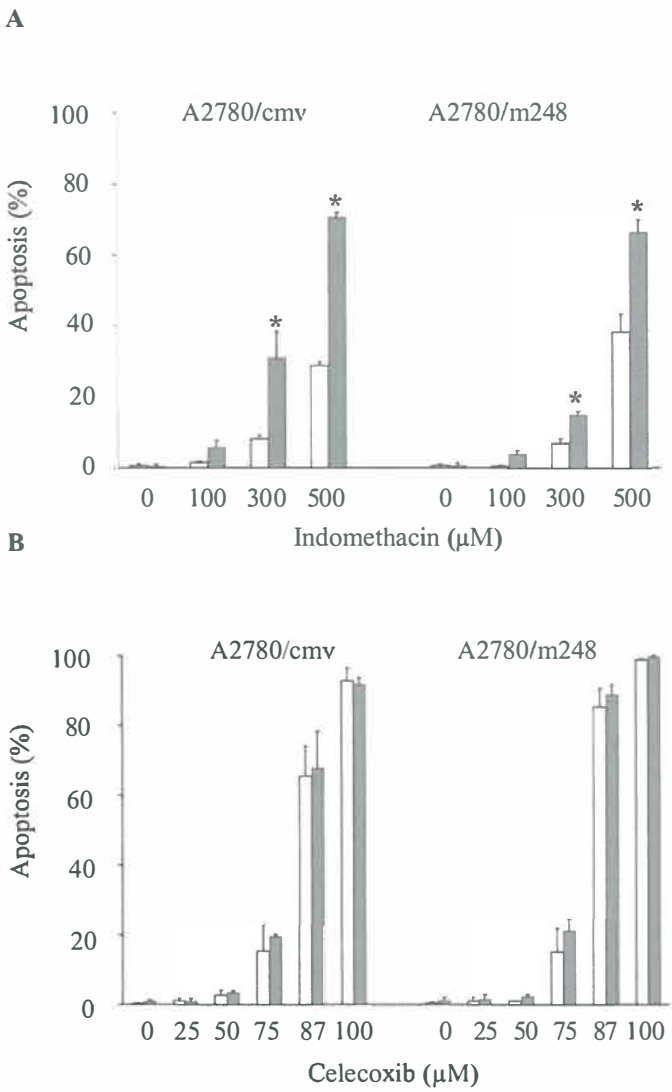


Figure 7. Percentage apoptosis in A2780/cmv and A2780/248 cells induced by cisplatin in combination with indomethacin or celecoxib. **(A)** Indomethacin incubation for 24 h (white bars) or indomethacin was added 2 h prior to cisplatin addition (grey bars). **(B)** Celecoxib incubation for 24 h (white bars) or celecoxib was added 2 h prior to cisplatin addition (grey bars). The figures represent the average apoptosis from three experiments ± SD. The asterisks indicate significant differences between control and cisplatin co-treated cells ($P < 0.05$).

Discussion

In order to circumvent chemotherapy resistance in ovarian cancer, alternative routes that result in apoptosis have become of interest. We demonstrated that the NSAIDs indomethacin and celecoxib induce apoptosis in the human ovarian cancer cell lines A2780/cmv and A2780/m248 in a caspase-dependent but p53-independent manner. Indomethacin partially utilizes the Fas-mediated apoptosis pathway via Fas FasL interaction at the cell surface, while celecoxib induces intracellular co-localization of Fas with FasL. Anti-Fas antibody in combination with indomethacin or celecoxib was only effective in A2780/m248 cells which have high Fas surface levels. A strong effect on apoptosis induction was observed with a combination of cisplatin and indomethacin, while cisplatin in combination with celecoxib had no additional effect on apoptosis compared to celecoxib in A2780/cmv and A2780/m248.

Our results suggest that indomethacin-induced apoptosis in the ovarian cancer cell lines is partially due to activation of the Fas apoptotic pathway. A similar effect has been reported for progesterone in normal and malignant human ovarian surface epithelial cells [18]. Indomethacin-induced apoptosis was blocked with the FasL antagonistic NOK-1 antibody and the Fas blocking ZB4 antibody, respectively, while no effect of these neutralizing antibodies was observed on celecoxib-induced apoptosis. Furthermore, indomethacin and celecoxib treatment induced caspase-8 and caspase-9 activation. Blocking of apoptosis induction by caspase-8 or caspase-9 specific inhibitors demonstrated the involvement of caspase activation in NSAID induced apoptosis. The indomethacin-induced FasL interaction with Fas was not the result of a strong induction of Fas or FasL membrane expression. Despite the difference in Fas expression on the membrane no major differences in apoptosis induction by either indomethacin or celecoxib were observed between A2780/cmv and A2780/m248. This may, for instance, be due to a limited FasL expression or a lack in signaling function of Fas on the membrane. The results with anti-Fas antibody 7C11 treatment alone indicated that in both cell lines trimerization of the Fas receptor is not sufficient to induce apoptosis. A possible explanation is that indomethacin treatment not only induces FasL secretion but also induces Fas relocation in rafts as observed with resveratrol and cisplatin in colon and ovarian cancer cells, respectively [19, 20], while FLIP is down regulated. This may even result in spontaneous formation of death-inducing signaling complexes that are no longer inhibited by NOK-1 and ZB4 at higher indomethacin concentrations or longer exposure to indomethacin similar to findings in previous studies using indomethacin or resveratrol [12, 19]. FasL secretion in microvescicles has also been described in ovarian cancer cell lines after lysophosphatidic acid exposure [21]. In contrast, celecoxib-induced apoptosis

was accompanied by a strong intracellular FasL staining. The increase was not at the protein level but probably due to a release of FasL from granules, which makes FasL more accessible to antibody. Compartmentalization of Fas and FasL is supposed to prevent autocrine or paracrine apoptosis in epithelial cells [22]. It is tempting to speculate that in case of celecoxib, the FasL interaction with Fas occurs intracellular and is therefor resistant to NOK-1 or ZB4 antibody. Intracellular FasL overexpression in prostate cancer cells induced Fas-mediated apoptosis possibly via FasL ligated to Fas within the Golgi or endoplasmic reticulum [23]. Storage and transport of intracellular FasL has been demonstrated in ascites-derived epithelial ovarian cancer cells. In these cells FasL is released in microvesicles to provide these cells with a mechanism by which tumors might counterattack Fas-bearing immune cells [24]. Further investigation of the role of the Fas apoptotic pathway in indomethacin- and celecoxib-induced apoptosis is thus required.

Anti-tumor effects observed with high NSAID concentrations (> 50 μM) are considered not to be attributed to COX inhibition only, but rather to COX-independent effects of NSAID treatment [25]. Other reports, however, showed that transfection of the COX-2 gene inhibited Fas-mediated apoptosis, while the COX-2 inhibitor NS-398 restores the Fas apoptosis pathway in COX-2 overexpressing cells [26]. Besides the possible induction of Fas-mediated apoptosis, several mechanisms may be involved in indomethacin- and celecoxib-induced apoptosis. These mechanisms include downregulation of the anti-apoptotic protein Bcl-X_L [27] and Mcl-1 [28] or upregulation of the pro-apoptotic proteins Bax and Bak [29, 30]. In addition, downregulation of beta-catenin [31, 32], activation of the MAP kinase pathway [33, 34], downregulation of 3-phosphoinositide-dependent kinase 1 (PDK1) activity [35] and Akt phosphorylation [36-38] have been reported. Although no upregulation of Bax was observed in A2780/cmv and A2780/m248, activation of caspase-9 by celecoxib treatment may not only be due to caspase-8 dependent Bid cleavage but also to changes in expression of other Bcl-2 family members. Similar findings were recently described for hepatocellular carcinoma cell lines [39]. Jendrossek et al. [40] also reported that celecoxib activated the mitochodrial apoptotic pathway. In contrast to our results they observed no inhibitory effect of caspase-8 specific inhibitors on celecoxib-induced apoptosis in Jurkat suggesting cell type specific effects of celecoxib. The effects of indomethacin and celecoxib seems to be largely p53-independent and Fas surface expression-independent, since we found no differences in apoptosis induction between A2780/cmv and A2780/m248 .

Indomethacin and celecoxib enhanced the sensitivity to 7C11-induced apoptosis only in A2780/m248 cells, i.e. the cell line with the highest Fas membrane level. None of the NSAIDs affected Fas membrane expression

suggesting that sensitization to 7C11-induced apoptosis especially occurred at the DISC level leading to enhanced caspase-8 activation and cleavage of cFLIP. Indomethacin-induced apoptosis was strongly elevated in combination with cisplatin in A2780/cmv and A2780/m248, while single treatment with cisplatin did not result in apoptosis. This effect was probably related to an enhanced Fas/FasL interaction at the cell surface, since cisplatin massively induced Fas membrane expression, while NOK-1 strongly inhibited apoptosis induced with this combination in both cell lines. Only few reports described the effects of indomethacin and cisplatin. Earlier reports demonstrated increased cytotoxicity of cisplatin in combination with indomethacin in uterine cervical cancer cells, associated with increased intracellular incorporation of free cisplatin [41]. In addition, an *in vivo* study showed that indomethacin and cisplatin only partially reduced the growth of colon tumors inoculated into mice after treatment with each drug on its own. However, a synergistic effect was observed when indomethacin in combination with cisplatin was given [42]. In contrast, the cytotoxicity of cisplatin was unaffected by indomethacin in lung cancer cells [43], malignant glioma cells [44] and liver tumor cells [45]. In contrast, apoptosis induction by celecoxib, a specific COX-2 inhibitor, was not enhanced in the presence of cisplatin. Interestingly, celecoxib-induced apoptosis was not sensitive to NOK-1, whereas apoptosis induced by celecoxib in combination with cisplatin was strongly inhibited. These results suggest that cisplatin treatment stimulated the Fas/FasL interaction at the cell surface, while at the same time the celecoxib-induced intracellular activation via Fas and/or FasL diminishes. The fact that cisplatin induces FasL as well as Fas membrane expression in ovarian cancer cells supports this observation [19], (Fig. 5A). The differences in mode of action between indomethacin and celecoxib needs further study, which has to take in account COX-dependent and COX-independent effects.

The abundant expression of Fas and FasL in human ovarian cancer, as we and other have previously demonstrated, and the p53-independence of indomethacin or celecoxib-induced apoptosis makes these drugs of clinical interest in combination with ligands activating the Fas pathway or in combination with chemotherapy [46, 47]. Although anti-Fas antibody is highly toxic to mice due to liver toxicity, FasL demonstrated much less liver toxicity. With the development of even more active FasL known as MegaFasL or FasL-based fusion protein one may envision a therapy based on intraperitoneally or even systemic application of FasL and indomethacin or celecoxib for ovarian cancer [48, 49]. However, based on our results such an approach is depending on the Fas surface expression. In contrast, indomethacin in combination with cisplatin induced apoptosis independently of the

p53 status and Fas surface expression. Thus, depending on the patient tumor characteristics different combinations can be envisioned.

Our results indicate that indomethacin and celecoxib exhibit different effects on the Fas apoptosis pathway in a p53-independent manner in ovarian cancer cell lines. These drugs may therefore find different applications in anti-cancer treatment as single agent and particularly in combination with cytostatic drugs.

Acknowledgments

We thank Dr. T.G. van Kooten (Department of Biomaterials) for his help using the confocal laser microscope. This study was supported by grant RUG 99-1880 of the Dutch Cancer Society.

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6

Estrogen antagonists enhance TNF related apoptosis inducing ligant (TRAIL) induced apoptosis in human breast cancer cells

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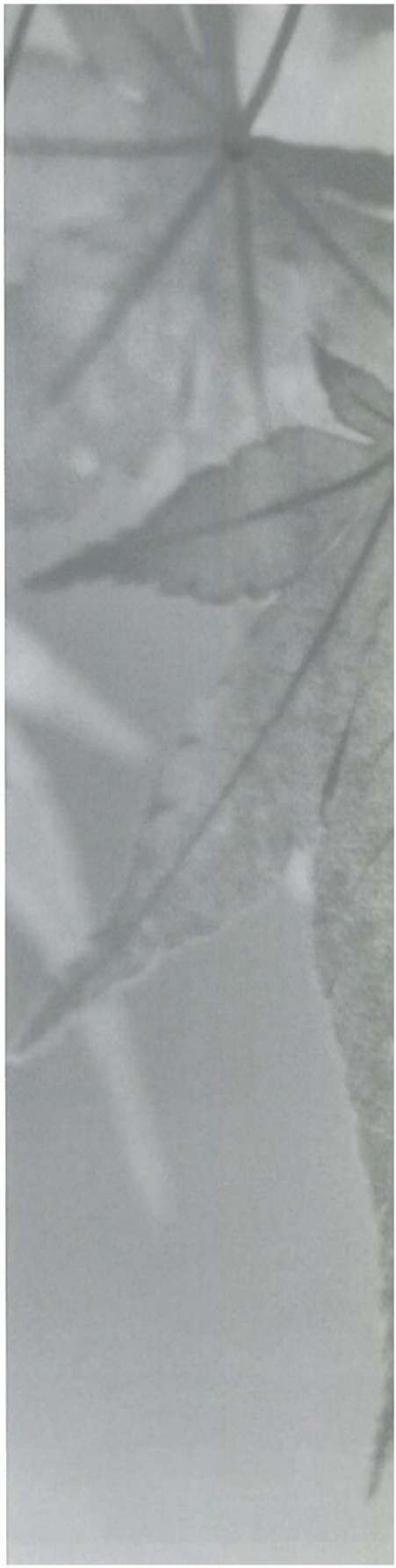

Abstract

Recombinant human TNF related apoptosis inducing ligand (rhTRAIL) is a novel potential anti-cancer drug showing activity against human breast cancer cell lines. This study analyzes whether the estrogen antagonists tamoxifen and fulvestrant, enhance rhTRAIL-induced apoptosis in the estrogen-receptor (ER) positive, caspase-3 positive T47D and in ER positive MCF-7 human breast cancer cell lines. No apoptosis induction occurred following 20 μM tamoxifen or 100 ng/mL rhTRAIL in T47D, while the combination induced 40 ± 16% (mean ± SD) apoptosis. The sensitizing effect is not due to increased TRAIL receptor cell surface expression. More important, tamoxifen and fulvestrant in combination with rhTRAIL resulted in stabilization of cleaved caspase-8 in T47D but not in MCF-7. Similar results were obtained with a proteasome inhibitor plus rhTRAIL. Since T47D in contrast to MCF-7 expresses caspase-3, we investigated whether estrogen inhibition affected the interaction between X-linked inhibitor of apoptosis (XIAP) and caspase-3. Immunoprecipitation demonstrated an interaction between XIAP and caspase-3 in T47D cells. Downregulation of XIAP with RNA interference in combination with rhTRAIL induced 27 ± 9% apoptosis in T47D but not in the caspase-3 negative MCF-7 cells. These results suggest that the modulating effect of estrogen antagonists on rhTRAIL mediated apoptosis in T47D cells is caused by increased stabilization of active caspase-8, which may be enhanced by a caspase-3 amplification loop. Thus, a combination of estrogen antagonists and rhTRAIL can be considered to enhance apoptosis in ER positive breast cancer cells for which the sensitizing effect depends on at least partially on caspase-3 expression.

Introduction

Anti-estrogens are widely used in the treatment of breast cancer. Estrogen is the primary stimulus for breast cell proliferation. Anti-estrogens such as tamoxifen induce growth inhibition of breast cancer cells through estrogen deprivation. Apart from the estrogen antagonist tamoxifen more recently also fulvestrant is used in the clinic. Fulvestrant binds, blocks and degrades the estrogen receptor (ER), thus downregulating cellular ER levels and has in contrast to tamoxifen no agonistic effects [1]. Although more than 60% of human breast cancers are ER-positive, no more than 50% of these ER-positive tumors respond to endocrine therapy. However also most of these patients develop tamoxifen resistance over time [2]. In addition, 5% to 10% of the patients designated ER-negative appear to initially respond to endocrine therapy [3]. Therefore, increased efficacy of anti-estrogens will have a major impact on breast cancer treatment. In cell line models apoptosis induction and growth inhibition have been described after anti-estrogens exposure [4].

The role of the tumor necrosis factor (TNF) family in inducing programmed cell death or apoptosis in breast cancer cells has gained increasing interest in recent studies. Both Fas Ligand (FasL) and TNF-Related Apoptosis Inducing Ligand (TRAIL) were overexpressed in malignant breast tissue compared to benign counterparts [5-9]. TRAIL has a family of agonistic receptors (DR4 and DR5), and antagonistic receptors (DcR1 and DcR2) [10-12] and can induce apoptosis in a fairly tumor specific fashion. Therefore, studies addressing TRAIL induced apoptosis in breast cancer cells are of interest [13-18].

TRAIL-mediated apoptosis can be executed via two different cellular pathways, the death receptor mediated (extrinsic) pathway and the mitochondrial (intrinsic) pathway [19, 20]. Following binding of rhTRAIL to DR4 and DR5, receptor trimerization induces the recruitment of FADD and caspase-8. Autocleavage of caspase-8 results in active caspase-8, which can then activate caspase-3. Cellular FLICE-inhibitory protein (c-FLIP) is an important inhibitor of caspase-8 activation. Caspase-8 can also indirectly activate caspase-3 via the mitochondrial pathway following Bid cleavage. In that case, cytochrome C and Smac/DIABLO, which are released from the mitochondria, induce caspase-9 activation and X-linked inhibitor of apoptosis (XIAP) inactivation, finally resulting in active caspase-3. XIAP is a key determinant of TRAIL-induced apoptosis [21-27]. Proteasomal degradation of caspase-3, caspase-9 and caspase-7 is one of the supposed mechanisms XIAP, and possibly c-IAP1 and cIAP2 as well, is mediating [18].

