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Synthesis and evaluation of cytostatic and antiviral activities of 3′ and 4′-avarone derivatives

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Summary

A series of 3′ and 4′-substituted avarone derivatives were synthesized and tested in culture systems as antitumour and antiviral agents in comparison to avarol and avarone. 3′-alkylamino derivatives showed potent cytostatic activities against murine L1210 and human B (Raji) and T (C8166, H9) lymphoblast cells (ID_{50} range 1.7–3.7 μM). Avarol and avarone were six times less active. While none of the derivatives showed anti-human immunodeficiency virus (HIV) activity superior to that of the parent compounds, most of them, avarol and avarone included, were potent and selective inhibitors of poliovirus multiplication.

Introduction

Avarol, a sesquiterpenoid hydroquinone, and its quinone derivative avarone, are secondary metabolites isolated from the sponge *Dysidea avara* (Minale *et al.*, 1974; De Rosa *et al.*, 1976). Both compounds were first described as potent antileukaemic agents *in vitro* and *in vivo* (Müller *et al.*, 1985), and then were shown to effectively inhibit human immunodeficiency virus type 1 (HIV-1) replication *in vitro* (Sarin *et al.*, 1987). Controlled studies, however, failed to confirm clinical usefulness of the compounds in acquired immunodeficiency syndrome (AIDS) patients.

Nevertheless, the potent 'T-lymphotropic' cytostatic activity of avarol and avarone, their low toxicity in mice, the lack of interference with antibody-mediated and delayed-type hypersensitivity reactions in animals, the penetration of the blood–brain barrier and the capacity to induce interferon (Müller *et al.*, 1986, 1987), make these compounds good candidates for structural rearrangements aimed at improving their cytostatic and/or antiviral activity.

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3′-methylamino (compound 3; fig. 1) and 4′-methylamino (5) derivatives of avarone have previously been shown to possess broad spectrum biological properties (Cimino *et al.*, 1982). This prompted us to investigate the effects of other amino derivatives of avarone.

The work reported here extends the *in vitro* testing of avarol and avarone as cytostatic and antiviral agents and includes an investigation on the activity of four new and seven recently synthesized avarone derivatives.

Results

Cytostatic activity

The compounds were evaluated *in vitro* for their ability to inhibit the growth of fibroblast-like cells, murine lymphocytic leukaemia and human B- and T-lymphoblast cells. The 50% inhibitory doses (ID_{50} values) for these compounds are summarized in Table 1.

While both avarol (compound 1) and avarone (2) showed similar inhibitory activity against all the cell lines tested (ID_{50} range 10–20 μM, compounds 3 and 4 were six times more potent against L1210 and B and T cells than against Vero cells. Compounds 5–7 and 9–11 showed cytostatic activity comparable to that of avarone, whereas 12, 13 and 8 were significantly less inhibitory to Vero cell proliferation.

Antiviral activity

Previous results by Sarin *et al.* (1987) showed that both avarol and avarone possessed significant anti-HIV-1 activity at concentrations as low as 0.3 μM, as measured by determination of reverse transcriptase activity and expression of the gag proteins p24 and p17. Under our assay conditions (Table 2), avarone showed an anti-HIV-1 activity more potent and selective than avarol. Among the avarone derivatives, none showed anti-HIV activity at concentrations lower than the ID_{50}, with the exception of compounds 11 and 12. Both were as potent as avarone and the latter was even more selective.

The compounds were also tested against African Swine Fever virus (ASFV), herpes simplex types 1 and 2 (HSV-1 and -2), polio type 1 (polio) and vesicular stomatitis virus (VSV) (Table 2). All compounds selectively inhibited the multiplication of poliovirus but were virtually inactive on the other viruses tested.

