Cancer Chemother Pharmacol (1993) 32: 39–45

Cancer Chemotherapy and Pharmacology

Pharmacological properties of the ornithine decarboxylase inhibitor 3-aminooxy-1-propanamine and several structural analogues

Helmut Mett[1], Jaroslav Stanek[1], Juan A. Lopez-Ballester[2], Juhani Jänne[3], Leena Alhonen[3], Riita Sinervirta[3], Jörg Frei[1], Urs Regenass[1]

[1] Research Laboratories, Pharmaceuticals Division, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland
[2] Department of Biochemistry, University of Murcia, Spain
[3] Department of Biochemistry and Biotechnology, University of Kuopio, SF-70211 Kuopio, Finland

Abstract. Analogues of 3-aminooxy-1-propanamine proved to be highly potent and selective inhibitors of ornithine decarboxylase (ODC). The compounds competed with ornithine for the substrate binding site of ODC, but resulted in progressive and apparently irreversible inactivation of the enzyme. Diamine oxidase was inhibited by these compounds to a lesser extent than ODC; the compounds were not metabolized by this enzyme. Several derivatives were growth-inhibitory for human T24 cells and for other mammalian cells, the most active compound being 3-aminooxy-2-fluoro-1-propanamine (AFPA). Growth-arrested cells were largely depleted of putrescine and spermidine. Cellular growth arrest could be antagonized by supplementation with spermidine. Selection for resistance against AFPA led to cells with amplified ODC genes and overexpression of the message. Some of the derivatives were tumoristatic at well-tolerated doses in mice bearing solid T24 tumours. The antiproliferative activity of these compounds appears to be mediated by polyamine depletion.

Introduction

Rapidly proliferating cells, like tumour cells, generally have much larger polyamine pools and higher specific activities of their biosynthetic enzymes than non-proliferating or fully differentiated cells (for reviews see Pegg [22, 23] and Seiler [32]). Cell growth appears to depend on cellular polyamine pools. Reduction of these pools by inhibition of polyamine biosynthesis has been shown to block cellular proliferation [18, 20, 24, 33]. However, the ultimate growth-regulatory function of the major polyamines putrescine, spermidine, and spermine is not yet fully understood.

Correspondence to: H. Mett, K-125.4.01, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland

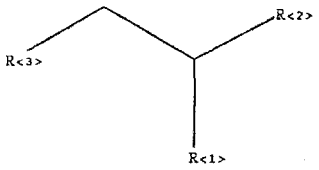
Two polyamine antimetabolites, the ornithine decarboxylase (ODC) inhibitor α-difluoromethylornithine and the *S*-adenosylmethionine decarboxylase (AdoMetDC) inhibitor methylglyoxal bis(guanylhydrazone), have already undergone clinical trials as antitumour agents [4, 6, 16, 17]. The results were rather unsatisfactory, mainly because of lack of potency (α-difluoromethylornithine) and serious side-effects after administration of methylglyoxal bis(guanylhydrazone).

The key enzymes in polyamine biosynthesis, ODC and AdoMetDC, are potential targets for new antiproliferative agents. Amongst the ODC inhibitors, α-difluoromethylornithine and several other ornithine and putrescine derivatives have been tested as antiproliferative and/or antitumour agents [2, 11, 18, 26, 28]. The structurally rather simple putrescine analogue 3-aminooxy-1-propanamine (APA) was first described by Khomutov et al. [12]. Several publications (e.g. [7, 8, 12]) describe various properties of this compound: it is a potent, irreversible inhibitor of ODC, the K_i being in the nanomolar range. It also showed inhibitory activity against AdoMetDC (K_i = 50 μM [12] or 1–2 mM [7]) and spermidine synthase (K_i = 2.3 μM), but was not a substrate for the latter enzyme. Its antiproliferative activity was rather weak [29]; the compound was apparently metabolized to a significant extent by hamster cells [9].

We wanted to evaluate this class of compounds as potential candidates for therapy of human tumours. A series of derivatives of the basic APA structure was synthesized with the aim to increase (1) its selectivity for ODC in comparison to the other above-mentioned targets of APA, (2) its potency as an antiproliferative agent, and (3) its antitumour activity in animal tumour models. These aims were best reached with 2-halogenated derivatives of APA.