Although sensitization of cancer cell lines to TRAIL-mediated apoptosis by co-exposure to chemotherapeutic agents has been well established [17, 28, 29],

the combination of anti-estrogens such as tamoxifen or fulvestrant with rhTRAIL has never been explored. Given the relevance of optimizing hormonal treatment of breast cancer patients and agonistic TRAIL death receptor antibodies this is of clear interest. In the present study, we used ER positive human breast cancer cell lines, and investigated the mechanism of apoptosis induction by estrogen antagonists in combination with rhTRAIL. Experiments were performed in a serum supplemented setting to mimic the physiological situation. Activation of caspase-8 and the importance of XIAP as an inhibitor of apoptosis in breast cancer cell lines were studied in detail using immunoprecipitation and RNA interference in a caspase-3 wild-type and mutant breast cancer cell line.

Materials & Methods

Antibodies and reagents

The antibodies used for Western blot analysis were all diluted in Tris buffered saline (TBS) buffer (20 mM Tris-HCl, 137 mM NaCl and 0.05% Tween 20) supplemented with 5% skim milk powder (Merck, Darmstadt, Germany), XIAP and caspase-3 (for immunoprecipitation) antibodies were purchased from Transduction Laboratories (Alphen a/d Rijn, the Netherlands), DR4, Bax-, Bcl-2-, and Bcl-$X_{S/L}$ antibodies from Santa Cruz (Heerhugowaard, the Netherlands), caspase-8 antibody from Cell Signalling (Leusden, the Netherlands), caspase-9, and caspase-3-antibodies were obtained from Pharmingen (Alphen a/d Rijn, the Netherlands), Bid antibody from Biosource (Etten-Leur the Netherlands) and DR5 antibody from Oncogene Research Products (Calbiochem-Novabiochem, Germany). The PARP antibody was obtained from Roche (Almere, the Netherlands), FLIP antibody from Alexis (Breda, the Netherlands) and β-actin antibody from ICN Biomedicals (Zoetermeer, the Netherlands). DR4, DR5, DcR1, and DcR2 antibodies used for flow cytometry were obtained from R&D systems (Abingdon, United Kingdom). All secondary antibodies were purchased from DAKO (Glostrup, Denmark). 2,5-diphenyltetrazolium bromide (MTT) and tamoxifen were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). The proteasome inhibitor MG132 was purchased from Calbiochem (Breda, The Netherlands). Fulvestant was purchased from Astra Zeneca Pharmaceuticals (Macclesfield, United Kingdom)

SDS gel electrophoresis and Western blot

Proteins for Western blot analysis were extracted by lysing cells with lysis buffer containing 0.125 M Tris HCl, 2% SDS, 10% glycerol and 0.001% bromophenol blue. Cells were harvested after 4 h rhTRAIL exposure to detect all possible caspase cleavage products. Samples were boiled for 5 min. Twenty μg of protein was resolved on 10% SDS polyacrylamide gels at 200 V and transblotted onto polyvinylidene difluoride membranes (PVDF) (Millipore, Bedford, United Kingdom) with a semi-dry blot system. Membranes were activated in methanol for 5 min and washed 3 times with H_2O and once with TBS without Tween 20. Membranes were then blocked for 1 h in TBS supplemented with 5% skim milk and probed with the primary antibody for 1 h. Membranes were washed 3 times with TBS and incubated with the horseradish peroxidase bound secondary antibody for 1 h at room temperature. Membranes were washed 3 times with TBS and bands were visualized with chemoluminiscence POD or Lumi-light$^+$ (Roche Diagnostics, Almere, the Netherlands). Experiments were performed three times.

Flow cytometry

To determine TRAIL receptor membrane expression, cells of both cell lines were incubated for 20 h with 20 μM of tamoxifen, harvested with trypsin and subsequently washed and stained as has been described previously [29]. Analysis was performed on a Coulter Elite Flow cytometer (Becton Dickinson, Mountain View, CA) with Winlist software (Verity, Nieuwegein, Netherlands). These experiments were performed three times.

Apoptosis assay

Cells ($1x10^4$ per well) were cultured in 96-wells plates and incubated with tamoxifen, rhTRAIL or combinations. Cells were exposed to tamoxifen (20 μM) for 16 h and subsequently for 6 h with the combination of rhTRAIL (0.1 μg/mL) and tamoxifen (20 μM). Caspase inhibitors were added 1 h before rhTRAIL exposure. Apoptosis was defined by the appearance of apoptotic bodies and/or chromatin condensation, using a fluorescence microscope. Results are expressed as the percentage of apoptotic cells in a culture by counting at least 400 cells per well. All apoptosis assays were performed three times.

Fulvestrant and MG132

Cells ($3x10^5$ per well) were cultured in 6-wells plates and incubated with fulvestrant, rhTRAIL or combinations. Cells were exposed to 10 μM fulvestrant for 16 h and subsequently for 6 h with the combination of rhTRAIL (0.1 μg/mL) and fulvestrant (10 μM). For the experiments with the proteasome inhibitor MG132, cells were cultured in 6-wells plates and pre-incubated with MG132 (10 μM) for 2 h after which the cells were exposed to rhTRAIL in combination with MG132 (10 μM). After exposure to the agents previously mentioned cells were lysed in SDS lysis buffer and protein expression was analyzed by Western blot analysis. Experiments were performed 3 times.

RNA interference

Small interfering RNAs (siRNAs) specific for human XIAP were produced by Eurogentec (Liege, Belgium) 5'-GUG GUA GUC CUG UUU CAG CdTdT-3' (sense) and GCU GAA ACA GGA CUA CCA CdTdT-3' (antisense). Double-stranded RNA molecules specific for the luciferase (Luc) gene served as control (30). The sequences for Luc RNA molecules were 5'-CUU ACG CUG AGU ACU UCG AdTdT-30 (sense) and 5'-UCG AAG UAC UCA GCG UAA GdTdT-30 (antisense). MCF-7 and T47D cells ($3x10^5$/well) were transfected in six-well plates with 10 μL of 20 μM siRNA duplexes using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV, Breda, The Netherlands). After 24

h, cells were harvested, and plated in 96 wells or six wells for an apoptosis assay or for protein isolation, respectively. At 6 h after rhTRAIL or medium exposure, acridine orange was added and apoptosis was determined by acridine orange apoptosis assay or the cells were lysed for protein analysis.

2,5-diphenyltetrazolium bromide (MTT) assay

The cell lines were cultured in HAM/F12 and DMEM medium (1:1) (Life Technologies) supplemented with 20% FCS. The effect of tamoxifen and rhTRAIL on survival was tested in the MTT assay as described previously [31]. Cells were incubated for 4 days at 37 ^{0}C and 5% CO_2 in a humidified environment with a range of tamoxifen or rhTRAIL concentrations. After a 4-day culture period, MTT (5 mg/mL in phosphate buffered saline (PBS): 6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 mM NaCl; 2.7 mM KCl; pH 7.2) was added and formazan crystal production was measured as described previously. Controls consisted of media without cells (background extinction) and cells incubated with medium instead of chemotherapeutic agents. Experiments were performed three times in quadruplicate.

Immunoprecipitation and Western blot analysis

Tamoxifen and rhTRAIL exposed T47D cells were harvested and washed once in cold PBS. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), and complete protease inhibitors (Roche Diagnostics) for 15 min on ice. After centrifugation at 2,500 g at 4 ^{0}C for 10 min, protein concentration of the supernatant was determined and equal amounts of proteins were used for the immunoprecipitation. Anti-caspase-3 (Transduction Laboratories; 2 μg/ml) was added to the lysates and reacted at 4 ^{0}C for 1 h. Immune complexes were precipitated using 30 μL protein A sepharose (Amersham, Buckinghamshire, United Kingdom) and washed three times in 1.0 mL lysis buffer. The precipitate was examined for the presence of caspase-3 and XIAP by Western blot analysis as described above. Goat HRP-conjugated secondary antibody specific for mouse IgG1 (Southern Biotechnologies, AL) was used for the detection of XIAP. Horseradish peroxidase-conjugated Protein G (Sigma Aldrich, Zwijndrecht, The Netherlands) was used for detection of the goat caspase-3 antibody. The anti-mouse secondary antibody was a horseradish peroxidase labeled rabbit anti-mouse, which was diluted 1:1500 in TBS supplemented with 5% milk. The secondary anti-rabbit antibody, a swine anti-rabbit, was diluted 1:1500. Against goat primary antibodies a rabbit anti-goat horseradish peroxidase (HRP) labeled antibody 1:2000 was used.

Statistics

All experiments were performed at least three times on different occasions. Analysis included double sided non-paired t-test. A P-value < 0.05 was considered significant.

Results

Growth inhibition and apoptosis induction by tamoxifen and rhTRAIL in human breast cancer cells

Cell survival assays were used to determine the sensitivity of T47D and MCF-7 for tamoxifen and rhTRAIL. T47D are more sensitive to tamoxifen than MCF-7 with 50% inhibitory concentrations (IC_{50}) values of 5.4 μM for T47D and 30.4 μM for MCF-7 following continuous treatment with tamoxifen for 4 days. The IC_{50} values of continuous incubation with rhTRAIL show a resistance for TRAIL with >1000 ng/mL for T47D and rhTRAIL sensitivity for MCF-7 of 3.52 ± 2.38 ng/mL (mean ± SD).

The constitutive expression levels of proteins involved in the TRAIL-mediated apoptosis pathway, were analyzed by Western blotting (Fig. 1A). Caspase-8 levels were much lower in T47D as compared to MCF-7, while caspase-3 was not expressed in MCF-7 at all. No differences were observed for the other proteins analyzed.

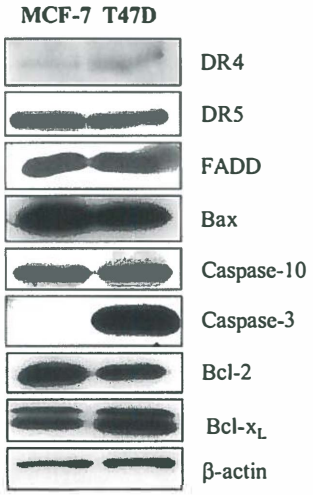

Figure 1A. Basic expression levels of pro-apoptotic and anti-apoptotic proteins in T47D and MCF-7 in the TRAIL-mediated apoptosis pathway.

TRAIL receptor expression at the cell surface was studied with flow cytometry. DR4 surface levels in T47D and MCF-7 were comparable, while DR5 expression was higher in T47D (Fig. 1B). DcR1 but not DcR2 membrane expression was significantly higher in T47D compared to MCF-7. Tamoxifen did not induce upregulation of DR4 or DR5 membrane expression in both cell lines (Fig. 1B). Rather, DR5 and DcR1 membrane expression was slightly decreased in MCF-7.

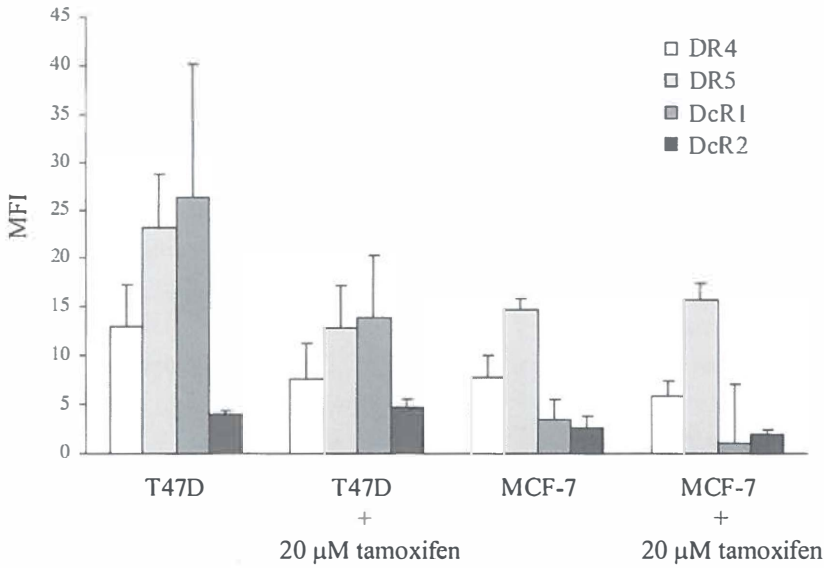

Figure 1B. TRAIL receptor membrane expression in T47D and MCF-7 cells measured by flow cytometric analysis after exposure to 20 μM of tamoxifen for 20 h.

The difference in rhTRAIL sensitivity between T47D and MCF-7 was reflected in the level of apoptosis induction in both cell lines. rhTRAIL or tamoxifen treatment only induced minimal apoptosis in T47D cells (Fig. 2A). In MCF-7 cells, apoptosis could not be quantified based on morphological changes. With PARP cleavage as a marker for apoptosis in MCF-7, rhTRAIL but not tamoxifen treatment was shown to result in substantial PARP cleavage (see Fig. 2B). Thereafter cells were pre-incubated for 16 h with tamoxifen and exposed to 0.1 μg/ml rhTRAIL in combination with tamoxifen for an additional 4 h. The combination resulted in 43 ± 16.1% (mean ± SD) apoptosis in T47D. Apoptosis induction was prevented by the caspase-8 inhibitor zIETD-fmk, the caspase-9 inhibitor zLEHD-fmk and the pan-caspase inhibitor zVAD-fmk (Fig. 2A).

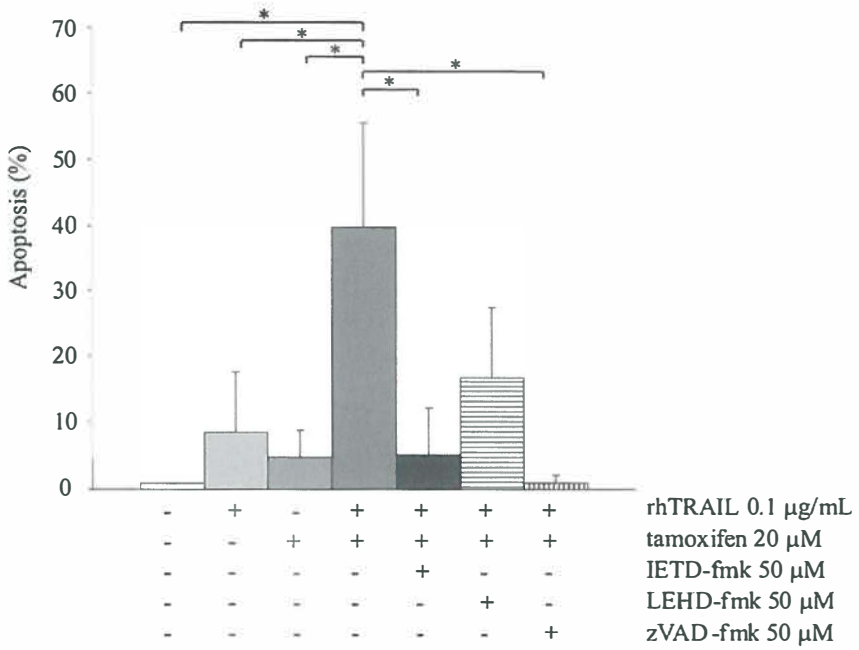

Figure 2A. TRAIL-mediated apoptosis induction. Apoptosis induction in T47D was determined after exposure to medium, 0.1 µg/mL TRAIL for 6 h, 20 µM tamoxifen for 20 h or both using the apoptosis assay. Cells were also exposed to tamoxifen and rhTRAIL in combination with a caspase-8, caspase-9 and pan-caspase inhibitor. Data represent the mean ± SD of three independent experiments (* $P < 0.05$).

In MCF-7 cells no increase in PARP cleavage was observed with a combination of tamoxifen and rhTRAIL compared to rhTRAIL alone (Fig. 2B).

Estrogen antagonists in combination with rhTRAIL strongly enhances active caspase-8 levels in T47D cells

The apoptosis-inducing effect of tamoxifen in combination with rhTRAIL was further investigated at the molecular level. Both cell lines were pre-incubated for 16 h with tamoxifen and exposed to 0.1 µg/mL rhTRAIL in combination with tamoxifen for 4 h. Tamoxifen induced no changes in expression of proteins involved in rhTRAIL-mediated apoptosis in T47D cells, while rhTRAIL treatment resulted in a slight increase in the intermediate p43/p41 cleavage product of caspase-8 but also in a complete cleavage of FLIP. Tamoxifen in combination with rhTRAIL resulted in a strong accumulation of active caspase-8 (p18 fragment) and the intermediate p43/p41 cleavage product. Procaspase-8 remained almost undetectable similar to procaspase-8 levels in control, tamoxifen or rhTRAIL treated T47D cells. More downstream proteins such as Bid and procaspase-3 were

then completely cleaved as determined by a decrease in full-length proteins and PARP cleavage. In MCF-7 cells rhTRAIL treatment resulted in the appearance of active caspase-8, cleaved FLIP and decreased Bid levels, while PARP cleavage was clearly detectable. In combination with tamoxifen no enhanced levels of active caspase-8 were observed in MCF-7 with rhTRAIL. Caspase-9 cleavage was not detected in either cell line (Fig. 2B).

