Role of diamine oxidase during the treatment of tumour-bearing mice with combinations of polyamine anti-metabolites

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Treatment of mice bearing L1210 leukaemia with 2-difluoromethylornithine, a specific inhibitor of ornithine decarboxylase (EC 4.1.1.17), produced a profound depletion of putrescine and spermidine in the tumour cells. Sequential combination of methylglyoxal bis(guanylhydrazone), an inhibitor of adenosylmethionine decarboxylase (EC 4.1.1.50), with difluoromethylornithine largely reversed the polyamine depletion and led to a marked accumulation of cadaverine in the tumour cells. Experiments carried out with the combination of difluoromethylornithine and aminoguanidine, a potent inhibitor of diamine oxidase (EC 1.4.3.6), indicated that the methylglyoxal bis(guanylhydrazone)-induced reversal of polyamine depletion was mediated by the known inhibition of diamine oxidase by the diguanidine. In spite of the normalization of the tumour cell polyamine pattern upon administration of methylglyoxal bis(guanylhydrazone) to difluoromethylornithine-treated animals, the combination of these two drugs produced a growth-inhibitory effect not achievable with either of the compounds alone.

Inhibitors of polyamine biosynthesis (polyamine anti-metabolites) have not only gained widespread experimental use as anti-proliferative agents (for references, see Heby & Jänne, 1981), but have recently also been used in pilot clinical trials for the evaluation of their anti-cancer potential in man (Siimes *et al.*, 1981). Especially interesting is the combination of difluoromethylornithine and methylglyoxal bis(guanylhydrazone). The growth-inhibitory effect of this combination is probably not based on an inhibition of polyamine biosynthesis at two different sites (ornithine decarboxylase and adenosylmethionine decarboxylase), but is more likely attributable to the fact that difluoromethylornithine-induced polyamine depletion strikingly enhances the cellular uptake of subsequently administered methylglyoxal bis(guanylhydrazone) (Alhonen-Hongisto *et al.*, 1980; Seppänen *et al.*, 1981; Siimes *et al.*, 1981).

Although the combination of the two polyamine anti-metabolites produces a synergistic or additive anti-tumoural effect in transplantable murine leukaemias (Burchenal *et al.*, 1981; Bartholeyns & Koch-Weser, 1981; Seppänen *et al.*, 1983), there are reports indicating that inhibitors of adenosylmethionine decarboxylase, such as methylglyoxal bis(guanylhydrazone) or its close derivative 1,1'-[(methylethanediyldene)-dinitrilo]bis-(3-amino-

guanidine), not only abolish difluoromethylornithine-induced growth inhibition but also result in a restoration of the normal intracellular polyamine pattern in an experimental murine sarcoma (Prakash *et al.*, 1980; Bartholeyns & Koch-Weser, 1981). The latter phenomenon has been attributed (Prakash *et al.*, 1980) to the strong inhibitory action exerted by the diguanidine derivatives on mammalian diamine oxidase activity (Hölttä *et al.*, 1973; Pegg & McGill, 1978).

We have now carried out experiments by combining difluoromethylornithine with methylglyoxal bis(guanylhydrazone) and with aminoguanidine, an inhibitor of diamine oxidase having no effect on the biosynthesis of the polyamines, and reached the conclusion that the antagonism, with respect to polyamine deprivation, between difluoromethylornithine and methylglyoxal bis(guanylhydrazone) is based on the marked inhibition of diamine oxidase activity under conditions where the transport of polyamines is greatly enhanced.

Experimental

Animals and cells

Murine L1210 leukaemia cells were propagated and maintained *in vivo* in female DBA mice. Cells were transferred weekly by intraperitoneal trans-

plantation to new hosts. Leukaemia-bearing mice were killed on the fifth day after inoculation and the cells were washed out from the intraperitoneal cavity with the aid of 0.15 M-NaCl.

Chemicals

2-Difluoromethylornithine was a gift from the Centre de Recherche Merrell International (Strasbourg, France). Methylglyoxal bis(guanylhydrazone) was synthesized by and obtained from Orion Pharmaceutical Company (Espoo, Finland). Aminoguanidine sulphate was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Administration of the drugs

Difluoromethylornithine was administered in the drinking water [2% (w/v) solution] of the mice starting on the day of inoculation. Aminoguanidine (35 mg/kg body wt.) and methylglyoxal bis(guanylhydrazone) (50 mg/kg body wt.) were injected intraperitoneally on days 2 and 4 after inoculation.

Analytical methods

Polyamines present in the HClO₄-soluble fraction were determined after dansylation (5-dimethylaminonaphthalene-1-sulphonylation) by the method of Seiler (1970) using the solvent system of Hölttä *et al.* (1979). The activity of diamine oxidase from mouse intestine homogenized in 4 vol. of 0.15 M-NaCl was assayed by the method of Tryding & Willert (1968). Protein content was determined by the method of Lowry *et al.* (1951).