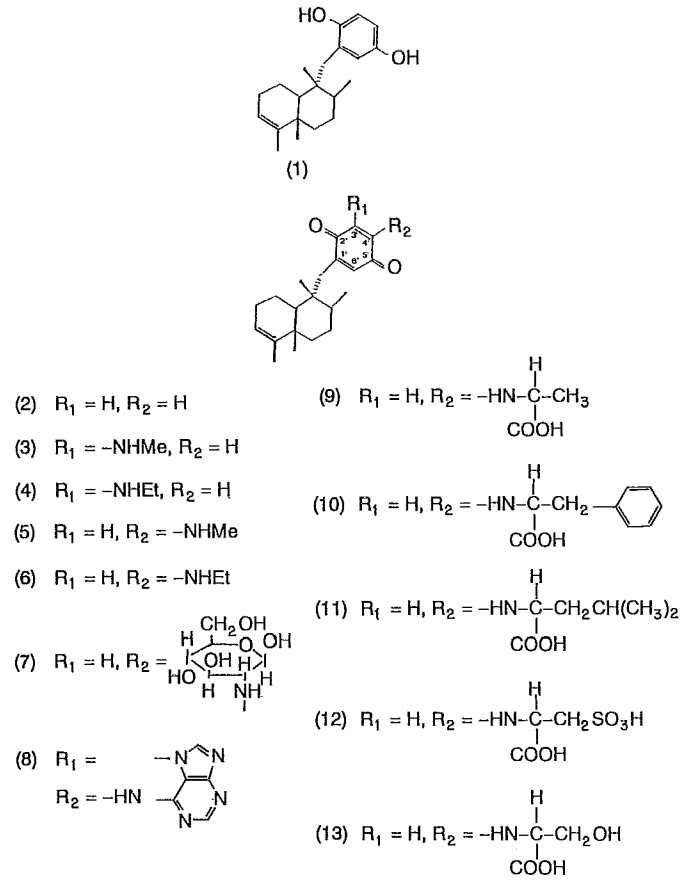

Fig. 1. Structural formulae of compounds 1–13.

Against poliovirus, avarol, avarone and compounds 3 and 4 emerged as the most potent and selective inhibitors, whereas compounds 12 and 13 retained a selectivity comparable to that of the parent compounds because of their low toxicity for Vero cells.

Discussion

In the present study we compared cytostatic and antiviral activities of avarol, avarone and a series of 3′- and 4′-substituted avarone derivatives.

The cytostatic activity of avarol and avarone in Vero cells is comparable to that previously reported by Müller *et al.* (1985) in HeLa cells. However, if compared to that in murine L5178Y lymphoma cells (ID_{50} reported in the range 0.6–0.9 μM), both compounds are considerably less active against L1210 and human B- and T-lymphoblast cells (ID_{50} in the range 10–20 μM). This discrepancy may be accounted for by the different experimental conditions used, such as type of cell line, initial cell number (5×10^3 versus 1×10^5 cells/ml), different length of treatment (seven versus three cell cycles).

The introduction of a methylamino (3) or an ethylamino (4) group in the 3′ position of the quinone ring of avarone results in compounds endowed with higher potency against L1210 and B- and T-lymphoblast cells. On the contrary, the introduction of the same alkylamino substituents in the 4′ position gives rise to compounds (5 and 6) with cytotoxicity comparable to that of avarone. This also held true with other 4′-substituted avarone derivatives (7, 9–11). However, 4′-serine- (12), 4′-cysteic acid- (13) and 3′,4′-adenine-avarone (8) showed a lower cytostatic effect against Vero cells while maintaining the toxicity of avarone against lymphoid cells. 3′-substituents other than methylamino (3) and ethylamino (4) could not be obtained, presumably because the introduction of substitutents in this position is regulated by steric hindrance and/or reactivity of substituents.

Under our experimental conditions, avarol and avarone show potent, although not very selective, anti-HIV activity. Modifications in the quinone ring of avarone always result in loss of anti-HIV activity with two excep-

Table 1. Cytostatic activity of compounds 1–13.

