Biochem. J. (1989) 257, 769–774 (Printed in Great Britain)

Regulation of polyamine biosynthesis in rat hepatoma (HTC) cells by a bisbenzyl polyamine analogue

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A bisbenzyl polyamine analogue, MDL 27695, rapidly repressed ornithine decarboxylase (ODC) and *S*-adenosyl-L-methionine decarboxylase (AdoMet DC) activity and depleted polyamines in rat hepatoma (HTC) cells. The suppression of ODC and AdoMet DC activity was temporally related to metabolism of MDL 27695 by intracellular polyamine oxidase to a free-amine analogue, MDL 26752, which, when added directly to HTC cells, suppressed ODC activity and polyamine biosynthesis more rapidly and to a greater extent than did the bisbenzyl analogue. The ODC suppression caused by MDL 27695 was completely blocked by the addition of a polyamine oxidase inhibitor to the HTC-cell cultures along with MDL 27695. These data suggested that MDL 27695 acted as a prodrug, with metabolism to an active analogue being necessary for ODC repression to occur. MDL 27695 and MDL 26752 completely abolished division of HTC cells when added to cultures at 1 μM. This established them as being among the most potent antiproliferative polyamine analogues yet described. MDL 27695 has also been shown to possess significant antimalarial effects both *in vitro* and *in vivo*, and it is possible that the marked suppression of polyamine biosynthesis described herein may contribute to its anti-malarial effects as well as its antiproliferative effects in mammalian cells.

INTRODUCTION

Numerous studies have conclusively shown that the natural polyamines putrescine, spermidine and spermine are important regulators of rapid cell growth (Pegg & McCann, 1982). Interference with polyamine biosynthesis, therefore, has been a chemotherapeutic approach for the treatment of various disorders ranging from various malignancies to disease caused by parasitic protozoa (Schechter *et al.*, 1987). The major effort in this chemotherapeutic approach has focused on the use of α-difluoromethylornithine (DFMO; eflornithine), an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC) (Metcalf *et al.*, 1978), the first enzyme of polyamine biosynthesis. Although it has not become a panacea for proliferative diseases, remarkable success with DFMO has been achieved in the treatment of African trypanosomiasis (sleeping sickness) and *Pneumocystis carinii* pneumonia of autoimmune deficiency syndrome ('AIDS') (Sjoerdsma, 1987), giving credence to the overall rationale. In recent years, other new strategies for interfering with polyamine biosynthesis and/or function have emerged, one significant approach being the use of analogues of the natural polyamines.

The ability of natural polyamines and non-physiological diamines to regulate ornithine decarboxylase (ODC) activity and polyamine biosynthesis has been known for some time (Kay & Lindsay, 1973; Clark & Fuller, 1975; McCann *et al.*, 1979, 1980; McCann, 1980). Addition of polyamines to cultured cells resulted in a rapid, profound loss of ODC activity. Several polyamine analogues have been synthesized in an attempt to find compounds which would depress polyamine biosynthesis but would not substitute for the natural polyamines in other growth-supportive functions. The bisethyl derivatives of spermidine and spermine are such compounds which down-regulate polyamine biosynthesis and inhibit cancer cell growth *in vitro* at micromolar concentrations (Porter *et al.*, 1987*a*,*b*).

We have recently identified a number of bisbenzyl polyamine analogues which have marked anti-malarial effects both *in vitro* against chloroquine-resistant *Plasmodium falciparum* and against *Plasmodium berghei* (malarial parasites) in mice (Bitonti *et al.*, 1988). The mechanism of action of these analogues has not been fully elucidated, but one potential mechanism which must be considered is that of regulation of polyamine biosynthesis. Unfortunately, malaria parasites cannot be easily grown in quantities sufficient for biochemical studies to be done and, further, the parasites grow only within a mammalian erythrocyte, a fact which can potentially cloud interpretation of many biochemical data. We have, therefore, as an initial step in the elucidation of the biochemical mechanism of action, examined the effects of one bisbenzyl polyamine analogue, MDL 27695 (see Fig. 1 below), on polyamine biosynthesis in cultured rat hepatoma (HTC) cells. In the present report we show that MDL 27695 does, in fact, down-regulate polyamine biosynthesis in HTC cells, an effect accompanied by polyamine depletion and growth inhibition. The potential contribution of these effects to the anti-malarial efficacy of MDL 27695 is discussed.