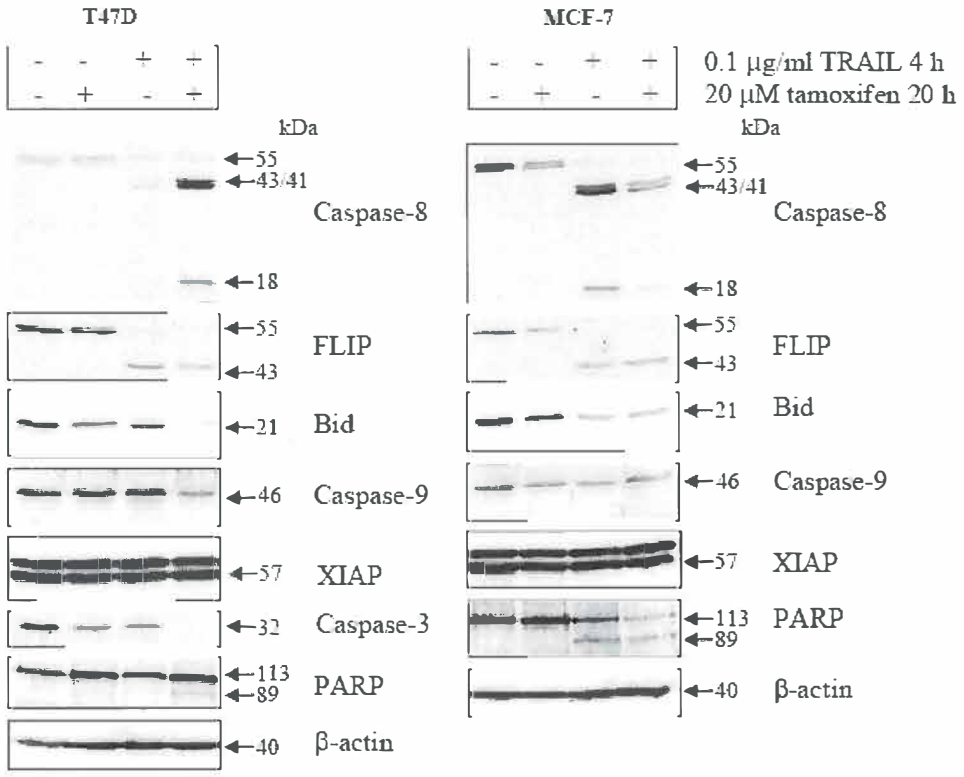

Figure 2B. TRAIL-mediated cleavage of PARP, Bid, FLIP, caspase-9 and caspase-8. Caspase cleavage was determined after exposing T47D and MCF-7 to TRAIL for 4h and tamoxifen for 16 h prior to TRAIL exposure. A representative example of three independent experiments is shown.

These results suggest that the sensitizing effect of tamoxifen in combination with rhTRAIL on apoptosis in T47D cells is related to an increased stabilization of the intermediate p43/p41 and the active p18 cleavage products of caspase-8.

To investigate whether the effect of tamoxifen in combination with rhTRAIL on T47D is estrogen inhibition dependent, cells were pretreated with the specific estrogen antagonist fulvestrant for 16 h prior to rhTRAIL exposure for 4 h.

Fulvestrant exposure alone did not result in caspase-8 activation or PARP cleavage, but in combination with rhTRAIL increased levels of p43/p41 and p18 caspase-8 cleavage products were observed, while PARP cleavage was increased. In MCF-7, rhTRAIL alone induced caspase-8 and PARP cleavage. Addition of fulvestrant does not enhance this effect (Fig. 3).

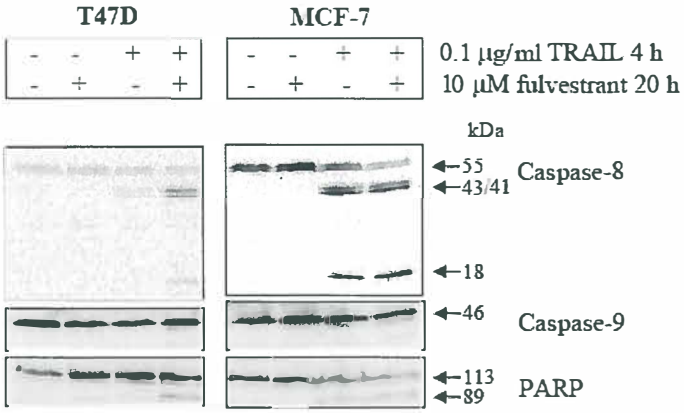

Figure 3. TRAIL-mediated cleavage of caspase-8, caspase-9 and PARP. Caspase cleavage was determined after exposing T47D and MCF-7 to TRAIL for 4 h and fulvestrant for 16 h prior to TRAIL exposure. A representative example of three independent experiments is shown.

Induction of active caspase-8 in T47D by estrogen antagonists in combination with rhTRAIL is due to protein stabilization

The caspase-8 mRNA expression levels were not different between T47D and MCF-7, despite the differences in protein level. Furthermore caspase-8 mRNA expression levels did not dramatically change in both cell lines following treatment with tamoxifen, rhTRAIL or a combination of these drugs (data not shown). The increase in caspase-8 cleavage products following estrogen antagonists in combination with rhTRAIL may thus be caused by an enhanced stabilization of the caspase-8 cleavage products. We therefore used MG132 in combination with rhTRAIL. Procaspase-8 levels did not increase after MG132 exposure alone in either cell line. However, exposure to the combination of MG132 and rhTRAIL resulted in accumulation of active caspase-8 in T47D. In MCF-7 exposure to the combination of MG132 and rhTRAIL did not result in strongly enhanced levels of p43/p41 and p18 caspase-8 cleavage products as compared to rhTRAIL treatment (Fig. 4).

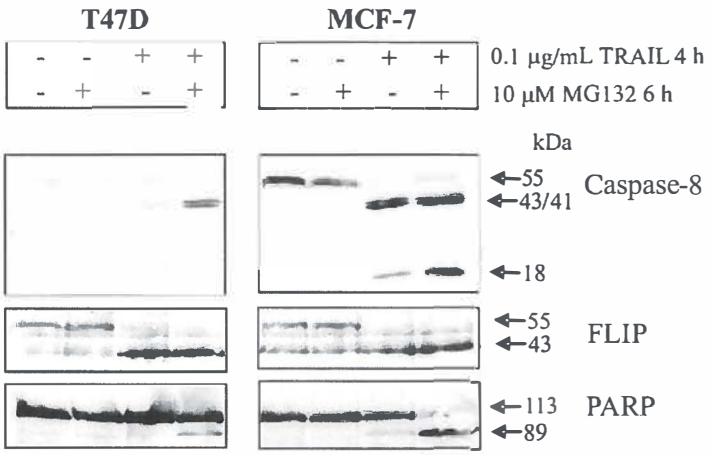

Figure 4. Caspase activation in T47D and MCF-7 after exposure to 10 μM MG132 in combination with 0.1 μg/mL of TRAIL. MG132 2h was followed by 6 h of TRAIL and MG132 exposure. A representative example of three independent experiments is shown.

XIAP and caspase-3 coprecipitate in T47D lysates following rhTRAIL treatment

A major difference between T47D and MCF-7 is the absence of caspase-3 in the latter. In addition, apoptosis induction by the combination of tamoxifen and rhTRAIL in T47D was blocked with the caspase-9 inhibitor LEHD-fmk suggesting the involvement of the mitochondrial pathway. Previous studies have demonstrated that XIAP is an important regulator of TRAIL sensitivity. Moreover, XIAP inhibits caspase-3 and caspase-9 activity via a direct interaction with these caspases. We thus hypothesized that tamoxifen may either directly or indirectly affect the interaction between caspase-3, caspase-9 and XIAP. Basal protein levels of Bcl-X_L and XIAP were higher in T47D, whereas cIAP-2 expression was lower in T47D compared to MCF-7 (Fig. 5A). No changes in protein levels of the most important antiapoptotic Bcl-2 and IAP family members, however, were observed following a 20 h tamoxifen exposure.

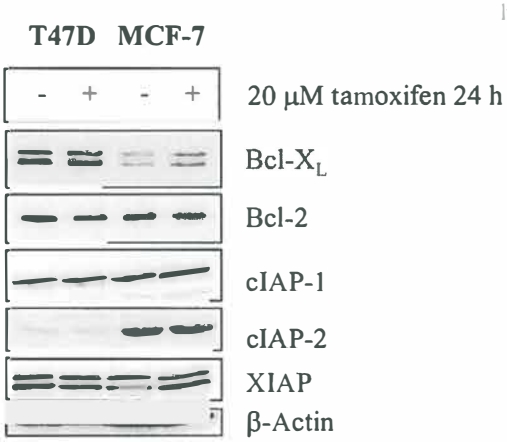

Figure 5A. Expression of inhibitors of apoptosis after exposure to 20 μM tamoxifen for 20 h. Representative examples of three independent experiments are shown.

To investigate whether XIAP directly interacts with caspase-3 and in T47D cells co-immunoprecipitation experiments were performed (Fig. 5B). Caspase-3 immunoprecipitation resulted in coprecipitation of XIAP in rhTRAIL exposed T47D cells. A slight reduction in immunoprecipitated caspase-3 and XIAP was observed in samples exposed to tamoxifen and rhTRAIL.

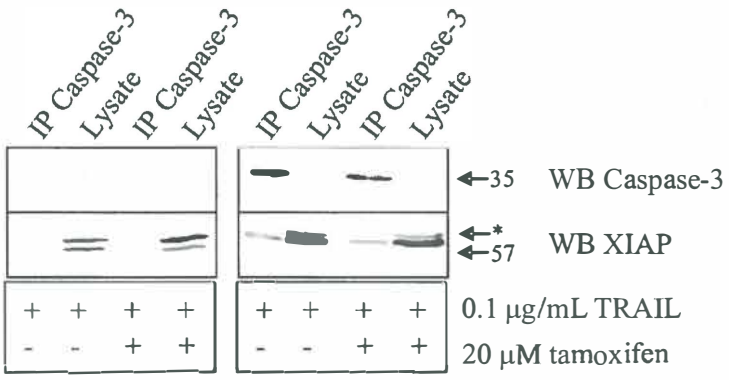

Figure 5B. Immunoprecipitation of XIAP in T47D cells after exposure to 0.1 μg/mL TRAIL for 6 h or to 20 μM tamoxifen for 16 h and co-exposure to 0.1 μg/mL TRAIL for an additional 6 h. A representative example of three independent experiments is shown.

XIAP RNA interference sensitizes T47D cells to rhTRAIL

Although immunoprecipitation clearly demonstrated the interaction of XIAP with caspase-3 in T47D cells following rhTRAIL treatment, the importance of XIAP binding to caspase-3 for inhibiting rhTRAIL-induced apoptosis was not demonstrated. We therefore used siRNA to suppress XIAP protein expression.

XIAP expression was strongly downregulated in the XIAP siRNA exposed T47D and MCF-7 cells after 24 h (Fig. 6A).

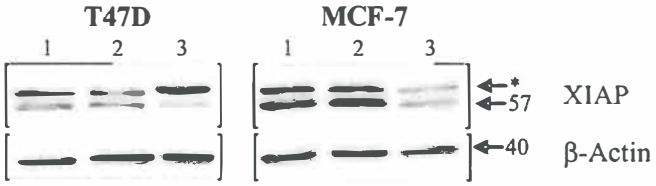

Figure 6A. Downregulation of XIAP protein expression using XIAP siRNA in T47D and MCF-7. A representative example of three independent Western blotting results of T47D and MCF-7 cells mock transfected (1), transfected with luciferase siRNA (2) or transfected with XIAP siRNA (3) is shown.

T47D cells, pretreated with XIAP or control luciferase siRNA (Luc siRNA) for 24 h, were exposed to rhTRAIL for 4 h. A strong induction of apoptosis was observed in XIAP suppressed cells, as reflected in enhanced PARP cleavage (Fig. 6B). T47D cells exposed to the transfection agent (control) or transfected with Luc siRNA were almost not sensitized to rhTRAIL.

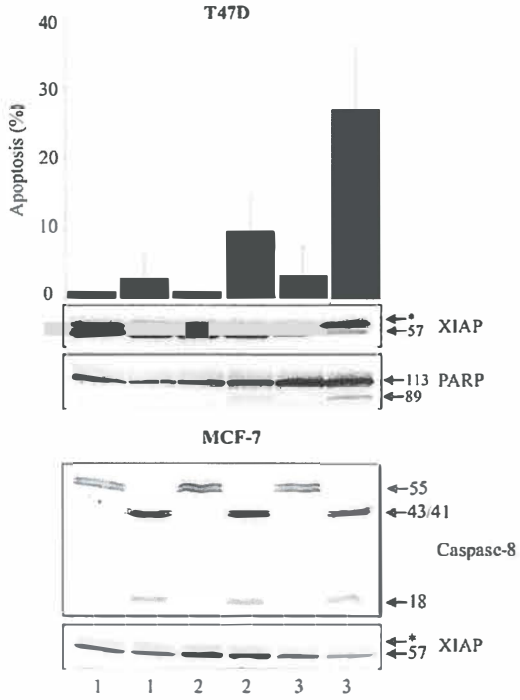

Figure 6B. Downregulation of XIAP sensitizes T47D cells to apoptosis induction by rhTRAIL.

Discussion

This is the first study that illustrates the apoptosis sensitizing effect of estrogen antagonists such as tamoxifen and fulvestrant to rhTRAIL. The molecular mechanism behind the apoptosis sensitizing effect has been revealed. Tamoxifen had no effect of death receptor surface expression, whereas tamoxifen as well as fulvestrant in combination with rhTRAIL resulted in stabilization of active caspase-8 in the ER-positive T47D breast cancer cell line. In addition, an interaction between caspase-3 and XIAP was observed in the caspase-3 expressing T47D cells. Moreover, downregulation of XIAP with siRNA enhanced rhTRAIL sensitivity in T47D but not in the caspase-3 negative MCF-7 cells. These results suggest that the modulating effect of estrogen antagonists on rhTRAIL mediated apoptosis induction in T47D cells is caused by an increased stabilization of active caspase-8, which may be enhanced by a caspase-3 amplification loop.

Tamoxifen is an anti-estrogen used in the treatment of hormone responsive breast cancer since 1971. It exerts its effects mainly via regulation of genes involved in apoptosis, cell cycle regulation and signal transduction. Caspase-9 among others is upregulated by estrogen signaling [32, 33]. The only data on antihormonal strategy in combination with TRAIL exposure comes from a study with the androgen dependent LNCaP prostate cancer cell line. Androgen withdrawal decreased TRAIL sensitivity by downregulation of DR4 and DR5 in combination with a less effective death inducing signaling complex (DISC) formation [34]. The effect of androgen withdrawal differs from our results with tamoxifen or fulvestrant in the MCF-7 and T47D breast cancer cell lines.

Exposure to tamoxifen and rhTRAIL resulted in strong apoptosis induction in the T47D cell line, whereas the combination had no additional effect on apoptosis in MCF-7 compared to rhTRAIL alone. In contrast to MCF-7, apoptosis induction in T47D was accompanied by the accumulation of activated caspase-8 p41/43 product following treatment with a combination of tamoxifen and rhTRAIL. $FLIP_L$, however, was fully cleaved in both cell lines after exposure to rhTRAIL alone and to a combination of tamoxifen and rhTRAIL. These results suggest that rhTRAIL induces DISC formation in T47D, as reflected by full $FLIP_L$ cleavage and some caspase-8 activation, without forwarding the apoptotic signaling. In MCF-7 cells activation of caspase-8 was accompanied by full $FLIP_L$ cleavage after exposed to rhTRAIL, whereas no additional effect was observed in combination with estrogen antagonists. Effective apoptosis signaling in MCF-7 by rhTRAIL was also reflected in activation of the proteins involved in the pathway downstream of the DISC. In several studies increased effectiveness of caspase-8 recruitment to and activation at the DISC was facilitated by the downregulation of cFLIP [35, 36]. Our results, however, suggest that $FLIP_L$ is not a rate limiting step

of caspase-8 activation in the breast cancer cells, but rather the lack of procaspase-8 protein expression. Although no difference was observed at the caspase-8 mRNA expression level between the two cell lines, basal procaspase-8 protein expression levels in T47D are much lower as compared to MCF-7 suggesting differences in procaspase-8 stability. Increased degradation of procaspase-8 has been described as a TRAIL resistance mechanism in an acquired TRAIL-resistance colon cancer cell line model [37]. A family of RING domain containing proteins that has been implicated in proteasomal degradation via ubiquitination of caspase-8 and -10 consists of the caspases-8- and -10-associated RING proteins (CARPs) [38]. These proteins can conjugate ubiquitin proteins to certain proteins and hereby target them for proteasomal degradation. In our cell lines procaspase-8 levels did not increase after tamoxifen exposure therefore the effect of tamoxifen must be on the degradation of the activated p41/p43 caspase-8 products. A similar effect was observed after CARP siRNA treatment of H460 human lung cancer cells, caspase-8 processing was increased, but basal levels did not [38]. In our study inhibition of the proteasome also increased the level of activated p41/p43 caspase-8 after rhTRAIL exposure indicating that the proteasome is involved in the effects of tamoxifen on TRAIL sensitivity.

The cell line panel we used includes a caspase-3 wild type cell line T47D and a caspase-3 deficient cell line MCF-7 [39]. The fact that the caspase-9 inhibitor reduced the sensitivity of T47D to tamoxifen and rhTRAIL, and that XIAP interacts with caspase-3 in T47D cells lead to the hypothesis that the sensitizing effect may also be due to an effect of tamoxifen on the interaction between XIAP, caspase-3 and or caspase-9 [40, 41]. Downregulation of XIAP resulted in an enhancement of rhTRAIL-induced apoptosis indicating that XIAP is an important inhibitory factor in TRAIL-mediated apoptosis. Although some of our results suggest a role for XIAP in the modulatory effect of tamoxifen on TRAIL-mediated apoptosis, the actual level of modulation is more likely to be at the level of active caspase-8 stability. A recent study showed that caspase-3 activation can result in proteasome inactivation, which may thus stimulate an amplification loop resulting in an increased stability of active caspase-8 but this needs to be investigated further [42].

