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Hydroxylamine-containing inhibitors of polyamine biosynthesis and impairment of colon cancer cell growth

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Abstract

Polyamine synthesis (by the action of ornithine decarboxylase [ODC] and *S*-adenosylmethionine decarboxylase [SAMDC]) and polyamine content are high in colon cancer. In addition, colonic lumen is rich in polyamines synthesised by colonic microflora; for this reason, polyamine depletion in colon cancer may be a logical approach to impair growth of colon cancer cells. We evaluated highly specific and reportedly non-toxic hydroxylamine-containing inhibitors of ODC (1-aminooxy-3-aminopropane, APA) and SAMDC (*S*-(5′-deoxy-5′-adenosyl)-methylthioethyl-hydroxylamine, AMA) in human colon cancer cells (Caco-2 and HT-29) in culture. APA depleted ODC activity within 24 hr, more rapidly than did difluoromethylornithine. APA and AMA in combination (100 μM each) reduced ODC and SAMDC activities to undetectable levels within 24 hr and intracellular polyamines to 8–23% of control. The resulting growth arrest could be reversed only by twice as much spermidine as is physiologically present in the colonic lumen. In concentrations sufficient to deplete growth, APA and AMA were not toxic. Simultaneous treatment with APA, AMA, and 5-fluorouracil reduced colon cancer cell survival more potently than treatment with 5-fluorouracil alone. The hydroxylamine-containing ODC and SAMDC inhibitors APA and AMA are potent inhibitors of colon cancer cell proliferation and might be therapeutically promising in colon cancer.

Keywords: Cell culture; Colon cancer; Polyamines

1. Introduction

The polyamines putrescine, spermidine, and spermine are aliphatic polycations necessary for cell growth. Polyamine content is high in rapidly proliferating normal and neoplastic cells. The cells stop dividing when deprived of polyamines, resuming growth after restoration of their polyamine levels. Intracellular polyamine content is regulated by the action of two rate-limiting enzymes: putrescine is synthesised from ornithine via ODC, whereas SAMDC is responsible for the synthesis of decarboxylated SAM, precursor of the 3-carbon fragment of spermidine and spermine backbones. In addition, all mammalian cells are equipped with an active polyamine uptake system; exogenous polyamines can completely substitute for those synthesised by the cell in the processes related to normal, adaptive, or neoplastic cell growth [1–4].

In colorectal cancer, the activities of ODC and SAMDC and the polyamine content are increased 3- to 4-fold over those found in the equivalent normal tissue [5–8]. Furthermore, and in contrast to all other cell systems in the body, colon cancer cells are exposed to high concentrations of putrescine from the lumen, synthesised by colonic microflora: faecal putrescine concentration is well within millimolar range, while spermidine and spermine concentrations are as low as 50–60 μmol/L [9]. Therefore, in addition to

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Abbreviations: AMA, *S*-(5′-deoxy-5′-adenosyl)-methylthioethyl-hydroxylamine; APA, 1-aminooxy-3-aminopropane; DFMO, alpha-difluoromethylornithine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; 5-FU, 5-fluorouracil; LDH, lactate dehydrogenase; MGBG, methyl-bisguanylhydrazone; SAM, *S*-adenosylmethionine; SAMDC, *S*-adenosylmethionine decarboxylase; and ODC, ornithine decarboxylase.

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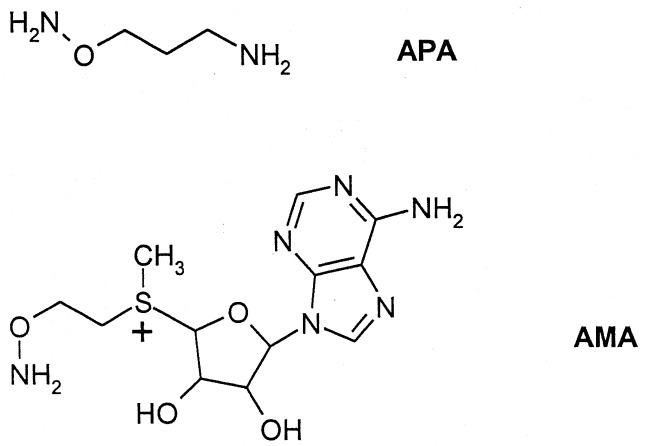