Compound	ID_{50} (μM) ± SD[a]				
	Vero	L1210	Raji	C8166	H9
1	19.5 ± 0.50	13.9 ± 0.39	11.7 ± 0.43	9.2 ± 0.09	13.5 ± 0.25
2	17.0 ± 1.15	15.6 ± 0.40	18.1 ± 0.69	12.9 ± 0.10	14.2 ± 0.39
3	14.0 ± 0.51	2.3 ± 0.08	2.0 ± 0.08	1.7 ± 0.08	2.3 ± 0.09
4	12.5 ± 0.34	3.4 ± 0.12	3.9 ± 0.10	2.5 ± 0.12	3.7 ± 0.13
5	10.2 ± 0.38	15.2 ± 0.21	18.1 ± 0.15	16.2 ± 0.19	22.0 ± 0.21
6	12.0 ± 0.31	28.2 ± 0.34	20.3 ± 0.33	18.6 ± 0.40	28.0 ± 0.19
7	21.3 ± 0.25	7.3 ± 0.39	8.4 ± 0.20	7.1 ± 0.10	8.9 ± 0.23
9	27.7 ± 0.46	8.3 ± 0.25	7.5 ± 0.21	5.7 ± 0.13	5.0 ± 0.15
10	32.9 ± 0.37	8.8 ± 0.18	7.1 ± 0.09	8.0 ± 0.20	10.1 ± 0.41
11	23.5 ± 0.32	11.6 ± 0.29	9.1 ± 0.15	8.8 ± 0.24	9.1 ± 0.18
12	>100.0	26.3 ± 0.21	24.0 ± 0.39	22.3 ± 0.35	30.1 ± 0.43
13	>100.0	27.5 ± 0.33	24.1 ± 0.41	23.5 ± 0.23	36.0 ± 0.53
8	>100.0	37.5 ± 0.45	30.5 ± 0.31	28.2 ± 0.41	28.9 ± 0.65
metotiexate (MTX)	0.02	0.01	0.01	0.01	0.007

a. ID_{50}: drug concentration required to reduce by 50% the number of cells under conditions allowing exponential cell growth for three cell cycles.

Table 2. Antiviral activity of compounds 1–13.

Compound	ED_{90}[a] (μM)	ED_{50}[b] (μM)					SI[c] (μM)	
	HIV-1	ASFV	HSV-1	HSV-2	Polio	VSV	HIV-1	Polio
1	2.9	19.0	10.5	7.6	0.8	>25.0	3.1	24.3
2	1.5	17.4	9.5	11.6	0.8	>17.0	8.6	21.8
3	>0.17	>14.0	4.4	6.7	0.6	>14.0	<1.0	23.3
4	>2.5	>12.5	>12.5	>12.5	0.8	>12.5	<1.0	15.6
5	>16.2	>10.2	>10.2	>10.2	2.0	>10.2	<1.0	5.1
6	>18.6	>12.0	>12.0	>12.0	3.3	>12.0	<1.0	3.6
7	>7.1	>21.0	17.0	>21.0	4.5	>21.0	<1.0	5.7
9	>5.7	15.0	10.5	15.0	4.0	13.2	<1.0	6.9
10	>8.0	>32.9	25.0	16.0	8.3	>32.9	<1.0	3.9
11	1.1	>23.5	23.5	23.5	2.7	>23.5	8.7	8.5
12	1.0	>100.0	>100.0	>100.0	3.9	>100.0	22.3	>25.6
13	>23.5	>100.0	>100.0	>100.0	5.8	>100.0	>1.0	>17.2
8	>28.2	>100.0	22.5	36.0	22.0	>100.0	>3.5	>4.5
dideoxyadenosine (ddAdo)	20.0	–	–	–	–	–	>50.0	
acyclovir (ACG)	–	>10.0	0.04	0.2	>10.0	>10.0	–	
Guanidine	–	>500.0	>500.0	>500.0	125.0	>500.0	–	130.0

a. ED_{90} (90% effective dose), drug concentration required to reduce by 1 log the HIV-1 yield in C8166 cells. Virus titre in untreated controls was 2×10^5 $CCID_{50}$/ml.
b. ED_{50} (50% effective dose), drug concentration required to reduce by 50% the number of plaques. Number of plaques in control cultures were: 92 (ASFV), 115 (HSV-1), 100 (HSV-2), 132 (polio), 91 (VSV).
c. SI (selectivity index), HIV = ratio ID_{50} C8166/ED_{90}; polio = ratio ID_{50} Vero/ED_{50}.

tions: 4′-leucine- (11) and 4′-serine-avarone (12). Both retain the potency of the parent compound, whereas the 4′-serine derivative shows a three-fold higher selectivity.