RhTRAIL or agonistic TRAIL antibodies in combination with anti-estrogens in breast cancer patients may be an effective addition to the current hormonal treatment.

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7

APO010, a Fas Ligand multimer, is an effective inducer of apoptosis in a subset of chemotherapy resistant human tumor cell lines

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Submitted

Abstract

The objective of this study was to investigate the apoptosis inducing potential of APO010, a hexameric type of soluble human Fas ligand, for its activity in chemotherapy resistant human cancer cell lines and their parental counterparts. The panel of human cell lines comprised: cisplatin, doxorubicin or taxol-resistant resistant small cell lung cancer (SCLC), testicular germ cell tumor (TGCT) and ovarian cancer cell lines. APO010 sensitivity of drug resistant cell lines and their parental drug sensitive counterparts were monitored using assays measuring survival, apoptosis and activated caspase-3. In addition, Fas membrane expression was determined with flow cytometry.

Our results showed that the cisplatin-resistant A2780-CP70 and the cisplatin-sensitive parental A2780 ovarian cancer cells, in contrast to the cisplatin-sensitive and -resistant TGCT cell lines Tera (NTera2/D1) and Tera-cp, were sensitive to APO010. The cisplatin-sensitive and -resistant SCLC cell lines GLC_4 and GLC_4-CDDP, as well as the taxol-resistant subline GLC_4-Pgp, overexpressing P-glycoprotein, were all resistant to APO010. The doxorubicin resistant subline GLC_4-Adr overexpressing MRP1 was, however, extremely sensitive to APO010. The sensitivity of APO010 was related to caspase-3 activity and apoptosis induction but less related to Fas membrane expression. Sensitivity to APO010 could be induced in drug-sensitive and drug-resistant cells by pretreating those cells with cisplatin or doxorubicin.

This study demonstrated that there is no consistent cross-resistance between APO010 and classical chemotherapeutic agents. Moreover, the sensitivity of cells overexpressing MRP1 for APO010, when confirmed in other tumor cell lines, could indicate an opportunity for overcoming a major tumor resistance mechanism.

Introduction

The occurrence of chemotherapy resistance is one of the major reasons for chemotherapy failure in patients treated for solid malignancies. Chemotherapy resistance is often not specific for one class of chemotherapeutic agents, but this resistance can occur for a number of unrelated agents. Targeting tumor cells in a different way by activating proapoptotic members of the tumor necrosis factor (TNF) receptor family such as Fas may therefore be a potential new cellular pathway that can be used to circumvent chemotherapy resistance.

The proapoptotic TNF family member Fas ligand (FasL) signals cell death by activation of its cognate receptor, Fas. The intracellular domain of Fas contains a death domain (DD), which interacts with the DD of the adaptor protein FADD [1, 2]. FADD recruits procaspase-8 and procaspase-10 upon activation and a death inducing signaling complex (DISC) is formed. Activated caspase-8 and 10 are initiator caspases that can activate a cascade of effector caspases and subsequently induce apoptosis.

Short-term cultures of patient-derived tumor cells and cell lines from various tumor types often express Fas on their membrane. A number of these cell lines are sensitive to exposure to agonistic anti-Fas antibodies or recombinant soluble FasL [3]. Tumor cell lines can be sensitized for anti-Fas antibodies or recombinant soluble FasL (sFasL) by exposure to chemotherapeutic agents. The Fas-mediated apoptosis pathway as well as the mitochondrial (intrinsic) pathway can be a target for modulation by chemotherapeutic agents. Sensitization can occur at the membrane level by upregulation of Fas or FasL [4] and at the initiator or effector caspase level by enhanced caspase activation. FasL or anti-Fas antibodies in combination with chemotherapeutic agents may therefore be a potential interesting method of killing both chemotherapy sensitive and chemotherapy resistant tumor cells. Unfortunately, anti-Fas antibody treatment appeared to be extremely toxic in mice specifically targeting the liver of these mice. sFasL, seemed to be less toxic in mice. Physiologically, FasL is mostly membrane-bound but can be processed to a soluble form and shed by the action of a metalloprotease. The sFasL is less effective in inducing apoptosis, but [5-7] cross-linking of sFasL restores the proapoptotic properties of FasL [5]. A hexamer of FasL, consisting of two trimers held in close proximity, is the minimal ligand structure required to induce apoptosis to a similar extend as membrane-bound FasL. The conservative TNF family domain of FasL has structural homology with the C-terminal domain of ACRP30, a member of the complement C1q family [8]. Therefore, a FasL trimer linked to another FasL trimer with the C-terminal domain of adiponectin (ARCP30) has been constructed. This so called MegaFasL or APO010 was highly effective against a number of tumor cell lines [9, 10].

The aim of the present study was to investigate the apoptosis inducing potential of APO010 in chemotherapy resistant cancer cell lines and their parental sensitive cell lines. Therefore, cell survival, caspase-3 activation, apoptosis induction and Fas membrane expression have been determined in cisplatin, doxorubicin and paclitaxel resistant small cell lung cancer (SCLC), testicular germ cell tumor (TGCT) and ovarian cancer cell lines. To elucidate whether APO010 can overcome death receptor pathway related resistance we also included a recombinant human (rh)TRAIL resistance and an anti-Fas antibody resistance cell line model. Finally, we investigated whether APO010 in combination with these chemotherapeutic drugs enhances cell kill in the chemotherapy resistant cell lines.

Materials & Methods

Cell lines

To investigate APO010 sensitivity of chemotherapy resistant cell lines, a cisplatin resistance cell line panel was used that included a cisplatin resistant SCLC cell line (GLC_4-CDDP) and its parental cell line GLC_4 [11]. Two GLC_4 sublines, overexpressing ATP binding cassette (ABC) transporters, were included: GLC_4-Adr is an endogenous MRP1 overexpressing cell line with acquired resistance to doxorubicin, while GLC_4-Pgp is a paclitaxel, vincristine, and doxorubicin resistant cell line caused by stably overexpression of P-glycoprotein (Pgp) following transfection with *MDR1*. [12-15]. The cell line panel also included a cisplatin resistant TGCT cell line (Tera-CP) and its parental cell line NTera2/D1 (Tera) [16-18], and a cisplatin resistant ovarian cancer cell line A2780-CP70 and its parental cell line A2780 [19]. The GLC_4 and Tera cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and the ovarian cancer cell lines were cultured in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine. A rhTRAIL resistant colon cancer cell line SW948-TR and its parental cell line SW948 were also investigated for APO010 sensitivity [20]. These cell lines were cultured in 50% myeloma and 50% Leibovitz medium supplemented with 10% FCS. A anti-Fas antibody sensitive and anti-Fas antibody resistant cervical cancer cell line, CaSki and SiHa were also investigated. Both cell lines were cultured HAM/F12 and DME medium supplemented with 20% FCS.

Chemicals, media and reagents

Minimal essential medium (MEM, supplemented with Earle's salts and L-glutamine), and Trizol were purchased from Invitrogen Life Technologies (Breda, The Netherlands). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and propidium iodide were obtained from Sigma-Aldrich BV (Zwijndrecht, The Netherlands), DNase-I from Roche Diagnostics (Mannheim, Germany), doxorubicin-HCl from Pharmachemie BV (Haarlem, The Netherlands), the RNeasy kit from Qiagen (Venlo, The Netherlands).

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

The cell lines were cultured in HAM/F12 and DMEM medium (1:1) (Life Technologies) supplemented with 20% FCS. The effect of APO010 on survival was tested by MTT assay as described previously [14]. Cells were incubated for 4 days at 37 °C and 5% CO_2 in a humidified environment with a range of APO010 concentrations. After a 4-day culture period, MTT (5 mg/mL in PBS) was added and formazan crystal production was measured as described previously. Controls consisted of media without cells (background extinction) and cells incubated with

medium instead of APO010. In the assays in which the combination of APO010 and cisplatin was investigated, cells were exposed to cisplatin for 4 h and cisplatin was removed using 3 wash steps. Experiments were performed at least three times in quadruplicate.

Apoptosis assay

Cells (1.5×10^4 per well) were cultured in 96-wells. Apoptosis was induced by adding APO010 for 1-4 h acridine orange was added subsequently. Apoptosis was defined as the appearance of apoptotic bodies and/or chromatin condensation, using a fluorescence microscope. Results were expressed as the percentage of apoptotic cells in a culture by counting at least 200 cells per well. All apoptosis assays were performed three times in twofold.

Activated caspase-3 assay

The cleavage assay was carried out in 6 well plates according to Thornberry et al [21]. Activity of caspase-3 was assayed according to the manufacturer's instructions using the fluorescence peptide substrate Ac-DEVD-AFC (Biomol Tebu-bio, Heerhugowaard, The Netherlands). Fluorescence from free 7-amino-4-trifluoromethyl coumarin (AFC) was monitored in a FL600 Fluorimeter Bio-tek plate reader (Beun de Ronde, Abcoude, The Netherlands) using 380 nm excitation and 508 nm emission wavelengths. Relative caspase-3 activity was calculated by the fluorescence of a sample of treated cells by a sample of untreated cells. Experiments were performed three times.

Flow cytometry

To determine Fas membrane expression cells were harvested from the culture medium by centrifugation at 110 g for 5 min and washed twice with cold PBS supplemented with 2% FCS and 0.1% sodium azide. Cells were then incubated for 1 h with the PE-labeled anti-human Fas DX2 antibody, which was diluted 1:10 in cold PBS supplemented with 2% FCS and 0.1% sodium azide for 1 h on ice in the dark and washed twice with cold PBS. Analysis was performed on a Coulter Elite Flow cytometer (Becton Dickinson, Mount View, CA) with Winlist and Winlist 32 software (Verity Software House, Inc., Topsham, ME). Fas membrane expression was determined as Mean Fluorescence Intensity (MFI). These experiments were performed twice.

RNA isolation and purification

After harvesting exponentially grown cells, pellets were resuspended in 5×10^6 cells/mL of Trizol Reagent (Invitrogen, Merelbeke, Belgium) and stored

until use at -80 °C. Subsequent isolation of total cellular RNA was performed by adding 200 µL of chloroform to each sample and inverting the tubes for 15 s, followed by incubation for 2-3 min at room temperature. After centrifugation (18,000 g for 5 min at 4 °C), the top aqueous layer was transferred to a new RNase free tube and 500 µL of isopropanol was added. Samples were mixed thoroughly and incubated for 10 min at room temperature before centrifugation (18,000 g for 20 min at 4 °C). The supernatant was subsequently removed and the pellet consisting of precipitated nucleic acids washed by adding 500 µL of 75% ethanol. Finally, the pellets were air dried at 37 °C and resuspended in a volume of 30 µL DEPC treated H_2O.

Total RNA was purified using the RNeasy mini Kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions. To remove trace amounts of DNA contamination, the optional on-column DNase I digestion was applied following the manufacturer's recommendations.

Yield and quality of the total purified RNA was assessed using spectrophotometer absorbance at 230, 260 and 280 nm (with 1 OD unit considered equal to 40 µg/mL) and RNA gel electrophoresis visualization of 18S and 28S rRNA bands.

Reverse Transcription (RT)

Single-strand mRNA was reverse transcribed in a reaction volume of 20 µL, containing 800 ng of purified RNA, 0.5 µg of oligo(dT)11 primer, 8 µL 5× 1st strand buffer, DTT (10 mM), dNTPs (0.5 mM) and 1 µL M-MLV reverse transcriptase (Invitrogen). Purified RNA was initially incubated with the oligo(dT)11 primer for 10 min at 65 °C. After cooling on ice, the remaining contents were added and the final reaction mixture was incubated for 60 min at 42 °C, followed directly by incubation for 10 min at 65 °C to inactivate the reverse transcriptase. Negative controls were obtained from a similar reaction without adding the M-MLV reverse transcriptase. A qualitative RT-PCR was performed for the housekeeping reference gene coding for the enzyme glycerylaldehyde-3-phosphate dehydrogenase (GAPDH). Obtained cDNA was stored at 4 °C.

Real-time RT-PCR

Table 1 shows the sequences of the primers used in this real-time RT-PCR assay. A gradient real time (RT)-PCR was performed to assess primer specificity and to optimize the annealing temperature (T_{ann}) for each set of gene specific primers (Table 1).

Table 1. Gene specific primer sequences

Gene	Primer	5'-sequence-3'	Length (bp)	Prod. length (bp)	T_{ann} (°C)
Caspase-8	sense	CTGCTTCATCTCTGTATCC	19	355	58
	antisense	GCAAAGTGACTGGATGTACC	20		
FADD	sense	AGCTCAAAGTCTCAGCACACC	21	250	58
	antisense	TCTGAGTTCCATGACATCGG	20		
Fap-1	sense	GGAGTTAGTCTAGAAGGAGC	20	276	58
	antisense	ACTGAATCCTAGACCTGAGC	20		
Fas	sense	CATGGCTTAGAAGTGGAAAT	20	338	58
	antisense	ATTTATTGCCACTGTTTCAGG	21		
$Flip_S$	sense	GAACATCCACAGAATAGACC	20	172	58
	antisense	GTATCTCTCTTCAGGTATGC	20		
$Flip_L$	sense	GAACATCCACAGAATAGACC	20	262	58
	antisense	TTTCAGATCAGGACAATGGG	20		
GAPDH	sense	CACCACCATGGAGAAGGCTGG	21	200	56-65
	antisense	CCAAAGTTGTCATGGATGACC	21		

Real-time RT-PCR was performed in 96-well optical plates (BIOplastics, Landgraaf, the Netherlands) using the iCycler iQ™ real-time PCR detection system (Bio-Rad, Veenendaal, the Netherlands). Amplification of the samples in duplicate was carried out in a final reaction volume of 25 µL, containing 12.5 µL iQ™ SYBR Green Supermix (Bio-Rad), 1 µL of each gene specific primer (5 µM) and 5 µL cDNA (1:50). The thermocycling program used for each real-time RT-PCR consisted of an initial 3 min denaturation at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C, 20 s primer annealing at the primer specific T_{ann} and 30 s fragment elongation at 72 °C. Fluorescence data were acquired during the fragment elongation step at 72 °C. A melting curve was obtained at the end of each 40 cycles of amplification in order to determine the presence of a unique reaction product.

To determine RT-PCR efficiency and initial starting quantity of the samples, a standard curve was generated using samples from a 1:3 dilution series of total starting cDNA. A sample without reverse transcriptase was also included, to check for contaminating genomic DNA. Furthermore, the glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) gene served as a housekeeping reference gene to correct for differences in the amount of starting cDNA between samples.

Results

Effect of APO010 on survival

APO010 sensitivity was investigated in the cell line models using a 4 day survival assay and exposing the cells to APO010 concentrations up to 200 ng/mL. From the cisplatin resistant cell line panels used (see table 2) only the cisplatin resistant A2780-CP70 ovarian cancer cell line and its parental A2780 cell line showed growth inhibition after APO010 exposure.

Table 2. APO010 IC50 values

Cell line	Cancer	Resistance (RF)[1]		APO010 IC_{50} (ng/mL)	SD
A2780	Ovarian cancer			1.362	0.340
A2780-CP70	Ovarian cancer	cisplatin	(5x)	4.722	2.284
Tera	Testicular cancer			>200	-
Tera-CP	Testicular cancer	cisplatin	(2.7x)	>200	-
GLC_4	SCLC			>200	-
GLC_4-CDDP	SCLC	cisplatin	(16x)	>200	-
GLC_4-Pgp	SCLC	taxol	(10x)	>200	-
GLC_4-Adr	SCLC	doxorubicin	(330x)	0.690	0.119
SW948	Colorectal cancer			2.466	1.237
SW948-TR	Colorectal cancer	TRAIL	(>100)[2]	53.305	0.486
CaSki	Cervical cancer			2.729	1.760
SiHa	Cervical cancer	anti-Fas	(>100)[2]	>200	-

[1]) Except for SiHa and GLC_4-Pgp that are intrinsic resistant, cell lines have been made resistant to the drug indicated. RF is the resistance factor based on the IC50 concentration.
[2]) >100x indicates that IC50 could not be achieved for the resistant lines. IC50 rhTRAIL in SW948 is 0.007 µg/mL, IC50 7C11 anti-Fas Ab in CaSki is 0.011 µg/mL.

No effect was observed in the TGCT or SCLC cell line models. We also tested a SCLC cell line panel with drug efflux related mechanisms of resistance. GLC_4-Adr was extremely sensitive to APO010 (IC_{50}=0.677 ng/mL), but the GLC_4-Pgp cell line was not sensitive at all (Fig. 1).