Materials and methods

Preparation of ODC and assay for ODC activity. The methods of Seely and Pegg [31] and Hayashi and Kameji [5] were used with minor modifications. In brief, ODC was extracted from livers of Wistar rats pre-



Compound	R_1	R_2	R_3
Putrescine	H	CH_2-NH_2	CH_2-NH_2
APA*	H	CH_2-NH_2	O-NH_2
1 (AFPA)	F	CH_2-NH_2	O-NH_2
2 (AHPA)	OH	CH_2-NH_2	O-NH_2
3	Cl	CH_2-NH_2	O-NH_2
4	OH	CH_2-NH_2	O-N=C(CH_3)-OC_2H_5
5	F	CH_2-NH_2	O-N=C-R^a
6	F	CH_2-NH_2	O-N=C-R^b
7	F	CH_2-NH_2	O-N=C-R^c
8	OH	CH_2-O-NH_2	O-NH_2
9	H	NH-NH_2	O-NH_2
10	F	CH_2-NH_2	NH-NH_2

Fig. 1. Chemical structures of 3-aminooxy-1-propanamine (APA) analogues used in this study. An ethyl core was substituted at one carbon with the residues R_1 and R_2, at the other carbon with residue R_3. R^a, 2-methyl-3-hydroxy-5-hydroxymethyl-pyridyl(4); R^b, *o*-hydroxyphenyl; R^c, 2-methyl-3-hydroxy-5-[(phosphonooxy)methyl]-pyridyl(4); AFPA, 3-aminooxy-2-fluoro-1-propanamine; AHPA, 3-aminooxy-2-hydroxy-1-propanamine

treated with thioacetamide. The supernatant fraction of liver homogenates was dialysed against 25 mM Tris·HCl pH 7.4, 0.1 mM EDTA, and 1 mM dithiothreitol and stored in aliquots at –70° C. These preparations resulted in a specific ODC activity of 30 ± 1.7 pmol·min^{-1}·mg^{-1} (see below).

The assay mixture for determination of ODC activity consisted of (final concentrations) 100 mM Tris·HCl pH 7.0, 4 mM EDTA, 4.4 mM dithiothreitol, 0.214 mM [1-^{14}C]ornithine (Amersham; 0.2 μCi/assay), 0.4 mM pyridoxal phosphate, and variable amounts of ODC in a total volume of 0.25 ml. After incubation for 15–60 min at 37° C, reactions were stopped by the addition of 0.17 ml 2 *N* HCl and incubated for 20 min. CO_2 trapped on Whatman 3MM filters humidified with 25 μl Soluene-100 (Packard) was counted in a toluene-based liquid scintillation cocktail (Irgaszint; Ciba-Geigy). Control assays lacking ODC preparation were usually well below 100 cpm.

The same assay with variable amounts of ornithine was used for kinetic experiments. Unless stated otherwise, inhibitors were added simultaneously with substrate, cofactor, and enzyme. The concentration of inhibitor inhibiting ODC by 50% (IC_{50}) under these experimental conditions was usually in the same order of magnitude as the K_i value.

The concentration of pyridoxal phosphate was kept well above the K_m (which was below 1 μM) in order to make the assay insensitive for the detection of pyridoxal phosphate analogues.

Preparation of other enzymes and assay for their activities. AdoMetDC was prepared from livers of Wistar rats and assayed essentially as described [25]. Aliquots of cell-free dialysed rat liver extracts were stored at –70° C. Assay mixtures for determination of AdoMetDC activity consisted of (final concentrations) 100 mM Tris·HCl pH 7.0, 0.7 mM EDTA, 7.5 mM dithiothreitol, 3.3 mM putrescine, 0.21 mM *S*-adenosyl[carboxyl-^{14}C]methionine (Amersham; 0.1 μCi/assay), and variable amounts of AdoMetDC (specific activity 54 ± 27 pmol·mg^{-1}·min^{-1}) in a total volume of 150 μl. Reactions were stopped and processed as described for ODC.

Rat small intestinal diamine oxidase (DAO) was prepared and assayed as described [34], and aliquots were stored at –70° C. The assay mixture for determination of DAO activity contained (final concentrations) 112 mM potassium phosphate pH 7.4, 0.4 mM [1,4-^{14}C]putrescine (Amersham; 40 nCi/assay), 0.02 mM EDTA, 2 mM mercaptoethanol, and various amounts of DAO in a total volume of 250 μl. After 30 min incubation at 37° C, reactions were stopped by chilling to 0° C and addition of 0.5 ml 1 M sodium carbonate containing 1 mM aminoguanidine. Radiolabelled product was collected by three cycles of toluene extractions (4 ml each, containing 5 g/l of 2,5-diphenyloxazole). Combined extracts were counted in a liquid scintillation counter. Control assays lacking DAO resulted in 200–300 cpm. Commercial porcine kidney DAO (Sigma) was assayed exactly as the rat enzyme using 0.5 mg DAO/assay.

Antiproliferative activity of ODC inhibitors. Antiproliferative effects on mouse L1210 cells, human T24 bladder carcinoma cells, and X63 cells were determined as described previously [19, 28]. Cell numbers were measured by staining with methylene blue.

Spermidine (10 μM) was added to the cells together with the antiproliferative agent to test whether antiproliferative effects could be reversed by polyamines.

Selection for resistance to 3-aminooxy-2-fluoro-1-propanamine. L1210 cells were exposed to concentrations of 3-aminooxy-2-fluoro-1-propanamine (AFPA) starting with 40 μM (3 weeks), then 100 μM (5 weeks), 200 μM (9 weeks), and 500 μM (6 months). Sultan cells [15] were first exposed to 10 μM, then 20 μM for 4 weeks each, and subsequently to 40 μM for 4 months and 100 μM for 6 months. Southern and Northern blot analysis were done as described elsewhere [15].