Abbreviations used: DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; AdoMet DC, *S*-adenosyl-L-methionine decarboxylase; IC_{50}, concentration causing 50% inhibition; MDL 27695, MDL 28560 and MDL 26752, polyamine analogues the structures of which are depicted in Fig. 1.

EXPERIMENTAL

Rat hepatoma (HTC) cells

HTC cells were grown in spinner cultures as described previously (Hershko & Tomkins, 1966), except that Earle's Minimal Essential Medium was used. In experiments designed to follow the short-term repression of ODC activity, HTC cells were grown to a density of approx. 1×10^6/ml in medium containing 10% (v/v) fetal-bovine serum then transferred at the same cell density into identical culture medium which was supplemented with 0.5% bovine serum albumin instead of fetal-bovine serum. After 24 h the cells were diluted to a cell density of $(1–1.5) \times 10^5$/ml in fresh medium containing 10% (v/v) horse serum to induce ODC activity and cell growth. After an additional 16–18 h, polyamine analogues were added to some cultures, and the effects of the analogues on ODC, *S*-adenosyl-L-methionine decarboxylase (AdoMet DC) and intracellular polyamines were measured. HTC cells were enumerated with a haemocytometer and their viability was assessed by Trypan Blue exclusion.

At selected times after addition of the polyamine analogues to the HTC-cell cultures, portions of the cell suspensions were removed and the cells were washed twice with phosphate-buffered iso-osmotic NaCl (Bitonti *et al.*, 1986*a*), placed in a solution of 0.1 mM-pyridoxal phosphate, 5 mM-dithiothreitol, 0.1 mM-EDTA and 50 mM-Tris/HCl, pH 7.2, and then sonicated for 3×30 s to disrupt the cells. The crude broken-cell preparations were used to assay ODC activity.

All of the experiments described were repeated at least twice, with similar results being obtained in the separate experiments.

ODC and AdoMet DC activity

ODC activity was measured by monitoring the release of CO_2 from DL-[1-^{14}C]ornithine as described (Bitonti *et al.*, 1985). Each ODC assay contained, in a total volume of 1 ml, 50 mM-Tris/HCl, pH 7.2, 0.1 mM-pyridoxal phosphate, 5 mM-dithiothreitol, 0.2 mM-ornithine, 2 μCi of DL-[1-^{14}C]ornithine and approx. 1 mg of protein. The assay was run for 60 min at 37 °C and then terminated by the addition of 1 ml of 40% (w/v) trichloroacetic acid to liberate CO_2, which was trapped on a filter paper saturated with methylbenzethonium hydroxide. The trapped radioactivity was quantified by liquid-scintillation counting in 10 ml of Omnifluor. Cells for the measurement of AdoMet DC activity were treated and collected in a manner similar to that for the measurement of ODC activity. AdoMet DC activity was assayed as described (Bitonti *et al.*, 1986*b*) at 37 °C in a total volume of 0.6 ml, which contained 3 mM-dithiothreitol, 3 mM-putrescine, 125 mM-sodium phosphate, pH 7.4, approx. 1 mg of protein and 0.2 μCi of *S*-adenosyl-L-[*carboxy*-^{14}C]methionine. Liberated CO_2 was trapped and quantified as described above for ODC. Protein concentrations in the enzyme preparations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Measurement of polyamines and polyamine analogues

HTC cells (a minimum of 1×10^6) were collected and washed as described above for ODC measurements and then were extracted with 0.5 ml of 0.2 M-$HClO_4$. Putrescine, spermidine, spermine and MDL 26752 (retention time 36 min) were assayed by h.p.l.c. using the method of Seiler & Knodgen (1980). After chromatographic separation on an Altex Ultrasphere ODS-IP (10 μm particle size) column (4.6 mm × 250 mm), the polyamines were derivatized with *o*-phthalaldehyde and detected with a fluorimeter.