Fig. 1. Aminooxy polyamine analogues. APA, 1-aminooxy-3-aminopropane; AMA, *S*-(5′-deoxy-5′-adenosyl)-methylthioethyl-hydroxylamine.

up-regulated ODC and SAMDC activity, colon cancer cells are "bathed" in large amounts of putrescine, which is, in turn, readily taken up by neoplastic colonocytes [10], then converted to metabolically active spermidine and spermine and utilised for cell growth, either directly or as an instant energy source [11].

Because of the essential role of polyamines in maintaining high cell proliferation rates, polyamine depletion has long been a desired goal in antitumour therapy. Initial attempts to inhibit polyamine biosynthesis by blocking ODC activity with a suicide inhibitor, DFMO, failed to demonstrate convincing antitumour effects in man, because increased uptake of exogenous polyamines overcame the inhibitory effects of the ODC blockade [12]. On the other hand, there is still a lack of a chemically stable, non-toxic, and specific SAMDC inhibitor. The traditionally used MGBG is of limited clinical applicability due to its general toxicity and side-effects (low specificity, antimitochondrial activity, general toxicity, interference with the polyamine transport system [13]). Recently designed aryl and heteroaryl analogues of MGBG such as CGP-48664 are less toxic, but still have side-effects similar to those of MGBG or exert their antiproliferative actions independently of the SAMDC blockade and polyamine depletion [14–16].

The aim of this study was to evaluate the effect of hydroxylamine-containing inhibitors of ODC and SAMDC (Fig. 1) on the growth of human colon cancer cells in culture. These compounds had not been tested in colon cancer cells previously. However, their evaluation in other cell types provided evidence of their high specificity, chemical stability, and absence of any detectable toxicity. APA is an irreversible inhibitor of ODC; it efficiently inhibits proliferation of both normal and neoplastic cells, reducing intracellular polyamines concomitantly with an arrest of cell growth [17]. AMA is an active, site-directed irreversible inhibitor of SAMDC, binding covalently to the pyruvate residue at the enzyme active site. With an IC_{50} of 0.1 mmol/L, AMA decreases spermidine and spermine concentration in leukaemic cells by more than 80%, resulting in growth arrest that could not be reversed by exogenous putrescine [18]. In the present study, APA and AMA were evaluated under experimental conditions closely resembling those in human colon; therefore, together with the inhibitors, the cells were exposed to natural polyamines in concentrations similar to those reportedly found in the colonic lumen.

2. Materials and Methods

2.1. Chemicals

APA and AMA were synthesised as described previously [19,20]. Other chemicals were purchased from Sigma Chemie. DL-[1-^{14}C]Ornithine (specific activity 43.8 mCi/mmol) was from Du Pont NEN, and [^{3}H]thymidine (specific activity 17.5 Ci/mmol) and *S*-adenosyl(carboxyl-^{14}C)-L-methionine (specific activity 59 mCi/mmol) were from Amersham Buchler. Cell culture media and supplements were purchased from GIBCO BRL.

2.2. Cell culture

Human colon cancer cell lines (Caco-2 and HT-29) were obtained from the European Animal Cell Culture Collection and used for experiments within five passages in culture. The cells were maintained in DMEM containing 4.5 g/L of glucose and 25 mM HEPES, supplemented with 10% foetal bovine serum, 100 U/mL of benzylpenicillin and 10 μg/mL of streptomycin, 1% non-essential amino acids, and 1% pyruvate. The medium was changed every second day. The cells were checked for mycoplasma at monthly intervals.

For experiments, the cells were expanded in tissue culture flasks (75-cm^2 growth area), detached by treatment with 0.5 g/L of trypsin and 0.2 g/L of EDTA in PBS, and, as dictated by the design of each particular experiment, reseeded to 6-, 24-, or 96-well tissue culture plates (Nunc) at a density indicated in the tables and figures. Cell morphology was routinely examined by phase contrast microscopy.