Cellular spermidine uptake. Possible interference of putrescine analogues with spermidine uptake was measured as described elsewhere [27]. T24 cells were grown to confluence in 24-well tissue-culture plates, washed once with phosphate-buffered saline, and supplied with serum-free culture medium. Unlabelled spermidine (0.2–2 μM) was added together with 1 μCi/well of [G-^{3}H]spermidine (Amersham, 21 Ci/mmol), and cells were incubated for 20 min at 37° C or at 4° C (controls). Cells were then washed with serum-free, ice-cold medium containing 10 mM

Table 1. Inhibition of ODC and DAO by APA derivatives

Compound no.	50% inhibitory concentration [μM]		
	ODC	Rat DAO	Porcine DAO
Putrescine[a]	300	–	–
APA[b]	0.035	0.69	0.01
1 (AFPA)	0.014	5.9	2.3
2	0.039	8.3	2.2
3	0.093	94	22
4	>50	>100	>100
5	23	>100	>100
6	11	>100	>100
7	5.2	>100	>100
8	670	24	9.0
9	60	1.7	0.73
10	3.3	0.19	0.19

Values are averages of at least three independent determinations. For structures of the compounds, see Fig. 1

[a] Putrescine is the substrate for DAO

[b] APA is 3-aminooxy-1-propanamine

Table 2. Kinetic analysis of ODC inhibition by APA analogues

Compound no.	app. K_m [μM]	app. v_{max} pmol·mg^{-1}·min^{-1}	Inhibition K_i [nM]
Control	210	67	–
APA	1710	73	2.8± 1.1
1 (AFPA)[a]	1340	76	4.6± 3.3
2 (AHPA)[b]	1710	45	31. ±13

ODC (3.5 mg protein, specific activity 7.1 pmol·mg^{-1}·min^{-1}) was incubated for 30 min at 37° C with various amounts of substrate ornithine in the absence (Control) or presence of 10-fold IC_{50} of inhibitor. Data were analysed according to Eady-Hofstee by plotting v versus v/[S]. The apparent K_m and v_{max} values of one individual experiment are given. K_i values are the averages of three to five independent determinations

[a] 3-Aminooxy-2-fluoro-1-propanamine

[b] 3-Aminooxy-2-hydroxy-1-propanamine

spermidine. dissolved by incubation with 1 *N* NaOH for 30 min at 60° C, and neutralized with an equal amount of 1 *N* HCl. Samples were analysed by liquid scintillation counting; the K_m of spermidine uptake was 0.23±0.01 μM, and the v_{max} was 156 pmol·h^{-1}·10^{-6} cells^{-1}.

Determination of polyamine pools and other biochemical tests. Polyamines were quantified by reversed-phase HPLC after pre-column derivatization essentially as already described [10]. Proteins were determined according to Bradford [3], using bovine serum albumin as a standard.

Chemicals. The ODC inhibitors mentioned in this study (Fig. 1, Table 1) were synthesized in the Ciba-Geigy Chemistry Department [35]. All other chemicals were from commercial sources and of the highest purity available.

Results and discussion

Inhibition of ODC

As shown in Table 1 and Fig. 1, all compounds with the basic structure of APA [12], irrespective of the substituent at carbon 2, were highly active as ODC inhibitors, with IC_{50} values below 100 nM; compounds nos. 1 (AFPA), 2, and 3 belonged to this group. Upon derivatization of the aminooxy group, compounds lost some two orders of potency as ODC inhibitors; this group included compounds 4, 5, 6, and 7. Replacement of the aminooxy group by a hydrazine group (compound 10) or the aminomethyl group by a hydrazine group (compound 9) caused a drop in ODC inhibitory potency to IC_{50} values of 3.3 μM and 60 μM, respectively. Changing the aminomethyl group to an aminooxymethyl group (compound 8) caused an almost complete loss of ODC-inhibitory potential.

All putrescine derivatives appeared to inhibit ODC competitively with respect to its substrate ornithine (Table 2). However, a more careful analysis showed that AFPA – like the ornithine analogue α-difluoromethylornithine [18] – progressively inactivated ODC. Preincubation of the enzyme with the compound prior to the addition of ornithine significantly changed the sensitivity of the enzyme for the compound and altered the mechanism of inhibition into a complex, rather non-competitive type: the apparent K_m for ornithine was increased by a factor of 5 and the apparent v_{max} was reduced 3-fold upon preincubation of ODC for 15 min with 45 nM AFPA. The inhibition by AFPA, once bound to the enzyme, was hardly reversible (Table 3), since compound-pretreated ODC did not regain much activity upon dialysis, similar to the other APA derivatives and α-difluoromethylornithine. Unbound compounds were completely removed by dialysis as tested by the addition of fresh ODC to dialysed samples: the fresh enzyme was not inhibited, and the activities of added ODC and dialysed preincubated ODC were additive (data not shown). The reference compound α-methylornithine has been described to inhibit ODC in a reversible manner [1]. This was the only compound allowing complete reactivation of ODC upon dialysis (Table 3).