Results

The marked depletion of putrescine and spermidine produced by difluoromethylornithine in L1210 leukaemia cells is shown in Table 1.

Aminoguanidine alone did not change the polyamine pattern, except for doubling the content of cadaverine, whereas in response to methylglyoxal bis(guanylhydrazone) the content of spermine was significantly decreased and that of putrescine markedly elevated (Table 1). The finding that methylglyoxal bis(guanylhydrazone) increased putrescine concentration by more than 10-fold, whereas aminoguanidine had no effect, is probably a result of blocked forward utilization of putrescine in response to methylglyoxal bis(guanylhydrazone).

Administration of aminoguanidine or methylglyoxal bis(guanylhydrazone) to difluoromethylornithine-treated animals corrected to large extent the polyamine deprivation in the tumour cells (Table 1). A marked difference between aminoguanidine and methylglyoxal bis(guanylhydrazone) was the large increase in spermidine content produced by the former compound, in contrast with a decrease in this polyamine in response to methylglyoxal bis(guanylhydrazone) (Table 1). This difference, however, can be explained in terms of the blockage of spermidine conversion into spermine by methylglyoxal bis(guanylhydrazone), but not by aminoguanidine. It is also interesting to note that, whereas difluoromethylornithine, when given together with aminoguanidine or methylglyoxal bis(guanylhydrazone), decreased the accumulation of putrescine, the combined treatments resulted in each case in a marked enhancement of cadaverine accumulation in the tumour cells (Table 1). By taking into consideration the proposal that the formation of cadaverine in animal tissues is catalysed by the action of ornithine decarboxylase on lysine (and is thus difluoromethylornithine-sensitive) (Pegg & McGill, 1979), it is likely that cadaverine and its aminopropyl derivative (Alhonen-Hongisto *et al.*, 1982a) of these tumour cells was derived from

Table 1. Effect of difluoromethylornithine, aminoguanidine, methylglyoxal bis(guanylhydrazone) or their combination *in vivo* on polyamine levels of L1210 tumour cells

L1210 tumour cells were grown in the intraperitoneal cavity of mice for 5 days. Aminoguanidine (35 mg/kg body wt.) and methylglyoxal bis(guanylhydrazone) were injected intraperitoneally into mice on days 2 and 4 after inoculation. Difluoromethylornithine (2%, w/v) was administered in the drinking water of the mice starting on the day of inoculation of L1210 tumour cells. There were four mice in each group. The significance of the differences was **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Abbreviations used: DFMO, difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone); Put, putrescine; Cad, cadaverine; Spd, spermidine; Apc, *N*-aminopropylcadaverine; Spm, spermine; n.d., not determined.

Treatment	Polyamine level (nmol/mg of protein)				
	Put	Cad	Spd	Apc	Spm
None	1.15 ± 0.18	0.38 ± 0.09	17.51 ± 2.25	n.d.	9.83 ± 1.10
DFMO	0.34 ± 0.31***	0.41 ± 0.08	1.20 ± 0.58***	0.96 ± 0.30	13.75 ± 3.35*
Aminoguanidine	1.06 ± 0.18	0.72 ± 0.19*	16.91 ± 0.26	n.d.	8.65 ± 2.05
MGBG	12.87 ± 2.40***	1.58 ± 0.29**	17.37 ± 3.90	n.d.	5.27 ± 1.04***
DFMO + aminoguanidine	0.79 ± 0.38	4.53 ± 1.39**	8.28 ± 1.49**	1.30 ± 0.46	16.52 ± 2.70**
DFMO + MGBG	4.36 ± 1.54**	5.28 ± 1.81**	11.06 ± 0.89**	n.d.	5.84 ± 1.29**

Table 2. *Inhibition of diamine oxidase activity by aminoguanidine and methylglyoxal bis(guanyldihydrazone) in the intestine of mice bearing L1210 tumour cells*

For details of the treatment of the mice, see the legend to Table 1. There were four mice in each group. The significance of the differences was: ** $P < 0.01$; *** $P < 0.001$. Abbreviations used: DFMO, difluoromethylornithine; MGBG, methylglyoxal bis(guanyldihydrazone).

Treatment	Diamine oxidase activity (nmol/mg of protein)
None	23.2 ± 6.68
DFMO	23.6 ± 4.36
Aminoguanidine	4.2 ± 0.91**
MGBG	0.8 ± 0.28***
MGBG + aminoguanidine	3.9 ± 0.96**
DFMO + MGBG	0.7 ± 0.07***

Table 3. *The anti-proliferative effect of the combination of difluoromethylornithine and methylglyoxal bis(guanyldihydrazone) on L1210 tumour cells in vivo*

For the treatment of the mice see the legend to Table 1. There were four mice in each group. The significance of the differences was: * $P < 0.05$. Abbreviations used: DFMO, difluoromethylornithine; MGBG, methylglyoxal bis(guanyldihydrazone).