2.3. ODC and SAMDC activity

ODC and SAMDC activity were assessed by measuring the amount of $^{14}CO_2$ liberated from L-[1-^{14}C]ornithine and *S*-adenosyl(carboxyl-^{14}C)-L-methionine, respectively, as described previously [17,18]. The final volume of incubation medium for ODC consisted of 50 mM Tris–HCl pH 7.2, 0.4 mmol/L of ^{14}C-labelled L-ornithine, 0.05 mmol/L of pyridoxal-5-phosphate, 2.5 mmol/L of DTT, and 0.1 mmol/L of EDTA. For the SAMDC assay, instead of L-[1-^{14}C]ornithine, *S*-adenosyl (carboxyl-^{14}C)-L-methionine was used at a concentration of 0.2 mmol/L and 1.5 mmol/L of putrescine. For both enzymes, the incubation period at 37°

was 60 min. Protein was measured by the method of Bradford [21], using the Bio-Rad commercial kit.

2.4. Intracellular polyamine concentration

The cells were harvested by scraping and permeabilised with 200 mmol/L of perchloric acid. Polyamines were analysed in the medium and in the cells by HPLC, as described previously [22]. The polyamine content of foetal bovine serum, used for maintaining the cells in culture, was found to be below the limit of detection when analysed by HPLC.

2.5. Cell proliferation

To drive the cells completely through the cycle, proliferation was evaluated by cell counting. Colourimetric assay after crystal violet staining was used [23]: for this assay, the cells were seeded on to 96-well plates (0.33 cm^2) in a volume of 100 μL medium at 10^4 cells/cm^2, followed by addition of substances in various concentrations to make a final volume of 200 μL. After incubation for a desired time period, the number of viable cells was evaluated by staining with 0.05% crystal violet in 2% ethanol. After washing with PBS, the dye was extracted in 100 μL citrate buffer, and the adsorbance at 560 nm was measured using a microplate ELISA reader. The number of cells per plate was measured in comparison to a standard growth curve determined separately for each cell line and each experiment. In a limited number of experiments, the cells were harvested by trypsinisation, stained with trypan blue, and counted in a haemocytometer. The correlation coefficient of the results from both methods was 95%.

2.6. Cytotoxicity

As a measurement of non-specific toxicity, membrane damage was estimated by measuring LDH in cell culture medium. LDH is an exclusively cytosolic enzyme that is absent in cell culture medium unless the cell membrane is damaged. In colon cancer cells, membrane damage occurs relatively late in apoptosis, but early after non-specific membrane damage, i.e. necrosis. To measure LDH release, the cells were incubated with the analogues for not longer than 24 hr. LDH in cell culture medium was measured using the Cytotoxicity Detection Kit (Boehringer) according to the manufacturer's instructions.

2.7. Statistics

Each data point presented in this paper is the mean ± SEM of 4–8 experiments. Unpaired one-way ANOVA was used to compare means, and $P < 0.05$ was taken as the level of significance required to reject the null hypothesis.

3. Results

3.1. Effect of enzyme inhibitors on ODC and SAMDC activities and polyamine content in human colon cancer cells

In homogenates of Caco-2 cells, increasing concentrations of the analogues inhibited the activity of their corresponding enzymes in a dose-dependent manner, with IC_{50} values of approximately 0.1 mmol/L. At this concentration, the enzymes were inhibited to undetectable levels, which was in agreement with the described effects of these substances on mammalian ODC and SAMDC [17,18,24]. Simultaneous treatment of both Caco-2 and HT-29 cells with the same concentration of APA and AMA (0.1 mmol/L) resulted in a complete blockade of both ODC and SAMDC within 24 hr.

The effects of a "traditional" ODC inhibitor, DFMO (5 mmol/L), and of APA (0.1 mmol/L) on ODC activity in Caco-2 cells were studied for up to 7 days in culture. APA inhibited ODC activity more rapidly than DFMO, the effect of the latter being comparable to APA after 3–4 days (Fig. 2, left panel). In the case of a "classical" SAMDC blocker, MGBG (1 μmol/L) compared to AMA (100 μmol/L), inhibition of SAMDC activity in Caco-2 cells was rapid in both (Fig. 2, right panel).