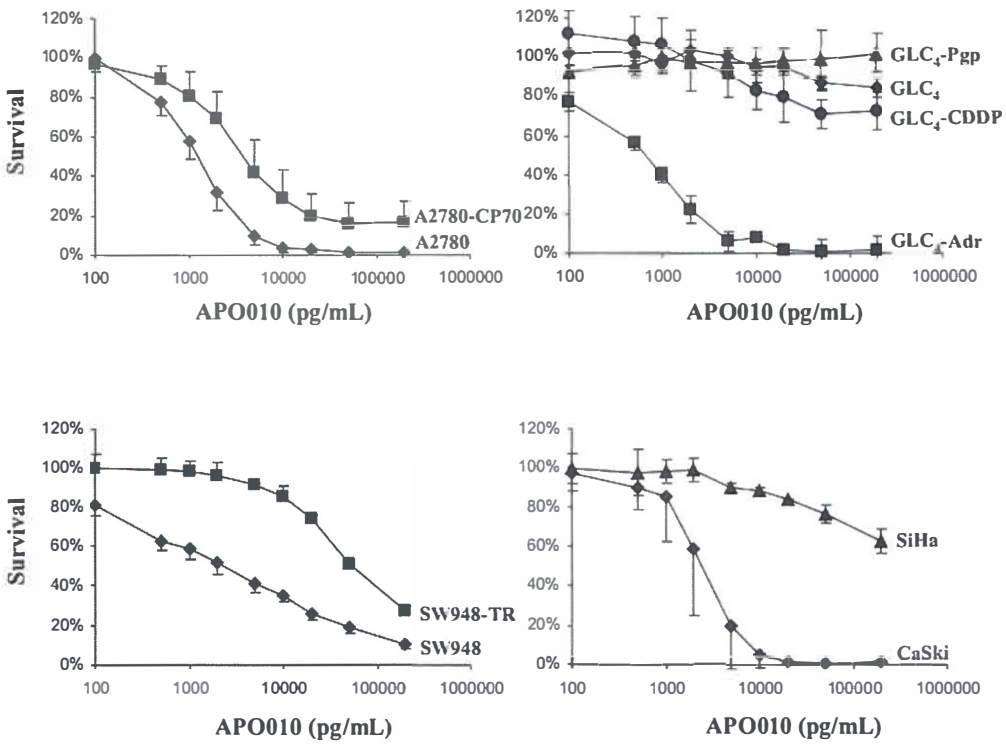

Figure 1. APO010-mediated growth inhibition after 96 h APO010 exposure. Data represent the mean ± SD of three independent experiments.

The TRAIL resistant colon cancer cell line SW948-TR and its parental cell line SW948 were both sensitive to APO010 exposure. The TRAIL resistant cell line, however, was 20-fold less sensitive to APO010 compared to the parental cell line. The anti-Fas antibody sensitive cervical cell line CaSki was also sensitive to APO010, while for the other cervical cancer cell line SiHa the IC_{50} of APO010 has not been reached. These results suggest that resistance to rhTRAIL and anti-Fas antibody is related to a reduced APO010 sensitivity.

APO010 –induced apoptosis

The ability of APO010 to induce apoptosis was measured both in a concentration-dependent as well as time-dependent manner in a cisplatin resistance model, a drug-efflux pump-mediated resistance model and a death receptor resistance model (Fig. 2). In A2780 and A2780-CP70 sensitive to APO010 apoptosis induction was reflected in a concentration dependence as well as a time dependence. Opposite to the results in the survival assays the A2780-CP70 was

less sensitive to APO010 compared to A2780. GLC_4-Adr was extremely sensitive to APO010 apoptosis induction in contrast to GLC_4 (note that for the latter much higher APO010 concentrations were used). Similar findings were observed with CasKi and SiHa. CasKi was extremely sensitive to APO010-induced apoptosis, while in SiHa only at high APO010 concentrations some apoptosis was detected (Fig. 2).

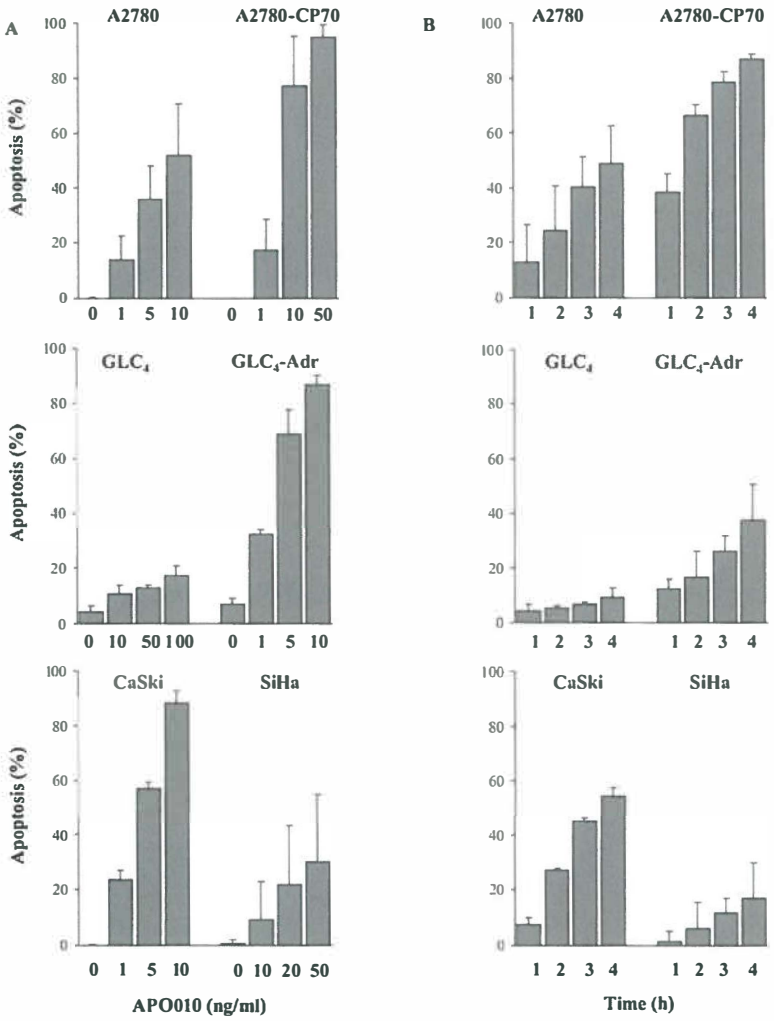

Figure 2. (A) Dose-dependent APO010-mediated apoptosis induction after 24 h exposure to different APO010 concentrations. Data represent the mean ± SD of three independent experiments. **(B)** Time-dependent APO010-mediated apoptosis induction after 1, 2, 3 and 4 h exposure to 100 ng/mL APO010. Data represent the mean ± SD of three independent experiments.

APO010 mediated caspase-3 activation

To confirm the apoptosis data, we performed fluorescent caspase-3 activity assays on all cell line panels. Active caspase-3 assays correlated well with the apoptosis assays for the cisplatin resistance model, the drug efflux-mediated resistance model and the death receptor resistance model as used in the previous apoptosis section. Again A2780-CP70 showed more APO010 induced caspase-3 activity then A2780. From the cell line models not included in Fig. 2, APO010 mediated caspase-3 activation was observed GLC_4-CDDP but not in GLC_4, no activity was observed in either Tera and Tera-CP, while caspase-3 activition was found in SW948 but not in the TRAIL-resistant SW948-TR cell line (Fig. 3A).

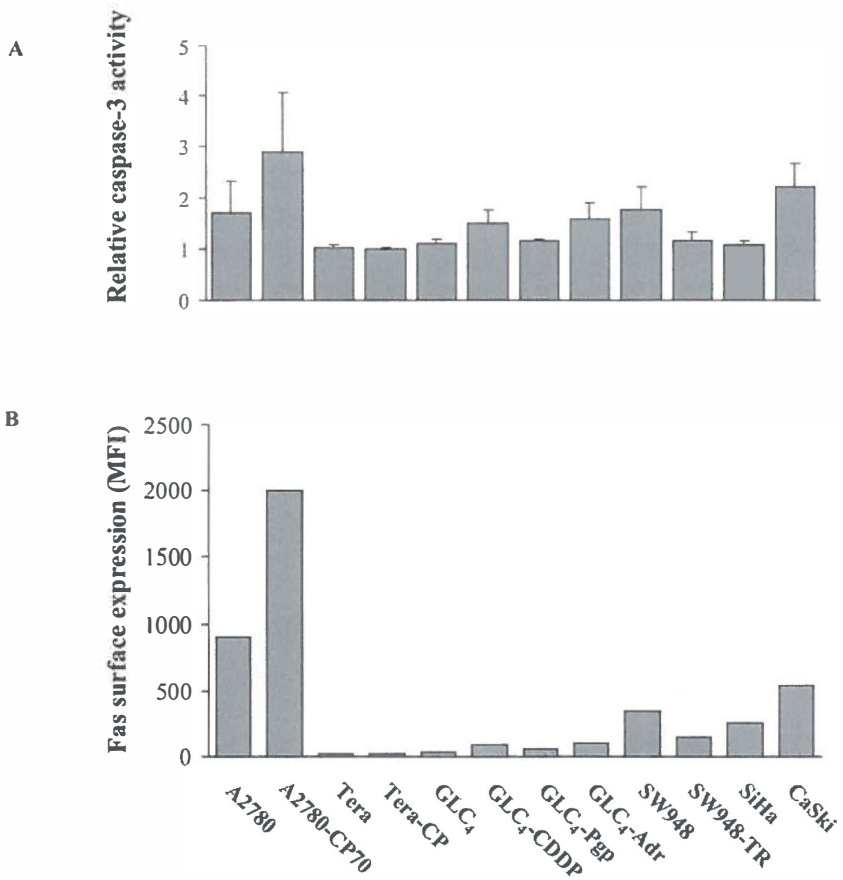

Figure 3. (A) Caspase-3 activation of cells exposed to 100 ng/mL for 4 h relative to untreated cells. Data represent the mean ± SD of three independent experiments. **(B)** Fas membrane expression of untreated cells measured by flow cytometry. Results show a representative example.

Fas membrane expression

Fas membrane expression was determined to investigate a possible correlation between Fas membrane expression levels and APO010 sensitivity at the level of cell survival and apoptosis induction (Fig. 3B). The absolute Fas membrane levels predicted sensitivity to APO010 in most cases, meaning that cell lines with high Fas membrane levels in general were sensitive to APO010 and the low Fas expressing cell lines were not sensitive. Exceptions were GLC_4-Adr, which had a relatively low Fas membrane expression compared to A2780 but was most sensitive to APO010, and SiHa, which showed a high level of Fas membrane expression, but was almost not sensitive to APO010 (Fig. 4). These resuls suggest that relative Fas membrane expression within each cell line panel, i.e. ovarian cancer, TGCT, SCLC, colon cancer and cervical cancer cell line panel, was associated with APO010–induced caspase-3 activation, apoptosis-induction and cell survival (see Fig. 1-3A). Thus, Fas membrane levels are important but other factors influencing APO010 sensitivity must also be considered.

RT-PCR

Expression of genes involved in DISC formation were investigated in a subset of cell line models with real-time RT-PCR to determine which genes are important determinants of sensitivity to APO010-mediated apoptosis. Of all the mRNA levels we have measured, only a reduction in FAP-1 mRNA expression was found in the less sensitive cell line A2780-CP70 compared to A2780. Caspase-8 mRNA levels were decreased and $FLIP_s$ mRNA levels elevated in GLC_4-CDDP compared to GLC_4, although both cell lines were resistant to APO010. Similarly, Fas mRNA levels were reduced in GLC_4-Pgp, while both GLC_4-Pgp and GLC_4 were resistant to APO010. Interestingly, Fas and FAP-1 mRNA expression levels were higher in the highly sensitive GLC_4-Adr cell line compared to GLC_4. In addition, in SW948-TR Fas mRNA expression was less than in the more sensitive SW948 cell line. These results demonstrate that only Fas and FAP-1 mRNA expression levels are associated with APO010 sensitivity in these cell line models (Table 3).

Table 3. Real-time RT-PCR of genes involved in DISC formation. Relative increases or decreases compared to parental cell lines are shown ($P < 0.05$).

	Fas	FAP-1	Caspase-8	FADD	$FLIP_S$	$FLIP_L$
A2780						
A2780-CP70	≈	↓	≈	≈	≈	≈
GLC_4						
GLC_4-CDDP	≈	≈	↓	≈	↑	≈
GLC_4-Pgp	↓	≈	≈	≈	≈	≈
GLC_4-Adr	↑	↑	≈	≈	≈	≈
SW948						
SW948-TR	↓	≈	≈	≈	≈	≈

Chemotherapy-induced sensitization to APO010

Finally, we investigated whether APO010 in combination with chemotherapy can enhance apoptosis induction in various cell line panels. Tera and Tera-CP were pretreated with cisplatin for 24 h resulting in a strong sensitization to APO010 (Fig. 4A) Cisplatin treatment also sensitized GLC_4 and GLC_4-CDDP to APO010 (Fig. 4B). This effect was not depending on cisplatin, since doxorubicin had a similar effect on APO010 induced apoptosis in GLC_4 and GLC_4-Adr (Fig. 4C).

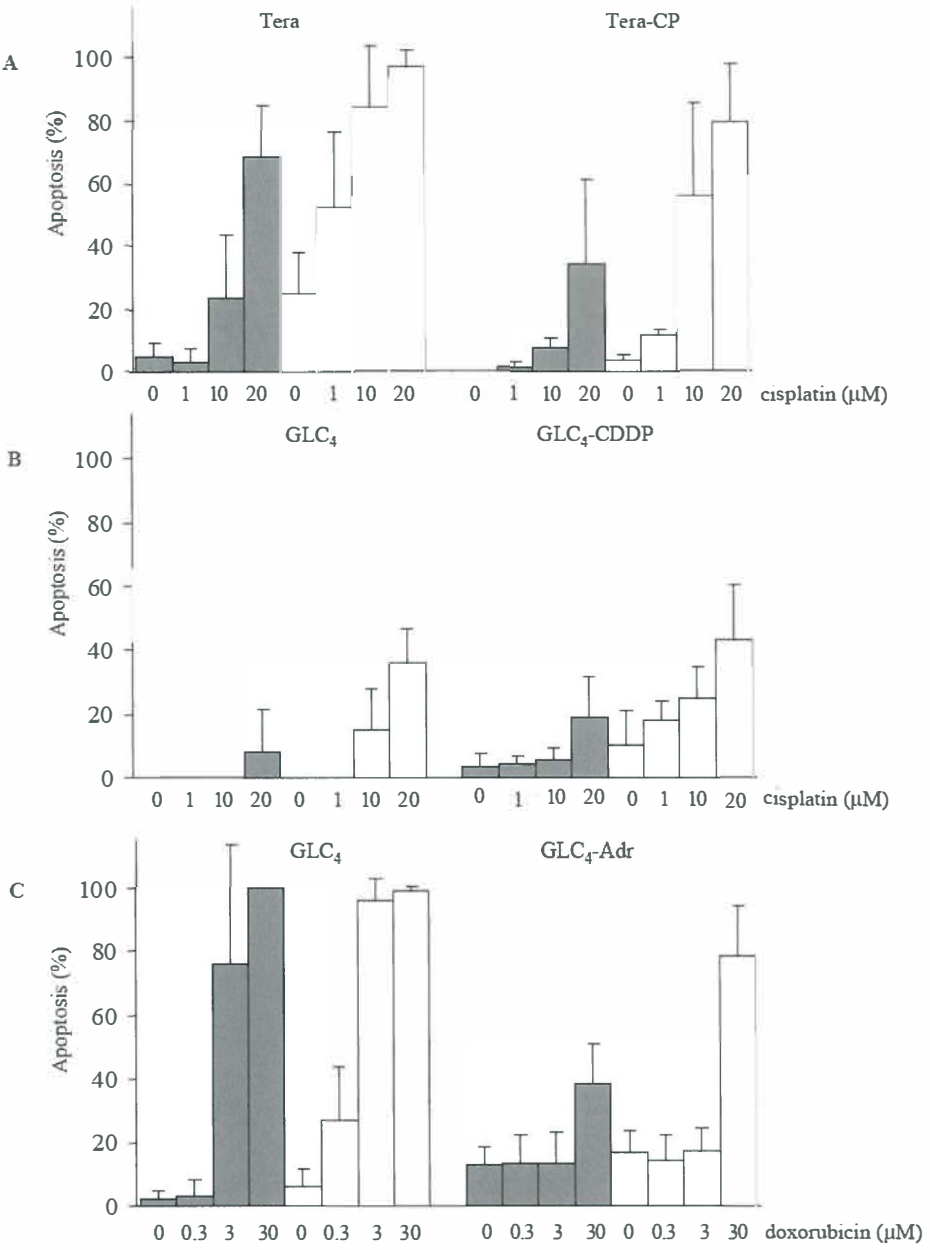

Figure 4. Apoptosis after 24 h cisplatin or doxorubicin pre-exposure and subsequent 4 h chemotherapy and APO010 co-exposure. White bars represent chemotherapy exposure alone, grey bars represent chemotherapy and APO010 co-exposure. APO010 concentrations were: 200 ng/mL for GLC_4 and GLC_4-CDDP, Tera and Tera-CP, 50 ng/mL for A2780 and A2780-CP70, SiHa and CaSki, 1 ng/mL for GLC_4 and GLC_4-Adr.

Discussion

This is the first study investigating the effectiveness of a multimer death ligand in chemotherapy resistant cell line models. APO010 is a hexamer FasL molecule that was shown to be more potent than anti-Fas antibodies or single FasL molecules in cell lines and primary cells representing multiple myeloma, acute myeloid leukaemia, acute lymphoblastic leukaemia and Burkitt's lymphoma [9].

In the present study solid tumor cell lines derived from different tumor types were investigated. Large differences in sensitivity to APO010 were found between the cell lines. High sensitivity to APO010 was observed in both A2780 and the cisplatin-resistant subline A2780-CP70. Sensitivity in survival assays was correlated with apoptosis induction in apoptosis assays and this apoptosis induction was correlated to caspase-3 activation. Survival, apoptosis assay and activated caspase-3 assay data were very predictive in isogenic cell line models, but failed to predict absolute apoptosis induction or caspase-3 activation. For instance GLC_4-Adr is the most sensitive cell line in the survival assay and has a higher caspase-3 activation than its parental cell line but the absolute caspase-3 activation is higher in A2780. The correlation between Fas membrane expression and FasL-mediated apoptosis has been investigated in *in vitro* experiments as well as in mathematical models. The theoretical model decribed by Bentele *et al* suggests that there is a minimal amount of Fas membrane expression necessary to induce apoptosis. In our cell lines the threshold amount of Fas membrane expression may be between the expression of GLC_4-CDDP and GLC_4-Adr [22].