The polyamine analogues, MDL 27695, MDL 28560 and MDL 26752, were also measured by h.p.l.c. and fluorescence detection after derivitization with dansyl chloride. Chromatographic separations were carried out using an Altex Ultrasphere ODS-IP column as described above. The gradient elution buffer system was essentially that described by Brown *et al.* (1982). The column was equilibrated at 50% solution A (heptane-1-sulphonate, pH 3.4) and 50% solution B (acetonitrile) for 10 min before each new run. Upon sample injection, solution B was increased to 80% over a 22 min period and then to 100% over 5 min, followed by an additional 15 min period at 100% acetonitrile. The retention times for the polyamine analogues were: MDL 27695, 34 min; MDL 28560, 32 min; and MDL 26752, 30 min.

Dexamethasone induction of HTC cells

Tyrosine transaminase activity was induced in HTC cells with 10 μM-dexamethasone as described by Tomkins *et al.* (1966) and the enzyme activity was measured by the method of Lin & Knox (1957). HTC cells were lysed by sonication in the same buffer used for ODC activity measurements and the lysates were centrifuged at 15000 *g* for 5 min before assay. Enzyme activity was measured by monitoring the increase in A_{310} for 20 min in a Perkin–Elmer spectrophotometer equipped with a cuvette holder heated at 37 °C. The assays contained, in a total volume of 1 ml, 0.5 M-sodium borate, pH 7.8, 5 μmol of tyrosine, 10 μl of porcine phenylpyruvate tautomerase (EC 5.3.2.1; phenylpyruvate keto–enol isomerase; 5.2 units/ml; Sigma Chemical Co.), 10 μmol of α-oxoglutarate, 0.2 μmol of pyridoxal phosphate and 0.15 M-sodium phosphate, pH 7.8. The molar absorption coefficient for the enol–borate complex of *p*-hydroxyphenylpyruvate was taken to be 10700 litre·mol^{-1}·cm^{-1}.

Chemicals

The polyamine analogues MDL 27695, MDL 28560 and MDL 26752, as well as the polyamine oxidase inhibitor MDL 72527 (N^1N^4-bis(butadienyl)butanediamine; Bey *et al.*, 1985), were synthesized at this Institute. *S*-Adenosyl-L-[*carboxy*-^{14}C]methionine (58.9 mCi/mmol) was purchased from New England Nuclear and DL-[1-^{14}C]ornithine (61 mCi/mmol) was from Amersham.

RESULTS

The polyamine analogues MDL 26752 and MDL 27695 (see Fig. 1) rapidly inhibited ODC activity when added to cultures of growing HTC cells (Fig. 2*a*). During the first hours after addition of the analogues to the cultures, 1 μM-MDL 26752 repressed ODC more rapidly and to a greater extent than did 1 μM-MDL 27695. ODC was inhibited by a maximum of 80% at 2 h by MDL 26752 and a maximum of 55% by MDL 27695 at 6 h after addition to the culture medium. At 24 h, ODC activity remained inhibited by 55 and 40% by MDL 26752 and MDL 27695 respectively. The polyamine analogues also inhibited AdoMet DC activity,

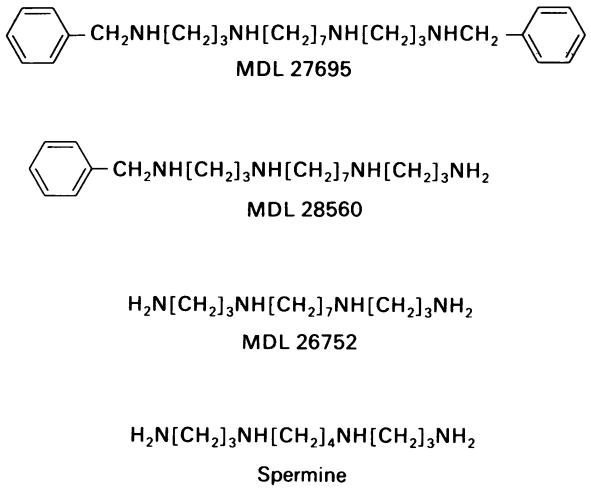

Fig. 1. Structures of polyamine analogues and spermine

but the inhibition was not as great as that seen with ODC activity (55% with MDL 26752 and 20% with MDL 27695 at 6 h), and AdoMet DC activity had returned to the control level by 24 h (Fig. 2*b*). It is important to note that the repression of ODC and AdoMet DC occurred in the absence of an effect of MDL 27695 and MDL 26752 on general protein synthesis as measured by [^{3}H]leucine incorporation (results not shown), suggesting that the inhibitory effect was specific for polyamine metabolism. Neither MDL 27695 nor MDL 26752 was directly inhibitory to ODC or AdoMet DC at concentrations up to 100 μM when added to the enzyme assay mixtures.