APA has been shown to react spontaneously with pyridoxal phosphate [14]. We have preincubated APA and several derivatives in the presence or absence of pyridoxal phosphate: a time-dependent change of the UV spectrum of pyridoxal phosphate was observed parallel to a reduction of the ODC-inhibitory potency of the compounds (Table 4). The UV spectrum indicated the formation of an oxime between the aminooxy group of the compounds and the carbonyl group of pyridoxal phosphate (comparison with the spectrum of reference substance). This reaction product (compound 7) was a reversible ODC inhibitor with low affinity (IC_{50} = 5.2 μM). Kinetic experiments using ODC purified to over 50% homogeneity by affinity chromatography on a pyridoxamine-Affigel column [13] showed that the K_i value of AFPA did not differ from that obtained with crude ODC (H. Mett et al., manuscript in preparation). A detailed study of the mode of interaction of AFPA and of compound 7 with ODC is required for showing whether these compounds interact primarily with the ornithine-binding site or the pyridoxal-phosphate-binding site of ODC. Under our experimental conditions, with a large excess of pyridoxal phosphate, ODC inhibition by our compounds is primarily due to interaction with the ornithine-binding site. In contrast to our compounds, α-difluoromethylornithine was covalently bound to lysine-69 or to cysteine-360 of ODC after going through a three-component complex involving ODC, its cofactor pyridoxal phosphate, and the inhibitor [30].

Table 3. Irreversible inhibition of ODC by APA derivatives

Compound no.	[μM][a]	ODC activity (% of control)		Activation by dialysis (-fold)
		before dialysis	after dialysis	
Control	–	100.(34)	100.(27)	0.8
α-Methylornithine	52,000	1.3	106	82
α-Difluoromethylornithine	60	3.6	16	4.4
APA	0.69	25	71	2.8
1 (AFPA)	0.14	10	40	4.0
2 (AHPA)	3.3	21	83	4.0

ODC was preincubated for 15 min at 37° C in the presence of the indicated compound concentrations. An aliquot was used for immediate testing of ODC activity, and the remainder dialysed twice against a 1000-fold volume each of Tris-EDTA-dithiothreitol (see ODC preparation) before testing

Figures in brackets give specific ODC activity (pmol·min·mg^{-1})

[a] Compound concentration during preincubation; the final concentration in the ODC assay was 40% of this value

Table 4. Loss of ODC-inhibitory potency of APA derivatives upon preincubation with pyridoxal phosphate

Compound no.	IC_{50} [nM]	
	Without PLP	With PLP
APA	33	>10,000
1 (AFPA)	6	3,700
2 (AHPA)	160	>10,000
7	5,200	4,000

Compounds were preincubated for 60 min at 37° C in the presence or absence of 1 mM pyridoxal phosphate (PLP); then enzyme and substrate were added, and after 15 min the reactions were terminated as usual. During enzymatic decarboxylation, all assays contained 0.4 mM PLP

Selectivity: inhibition of AdoMetDC and DAO

None of the compounds used in this study inhibited AdoMetDC by more than 15% at 50 μM, which is in line with data published recently [7] showing that APA has only a low affinity to AdoMetDC (K_i = 1.5–2 mM).

Rat small intestinal DAO was inhibited with IC_{50} values ranging from 0.6 to 9 μM, and porcine DAO was even more sensitive (Table 1). APA and compound 10 appeared to be the most potent inhibitors of DAO, thus exerting the lowest degree of selectivity amongst the ODC inhibitors tested. Preincubation of these compounds with DAO did not change the IC_{50}, indicating that the compounds were not metabolized by DAO, nor did they inactivate rat or porcine DAO in a progressive manner. Kinetic studies indicated that AFPA inhibited porcine DAO competitively with respect to its substrate putrescine (K_i = 1.6 μM).

It is interesting to note that, in addition to APA, only the two hydrazine derivatives, compounds 9 and 10, belonged to the highly potent group of DAO inhibitors with IC_{50} below 2 μM. Compounds 9 and 10, in contrast to APA, had low inhibitory activity against ODC; thus, ODC inhibition and DAO inhibition are not necessarily based on the same structural features of the inhibitor.

Antiproliferative effects

Assuming that cells would not catabolize intracellular polyamines or release them into the surrounding medium nor form new polyamines from precursors other than putrescine and would grow in a polyamine-depleted medium, total inhibition of ODC should block putrescine formation and thus cause a 50% reduction in overall polyamine pools per cell generation. Since polyamine pools need to be reduced to a large extent before cell growth stops, a compound exclusively interfering with polyamine biosynthesis may not block cellular growth in the first one or two generations after addition to the culture. Therefore, we tested the antiproliferative potency of our compounds in 120-h growth incubations, allowing some five cell divisions to take place in control cultures.