In survival assays A2780 was more APO010 sensitive whereas A2780-CP70 was more sensitive in the apoptosis related assays. These results may be explained by the difference in Fas membrane expression level, which was much higher in A2780-CP70. Since the concentrations of APO010 used in the apoptosis and caspase-3 activation assays (100 ng/mL) were much higher than those used in the survival assay, our results suggest that at higher doses of APO010 Fas membrane expression is an important factor determining apoptosis induction. At lower doses of APO010 other factors influencing APO010 sensitivity must be considered for instance DISC formation, Fas clustering and internalization. Preliminary data in our laboratory have indicated that the caspase-8 level in A2780-CP70 is much lower than in A2780, which may explain the results in the APO010 survival assay. The role of FAP-1 expression in tumor cell lines in relation to Fas sensitivity is still controversial. A pro-apoptotic effect of FAP-1 expression in tumor cell lines has been described in Fas-resistant human SW480 colon carcinoma cells transfected with a FAP-1 construct, perhaps through increased p21 expression [23]. In contrast, forced FAP-1 overexpression has also been associated with decreased Fas membrane trafficking and therefore decreased

FasL sensitivity in human OM431 melanoma, TIG3 human embryonic lung fibroblast, and HeLa cervical cancer cells [24]. Elevated FAP-1 protein levels correlated with FasL sensitivity in a panel of ovarian cancer cell lines. Decreased FAP-1 expression and therefore FasL sensitivity was observed in HEY and BG-1 ovarian cancer cell lines and elevated FAP-1 expression and therefore FasL resistance was observed in the OVCAR-3 FR and SK-OV-3 cell lines.

In the cisplatin resistance cell line models, both GLC_4 and its cisplatin resistant subline were resistant for APO010. Similar findings were obtained for Tera and the cisplatin resistant subline Tera-cp. When these cell lines were pretreated with cisplatin, cells were clearly sensitized to APO010. This discrepancy in sensitization can be explained by differences in kinetics of APO010 and cisplatin exposure. Sensitization to Fas-mediated apoptosis can be induced by cisplatin by upregulation of Fas, which was observed in several tumor cell lines after treatment with chemotherapeutic drugs such as cisplatin [17, 25-28]. In addition, anticancer agents may sensitize tumor cells for death receptor mediated apoptosis by upregulating proapoptotic molecules including caspases or FADD [29, 30]. Chemotherapy-induced decreases in anti-apoptotic proteins such as FLIP, Bcl-2 Bcl-X_L have also been described [31, 32]. Further investigations are required to identify the mechanism(s) of sensitization that may depend on tumor type or on the resistance to a specific drug.

Apoptosis and survival assays in the MRP1 and Pgp overexpressing cell lines GLC_4-Adr and GLC_4-Pgp showed different results. The MRP1 overexpressing doxorubicin resistant GLC_4-Adr cell line is very APO010 sensitive whereas the paclitaxel, vincristine, and doxorubicin resistant GLC_4-Pgp, transfected with a *MDR1* gene construct encoding Pgp, is relatively resistant to APO010. These results are in agreement with a previous study demonstrating that GLC_4-Adr in contrast to GLC_4 was sensitive to anti-Fas antibody. APO010, however, is much more effective than anti-Fas antibody, which only demonstrated activity in GLC_4-Adr in the presence of cycloheximide [13]. Interestingly, the sensitivity to APO010 is very similar to our findings with indomethacin, which induced apoptosis in GLC_4-Adr but not in the parental cell line. Doxorubicin was an efficient enhancer of APO010 sensitivity in GLC_4-Adr and less in GLC_4 although higher concentrations of doxorubicin were needed to modulate APO010 sensitivity in GLC_4-Adr. Indomethacin, however, which also induces apoptosis via the Fas pathway, only modulated doxorubicin sensitivity in GLC_4-Adr, suggesting different mechanisms involved in the modulation [13]. Thus a combination of APO010 with indomethacin may be even more effective in these MRP expressing cells. Contrary results regarding ABC transporter overexpression and FasL sensitivity have been published before [33, 34]. No anti-Fas antibody and

chemotherapy cross resistance occurred in parental and chemotherapy resistant leukemia cell lines. Interestingly, MCF-7 breast cancer cells transfected with a *MDR1* gene construct revealed increased sensitivity to TRAIL-induced apoptosis compared to the parental cell line. Taken together, we found no association between APO010 and chemotherapy resistance within the cell line models used in the present study. Whether sensitivity to APO010 is associated with MRP overexpression and TRAIL sensitivity with Pgp overexpression needs to be established in larger cell line panels.

With the recent development of death receptor-targeting drugs such as rhTRAIL or pro-apoptotic antibodies against TRAIL receptors that are in phase I/II clinical trials it is of interest whether resistance to rhTRAIL or antibody-mediated apoptosis was accompanied by partial resistance to APO010. In the rhTRAIL-resistant subline SW948-TR treatment with APO010 induced apoptosis and resulted in growth inhibition but less effective then in SW948. This is probably due to a decreased caspase-8 expression in SW948-TR compared to SW948, however we can not exclude differences in Fas membrane expression as a causing factor [20]. Although the Fas-mediated apoptosis pathway and the TRAIL-mediated apoptosis pathway show many similarities, the level of resistance to APO010 is much less then the resistance factor observed for rhTRAIL. The cervical cancer cell lines SiHa and CaSki showed APO010 sensitivities that were similar to anti-Fas antibody sensitivity [35] with SiHa being much less sensitive for APO010 compared to CaSki. In addition to differences in Fas membrane levels, caspase-8 levels are also lower in SiHa [35]. In contrast to anti-Fas antibodies, SiHa demonstrated some sensitivity to APO010 at higher concentrations. These results suggest that APO010 can partially overcome resistance to rhTRAIL or anti-Fas antibody, but this needs to be confimed in a larger panel of cell lines. The therapeutic window for APO010, namely its antitumor activity versus its toxicity on normal tissues, will finally determine the applicability of this drug in the clinic.

In conclusion, APO010 is a potent apoptosis inducing ligand in a number of drug sensitive and drug-resistant solid tumor cell lines. The sensitivity to APO010 is partially depending on the Fas membrane level. The addition of chemotherapy can further enhance APO010 apoptosis induction.

References

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8

Summary and future perspectives

In recent years much effort has been put in identifying new and drugable targets in solid malignancies. The focus of this thesis is on different approaches to induce apoptosis using death receptor pathways. These approaches include modulation of the TNF related apoptosis inducing ligand (TRAIL) pathway with anti-estrogens, increased death receptor activation using modified death ligands and activation of death receptor pathways using nonsteroidal anti-inflammatory drugs (NSAIDs) alone as well as in combination with chemotherapeutic drugs.

The first approach discussed is the use of selective and non-selective COX inhibitors. In chapter 2 a review is given of the literature concerning these drugs. Most solid tumors express the cyclooxygenase-2 (COX-2) protein, a target of NSAIDs. A number of studies showed that COX-2 overexpression in solid tumors was a predictor of more advanced stage disease and of worse prognosis. Therefore, NSAIDs are evaluated as anticancer drugs. NSAIDs inhibit proliferation, invasiveness of tumors, and angiogenesis and can overcome apoptosis resistance in a COX-2 dependent and independent manner. Studies investigating anti-cancer effects of NSAIDs on cell lines and xenograft models have shown modulation of the Akt, NF-κB, tyrosine kinases and the death receptor-mediated apoptosis pathways. Despite the increased risk of cardiovascular toxicity induced by selective COX-2 inhibitors, several ongoing clinical trials are still investigating the therapeutic benefits of NSAIDs in oncology. The antitumor effects in these trials balanced with the side effects data will finally define the precise future of NSAIDs including COX-2 inhibitors in cancer treatment.

A pathway that has several important functions in apoptosis regulation and therefore tumor growth is the TNF family of death ligands. Members of the TNF family include TNF, Fas ligand (FasL) and TRAIL. The receptors of these ligands, TNFR1 and TNFR2 for TNF, Fas for FasL, death receptor 4 and death receptor 5 for TRAIL are often (over)expressed in solid tumors and thus targeting these pathways may be a successful strategy to circumvent drug resistance.

In Chapter 3 we describe our study in which we modulate the Fas-mediated apoptosis pathway with indomethacin (an NSAID) in a small cell lung cancer cell line model. Small cell lung cancers initially respond to chemotherapy but are often resistant at recurrence. A common mechanism of drug resistance is the overexpression of drug transporters and the failure to induce apoptosis in tumor cells. The major family of drug transporters is the ATP binding cassette family of transport proteins. Multidrug resistance protein 1 (MRP1), a member of this family of drug transporters, can act as an efflux pump for a number of chemically unrelated agents. A potentially new method to overcome resistance is to combine classical chemotherapeutic drugs with apoptosis induction via the TNF death receptor family. The doxorubicin resistant MRP1 overexpressing human small cell

lung cancer cell line GLC_4-Adr and its parental doxorubicin-sensitive line GLC_4 were used to analyze the potential of the Fas-mediated apoptotis pathway and the mitochondrial apoptotis pathway to modulate doxorubicin resistance in small cell lung cancer. Western blotting showed that all proteins necessary for death inducing signaling complex formation and several inhibitors of apoptosis were expressed in both lines. The proapototic proteins Bid and caspase-8, however, were higher expressed in GLC_4-Adr. In addition, GLC_4-Adr expressed 3.1-fold more Fas at the cell membrane. Both lines were resistant to anti-Fas antibody, but in combination with the protein synthesis inhibitor cycloheximide 40% apoptosis was induced in GLC_4-Adr. Indomethacin, a well-known inhibitor of MRP1 function, which targets the mitochondrial apoptotic pathway, induced apoptosis in GLC_4-Adr but not in GLC_4 cells. Surprisingly, in GLC_4-Adr indomethacin induced caspase-8 and caspase-9 activation as well as Bid cleavage, while both caspase-8 and caspase-9 specific inhibitors blocked indomethacin-induced apoptosis. In GLC_4-Adr, doxorubicin plus indomethacin resulted in elevated caspase activity and a 2.7-fold enhanced sensitivity to doxorubicin. In contrast, no effect of indomethacin on doxorubicin sensitivity was observed in GLC_4. Our findings show that indomethacin increases the cytotoxic activity of doxorubicin in a doxorubicin resistant small cell lung cancer cell line, this occurs partly via the death receptor apoptosis pathway, independent of Fas.

The MRP1 drug efflux pump can transport drugs that are conjugated to glutathione by glutathione S-transferase, it can also transport unconjugated drugs and glutathione alone. The doxorubicin resistant small cell lung cancer cell line GLC_4-Adr mentioned in chapter 3 overexpresses MRP1, which causes part of the doxorubicin resistance. To investigate the relation between MRP1 overexpression and indomethacin-induced apoptosis we performed the study described in chapter 4. GLC_4 and GLC_4-Adr cells were exposed to indomethacin and the effect of MRP1 downregulation using siRNA on indomethacin-induced cell survival and apoptosis was analyzed. In addition the effect of indomethacin on glutathione levels and mitochondrial membrane potential was investigated. Si-MRP1 reduced 2-fold MRP1 mRNA and diminished efflux pump function of MRP1, reflected in a 1.8-fold higher accumulation of MRP1 substrate carboxyfluorescein, in si-MRP1 versus si-Luciferase transfected GLC_4-Adr cells. MRP1 down-regulation decreased 2-fold initial high apoptosis levels in GLC_4-Adr after indomethacin treatment for 24 h, and increased cell survival (IC_{50}) from 22.8 ± 2.6 to 30.4 ± 5.1 μM following continuous indomethacin exposure. MRP1 down-regulation had no effect on apoptosis in GLC_4 or on glutathione levels in both lines. Although indomethacin (20 μM) for 2 h decreased glutathione levels by 31.5 % in GLC_4-Adr, complete depletion of cellular glutathione by L-buthionine (S,R)-sulfoximine only resulted in

a small increase in indomethacin-induced apoptosis in GLC_4-Adr demonstrating that a reduced cellular glutathione level is not the primary cause of indomethacin-induced apoptosis. Indomethacin exposure decreased mitochondrial membrane potential in GLC_4-Adr cells, suggesting activation of the mitochondrial apoptosis pathway. Indomethacin exposure decreased the mitochondrial membrane potential in GLC_4-Adr cells only. Indomethacin induces apoptosis in a doxorubicin resistant SCLC cell line through an MRP1 dependent mechanism, which may have implications for the treatment of patients with MRP1 overexpressing tumors.

In small cell lung cancer as well as in ovarian cancer treatment, drug resistance is the major reason for chemotherapy failure. A common drug resistance mechanism of tumor cells is the inability to go into apoptosis. To circumvent this apoptosis resistance we performed a study, described in chapter 5, which analyzed the apoptosis inducing effect of indomethacin and the selective COX-2 inhibitor celecoxib. The stable transfected human ovarian cancer cell lines A2780/cmv, transfected with an empty control plasmid, and A2780/m248, transfected with p53 mutated at codon 248, differ in Fas surface levels and were used to combine these drugs with agonistic anti-Fas antibody and the chemotherapeutic drug cisplatin. Celecoxib induced higher levels of apoptosis in A2780/cmv and A2780/m248 as compared to indomethacin. Celecoxib was also a stronger inducer of caspase-8 and caspase-9 activation, while caspase-8 and caspase-9 specific inhibitors blocked celecoxib and indomethacin-induced apoptosis. However, only indomethacin-induced apoptosis could be blocked up to 50% by antagonistic anti-FasL (NOK-1) and anti-Fas (ZB4) antibodies in both cell lines. Confocal laser microscopy revealed that treatment with celecoxib, in contrast to indomethacin resulted in a marked increase in granular cytoplasmic FasL colocalizing with Fas. Both indomethacin and celecoxib increased the sensitivity of A2780/m248, the cell line with the highest Fas membrane level, to agonistic anti-Fas antibody without increasing Fas membrane expression. In contrast, cisplatin potentiated indomethacin-induced apoptosis but not celecoxib-induced apoptosis in A2780/cmv and A2780/m248. Our results indicate that indomethacin and celecoxib differently affect the Fas apoptosis pathway in a p53-independent manner. These drugs may therefore have different applications in anti-cancer treatment combinations.

Modulation of death receptor apoptosis pathways can also be achieved by targeting tissue specific pathways such as the estrogen receptor in breast cancer. Recombinant human TRAIL (rhTRAIL) has been known to induce apoptosis in several breast cancer cell lines. In chapter 6, we investigated whether the estrogen antagonists tamoxifen and fulvestrant, enhance rhTRAIL-induced apoptosis in the estrogen-receptor positive, caspase-3 positive T47D and in estrogen receptor

positive MCF-7 human breast cancer cell lines. The estrogen antagonist fulvestrant only has antagonistic properties and decreases estrogen receptor expression, whereas the estrogen antagonist tamoxifen has also agonistic properties. No apoptosis induction occurred following 20 μM tamoxifen or 100 ng/mL rhTRAIL in T47D, while the combination induced 40 ± 16% (mean ± SD) apoptosis. The sensitizing effect is not due to increased TRAIL receptor cell surface expression. More important, tamoxifen and fulvestrant in combination with rhTRAIL resulted in stabilization of cleaved caspase-8 in T47D but not in MCF-7. Similar results were obtained with a proteasome inhibitor plus rhTRAIL. Since T47D in contrast to MCF-7 expresses caspase-3, we investigated whether estrogen inhibition affected the interaction between a member of the inhibitors of apoptosis (IAPs) X-linked inhibitor of apoptosis (XIAP) and caspase-3. Immunoprecipitation demonstrated an interaction between XIAP and caspase-3 in T47D cells. Downregulation of XIAP with RNA interference in combination with rhTRAIL induced 27 ± 9% apoptosis in T47D but not in the caspase-3 negative MCF-7 cells. These results suggest that the modulating effect of estrogen antagonists on rhTRAIL mediated apoptosis in T47D cells is caused by increased stabilization of active caspase-8, which may be enhanced by a caspase-3 amplification loop. Thus, a combination of estrogen antagonists and rhTRAIL can be considered to enhance apoptosis in estrogen receptor positive breast cancer cells for which the sensitizing effect depends on at least partially on caspase-3 expression.

Besides enhancement of death receptor-mediated apoptosis with NSAIDs, improving death receptor activation may be another potentially successful strategy to enhance apoptosis induction. The effect of multimerization (hexamers) of death receptor ligands such as FasL was investigated in a number of chemotherapy and death ligand resistant cell lines. The objective of this study described in chapter 7 was to investigate the apoptosis inducing potential of APO010, a hexameric type of soluble human FasL, for its activity in chemotherapy resistant human cancer cell lines and their parental counterparts. The panel of human cell lines comprised: cisplatin, doxorubicin or taxol-resistant resistant small cell lung cancer, testicular germ cell tumor and ovarian cancer cell lines. APO010 sensitivity of drug resistant cell lines and their parental drug sensitive counterparts were monitored using assays measuring survival, apoptosis and activated caspase-3. In addition, Fas membrane expression was measured with flow cytometry. Our results showed that the cisplatin-resistant A2780-CP70 and the cisplatin-sensitive parental A2780 ovarian cancer cells, in contrast to the cisplatin-sensitive and -resistant testicular germ cell tumor cell lines Tera (NTera2/D1) and TeraCP, were sensitive to APO010. The cisplatin-sensitive and -resistant small cell lung cancer cell lines GLC_4 and GLC_4-CDDP, as well as the taxol-resistant subline GLC_4-Pgp,

overexpressing Pgp, were all resistant to APO010. The doxorubicin resistant subline GLC_4-Adr overexpressing MRP1, in contrast, was extremely sensitive to APO010. The sensitivity of APO010 was related to caspase-3 activity and apoptosis induction but less related to Fas membrane expression. Sensitivity to APO010 could, however, be induced in drug-sensitive and drug-resistant cells by pretreating those cells with cisplatin or doxorubicin.