Changes in the intracellular content of putrescine correlated well with the changes in ODC activity. As seen in Table 1, the intracellular putrescine content decreased, after a slight lag period, in parallel with ODC activity. The spermidine and spermine levels did not change as markedly as those of putrescine. Spermidine was decreased by MDL 27695 by 24 h, whereas MDL 26752 had less of an effect. Spermine remained unchanged in the presence of either drug. By 24 h in this experiment, MDL 27695 was equal to MDL 26752 at preventing putrescine synthesis and was somewhat superior to MDL 26752 at preventing the synthesis of spermidine.

We also measured the accumulation of MDL 26752 and MDL 27695 by the HTC cells during these experiments. Both polyamine analogues were rapidly taken up by the cells (Fig. 3), reaching levels approximately equal to those of spermidine and spermine by 6 h. MDL 27695, a bisbenzyl polyamine, was metabolized in the HTC cells by way of sequential loss of its two benzyl groups, forming the monobenzyl (MDL 28560) and free-amine (MDL 26752) derivatives (see Fig. 1). The time course of appearance of MDL 26752 within the HTC cells treated with MDL 27695 was closely correlated with the repression of ODC activity due to MDL 27695 treatment, raising the possibility that the metabolite (MDL 26752), not the parent compound (MDL 27695), was responsible for ODC repression. To test this possibility, an experiment was done in which a specific polyamine oxidase inhibitor, namely N^1N^4-bis(butadienyl)butanediamine (MDL 72527), was added to HTC cells along with MDL 27695. It was found that 10 μM-MDL 72527 completely blocked the repression of ODC normally caused by MDL 27695 (Fig. 4), suggesting that metabolism of MDL 27695 within the HTC cells was crucial for the polyamine analogue's effects on polyamine biosynthesis.

We next carried out studies to assess the ability of the polyamine analogues to affect HTC-cell proliferation and the relationship of any effects to polyamine biosynthesis. Either 1 μM-MDL 26752 or 1 μM-MDL 27695 was markedly inhibitory to growth (Table 2). Both drugs appeared to be cytostatic rather than cytotoxic, since no cell death occurred during the 96 h of the experiment. The synthesis of putrescine, spermidine and spermine was clearly inhibited (Table 2), and MDL 26752 eventually resulted in complete depletion of

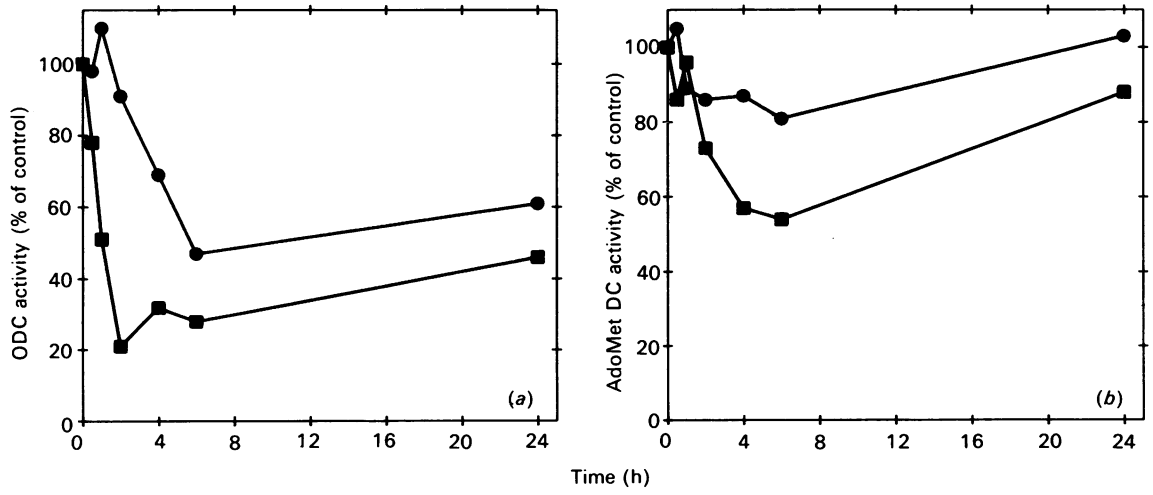

Fig. 2. Repression of HTC-cell ODC (*a*) and AdoMet DC (*b*) activity by MDL 27695 and MDL 26752