Polyamine content in Caco-2 and HT-29 cells decreased concomitantly with the fall in the activities of polyamine-synthesising enzymes. Likely due to their higher proliferative capacity, HT-29 cells expressed higher basal polyamine content than Caco-2 cells. Treatment with APA alone resulted in a decrease in putrescine content to non-detectable levels in both cell lines; spermidine was depleted moderately but significantly, while spermine content remained almost unchanged. AMA alone induced a compensatory increase in putrescine content, while spermidine and spermine levels were impaired to 30–50% of control. In the cells treated simultaneously with APA and AMA, all three polyamines decreased to minimal values after 72 hr of treatment (Table 1), and these values remained unchanged for up to five days in culture.

3.2. Effect of APA and AMA on colon cancer cell growth

The reduction in polyamine content had a considerable impact on the colon cancer cell growth rate. In Caco-2 and HT-29 cells treated with APA and AMA, cell counts remained relatively constant over time and corresponded to initial seeding density over 72 hr in culture (Fig. 3). As assessed by trypan blue exclusion, there were no changes in cell viability at either time point studied, since the number of cells taking up this vital dye did not exceed 5% in any

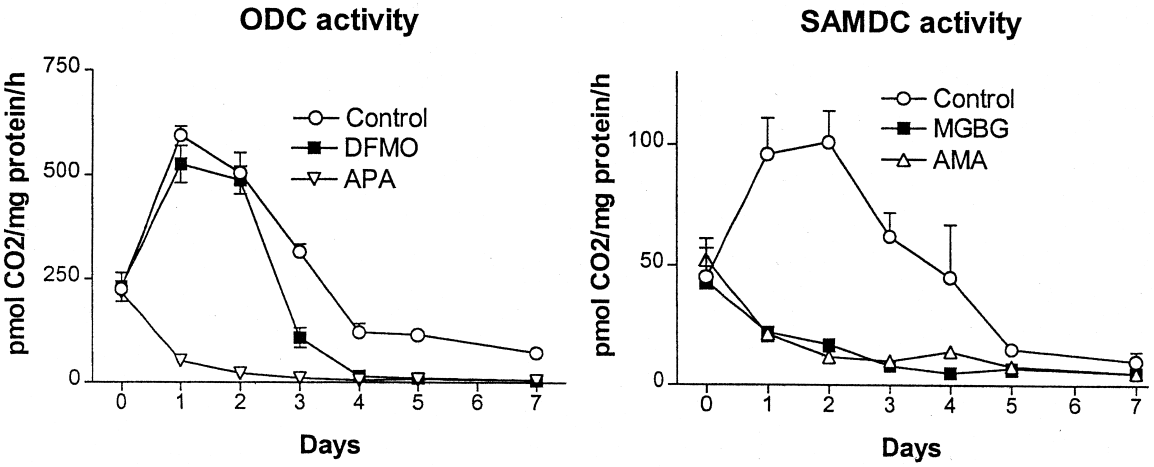

Fig. 2. Effect of APA, AMA, DFMO, and MGBG on ODC and SAMDC activities in Caco-2 cells over time. The cells were seeded at a density of 10,000 cells/cm^2 and allowed to attach for 24 hr. Treatment (0.1 mmol/L of APA; 5 mmol/L of DFMO; 0.1 mmol/L of AMA; 1 μmol/L of MGBG) was carried out over time. Results (means ± SEM, N = 4) are expressed in enzyme units (pmol of released CO_2) per milligram cellular protein per hour.

sample. Cells rounding up and floating in the medium (which indicates cell death) were not observed after APA and AMA treatment over 72 hr.

Growth inhibition after polyamine deprivation by APA and AMA could not be reversed by exogenous putrescine, but only if spermidine was added to cell culture medium. As shown in Table 2, in the cells treated with APA only, even low concentrations of both spermidine and putrescine were sufficient to restore proliferation. However, in the cells treated with APA together with AMA, only spermidine in concentrations exceeding those reportedly present in the colonic lumen was able to resume growth.