The two cell lines T24 and L1210 showed similar sensitivities to the compounds; IC_{50} values of the four most active compounds gave the same ranking, headed by AFPA. The most potent ODC inhibitors (Table 1; compounds with IC_{50} values below 100 nM) were 55- to 330-fold less active against growing cells, whereas the weaker ODC inhibitors (IC_{50} > 3 μM) showed comparable or higher antiproliferative activity. Thus, ODC-inhibitory potency apparently could not be directly translated into antiproliferative activity. The weaker ODC inhibitors probably blocked cell growth by mechanisms different from polyamine depletion, since growth inhibition by these compounds was hardly reversed by spermidine. However, incubation of the cells in the presence of spermidine could completely protect them from the antiproliferative effect of the highly potent ODC inhibitors APA, AFPA, and compounds 2 and 3 (Table 5).

Both the rate of uptake of compounds and their intracellular accumulation might also contribute to their antiproliferative potency. APA has been shown to be degraded in cells at a considerable rate [9]. We have not studied the metabolic fate of our compounds in detail; preliminary experiments indicated that homogenates from T24 cells degraded AFPA rather slowly.

Interference with cellular spermidine uptake

Eukaryotic cells have at least one specific polyamine uptake system [23]. We have studied the interference of some of our compounds with spermidine uptake by T24 cells as a model system for functional analogy of our compounds to natural polyamines. None of the compounds significant-

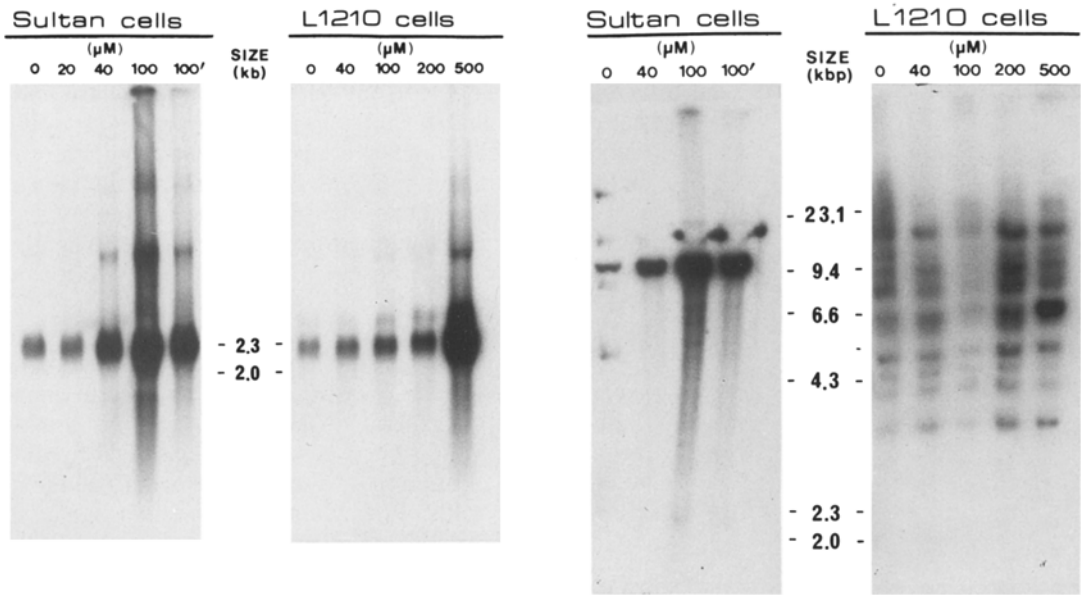


Fig. 2 A, B. ODC gene amplification and ODC mRNA overexpression by AFPA-resistant L1210 and Sultan cells. Cells underwent several rounds of selection for resistance against AFPA as described in the experimental section and were finally exposed to 100 μM AFPA (Sultan cells) or 500 μM AFPA (L1210 cells) for 6 months. **A** Left half: For Northern blot analysis, 10 μg of total RNA was fractionated by electrophoresis on formaldehyde-containing agarose gels and probed with a nick-translated ODC-probe [15]; molecular size markers are shown in the centre. **B** For Southern blot analysis (B) right half: 10 μg of *Eco*RI-digested DNA was fractionated by electrophoresis and probed with the same ODC probe; molecular size markers are shown in the middle

ly inhibited spermidine uptake at growth-inhibitory concentrations indicating that the compounds were not recognized by the cellular spermidine uptake system. Hyvönen et al. [8] have also shown that APA does not interfere with polyamine uptake in BHK cells. These compounds may enter the cells via a different uptake route. From rat cell culture experiments published by Nicolet et al. [21], one would have postulated that putrescine analogues would at least partly antagonize the uptake of spermidine.