HTC cells were grown to 1 × 10^6 cells/ml, incubated for 24 h in serum-free medium and then stimulated for 18 h by dilution into fresh serum-containing medium. MDL 27695 (●) and MDL 26752 (■) were added at 1 μM and, at the times indicated, HTC cells were collected, washed and assayed for ODC and AdoMet DC activity as described in the Experimental section.

Table 1. Acute effects of MDL 27695 and MDL 26752 on HTC-cell polyamines

HTC cells were cultured with polyamine analogues, and intracellular polyamines were measured as described in the Experimental section. Lack of cytotoxicity of the analogues was confirmed at 6 and 24 h by Trypan Blue exclusion.

Time (h)	Additions		[Polyamine] (pmol/10^6 cells)		
	MDL 27695 (1 μM)	MDL 26752 (1 μM)	Putrescine	Spermidine	Spermine
0	−	−	241	1670	1430
	+	−	241	1530	1290
	−	+	241	1440	1290
0.5	−	−	302	1690	1530
	+	−	302	1570	1410
	−	+	241	1610	1430
1	−	−	363	1890	1790
	+	−	363	1720	1610
	−	+	301	1810	1760
2	−	−	387	1820	1730
	+	−	416	1740	1730
	−	+	213	1340	1370
4	−	−	387	1940	1920
	+	−	387	1840	1950
	−	+	170	1640	1860
6	−	−	387	2000	1950
	+	−	277	1790	1970
	−	+	170	1520	2030
24	−	−	730	1950	1980
	+	−	99	980	1510
	−	+	108	1340	2060

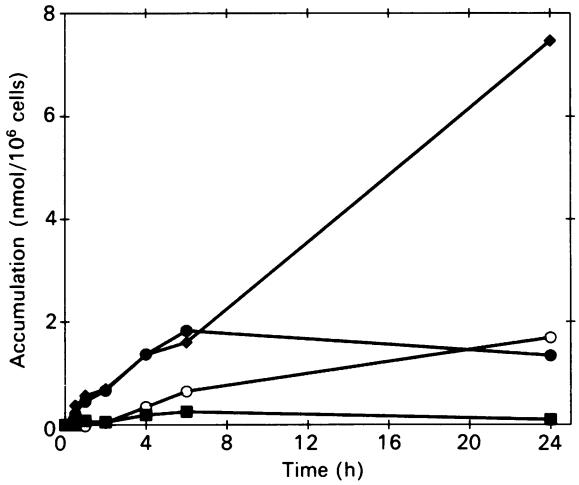

Fig. 3. Accumulation of MDL 27695 and its metabolites, MDL 28560 and MDL 26752, by HTC cells

HTC cells were treated as described in the legend to Fig. 2, but the cells were extracted with $HClO_4$ for the measurement of the polyamine analogues by h.p.l.c. MDL 27695 (○), MDL 28560 (■) and MDL 26752 (●) were measured in cells treated with 1 μM-MDL 27695. Additionally, MDL 26752 (◆) was measured in cells treated with MDL 26752.

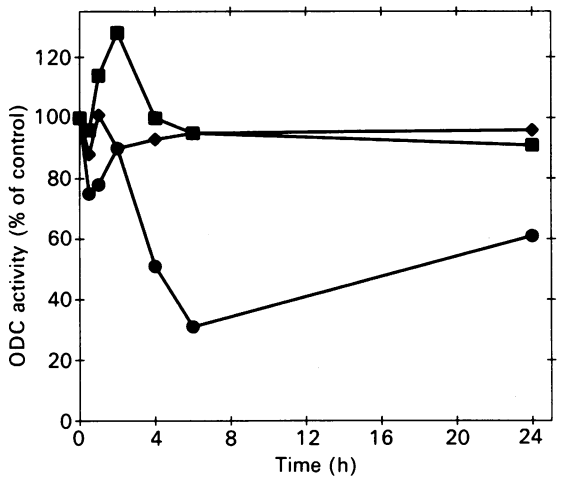

Fig. 4. Repression of ODC activity by MDL 27695 and inhibition of this effect by a polyamine oxidase inhibitor, MDL

HTC cells were grown as described in the legend to Fig. 2 and then either MDL 27695 (●), MDL 72527 (◆) or MDL 27695 plus MDL 72527 (■) were added to the cultures and ODC activity was measured in the treated cells as described in the Experimental section.

putrescine, but not until 72 h. Whereas spermidine and spermine syntheses were inhibited, neither of the drugs fully depleted these polyamines.