Both avarol and avarone show more potent and selective activity against poliovirus than against HIV. None of the modifications in the quinone ring result in improved anti-polio activity. The 3′-derivatives retain the same potency and selectivity of avarol and avarone, whereas all the 4′-derivatives show a significant reduction in potency and, in most cases, also in selectivity. 4′-serine- and 4′-cysteic acid-avarone are the only exceptions, with lack of toxicity for Vero cells.

The improvement in selectivity obtained against HIV-1 with compound 12 and against polio with compounds 12 and 13 suggests further investigations on avarone derivatives bearing substituents in different positions of the quinone ring.

Materials and Experimental procedures: chemistry

Melting points were determined using a Kofler hot-stage microscope and are uncorrected. 1H-NMR spectra were measured on a WM 500 Bruker spectrometer (TMS as internal standard). Only chemical shifts of quinone ring protons are reported since all the other signals belonging to the sesquiterpenoid and other structural moieties have been resported elsewhere by Minale *et al.* (1974) and De Rosa *et al.* (1976) or are generally known. UV spectra were obtained on a Varian DMS 90 spectrophotometer. Column chromatography was carried out on Merck Si gel 60 and Sephadex LH-20. Avarol (1) was isolated from *D. avara* (Minale *et al.*, 1974), which was collected in the Bay of Naples (Italy). Avarone (2) was prepared from avarol by oxidation with Ag_2O, as described previously (Minale *et al.*, 1974). Methylamino (3, 5), ethylamino (4, 6), glucosamino (7), adenine (8) and alanine (9) derivatives of avarone were obtained by slowly adding the amino reagent, dissolved in basic solution, to a dilute solution of avarone in ethanol or ethanol/water (1 : 1), as described elsewhere (Cozzolino *et al.*, 1990). D,L-Phenylalanine (Fluka, A.G. Bucks, OK), D,L-leucine (Fluka, A.G.), D,L-cysteic acid (Fluka, A.G.) and D,L-serine (Calbiochem, La Jolla, C.A.), were used for the syntheses.

Synthesis of compound 10

D,L-Phenylalanine (1 g) was dissolved in a saturated solution of $NaHCO_3$ (100 ml) and added to a solution of avarone (280 mg) in EtOH (100 ml) and stirred for 3 h at 60°C. After elimination of EtOH, the remaining aqueous solution was extracted with *n*-butanol; the extract was chromatographed on Si gel column to give, by elution with $CHCl_3$/MeOH (9 : 1), compound 10 (130 mg): m.p. > 300° with decomposition ($CHCl_3$-MeOH); UV max (ε) (MeOH) 292 (3350), 378 (750), 490 (1150) nm; 1H-NMR ($CDCl_3$ + CD_3OD) δ 6.21 (H-6′,s), 5.25 (H-3′, s).

Synthesis of compound 11

D,L-Leucine (650 mg) was dissolved in a saturated solution of $NaHCO_3$ (100 ml) and added to a solution of avarone (200 mg) in EtOH (100 ml). After the usual workup, the *n*-butanol extract was chromatographed on a Sephadex LH-20 column (2 × 100 cm) to give, by elution with MeOH, compound 11 (110 mg): m.p. > 300° with decomposition ($CHCl_3$-MeOH); UV λ max (ε) (MeOH) 286 (2150), 488 (700); 1H-NMR ($CDCl_3$ + CD_3OD) δ 6.78 (H-6′, s), 5.33 (H-3′, s).

Synthesis of compound 12

D,L-Cysteic acid (1 g) was dissolved in a solution of $NaHCO_3$ (100 ml) and added to solution of avarone (300 mg) in EtOH (100 ml). After the usual workup, the *n*-butanol extract was chromatographed on a Sephadex LH-20 column (2 × 100 cm) to give, by elution with MeOH, compound 12 (100 mg): m.p. >300° with decomposition ($CHCl_3$ – MeOH); UV λ max (ε) (MeOH) 283 (2750), 485 (950); 1H-NMR ($CDCl_3$ + CD OD) δ 6.10 (H-6′, s), 5.23 (H-3′, S).