3.3. Cytotoxicity

As assessed by LDH activity in cell culture medium over 24 hr of treatment, APA and AMA were not toxic in either colon cancer cell line within the concentration range sufficient to impair enzyme activities and inhibit growth. Significant increases in LDH release were not observed with either agent until millimolar concentrations were used (Fig. 4).

3.4. Polyamine deprivation with APA and AMA and treatment with 5-fluorouracil

The effect of polyamine depletion by APA and AMA on the sensitivity of HT-29 cells to 5-FU was tested after the cells were treated with APA and AMA for 12 hr. Polyamine depletion with APA and AMA potentiated antineoplastic effects of 5-FU in a dose-dependent manner. The percentage of surviving cells after 5-FU in polyamine-depleted cells was lower than in the cells in which the polyamine pool was not depleted, i.e. those not treated with APA and AMA (Fig. 5).

4. Discussion

Our data provide evidence that the hydroxylamine-containing ODC and SAMDC blockers APA and AMA are potent growth inhibitors in colon cancer cells, and that they may be a successful alternative to the traditional inhibitors of polyamine-synthesising enzymes used to impair cancer cell growth up to now. First, APA exerts a more rapid ODC

Table 1
Effect of APA and AMA on polyamine content in human colon cancer cells

Cell line	Control	APA	AMA	APA + AMA (% control)		
Caco-2	3.6 ± 0.2	ND	10.5 ± 1.1	0.9 ± 0.1	(25.0)	Putrescine
	12.3 ± 0.7	6.1 ± 0.1	5.8 ± 0.4	3.3 ± 0.1	(26.8)	Spermidine
	6.5 ± 0.4	6.0 ± 0.2	3.3 ± 0.2	2.1 ± 0.2	(32.0)	Spermine
HT-29	2.6 ± 0.2	ND	8.8 ± 0.7	0.3 ± 0.1	(11.5)	Putrescine
	21.5 ± 0.3	11.3 ± 0.6	11.4 ± 0.8	1.9 ± 0.3	(8.8)	Spermidine
	11.4 ± 0.1	10.2 ± 0.3	4.4 ± 0.2	3.1 ± 0.1	(27.2)	Spermine

The cells were seeded at a density of 10,000 cells/cm^2 and allowed to attach for 24 hr. Treatment with 0.1 mmol/L of APA, 0.1 mmol/L of AMA, or with both analogues in combination was carried out over the following 72 hr; medium with analogues was changed daily. Results (means ± SEM, N = 4) are expressed in nanomoles per milligram cellular protein, or as % of control. ND, not detectable.