Table 5. Antiproliferative activities of APA derivatives against T24 and L1210 cells

Compound no.	Cell growth inhibition (IC_{50}, μM ± SD)		Protection by spermidine[a]
	T24	L1210	
APA	5.1 ± 4.4	9.6 ± 0.49	+
1 (AFPA)	0.79 ± 0.5	2.0 ± 0.31	+
2	13. ± 7.4	14. ± 1.2	+
3	10. ± 4.9	89. ± 0.92	+
4	35. ± 20	25. ± 1.9	n. d.
5	0.83 ± 0.3	2.9 ± 0.57	n. d.
6	8.0 ± 4.7	22. ± 0.68	n. d.
7	1.4 ± 0.6	3.2 ± 0.65	n. d.
8	156. ± 34	<256	n. d.
9	178. ± 32	202. ± 2.3	n. d.
10	6.5 ± 5.4	20. ± 0.33	n. d.

Values give averages of at least three individual determinations ± standard deviation. For experimental conditions, see "Materials and methods". Growth inhibition was recorded after 48 h (L1210 cells) or 120 h (T24 cells).

Increase in IC_{50}: +, >10-fold; ±, 3- to 10-fold; –, ≤2-fold; n. d., not determined

[a] Spermidine (10 μM) was added together with the ODC inhibitor

Influence on intracellular polyamine pools

The well-studied specific ODC inhibitor α-difluoromethylornithine caused significant depletion of cells of putrescine and spermidine, but not of spermine [18]. The effect of AFPA and APA on polyamine pools of L1210 cells and of T24 cells was similar; putrescine and spermidine, but not spermine were depleted after 48–96 h exposure of the cells to the compound (Table 6).

ODC inhibitors of lower potency, e. g. compound 7, did not significantly affect cellular polyamine pools at concentrations inhibiting cell growth by 50% (data not shown).

Mechanism of cellular AFPA resistance

Both mouse L1210 cells and human Sultan cells could be adapted to growth in the presence of 100 μM AFPA by stepwise increase of drug concentration in the medium. After extended times of culture in the presence of high drug concentration, the cells apparently had amplified their ODC gene(s), as shown by Southern blot analysis (Fig. 2B) using the same probe as described previously [15]. Also the cellular content of ODC mRNA was clearly elevated compared with that of untreated cells (Fig. 2A). Since overexpression of ODC message (and probably also of the ODC protein) appeared to protect the cells from the growth-inhibitory action of AFPA, this drug seems to affect proliferation primarily by interference with cellular ODC. These results are pretty much the same as those described for cells resistant to α-difluoromethylornithine, which also had amplified ODC genes and overexpressed the ODC mRNA [15].

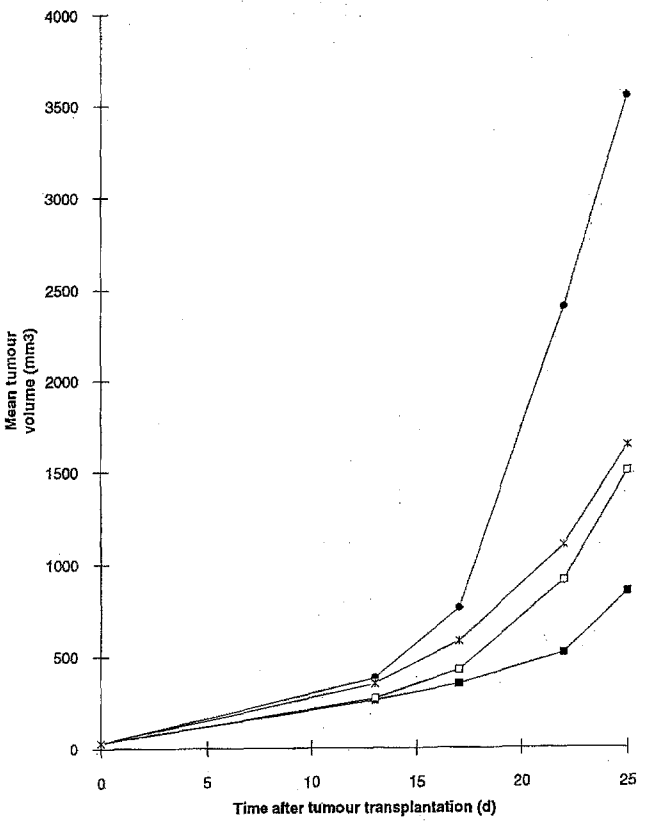


Fig. 3. Antitumour activity of AFPA in comparison with α-difluoromethylornithine (DFMO) in female CD-1 nude mice bearing human bladder carcinoma T24. Tumour cells (10^7/mouse) were injected at day 0, and starting at day 13 AFPA was given orally once daily for 10 days at a dose of 25 mg/kg (1/20 of the MTD; □) or 50 mg/kg (1/10 of the MTD; ■). Control animals received saline treatment instead (●), and the reference compound DFMO was given at a dose of 1000 mg/kg (1/5 of the MTD; x). Mean tumor volumes were measured at days 13 (onset of treatment), 17, 22 (last treatment) and 25 as described [19]

Antitumour activity

AFPA was tested for antitumour activity in female CD-1 nude mice bearing transplantable human bladder carcinoma T-24. As shown in Fig. 3, AFPA was active in this tumour model following oral administration when given once daily at 25 or 50 mg/kg (1/20 or 1/10 of the maximally tolerated dose) for 10 days. The compound had a tumoristatic action, blocking tumour growth by some 90% by the end of treatment; tumour regrowth was observed after the end of antitumour treatment. The antitumour activity of AFPA was much higher than that of the reference ODC inhibitor α–difluoromethylornithine, which had to be given at a 20-fold higher dosage for any antitumour effect to be seen in this model.