This study demonstrated that there is no consistent cross-resistance between APO010 and classical chemotherapeutic agents. Moreover, the sensitivity of cells overexpressing MRP1 to APO010, when confirmed in other tumor cell lines, could indicate an opportunity for overcoming a major tumor resistance mechanism.

NSAIDs as anti-tumor agents

Since the discovery of the involvement of the COX-2 enzyme in the carcinogenesis of colorectal cancer in familial adenomatous polyposis patients interest has been raised for the role of the *COX-2* gene in solid tumors. NSAIDs, the inhibitors of COX-1 and COX-2 were discovered to have more anti-cancer effects in preclinical models than can be explained by COX-1 and COX-2 inhibition alone. They also inhibit pathways involved in apoptosis regulation, angiogenesis, and tumor progression such as the Akt, NF-κB, tyrosine kinases, and the death receptor-mediated apoptosis pathways (chapter 2). Because of the plural mechanisms involved in NSAIDs antitumor activity it may be hard to design tests to predict efficacy in the clinic of NSAIDs in combination with classical chemotherapeutic agents. However, their role in the clinic has to be established first. There is currently still uncertainty whether NSAIDs can potentiate the effect on chemotherapy in solid tumor patients. Also the precise role of COX-2 inhibitors in this setting is unknown. Ongoing clinical studies will shed light on this in the near future. In addition, recent studies in non-cancer patients investigating the safety of NSAIDs showed an increase in cardiovascular events in patients using selective COX-2 inhibitors. To decrease the amount of cardiovascular events caused by selective COX-2 inhibitors, studies are in progress to determine which patients have an increased risk of developing cardiovascular complications after COX-2 inhibitor use. Given the limited cardiovascular toxicity of non-selective NSAIDs, these drugs may especially be of potential interest to modulate death receptor pathways in the future.

Utilization of drug resistance mechanisms to enhance apoptosis

Acquired drug resistance is one of the major problems in the treatment of patients with solid tumors. In chapter 3 we described that the COX inhibitor indomethacin induced apoptosis in a MRP1 overexpressing doxorubicin resistant small cell lung cancer cell line. Apoptosis induction was caspase-8 and caspase 9 dependent, but the actual target of indomethacin in this model has not been elucidated yet. To determine whether the death inducing signaling complex or caspase-8 is the target of indomethacin an inducible Bid RNAi system, FADD dominant negative or caspase 8 dominant negative can provide the answer. In chapter 4 we described that the depletion of glutathione by indomethacin induces apoptosis in an MRP1 overexpressing doxorubicin resistant small cell lung cancer cell line. Drug induced efflux of unconjugated glutathione has also been described for the ACE-inhibitor verapamil [1]. Studies combining P-glycoprotein inhibitors with chemotherapy have not shown an increased antitumor efficacy in the clinic. However, combining indomethacin with chemotherapy might still be of interest in tumors that do overexpress MRP1 as indomethacin has as additional effect on glutathione efflux. Tumor cells that overexpress ABC transporters such as MRP1 have an elevated glutathione turnover. Therefore, stimulation of glutathione efflux with indomethacin, would deplete these cells from glutathione much faster sensitizing them for cytotoxic agents. An increase in TRAIL-mediated apoptosis was observed in different P-glycoprotein overexpressing cell lines compared to their parental cell lines [2]. The concept of utilizing a drug resistance mechanism to enhance anti-cancer therapy is a potentially interesting new approach to an old problem and deserves further research.

Enhancement of death ligand and chemotherapy efficacy

As previously mentioned death receptor-mediated apoptosis can be enhanced by increasing intracellular signaling, but increasing death receptor activation can also be a method of inducing more apoptosis in tumor cells. This can be achieved by designing more potent death ligands such as APO010. APO010 is a Fas ligand hexamer and is much more effective inducer of apoptosis compared to the native Fas ligand or agonistic anti-Fas antibodies (chapter 7). The clinical benefit of multimer Fas ligand molecules remains uncertain due to their apoptosis inducing effects on hepatocytes. The drug will need a large potential therapeutic window before it can be considered for clinical testing. This perhaps can be circumvented by local application, for instance in the peritoneal cavity. This multimer has two advantages, it has a larger molecular weight to prevent diffusion into the systemic circulation and it is more effective at inducing apoptosis. To circumvent potential toxicity issues of Fas agonists in humans, APO010 has also

been investigated as an *ex vivo* purging agent in autologous haematopoietic stem cell transplantation following myeloablative high-dose chemotherapy.

The introduction of agonistic anti-TRAIL antibodies or recombinant human TRAIL (rhTRAIL) in clinical use seems to be more feasible. Currently phase I and phase II clinical trails investigating the safety of rhTRAIL and agonistic antibodies are being conducted. Identification of key factors in cell survival and apoptosis resistance in solid tumors are crucial for developing targeted therapies to treat these tumors. Considering the increasing interest in targeting death receptor pathways in solid tumors, the identification of the significant inhibitory factors in these pathways is of crucial importance. One group of apoptosis inhibitory factors described in this thesis is the IAP family. The most important member of this family involved in direct inhibition of apoptosis through binding of pro-apoptotic proteins such as caspase-3, caspase-9 and caspase-7, is XIAP. In chapter 6 the role of XIAP as a key inhibitor of TRAIL-mediated apoptosis was established. Therefore targeting XIAP with synthetic XIAP inhibitors is an example of enhancing death ligand efficacy. Currently anti-sense inhibition of XIAP expression and small molecule XIAP inhibitors are being evaluated in clinical trials. Another important family of inhibitors of apoptosis involved in apoptosis induced by the TNF family of death ligands is the Bcl-2 family. The anti-apoptotic members of the Bcl-2 family prevent the release of pro-apoptotic proteins such as SMAC/DIABLO, thereby preventing activation of effector caspases and thus apoptosis [3, 4]. Targeting these Bcl-2 family members in combination with an apoptosis inducing ligand or antibody can be an interesting anti-cancer strategy. A number of NSAIDs have also been described to inhibit bcl-2 family members en subsequently promote apoptosis. Therefore the combination of NSAIDs and activation of death receptors is a rational combination worth further investigation.

Conclusion

There is an abundance of new drugs currently being investigated in preclinical studies. These drugs finally have to prove themselves in clinical trials. Based on the mechanisms involved in the activity of several of these drugs it may well be that better characterization of the tumor can help to develop patient-tailored therapies. In addition, given the mechanisms involved in the activity of these drugs combinations of different drugs may be crucial to prove their relevance in the clinic.

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Samenvatting en toekomstperspectieven

Samenvatting

De afgelopen jaren is er veel aandacht voor het onderzoek naar signaaltransductieroutes in tumoren waartegen nieuwe middelen ontworpen kunnen worden. Doel van dit proefschrift is te identificeren op welke manieren de death receptor apoptose route geactiveerd kan worden. De Tumor Necrosis Factor (TNF) familie van apoptose inducerende death receptoren bestaat uit verschillende receptoren en bijbehorende liganden; ten eerste TNF met de receptoren TNF receptor 1 en TNF receptor 2, ten tweede Fas Ligand (FasL) en de bijbehorende pro-apoptotische receptor Fas en de anti-apoptotische receptor DcR3, en ten derde TNF Related Apoptosis Inducing Ligand (TRAIL) met de pro-apoptotische receptoren DR4 en DR5, de anti-apoptotische receptoren DcR1 en DcR2 en de niet-membraangebonden receptor osteoprotegrin. Apoptose wordt geïnduceerd door binding van de ligand aan een apoptotische celmembraanreceptor waardoor trimerisatie van de receptor ontstaat. Aan de binnenkant van de celmembraan kan nu het adapter eiwit Fas Associated Death Domain (FADD) en/of het adapter eiwit TNF Associated Death Domain (TRADD) samen met een initiator cysteïne protease (caspase), caspase-8 binden aan deze receptor. Dit complex geeft een apoptose signaal door, waardoor een cascade van caspases geactiveerd wordt in de cel.

De eerste methode, beschreven in dit proefschrift, om apoptose te induceren is activatie van de death receptor apoptose route met behulp van selectieve en niet-selectieve cyclooxygenase (COX) remmers. Het COX-2 enzym komt in veel tumoren tot expressie en kan een voorspeller van een slechte prognose zijn. De COX-1 en COX-2 enzymen kunnen worden geremd door nonsteroidal anti-inflammatory drugs (NSAID's). Daarom wordt de rol van NSAID's voor de behandeling van solide tumoren nu uitgezocht. Uit *in vitro* experimenten is gebleken dat NSAID's angiogenese en proliferatie remmen, apoptose induceren en verhinderen dat tumoren invasief gaan groeien. De moleculaire routes die hiervoor worden gebruikt zijn onder andere de AKT of protein kinase B route, de nuclear factor kappa B route, de tyrosine kinases en de death receptor gemediëerde apoptose route. De laatste jaren komen er steeds meer aanwijzingen dat het gebruik van NSAID's een verhoogd risico op cardiovasculaire toxiciteit geeft. Dit geldt in het bijzonder voor selectieve COX-2 remmers. Met behulp van klinische studies zal moeten blijken of het anti-tumor effect van NSAID's opweegt tegen de cardiovasculaire toxiciteit.

In hoofdstuk 3 wordt een studie beschreven waarin de Fas-gemediëerde apoptose route wordt geactiveerd door indomethacine (een NSAID) in een kleincellig longkankercellijn model. Bij veel patiënten met een kleincellig longcarcinoom is in eerste instantie vaak een goede respons op chemotherapie

zichtbaar, maar treedt er daarna vaak een recidief op, waarbij de tumor resistent is geworden voor chemotherapie. Een veelvoorkomend resistentiemechanisme voor chemotherapie is de verhoogde aanwezigheid van drug transporters in de tumorcelmembraan. De grootste familie van drug transporters is de ATP binding cassette familie van transporteiwitten (ABC familie). Multidrug resistance protein 1 (MRP1) is een lid van deze familie en kan fungeren als een efflux pomp in de celmembraan voor een aantal chemisch verschillende stoffen. Een potentieel nieuwe methode om deze resistentie voor klassieke chemotherapie te omzeilen is het induceren van apoptose via de TNF familie van death liganden. De doxorubicine resistente, MRP1 tot overexpressie brengende, kleincellig longkankercellijn GLC_4-Adr en de doxorubicine gevoelige moedercellijn GLC_4 zijn bestudeerd om uit te zoeken of de Fas-gemediëerde apoptose route gebruikt kan worden om doxorubicine resistentie te omzeilen. Alle eiwitten die nodig zijn voor de vorming van een death inducing signaling complex zijn aangetoond in beide cellijnen met behulp van Western blot. Er was een hogere expressie van de pro-apoptotische eiwitten Bid en caspase-8 in GLC_4-Adr. Tevens was er een 3,1 keer hogere Fas membraan expressie in GLC_4-Adr cellen dan in de moedercellijn. Beide cellijnen waren resistent voor agonistische Fas antilichamen, maar de combinatie van anti-Fas antilichamen met de eiwitsynthese remmer cycloheximide resulteerde in 40% apoptose in GLC_4-Adr. Apoptose kon ook geïnduceerd worden door indomethacine in GLC_4-Adr, maar niet in GLC_4. Indomethacine is niet alleen een COX-remmer, maar tevens een bekende remmer van MRP1. Caspase-8, caspase-9 en Bid werden geactiveerd door indomethacine, terwijl caspase-8 en caspase-9 specifieke remmers de indomethacine-geïnduceerde apoptose blokkeerden. De doxorubicine gevoeligheid in GLC_4-Adr maar niet in GLC_4 nam 2,7 keer toe door indomethacine. Uit deze gegevens kan worden geconcludeerd dat indomethacine de cytotoxische activiteit van doxorubicine in deze doxorubicine resistente kleincellig longkankercellijn vergroot. Dit gebeurt voor een deel via de death receptor apoptose route onafhankelijk van Fas.

De MRP1 drug efflux pomp kan drugs transporteren die geconjugeerd zijn aan glutathion door glutathion S-transferase, maar het kan ook niet-geconjugeerde drugs en ongebonden glutathion beide apart transporteren. De doxorubicine resistente kleincellig longkankercellijn GLC_4-Adr, zoals beschreven in hoofdstuk 3, brengt MRP1 tot overexpressie, hetgeen een groot deel van de doxorubicine resistentie kan verklaren. Om de relatie tussen de MRP1 overexpressie en indomethacine-geinduceerde apoptose te onderzoeken zijn de experimenten zoals beschreven in hoofdstuk 4 uitgevoerd. MRP1 eiwitexpressie werd verminderd in GLC_4 and GLC_4-Adr cellen door middel van RNA interference (RNAi). Hierna werden de cellen blootgesteld aan indomethacine en werd het effect hiervan op

celoverleving, MRP1 activiteit, totale glutathion hoeveelheid en mitochondriële membraan potentiaal geanalyseerd. GLC_4-Adr cellen die getransfecteerd waren met small interfering MRP1 (si-MRP1) hadden een 84% lagere accumulatie van het MRP1 substraat carboxyfluoresceine vergeleken met de controle si-Luci getransfecteerde cellen. MRP1 eiwitreductie leidde tot een tweevoudige reductie in apoptose bij een concentratie van 75 μM indomethacine en een betere celoverleving tijdens indomethacine blootstelling met een toename van de IC_{50} van 22,8 ± 2,6 tot 30,4 ± 5,1 μM. Het verlagen van MRP1 expressie met RNAi leidde in GLC_4 niet tot een verandering in indomethacine-geïnduceerde apoptose. Si-MRP1 had, in beide cellijnen, geen effect op de hoeveelheid glutathion per cel, maar blootstelling aan 20 μM indomethacine gedurende 2 uren verlaagde de hoeveelheid glutathion met 31,5% in GLC_4-Adr, maar niet in GLC_4. Het verlagen van de glutathion in de cel met L-buthionine (S,R)-sulfoximine resulteerde eveneens in een verdubbeling van indomethacine-geïnduceerde apoptose. Glutathion verlaging door L-buthionine (S,R)-sulfoximine had echter geen effect op indomethacine-geïnduceerde apoptose in GLC_4 cellen. De mitochondriële membraanpotentiaal werd door indomethacine alleen verlaagd in GLC_4-Adr cellen. Onze studie laat zien dat indomethacine-gemediëerde apoptose MRP1 afhankelijk is in de GLC_4-Adr doxorubicine resistente kleincellig longkankercellen. Deze bevinding kan implicaties hebben voor de behandeling van patiënten met tumoren die MRP1 tot overexpressie brengen.

Chemotherapie resistentie is de belangrijkste reden voor het falen van therapie in o.a. kleincellig longkanker evenals en in ovariumkanker. Een veelvoorkomende reden voor resistentie is een falende apoptose inductie. Om deze apoptose resistentie te omzeilen werd de studie die beschreven staat in hoofdstuk 5 uitgevoerd. Hierin werd het apoptose inducerende effect van indomethacine en de selectieve COX-2 remmer celecoxib onderzocht in combinatie met agonistische anti-Fas antilichamen en cisplatin, een veelgebruikt cytostaticum. De ovariumcarcinoom cellijnen A2780/m248, die getransfecteerd is met een p53-expressie vector waarin een mutatie op codon 248 van het p53 gen zit en A2780/cmv, die een lege controle vector bevat, zijn gebruikt in deze studie. A2780/m248 heeft een hogere Fas membraan expressie dan A2780/cmv. Celecoxib induceerde meer apoptose in A2780/m248 en A2780/cmv dan indomethacine. Celecoxib was tevens een sterkere caspase-8 en caspase-9 activator dan indomethacine. Caspase-8 en caspase-9 activatie door celecoxib kon worden voorkomen door zowel caspase-8 als caspase-9 specifieke remmers. Alleen indomethacine-geïnduceerde apoptose nam af in beide cellijnen en wel met 50% door co-incubatie van indomethacine met antagonistische anti-FasL (NOK-1) en anti-Fas (ZB4) antilichamen. Door middel van confocale lasermicroscopie werd

aangetoond dat granulaire co-lokalisatie van FasL en Fas optrad in beide cellijnen na celecoxib blootstelling. Dit effect werd niet gezien na indomethacine blootstelling. Zowel indomethacine als celecoxib verhoogden de gevoeligheid voor agonistische anti-Fas antilichamen in A2780/m248, de cellijn met de hoogste Fas membraanexpressie, zonder dat de Fas membraanexpressie was verhoogd. Cisplatin daarentegen verhoogde in beide cellijnen de indomethacine-gemediëerde apoptose maar had geen effect op de celecoxib-gemediëerde apoptose. Uit deze resultaten kan worden afgeleid dat indomethacine en celecoxib beide p53 onafhankelijk maar op verschillende wijze de Fas apoptose route beïnvloeden. Deze middelen hebben daarom mogelijk verschillende toepassingen in anti-kanker behandelcombinaties.