Tyrosine transaminase was induced in HTC cells by treatment of the cells with 10 μM-dexamethasone. Neither MDL 26752 nor MDL 27695 had any inhibitory effect on the induction of tyrosine transaminase, but, in these same cells, MDL 26752 and MDL 27695 markedly repressed ODC activity (Table 3).

Table 2. Effects of MDL 27695 and MDL 26752 on HTC-cell growth and intracellular polyamines during chronic treatment

HTC cells were grown to a high density (10^6 cells/ml) and then diluted to 10^5 cells/ml in fresh medium containing 10% horse serum. An aliquot of cells was taken for the 0 h time point and then the polyamine analogues were added. Aliquots of the cells were taken at 24 h intervals and polyamine content was measured as described in the Experimental section. Cell viability, assessed daily by Trypan Blue exclusion, was more than 90%. Polyamine analogues were added fresh every 24 h. Abbreviation: N.D., not detected (< 50 pmol/10^6 cells).

Time (h)	Additions		[Polyamine] (pmol/10^6 cells)			$10^{-5}\times$ Cell no.
	MDL 27695 (1 μM)	MDL 26752 (1 μM)	Putrescine	Spermidine	Spermine	
0	−	−	N.D.	370	1000	0.9
	+	−	N.D.	340	890	0.9
	−	+	N.D.	370	890	1.0
24	−	−	90	510	1020	2.4
	+	−	160	530	1720	0.9
	−	+	120	430	1880	0.8
48	−	−	390	1220	2620	2.8
	+	−	140	620	1610	1.4
	−	+	87	247	1030	1.5
72	−	−	570	1560	3290	5.2
	+	−	190	840	2210	1.4
	−	+	N.D.	277	1350	0.8
96	−	−	190	1110	2460	15.0
	+	−	90	690	2090	1.8
	−	+	N.D.	249	1460	0.9

Table 3. Effects of dexamethasone, MDL 27695 and MDL 26752 on HTC-cell ODC and tyrosine transaminase activities

HTC cells were grown to a density of 1×10^6 cells/ml and then diluted into fresh medium with or without 10 μM-dexamethasone (DEX) and 1 μM of either MDL 27695 or MDL 26752 to a density of approx. 1×10^5 cells/ml. At the indicated times after dilution, HTC cells were sedimented from the medium, washed and analysed for ODC activity or tyrosine transaminase activity as described in the Experimental section. Values in parentheses are percentages of control (no addition) at each time point.

Time (h)	Additions			ODC activity (nmol·h^{-1}·mg of protein^{-1})	Tyrosine transaminase activity (pmol·min^{-1}·mg of protein^{-1})
	DEX (10 μM)	MDL 27695 (1 μM)	MDL 26752 (1 μM)		
0	−	−	−	1.25 (100)	87 (100)
	+	−	−	1.53 (122)	107 (122)
	+	+	−	1.42 (113)	98 (112)
	+	−	+	1.14 (91)	80 (92)
4	−	−	−	0.90 (100)	111 (100)
	+	−	−	1.09 (121)	347 (310)
	+	+	−	0.54 (60)	306 (280)
	+	−	+	0.10 (11)	341 (307)
8	−	−	−	0.95 (100)	122 (100)
	+	−	−	0.82 (86)	684 (561)
	+	+	−	0.27 (28)	574 (470)
	+	−	+	0.19 (20)	572 (470)

DISCUSSION

As an initial step towards elucidating the biochemical mechanism(s) of the antiproliferative action of bisbenzyl polyamine analogues, we have studied the effects of one of these compounds on polyamine biosynthesis and cell growth in cultured HTC cells. MDL 27695 (1 μM) rapidly repressed ODC activity in HTC cells, but this repression apparently correlated with the metabolism of the bisbenzyl polyamine, MDL 27695, to its free-amine counterpart, MDL 26752, since addition of an inhibitor of the metabolism completely blocked the suppressive effects on ODC activity. Also, direct addition of 1 μM of MDL 26752 to HTC cells resulted in a much more rapid repression of ODC activity than did addition of MDL 27695. These data suggest that the effects of the bisbenzyl polyamine are indirect and therefore the compound can be thought of as a prodrug with regard to its ODC-

suppressive activity. It is unclear whether there are antiproliferative effects of MDL 27695 which are not due to its metabolism to MDL 26752, but rather due to the bisbenzyl analogue itself.