It thus appears that potent ODC inhibitors such as the APA derivatives used in this study are potential antitumour agents. Further studies investigating the mechanism of antitumour action in experimental animals are in progress. If the therapeutic window between toxic effects of these compounds and their antitumour activity turns out to be broad enough, compounds of this class should be investigated in clinical trials.

Acknowledgements. Technical assistance from Sandra Crivelli, Marcel Müller, Iris Oberkirch, Robert Reuter and Barbara Schacher is gratefully acknowledged. We thank R. Cozens for careful revision of the manuscript.

References

1. Abdel-Monem MM, Newton NE, Weeks C (1974) Inhibitors of polyamine biosynthesis. 1. Alpha-methyl-(+–)-ornithine, an inhibitor of ornithine decarboxylase. J Med Chem 17: 447
2. Bowlin TL, Hoeper BJ, Rosenberger AL, Davis GF, Sunkara PS (1990) Effects of three irreversible inhibitors of ornithine decarboxylase on macrophage-mediated tumoricidal activity and antitumor activity in B16F1 tumor-bearing mice. Cancer Res 50: 4510
3. Bradford MM (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248
4. Forastiere AA, Natale RB, Wheeler RR (1986) Phase II trial of methylglyoxal bis(guanylhydrazone) (MGBG) in advanced head and neck cancer. Cancer 58: 2585
5. Hayashi S, Kameji T (1983) Ornithine decarboxylase (rat liver). In. Tabor H, White-Tabor C (eds) Methods in enzymology, vol 94: Polyamines. Academic Press, New York, p 154
6. Horn Y, Schechter PJ, Marton LJ (1987) Phase I–II clinical trial with alpha-difluoromethylornithine, an inhibitor of polyamine biosynthesis. Eur J Cancer Clin Oncol 23: 1103

Table 6. Depletion of intracellular polyamine pools by cell growth in the presence of ODC inhibitors

Cell line	Compound	Treatment Conc. (μM)	Time (h)	Polyamine (% of untreated control)		
				Putrescine	Spermidine	Spermine
L1210	2	5	48	61	64	63
	AFPA	100	48	0	0	115
			96	0	0	65
T24	APA	10	48	0	14	86
			96	0	31	171
	AFPA	23	48	0	21	79
			96	0	6	58

Values are given as averages of two independent experiments. Control levels of polyamines in untreated cells were similar in both cell types; putrescine was 1.1 nmol/10^6 cells, spermidine 3.2 nmol/10^6 cells, and spermine 1.2 nmol/10^6 cells