Weefselspecifieke signaaltransductieroutes kunnen ook gebruikt worden om een death receptor apoptose route te moduleren. Van recombinant humaan TRAIL (rhTRAIL) is bekend dat het apoptose kan induceren in een aantal borstkankercellijnen. De mogelijkheid om rhTRAIL-gemediëerde apoptose inductie te moduleren door het benutten van de invloed van oestrogenen op borstkankercellen is onderzocht in hoofdstuk 6. De oestrogeen antagonist fulvestrant heeft alleen antagonistische eigenschappen terwijl tamoxifen ook agonistische eigenschappen bezit. Voor dit onderzoek zijn de caspase-3 mutante oestrogeenreceptor positieve borstkanker cellijn MCF-7 en de caspase-3 wild-type oestrogeenreceptor positieve borstkanker cellijn T47D gebruikt. Twintig μM tamoxifen net als 100 ng/ml rhTRAIL induceerde geen apoptose terwijl de combinatie van tamoxifen en rhTRAIL resulteerde in 40 ± 16% apoptose. Dit modulerende effect werd niet verklaard door toegenomen TRAIL receptorexpressie. Zowel tamoxifen als fulvestrant in combinatie met rhTRAIL zorgden voor een stabilisatie van geactiveerd caspase-8 in T47D, maar niet in MCF-7. Dezelfde stabilisatie van actief caspase-8 werd gezien na blootstelling aan de combinatie van een proteasoomremmer en rhTRAIL. De interactie tussen caspase-3 en een lid van de inhibitors of apoptosis en X-linked inhibitor of apoptosis (XIAP) werd onderzocht omdat MCF-7 in tegenstelling tot T47D geen functioneel caspase-3 tot expressie brengt en oestrogeenremming mogelijk de interactie tussen XIAP en caspase-3 beïnvloedde. Immunoprecipitatie liet een interactie tussen caspase-3 en XIAP zien, maar de ratio XIAP met caspase-3 na immunoprecipitatie veranderde niet door blootstelling aan rhTRAIL of tamoxifen. Het verminderen van XIAP expressie door RNAi in combinatie met blootstelling van T47D cellen aan 100 ng/ml rhTRAIL leverde meer apoptose op dan met rhTRAIL alleen, namelijk van 0 naar 27 ± 9% apoptose. Deze resultaten suggereren dat het modulerend effect van oestrogeenantagonisten op rhTRAIL-gemediëerde apoptose in T47D wordt veroorzaakt door toegenomen stabilisatie

van geactiveerd caspase-8. Dit zou nog kunnen worden versterkt door een caspase-3 amplificatie loop. De combinatie van een oestrogeenantagonist met rhTRAIL kan dus apoptose bevorderen in oestrogeenreceptor positieve borstkankercellen waarbij vermeld moet worden dat het modulerende effect in ieder geval gedeeltelijk caspase-3 afhankelijk is.

Behalve het activeren van de death receptor gemediëerde apoptose route op verschillende aangrijpingspunten in de cel, kan verhoogde activatie van de death receptor zelf ook een potentieel succesvolle strategie zijn. Het doel van deze studie zoals beschreven in hoofdstuk 7 was het onderzoeken van het apoptose inducerende potentieel van APO010, een hexamere vorm van oplosbaar humaan FasL, in chemotherapie resistente cellijnen en hun gevoelige moedercellijnen. Het panel bestond uit cisplatine, doxorubicine en taxol resistente kleincellig longkankercellijnen, testiculaire kiemceltumorcellijnen en ovariumkankercellijnen. Van alle cellijnen werd de gevoeligheid voor APO010 bepaald door middel van celoverlevingsexperimenten, apoptose proeven en geactiveerd caspase-3 bepalingen. Fas membraan expressie werd gemeten met behulp van flowcytometrie. De cisplatin resistente ovariumcarcinoom cellijn A2780-CP70 en de cisplatine gevoelige moedercellijn A2780 waren beide gevoelig voor APO010. De cisplatine resistente en gevoelige testiculaire kiemceltumorcellijnen Tera-CP en Tera daarentegen waren ongevoelig voor APO010. De cisplatine resistente sublijn GLC_4-CDDP, de taxol resistente sublijn GLC_4-Pgp, die P-glycoprotein tot overexpressie brengt, en de cisplatin gevoelige kleincellige longkankermoedercellijn GLC_4 waren eveneens ongevoelig voor APO010. In tegenstelling tot de hiervoor genoemde GLC_4 cellijn en de sublijnen, was de doxorubicine resistente, MRP1 tot overexpressie brengende, sublijn GLC_4-Adr extreem gevoelig voor APO010. De gevoeligheid voor APO010 correleerde met de gemeten caspase-3 activiteit en apoptose inductie, maar niet met de Fas membraanexpressie. Apoptose kon wel geïnduceerd worden in APO010 ongevoelige cellen na blootstelling aan cisplatine of doxorubicine.

Deze studie toont aan dat er geen duidelijke kruisresistentie tussen APO010 en klassieke chemotherapeutica bestaat. Zelfs is het zo, dat de extreme gevoeligheid voor APO010 van MRP1 tot overexpressie brengende tumorcellen een potentiële nieuwe mogelijkheid biedt voor het omzeilen van een belangrijke tumorresistentie mechanisme.

Toekomstperspectieven

NSAIDs als anti-tumor middelen

Sinds het aantonen van de betrokkenheid van het COX-2 enzym bij de carcinogenese van colorectale tumoren in familiaire adenomateuze polyposis patiënten, is er veel aandacht voor de rol van het *COX-2* gen in solide tumoren. Van NSAID's, de remmers van COX-1 en COX-2, werd ontdekt dat deze meer anti-kanker effecten hebben dan verklaard kan worden door hun COX-1 en COX-2 remming alleen. Ze remmen tevens signaaltransductieroutes die belangrijk zijn voor apoptose, angiogenese en tumorprogressie zoals de Akt, NF-κB, tyrosine kinase, en de death receptor-gemediëerde apoptose routes (hoofdstuk 2). Op dit moment bestaat er nog onzekerheid over hoe NSAID's het effect van klassieke chemotherapeutica potentieren. Het effect op de verschillende signaaltransductieroutes zal waarschijnlijk ook afhangen van het soort NSAID, het tumortype en de gebruikte chemotherapeutica. Tevens moet nog in de kliniek bewezen worden of, en zo ja bij welk type tumor NSAIDs het effect van chemotherapeutica versterken. Dit alles maakt het voorschrijven van een NSAID bevattende therapie gebaseerd op de tumorkarakteristieken van de individuele patiënt op korte termijn niet haalbaar. Recente studies die de veiligheid van selectieve COX-2 remmers in niet-kanker patiënten onderzochten, lieten een toegenomen cardiovasculaire toxiciteit zien vergeleken met niet-selectieve NSAID's. Het is mede gezien de cardiotoxiciteit van selectieve COX-2 remmers de vraag of deze middelen een rol in anti-kanker behandeling krijgen. Niet-selectieve NSAID's hebben weinig cardiovasculaire toxiciteit en zijn mogelijk interessantere middelen om death receptor routes te moduleren.

Het gebruik van drug resistentie mechanismen om apoptose te bevorderen

Verworven chemotherapie resistentie is een belangrijk obstakel voor een effectieve behandeling van patiënten met solide tumoren. In hoofdstuk 3 werd beschreven dat de COX remmer indomethacine, apoptose induceert in een verworven doxorubicine resistente kleincellig longkankercellijn. Deze apoptose was caspase-8 en caspase-9 afhankelijk, maar de directe aangrijpingsplek van indomethacine in de cel is nog niet gevonden. Een induceerbaar Bid RNA interference systeem of een dominant negatieve vorm van FADD zou gebruikt moeten worden om te bepalen of het death inducing signaling complex het aangrijpingspunt is van indomethacine. In hoofdstuk 4 wordt beschreven dat de gevoeligheid voor indomethacine van een MRP1 tot overexpressie brengende kleincellig longkankercellijn waarschijnlijk veroorzaaakt wordt door deze overexpressie van MRP1 en mogelijk door toegenomen efflux van glutathion. Efflux van ongeconjugeerd glutathion onder invloed van bepaalde geneesmiddelen

is al beschreven voor verapamil [1]. Studies waarin P-glycoproteine remmers werden gecombineerd met chemotherapie lieten geen toename van het anti-tumor effect zien. De combinatie van indomethacine met chemotherapie in tumoren die MRP1 tot overexpressie brengen zou wel interessant kunnen zijn. Tumorcellen die ABC transporters zoals MRP1 tot overexpressie brengen hebben een hogere glutathion productie en verbruik. Hierdoor zal stimulatie van glutathionefflux in deze cellen eerder leiden tot glutathiondepletie en daarmee een verhoogde gevoeligheid voor chemotherapie. In verschillende cellijnen die P-glycoprotein tot overexpressie brengen, is een verhoogde gevoeligheid voor rhTRAIL-gemediëerde apoptose gevonden [1]. Deze resultaten geven aan dat het gebruik maken van drugresistentie mechanismen zoals P-glycoproteïne en MRP1 om het effect van anti-kanker therapie met NSAID's en death liganden te potentiëren mogelijk een interessante nieuwe benadering is van een oud probleem en verder onderzoek verdient.

Vergroten van death ligand en chemotherapie effectiviteit

Zoals eerder vermeld kan death receptor gemediëerde apoptose versterkt worden door het verbeteren van de intracellulaire signaaltransductie, maar ook het verbeteren van death receptor activatie kan een manier zijn om meer apoptose te induceren in tumorcellen. Dit kan bereikt worden door het ontwerpen van potentere death liganden zoals APO010. APO010 is een hexameer van Fas Ligand en een veel potentere apoptose induceerder dan het normale trimere Fas Ligand of agonistische anti-Fas antilichamen (hoofdstuk 7). Of een multimeer Fas Ligand molecuul in de kliniek gebruikt kan worden is nog onduidelijk vanwege het apoptose inducerende effect op hepatocyten. APO010 zal dus veel effectiever moeten zijn tegen kankercellen dan normale cellen om het systemisch te kunnen gebruiken bij patiënten. Als het niet systemisch gegeven kan worden, kan APO010 mogelijk nog voor lokaal gebruik toegepast worden. Het multimere Fas Ligand heeft tevens als voordeel ten opzichte van het normale Fas Ligand een groter molecuulgewicht waardoor het minder snel diffundeert naar de circulatie. Om levertoxiciteit van Fas agonisten in patiënten te voorkomen is ook onderzocht of APO010 *ex vivo* gebruikt kan worden om tumorcellen uit het autologe hematopoëtische stamceltransplantaat te verwijderen voor reïnfusie na hoge dosis chemotherapie voor hematologische maligniteiten. Hierbij waren hematopoetische stamcellen niet gevoelig voor APO010, maar multipel myeloom cellen en acute myeloïde leukemie cellen wel [3].

De ontwikkeling van agonistische anti-TRAIL antilichamen of rhTRAIL is al veel verder. Fase I en fase II klinische studies worden op dit moment uitgevoerd om de veiligheid en effectiviteit van agonistische anti-TRAIL antilichamen en

rhTRAIL te onderzoeken. Het identificeren van belangrijke factoren die betrokken zijn bij overleving van kankercellen is cruciaal om gerichte therapieën tegen solide tumoren te kunnen ontwikkelen. Gezien de toegenomen interesse voor apoptose inductie via death receptoren in solide tumoren is ook het opsporen van belangrijke remmers in deze routes in tumorcellen van eminent belang. Een groep apoptoseremmers die beschreven werden in dit proefschrift is de IAP familie. XIAP is een belangrijk lid van deze familie dat betrokken is bij directe binding en inactivatie van de pro-apoptotische eiwitten caspase-9, caspase-3 en caspase-7. In hoofdstuk 6 wordt de rol van XIAP als belangrijk remmer van TRAIL-gemediëerde apoptose beschreven. Daarom zou remming van XIAP activiteit met bijvoorbeeld "small molecule" XIAP remmers tijdens rhTRAIL behandeling een potentieel interessante optie zijn om de effectiviteit van het death ligand te verhogen. Op dit moment wordt de veiligheid en effectiviteit van anti-sense remming van XIAP expressie en "small molecule" remming van XIAP in klinische studies onderzocht. Een andere familie van remmers van death receptor gemediëerde apoptose is de Bcl-2 familie. De anti-apoptotische leden van de Bcl-2 familie remmen het vrijkomen van pro-apoptotische eiwitten zoals SMAC/DIABLO uit de mitochondriën en remmen zo de activatie van effector caspases en dus apoptose [4, 5]. Het remmen van de functie van deze eiwitten in combinatie met een apoptose inducerend ligand of antilichaam kan ook potentieel een interessante anti-kanker strategie zijn. Van een aantal NSAID's is ook beschreven dat ze leden van de Bcl-2 familie remmen en zo apoptose induceren. De combinatie van NSAID's en death receptor activatie is derhalve een rationele combinatie die de moeite van het verder onderzoeken waard is.

Er zijn zeer veel nieuwe middelen die op dit moment onderzocht worden in klinische studies als anti-kankerbehandeling. Het beter karakteriseren van tumoren en meer inzicht in de mechanismen waarop de nieuwe middelen aangrijpen kan mogelijk helpen om gerichtere therapieën aan te bieden.

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Dankwoord

In mijn min of meer wanhopige zoektocht naar een studieplaats geneeskunde, kwam ik in mijn laatste jaar van de studie Medisch Biologie in contact met Professor Dr. E.G.E. de Vries Hoofd afdeling Medische Oncologie. Eigenlijk was het mijn bedoeling om de tijd tot een uiteindelijke inloting nuttig te besteden en wellicht ook het (nood)lot in positieve richting te manipuleren. En zo geschiedde. Niet voor de inloting, want die vond uiteindelijk spontaan plaats. Maar wel voor de jaren dat ik naast de geneeskundige studie in het laboratorium mocht werken onder de bezielende leiding van mijn begeleiders prof. dr. E.G.E. de Vries en dr S. de Jong.

Liesbeth, ik heb enorm veel geleerd van jouw niet aflatende optimisme en enthousiasme over onderzoeksresultaten, waarvan mij in ieder geval aanvankelijk de importantie nog wel eens niet helemaal duidelijk was. Ook de vele uitdagingen waarmee je mij rondom het laboratoriumonderzoek confronteerde zijn een enorme stimulans geweest. Mij past heel veel dank.

Steven, jij als mijn directe begeleider in het laboratorium, van jou heb ik erg veel geleerd op het gebied van de moleculair biologische techniek, maar ook op het gebied van wetenschapsmethodiek en wetenschapsethiek. De visie op de wereld werd natuurlijk eveneens op gepaste momenten besproken. Voor dit alles wil ik je ook graag bedanken.

De leden van de leescommissie prof. dr. H. Moshage, prof. dr. R.O.B. Gans en prof. dr. E.F. Smit ben ik zeer erkentelijk voor het beoordelen van het manuscript.

Dankzij het MD/PhD traject van de Junior Scientific Masterclass van het UMCG en onderzoeksinstituut GUIDE ben ik staat gesteld de klinische fase van de geneeskunde opleiding te combineren met een promotieonderzoek waarvoor dank.

De hoofdsponsoren van dit proefschrift wil ik hierbij ook van harte bedanken. Zij hebben gedurende 11,5 jaar ervoor gezorgd dat ik voorzien was van: een dak boven mijn hoofd, eten, wijze adviezen, enzovoort. Pap en mam dankjewel.

In het lab wil ik uiteraard alle mensen bedanken die mij de afgelopen jaren geholpen hebben met mijn experimenten. In het bijzonder Tineke Timmer, onder jouw leiding heb ik de eerste stapjes in het lab gezet. In die begin periode hebben Diana Spierings, Hetty Timmer, Coby Meijer, Bea Wisman en Phuong Le me, met veel geduld, de fijne kneepjes van het doen van experimenteel onderzoek geleerd.

Ook wil ik de promovendi met wie ik in de afgelopen jaren zo prettig heb mogen samenwerken bedanken; Dianne Bouis, Anne Crijns, Margaretha van der Deen, Ingrid van Dillen, Evelien Duiker, Caroline van Geelen, Pauline de Graeff, Esther de Haas, Hermien Hartog, Brigitte Hougardy, Dianne Heijink, Hilde Jalving, Xiang Yi Li, Sander de Korte, Roelof Koster, John Maduro, Wouter

Nagengast, Yang Nan, Janine Nuver, Bodvaël Pennanrun, Patrick Perik, Alessandra de Pietro, Bart Rikhof, Carolien Schröder, Ineke Sloots, Janet Stegehuis en Shinta Tan. Hilde jij bent 3 maanden eerder begonnen dan ik en ik heb erg veel aan jouw kennis van sollicitatieprocedures en subsidie aanvragen gehad dankjewel.

Met John, Martine, Sander, Phuong en Haukeline had ik het voorrecht in mijn laatste onderzoeksjaar een kamer te mogen delen en dat vergrootte mijn werkvreugde enorm, allen bedankt voor een geweldige tijd.

Zonder de ondersteuning in het lab van Wendy Dam, Gert-Jan Meersma, Wytske Boersma-Van Ek, Martine van Zweeden, Alice Koning, Edmond de Cuba, Mirjam Boelens, en Agnes Lendvai, Haukeline Volders en Dorenda Oosterhuis zouden er helemaal geen experimenten worden gedaan, dus daar wil ik jullie ook van harte voor bedanken.

In mijn promotie periode heb ik een aantal studenten van verschillende opleidingen mogen begeleiden en bij dezen wil ik Kim, Susan, Janny, Anouk en Arne ook graag bedanken voor de grote hoeveelheid werk die zij hebben verzet en de prettige samenwerking.

Bianca Smit en Gretha Beuker jullie wil ik bedanken voor het feit dat jullie altijd bereid waren om “even” een manuscript aan te passen. Geert en Henk bedankt voor jullie hulp bij alle flowcytometrie experimenten. Theo van Kooten dank voor de fantastische confocale lasermicroscopische opnamen. Als laatste wil ik mijn familie en vrienden bedanken voor het begrip tijdens weekenden en feestdagen waarop ik weer eens naar het lab moest of, zoals jullie het plachten te noemen, “slimme” stukjes moest schrijven.