Synthesis of compound 13

D,L-Serine (1 g) was dissolved in a saturated solution of $NaHCO_3$ (100 ml) and added to a solution of avarone (300 mg) in EtOH (100 mg). After the usual workup, the *n*-butanol extract was chromatographed on a Si gel column to give, by elution with $CHCl_3$-MeOH (8:2), compound 13 (270 mg): m.p. > 300° with decomposition ($CHCl_3$ – MeOH); UV λ max (ε) (MeOH) 286 (4300), 378 (950), 488 (1500) nm; 1H-NMR ($CDCl_3$ + CD_3OD) δ 6.34 (H-6′,s), 5.42 (H-3′, s).

Materials and Experimental procedures: virology

Cells

The following cells were used: H9 cells, a $CD4^+$ T-cell line that is permissive to HIV replication but partially resistant to its cytopathic effect (CPE); H9/IIIB cells, an H9 subline which is persistently infected with HTLV-IIIB; C8166 cells, a $CD4^+$ T-cell line containing a genome of HTLV-I and expressing only the *tat* gene, in which HIV induces an easily detectable, syncytium-forming CPE; L1210, lymphocytic mouse leukaemia cells; Raji, human lymphoblastoid B cells from a Burkitt lymphoma.

All these cell lines were mycoplasma-negative and were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 U-ml penicillin and 100 μg/ml streptomycin, at 37°C in a CO_2 incubator. In addition, Vero (African green monkey kidney) monolayers were used. These cells were grown in Dulbecco's modified minimal essential medium (MEM) supplemented with 10% newborn calf serum (NCS).

Viruses

The HIV-1 used in the assays was obtained from culture supernatants of H9/IIIB cells collected at the end of an exponential growth phase. The titre of virus stock solutions varied between 2 and 4 × 10^5 50% cell culture infectious doses fifty, ($CCID_{50}$) per ml. Virus stocks of HSV-1 (ATCC VR 733; Rockville, MD), HSV-2 (ATCC VR 734), ASFV (Istituto Zooprofilattico di Sassari), VSV (ATCC VR 158) and polio (Sabin strain) were obtained in Vero cells and had a titre of 5 × 10^7 plaque-forming units (PFU) per ml, 2 × 10^7 PFU ml, 3 × 10^7 PFU/ml, 1 × 10^8 PFU/ml and 5 × 10^8 PFU/ml, respectively.

Toxicity tests

L1210, Raji, C8166 and H9 cells were seeded at a density of 1 × 10^5 cells/ml in growth medium and cultured at 37°C with various concentrations of the compounds. Cell numbers were determined with a Coulter counter after 36 h (L1210) or 96 h (Raji, C8166 and H9), corresponding to three cell cycles.

Vero cells were seeded at a density of 1 × 10^5 cells/ml and allowed to adhere overnight. Growth medium containing various concentrations of the compounds was then added. After a 4-day incubation at 37°C (three cell cycles), the number of cells was determined with a Coulter counter after trypsinization of the monolayers.

In all cases cell viability was determined by the Trypan blue dye exclusion test.

Anti-HIV assays

Exponentially growing C8166 cells were seeded at a density of 1 × 10^6 cells/ml and then infected with 1 × 10^5 $CCID_{50}$ of HIV-1. After a 2-h incubation at 37°C the inoculum was removed, the cells were washed three times and then resuspended at 1 × 10^5/ml in RPMI-1640 containing 10% FCS, in the absence or in the presence of the test compounds. After a 4-day incubation at 37°C, the presence of syncytia was evaluated at the inverted microscope and the amount of infectious virus produced was determined by end-point titration.

HIV titration

Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, four replica wells per dilution) in 96-well plates. The infectious virus titre was determined by light microscope scoring of syncytia after 4 days of incubation, and virus titre was calculated as $CCID_{50}$ by the Reed and Muench method.

Anti-RNA and -DNA virus assays

Plaque reduction tests were performed according to Collins and Bauer (1977) in Vero cell monolayers. The plaque counts obtained in the presence of the compounds were expressed as a percentage of those obtained in untreated controls and plotted against the logarithm of drug concentrations. Dose–response lines were drawn by linear regression technique and 50% effective doses (ED_{50}) were calculated.

It should be noted that the concentrations of the compounds capable of inducing a 50% cytotoxic effect on the monolayers (by visual observation) after 5 days of incubation (time of incubation for ASFV) were always considerably higher than the ID_{50} values.

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