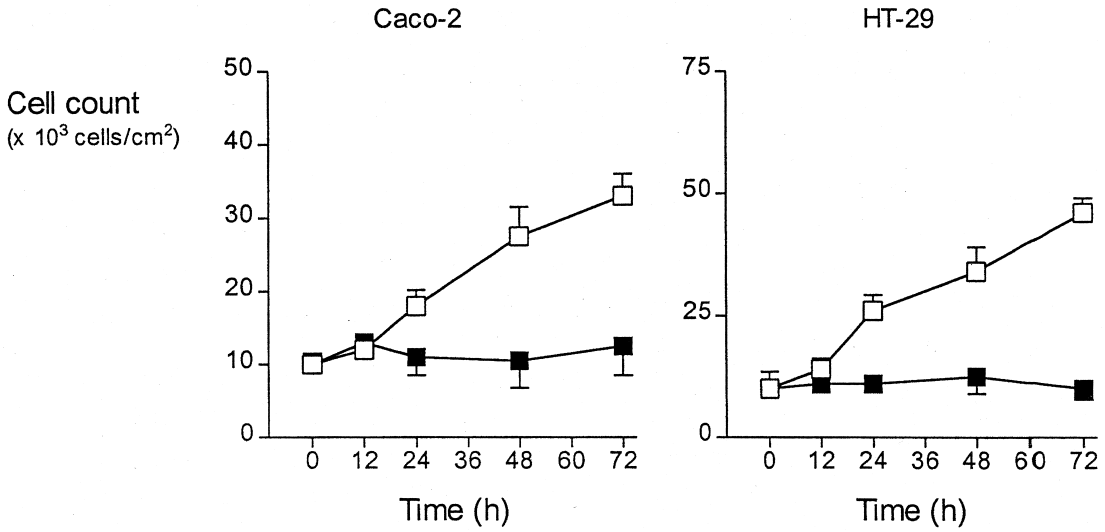

Fig. 3. Effect of simultaneous ODC and SAMDC blockade with APA and AMA on human colon cancer cell growth. The cells were seeded at a density of 10,000 cells/cm^2 and allowed to attach for 24 hr. Treatment (0.1 mmol/L of APA plus 0.1 mmol/L of AMA) was carried out up to 72 hr. Open squares: non-treated cells; full squares: cells treated with APA and AMA. Cell counts were measured using crystal violet staining. Means ± SEM, N = 8.

inhibition than DFMO. Second, in concentrations sufficient to deplete enzyme activities and arrest growth, APA and AMA are apparently non-toxic. Third, growth arrest due to polyamine deprivation by APA and AMA could be reversed with exogenous spermidine at higher concentrations than those reportedly present in the colonic lumen. And fourth, polyamine deprivation with APA and AMA enhances the cytostatic effect of a drug traditionally used in the treatment of colorectal cancer, 5-FU.

Because of their roles in normal and neoplastic cell growth, polyamines have long been recognised as a logical, though not specific target, in cancer treatment [25,26]. In gastrointestinal cancer, the rationale to deplete intracellular polyamine pools and inhibit cell growth is particularly well justified: neoplastic cells of the gastrointestinal tract are exposed to high amounts of luminal putrescine. Putrescine is actively taken up by epithelial cells. The uptake is saturable, temperature-dependent, and inhibited by structurally and chemically related substances [27,28]. It is dependent on amino acid supply [29,30] and up-regulated by growth stimuli such as EGF [31] or deoxycholic acid [32]. Both synthesised and absorbed putrescine is utilised completely,

Table 2
Effect of polyamine supplementation on analogue-induced growth arrest in HT-29 cells

Treatment	Population doublings (72 hr)	*P*
None	2.34 ± 0.27	
APA	0.19 ± 0.01	0.0006
APA + 100 μM putrescine	2.18 ± 0.11	0.7225
APA + 1 mM putrescine	1.77 ± 0.49	0.2223
APA + 10 μM spermidine	2.13 ± 0.27	0.6419
APA + 100 μM spermidine	2.20 ± 0.71	0.7558
AMA	0.25 ± 0.10	0.0008
AMA + 100 μM putrescine	0.18 ± 0.01	0.0006
AMA + 1 mM putrescine	0.14 ± 0.01	0.0005
AMA + 10 μM spermidine	1.14 ± 0.27	0.0208
AMA + 100 μM spermidine	1.90 ± 0.51	0.3388
APA + AMA	0.05 ± 0.01	0.0004
APA + AMA + 100 μM putrescine	0.09 ± 0.01	0.0004
APA + AMA + 1 mM putrescine	0.06 ± 0.02	0.0004
APA + AMA + 10 μM spermidine	1.08 ± 0.22	0.0165
APA + AMA + 100 μM spermidine	1.63 ± 0.49	0.1361

The cells were seeded at a density of 10,000 cells/cm^2 and allowed to attach for 24 hr. Treatment with 0.1 mmol/L of APA alone, 0.1 mmol/L of AMA alone, or the same concentrations of APA and AMA in combination was carried out over the following 72 hr; medium was changed daily. Putrescine and spermidine were added simultaneously with APA and/or AMA. One-way ANOVA was calculated in comparison to non-treated cells. Means ± SEM, N = 6.