7. Hyvönen T, Eloranta TO (1990) Regulation of *S*-adenosyl-L-methionine decarboxylase by 1-aminooxy-3-aminopropane: enzyme kinetics and effects on the enzyme activity in cultured cells. J Biochem 107: 339
8. Hyvönen T, Alakuijala L, Andersson L, Khomutov AR, Khomutov RM, Eloranta TO (1988) 1-Aminooxy-3-aminopropane reversibly prevents the proliferation of cultured baby hamster kidney cells by interfering with polyamine synthesis. J Biol Chem 263: 11 138
9. Hyvönen T, Khomutov AR, Khomutov RM, Lapinjoki S, Eloranta TO (1990) Uptake of ^{3}H-labeled 1-aminooxy-3-aminopropane by baby hamster kidney cells. J Biochem 107: 817
10. Kabra PM, Lee HK, Lubich WP, Marton LJ (1986) Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. J Chromatogr 380: 19
11. Kendrick DA, Danzin C, Kolb M (1989) 2,2-Difluoro-5-hexyne-1,4-diamine: a potent enzyme-activated inhibitor of ornithine decarboxylase. J Med Chem 32: 170
12. Khomutov RM, Hyvönen T, Karvonen E, Kauppinen L, Paalanen T, Paulin L, Eloranta TO, Pajula R-L, Andersson LC, Pösö H (1985) 1-Aminooxy-3-aminopropane, a new and potent inhibitor of polyamine biosynthesis that inhibits ornithine decarboxylase, adenosylmethionine decarboxylase and spermidine synthase. Biochem Biophys Res Commun 130: 596
13. Kopitz J, Adam G, Bohley P (1990) Very fast purification of ornithine decarboxylase with high yield from mouse kidney and generation of a monoclonal antibody. Biol Chem Hoppe Seyler 371: 363
14. Lapinjoki S, Eloranta TO, Pösö H (1987) On the mechanism of ornithine decarboxylase inhibition by 3-aminooxypropylamine. In: Korpela T, Christen P (eds) Biochemistry of vitamin B6. Birkhäuser, Basel, p 321
15. Leinonen P, Alhonen-Hongisto L, Laine R, Jänne OA, Jänne J (1987) Human myeloma cells acquire resistance to difluoromethylornithine by amplification of ornithine decarboxylase gene. Biochem J 242: 199
16. Lipton A, Harvey HA, Glenn J, Weidner WA, Strauss M, Miller SE, Taylor JB, White-Hershey D, Barlow JLR (1989) A phase I study of hepatic arterial infusion using difluoromethylornithine. Cancer 63: 433
17. Maddox AM, Freireich EJ, Keating MJ, Haddox MK (1988) Alterations in bone marrow and blood mononuclear cell polyamine and methylglyoxal bis(guanylhydrazone) levels: phase I evaluation of alpha-difluoromethylornithine and methylglyoxal bis(guanylhydrazone) treatment of human hematological malignancies. Cancer Res 48: 1367
18. Mamont PS, Duchesne M-C, Grove J, Bey P (1978) Antiproliferative properties of DL-alpha-difluoromethylornithine in cultured cells. A consequence of the irreversible inhibition of ornithine decarboxylase. Biochem Biophys Res Commun 81: 58
19. Meyer T, Regenass U, Fabbro D, Alteri E, Rösel J, Müller M, Caravatti G, Matter A (1989) A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and in vitro antiproliferative as well as in vivo anti-tumor activity. Int J Cancer 43: 851
20. Nagarajan S, Ganem B, Pegg AE (1988) Studies of non-metabolizable polyamines that support growth of SV-3T3 cells depleted of natural polyamines by exposure to alpha-difluoromethylornithine. Biochem J 254: 373
21. Nicolet TG, Scemama J-L, Pradayrol L, Seva C, Vaysse N (1990) Characterization of putrescine and spermidine transport systems of a rat pancreatic acinar tumor cell line (AR4-2J). Biochem J 269: 629
22. Pegg AE (1986) Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 234: 249
23. Pegg AE (1988) Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. Cancer Res 48: 759
24. Pegg AE, Jones DB (1988) Effect of inhibitors of *S*-adenosylmethionine decarboxylase on polyamine content and growth of L1210 cells. Biochemistry 27: 1408
25. Pegg AE, Pösö H (1983) *S*-Adenosylmethionine decarboxylase (rat liver). In: Tabor H, White-Tabor C (eds) Methods in enzymology, vol 94: Polyamines. Academic Press, New York, p 234
26. Pegg AE, Madhubala R, Kameji T, Bergeron RJ (1988) Control of ornithine decarboxylase activity in alpha-difluoromethylornithine-resistant L1210 cells by polyamines and synthetic analogs. J Biol Chem 263: 11 008
27. Pleshkewych A, Kramer DL, Kelly E, Porter CW (1980) Independence of drug action on mitochondria and polyamines in L1210 leukemia cells treated with methylglyoxal bis(guanylhydrazone). Cancer Res 40: 4533
28. Porter CW, McManis J, Casero RA, Bergeron RJ (1987) Relative abilities of bis(ethyl) derivatives of putrescine, spermidine, and spermine to regulate polyamine biosynthesis and inhibit L1210 leukemia cell growth. Cancer Res 47: 2821
29. Poulin L, Secrist JA III, Pegg AE (1989) Effect of 1-amino-oxy-3-aminopropane on polyamine metabolism and growth of L1210 cells. Biochem J 263: 215
30. Poulin R, Lu L, Ackermann B, Bey P, Pegg AE (1992) Mechanism of irreversible inactivation of mouse ornithine decarboxylase by alpha-difluoromethylornithine. Characterization of sequences at the inhibitor and coenzyme binding sites. J Biol Chem 267: 150
31. Seely JE, Pegg AE (1983) Ornithine decarboxylase (mouse kidney). In: Tabor H, White-Tabor C (eds) Methods in enzymology, vol 94: Polyamines. Academic Press, New York, p 158
32. Seiler N (1990) Polyamine metabolism. Digestion 46 [Suppl 2]: 319
33. Seppänen P, Alhonen-Hongisto L, Jänne J (1980) Relation of the antiproliferative action of methylglyoxal bis(guanylhydrazone) to the natural polyamines. Eur J Biochem 110: 7
34. Seppänen P, Alhonen-Hongisto L, Käpyaho K, Jänne J (1983) Two enzyme inhibition assays for methylglyoxal bis(guanylhydrazone). In: Tabor H, White-Tabor C (eds) Methods in enzymology, vol 94: Polyamines. Academic Press, New York, p 247
35. Stanek J, Frei J, Mett H, Schneider P, Regenass U (1992) 2-Substituted 3-(aminooxy)propanamines as inhibitors of ornithine decarboxylase: synthesis and biological activity. J Med Chem 35: 1339