The unique type of metabolism represented by the conversion of the bisbenzyl polyamine analogue to its free-amine counterpart has not been described before. Since the metabolic conversion was apparently inhibited by a specific inhibitor of polyamine oxidase, it is possible that the latter enzyme is responsible for the conversion, although this has not been rigorously tested. The involvement of polyamine oxidase in this metabolism is noteworthy because the usual metabolism of polyamines by polyamine oxidase involves the loss of an aminopropionaldehyde moiety, whereas, in the metabolic scheme described for MDL 27695, the polyamine backbone remains intact while the terminal benzyl groups are cleaved. Additional work is needed to further characterize this novel metabolic route and the enzyme(s) involved.

MDL 27695 has potent antimalarial effects *in vitro* against *Plasmodium falciparum* and, in combination with DFMO, cures virtually 100% of mice with an otherwise lethal *Plasmodium berghei* infection (Bitonti *et al.*, 1988). The question as to whether MDL 27695 or its metabolite MDL 26752 is the active growth inhibitor in plasmodia awaits the determination of whether or not these parasites have an enzyme activity capable of metabolizing the bisbenzyl compound to the free amine. If this enzymic activity is not present in plasmodia, then it is doubtful that suppression of polyamine biosynthesis would play a role in the antiproliferative effects of MDL 27695 against malaria parasites. Further studies need to be directed towards answering this question.

Besides the possible impact of our present findings on understanding the mechanism of action of the bisbenzyl analogue against plasmodia, the effects of the compounds in HTC cells are noteworthy *per se*. MDL 27695 and MDL 26752 are extremely potent polyamine analogues, with almost complete arrest of HTC cell growth occurring at 1 μM. Previously the most potent polyamine analogue described, bis(ethyl)spermine, had an IC_{50} against L1210 leukaemia cells of 1 μM (Porter *et al.*, 1987*a*). Given that there may be differences in sensitivities between HTC cells and L1210 cells, it remains that MDL 27695 and MDL 26752 are very effective inhibitors of mammalian cell growth. One difference between MDL 27695 and MDL 26752 and the bis(ethyl)spermidine and spermine derivatives seems to lie in specificity of the effects on polyamine biosynthesis. We have demonstrated that MDL 27695 and MDL 26752 are relatively specific regulators of ODC activity and polyamine biosynthesis. These analogues inhibited neither [^{3}H]leucine incorporation nor the induction by dexamethasone of tyrosine transaminase in HTC cells at times when ODC activity was maximally suppressed. However, almost complete inhibition of HTC cell growth occurred without complete depletion of spermidine or spermine. This suggests that there may be other antiproliferative effects of the compounds in addition to inhibition of polyamine biosynthesis. The bisethyl polyamines apparently more effectively deplete intracellular polyamines (Porter *et al.*, 1987*a*) and therefore may be more specific for this metabolic pathway. We have also found that MDL 26752 is as effective as spermine at stabilizing calf-thymus DNA against thermal denaturation (A. J. Bitonti, unpublished work), suggesting that this analogue can replace polyamines at their potential intracellular binding sites. MDL 27695 also stabilized DNA against thermal denaturation, but it was not as effective as MDL 26752. Thus MDL 26752 and MDL 27695 may not only inhibit cell growth through inhibition of polyamine metabolism, but likely interfere with polyamine function as well. The questions raised by the present study as to the relationship of the antimalarial effects to MDL 27695 to polyamine metabolism, and the mechanism(s) of the antiproliferative effects of MDL 27695 and MDL 26752 in mammalian cells, should stimulate more work in this important research area.

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Received 5 May 1988/22 August 1988; accepted 24 August 1988