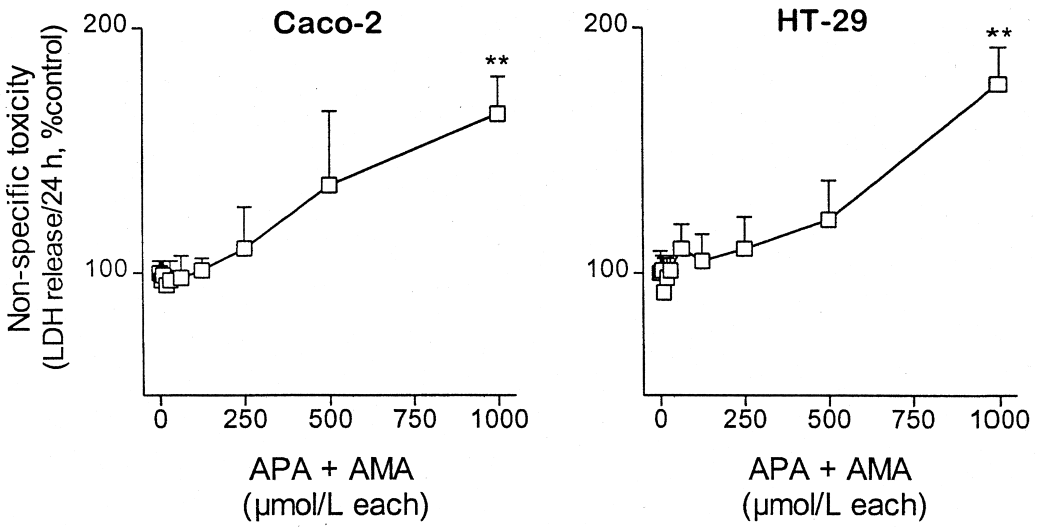

Fig. 4. Non-specific cytotoxicity (LDH release in cell culture medium over 24 hr) of APA and AMA in combination in Caco-2 and HT-29 cells. Mean ± SEM, N = 8; **, $P < 0.01$.

since it is, via SAMDC, rapidly converted to metabolically active spermidine and spermine [33].

ODC blockade after 24-hr treatment with APA, in contrast to a delayed action of DFMO, may be unexpected, since both APA and DFMO act in the same way and inhibit ODC after binding to the active site of the enzyme [12,17]. However, APA and DFMO may be transported into the cell via different pathways. APA is taken up into mammalian cells by passive diffusion, and it utilises neither the polyamine transport system (i.e. it does not compete with putrescine for uptake in colon cancer cells) nor amino acid transporters [34]. The sustained effect of DFMO in depleting ODC activity has already been observed in cell lines of intestinal origin, such as the IEC-6 cell line, which have a well-developed amino acid transport system [35]. Being a structural analogue of ornithine, DFMO is presumably taken up via the dibasic amino acid transport system also utilised by ornithine. Its transport is likely to be slowed down by the presence of the -CHF_2 group on the C_2 position of the ornithine molecule. As is colonic luminal content, cell culture media are rich in amino acids that can additionally compete with DFMO for uptake into colon cancer cells. Solely due to its rapid mode of action, APA may be a more suitable inhibitor of ODC activity than DFMO, at least in neoplastic cells of intestinal and colonic origin.

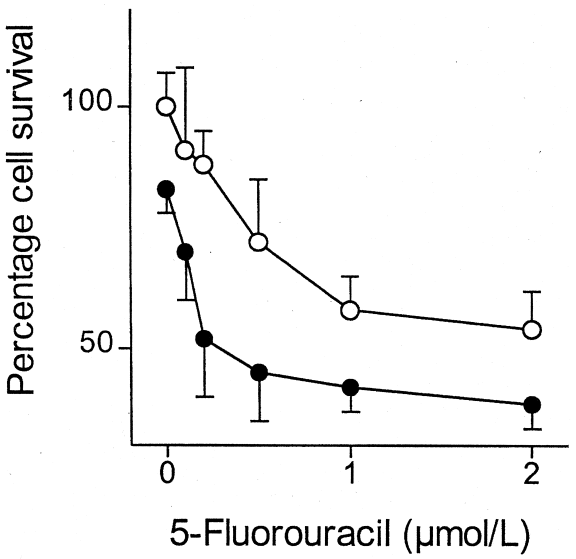

Fig. 5. Effect of 5-fluorouracil (5-FU) on HT-29 cell survival after polyamine depletion by APA and AMA. HT-29 cells were seeded at a density of 10,000 cells/cm^2, allowed to attach for 6 hr, and then exposed to increasing concentrations of 5-FU alone (open circles) or together with APA and AMA (0.1 mmol/L each; full circles) for the following 12 hr. Percentage cell survival was calculated after cell counting using crystal violet staining. Mean ± SEM, N = 8.