Treatment	Cell mass (mg wet weight)
None	723 ± 186
DFMO	702 ± 279
Aminoguanidine	851 ± 234
MGBG	573 ± 265
DFMO + aminoguanidine	627 ± 186
DFMO + MGBG	450 ± 64*

circulation and was possibly formed by the action of (intestinal?) bacteria.

Determination of diamine oxidase activity in intestinal tissue (which is the richest source of this enzyme in rodents) revealed that the enzyme activity was inhibited by 80–95% by any drug regimens containing aminoguanidine or methylglyoxal bis(guanyldihydrazone) (Table 2).

In spite of the fact that methylglyoxal bis(guanyldihydrazone) largely restored difluoromethylornithine-induced polyamine depletion towards the normal values, the combination of these two anti-metabolites produced an anti-proliferative action not achievable with either of the agents alone, as illustrated in Table 3.

Discussion

The present results, together with those published previously (Prakash *et al.*, 1980), strongly suggest that the ability of methylglyoxal bis(guanyldihydrazone) to inhibit diamine oxidase activity contributes to the paradoxical restoration of normal or near

normal polyamine pattern during combined difluoromethylornithine/methylglyoxal bis(guanyldihydrazone) treatment. In line with this view are the experimental findings recently reported by Heston *et al.* (1981). The latter authors exposed prostate-derived tumour cells (grown in the presence of calf serum, which contains high amine oxidase activity) to difluoromethylornithine and observed that the growth inhibition in response to the inhibitor of ornithine decarboxylase was largely, if not totally, reversed upon addition of aminoguanidine (Heston *et al.*, 1981).

Although the inhibition of diamine oxidase activity by diguanidines undoubtedly has a major impact on the restoration of near normal intracellular polyamine pools during polyamine deprivation, it is certainly not the only reason. The difluoromethylornithine-induced state of polyamine deprivation is characterized by markedly enhanced uptake of exogenous polyamines (including cadaverine) (Alhonen-Hongisto *et al.*, 1980, 1982a). Moreover, methylglyoxal bis(guanyldihydrazone) alone similarly stimulates the uptake of polyamines, most notably that of putrescine and cadaverine (Alhonen-Hongisto *et al.*, 1982b). Thus the inhibition of diamine oxidase combined with a strong stimulation of cellular transport of the polyamines in response to the polyamine anti-metabolites offers the most likely explanation for the paradoxical restoration of normal polyamine pattern during combined difluoromethylornithine and diguanidine treatment.

An interesting question remains: what is the origin of cadaverine seen in tumour cells exposed to difluoromethylornithine combined with aminoguanidine or methylglyoxal bis(guanyldihydrazone)? It is highly unlikely that this diamine is produced by the leukaemia cells *in vivo*, since cultured L1210 cells exposed to difluoromethylornithine, methylglyoxal bis(guanyldihydrazone) or to their combination do not contain detectable levels of cadaverine (Jänne *et al.*, 1982). The present results (Table 1) also suggested that the accumulation of cadaverine was not sensitive to difluoromethylornithine, as indicated by a further increase in tumour cadaverine (but not putrescine) content upon the addition of difluoromethylornithine to aminoguanidine- or methylglyoxal bis(guanyldihydrazone)-containing regimens (Table 1).

It is tempting to speculate that cadaverine was derived from circulation and primarily formed by the action of intestinal bacteria. Intestinal tissue is the richest source of diamine oxidase activity in rodents (to degrade bacteria-derived amines?) and, as shown in Table 2, aminoguanidine and methylglyoxal bis(guanyldihydrazone) almost completely inhibited the enzyme activity. The appearance of cadaverine in the tumour cells is not just an academic curiosity,

since cadaverine and its aminopropyl derivatives can support the growth of the tumour cells even in the absence of the natural polyamines (Alhonen-Hongisto *et al.*, 1982a).

As regards the combined use of difluoromethylornithine and methylglyoxal bis(guanylhydrazone), an important practical consideration deserves attention. If a tumour is sensitive to polyamine-deprivation but resistant to the action of methylglyoxal bis(guanylhydrazone) (regardless of whether or not mediated by polyamine metabolism) the combination of difluoromethylornithine and diguanidines is clearly disadvantageous, as has been shown previously (Prakash *et al.*, 1980; Bartholeyns & Koch-Weser, 1981). If the tumour is sensitive to methylglyoxal bis(guanylhydrazone) such a combination may be beneficial, yet the anti-proliferative action based on polyamine deprivation is possibly lost.

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