The essential role of ODC blockade in preventing cell growth has been well documented [36]. However, the inhibition of ODC activity in itself is unlikely to be therapeutically beneficial in patients with cancer for two reasons: first, ODC blockade depletes only putrescine and spermidine content, while intracellular spermine remains unchanged [25]; and second, impaired putrescine synthesis can be easily compensated for by increased uptake from the extracellular space. Simultaneous inhibition of ODC and SAMDC impairs growth more efficiently, since it depletes intracellular spermine and subsequently blocks DNA synthesis, arresting the cells in G_1 phase of the cell cycle [37]. These findings were additionally confirmed in our study. Inhibition of ODC by APA was easily overcome by adding physiological amounts of both putrescine and spermidine (Table 2). In turn, combined application of APA and AMA, depleting putrescine to 11.5%, spermidine to 8.8%, and spermine to 27.2% of control, was not compensated for by addition of exogenous putrescine and spermidine at physiological concentrations. Likely due to marked disturbances

of the cell cycle as a consequence of deep polyamine depletion, physiological concentrations of spermidine could reverse the antiproliferative effect of APA and AMA by only 50%.

In concentrations sufficient to inhibit ODC and SAMDC activity completely and arrest growth in colon cancer cells, APA and AMA were non-toxic. This was determined by both trypan blue incorporation (measurement of cell viability) and LDH release into cell culture medium over the initial 24 hr of treatment. The non-specific effects and toxicity of the substances used to inhibit polyamine-synthesising enzymes have been well documented [13,16], largely preventing their clinical use in the treatment of cancer. Of the few reports concerning AMA published to date, none have provided any evidence as to its toxicity [18,24]. As for APA, it has been shown that, like DFMO, it causes swelling of endoplasmic reticulum and Golgi cisternae, disappearance of actin stress fibres, and involution of rough endoplasmic reticulum in BHK cells [38]. In this study, however, APA was used at a concentration 10-fold higher than that necessary to inhibit ODC and deplete intracellular putrescine; at this concentration (1 mM), it was also toxic in Caco-2 and HT-29 cells in our study. Therefore, the effects of both DFMO and APA on BHK cell ultrastructural morphology, as described in the study of Parkkinen *et al*. [38], is likely to resemble toxic effects of both compounds than be a consequence of polyamine deprivation.

In the treatment of colorectal cancer, the poor efficiency of conventional chemotherapeutic drugs such as 5-FU is well known. Alterations in drug influx, drug efflux, intracellular metabolic activation, and intracellular catabolism, or alterations in the drug's target have generally been attributed as reasons for therapeutical failure [39]. To overcome this, combined therapy has been suggested; ideally, antiproliferative drugs, used in combination, should have different but complementary metabolic targets, and achieve synergistic, or at least additive effects. In an attempt to explore whether polyamine depletion with APA and AMA may enhance the therapeutic efficiency of conventionally used cytotoxic drugs, we used 5-FU (inhibitor of thymidilate synthase) in combination with APA and AMA. Polyamine depletion by APA and AMA enhanced the effect of 5-FU and reduced cell survival.

In summary, our data provide evidence that, in colon cancer cells, polyamine deprivation by potent and non-toxic hydroxylamine-containing ODC and SAMDC inhibitors (APA and AMA) induces growth arrest and enhances the cytostatic effect of traditionally used cytostatic drugs such as 5-FU. Therefore, hydroxylamine-containing ODC and SAMDC inhibitors may be a useful alternative to the ODC and SAMDC inhibitors used to date. Together with reduction of luminal polyamines (by a low-polyamine diet [40] or by eradicating colonic bacteria [41]), treatment with hydroxylamine-containing inhibitors of ODC and SAMDC may be therapeutically successful in colorectal cancer.

Acknowledgments

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