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Transfer of intestine-derived diamines into tumour cells during treatment of Ehrlich-ascites—carcinoma-bearing mice with polyamine anti-metabolites

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Treatment of Ehrlich-ascites-carcinoma-bearing mice with methylglyoxal bis(guanylhydrazone) alone or in combination with 2-difluoromethylornithine greatly enhanced the transfer of intragastrically administered radioactive putrescine and cadaverine into the carcinoma cells. Difluoromethylornithine alone did not have any effect on the accumulation of intestine-derived diamines in the tumour cells. The frequently reported restoration of difluoromethylornithine-induced polyamine depletion on administration of methylglyoxal bis(guanylhydrazone) is in all likelihood attributable to a profound inhibition of intestinal diamine oxidase (EC 1.4.3.6), resulting in an enhanced entry of intestinal (bacterial) diamines into general circulation and finally into tumour cells.

2-Difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase (EC 4.1.1.17) (Metcalf *et al.*, 1978), and methylglyoxal bis(guanylhydrazone), a competitive inhibitor of *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) (Williams-Ashman & Schenone, 1972; Hölttä *et al.*, 1973), inhibit the two key enzymes of polyamine biosynthesis. Thus, taking into consideration the fact that undisturbed polyamine biosynthesis appears to be required for cell division to occur (for references see Heby & Jänne, 1981), a combination of these two drugs seems to be a useful approach to anti-cancer therapy. A further attraction of the combination is the fact that difluoromethylornithine-induced depletion of putrescine and spermidine strikingly enhances the cellular accumulation of methylglyoxal bis(guanylhydrazone) (Alhonen-Hongisto *et al.*, 1980), and the combination results in a synergistic growth inhibition (Alhonen-Hongisto *et al.*, 1980; Seppänen *et al.*, 1981). The combination indeed works as expected in cultured cells: it depletes polyamines and exerts a profound and irreversible anti-proliferative effect (Seppänen *et al.*, 1981). Similarly, methylglyoxal bis(guanylhydrazone) alone is a potent inhibitor of accumulation of spermidine and spermine in cultured cells, yet in experimental animals it is remarkably difficult to achieve any polyamine depletion (with the possible exception of a slight decrease in spermine) even with high doses of this drug (Hölttä *et al.*, 1973; Alhonen-Hongisto *et al.*, 1982; Kallio & Jänne, 1983). When combined with difluoromethylornithine, methylglyoxal bis(guanylhydrazone) paradoxically restores normal or near-normal polyamine patterns in polyamine-depleted cells (Prakash *et al.*, 1980; Bartholeyns & Koch-Weser, 1981; Alhonen-Hongisto *et al.*, 1982; Jänne *et al.*, 1983). The phenomena described above, i.e. the ineffectiveness of methylglyoxal bis(guanylhydrazone) to induce polyamine depletion in experimental animals and the paradoxical restoration of normal polyamine contents during combined treatments, are almost certainly attributable to the known inhibition of diamine oxidase activity by methylglyoxal bis(guanylhydrazone) (Hölttä *et al.*, 1973; Pegg & McGill, 1978), as shown by Kallio & Jänne (1983).

We have now used a more direct experimental approach, and show here that treatment of tumour-bearing animals with methylglyoxal bis(guanylhydrazone), alone or in combination with difluoromethylornithine, greatly enhances the resorption of intra-intestinal diamines into the circulation and stimulates their transfer into the tumour cells.

Experimental

Animals, cells and drug treatments

Ehrlich-ascites-carcinoma cells were maintained *in vivo* in female NMRI mice. The treatments were started 2 days after inoculation. The animals received difluoromethylornithine (2%, w/v) in their drinking water and/or methylglyoxal bis(guanylhydrazone) as daily intraperitoneal in-

jections (25 mg/kg) for 4 days. On day 4 the animals received 1 μCi of either [^{14}C]putrescine or [^{14}C]-cadaverine through a stomach tube. The first blood sample was taken from the retro-orbital plexus 2 h after the administration of labelled diamines, and the second blood sample at 5 h, after which the mice were killed and the tumour cells harvested.

Chemicals

2-Difluoromethylornithine was a gift from the Centre de Recherche Merrell International (Strasbourg, France). Methylglyoxal bis(guanylhydrazone) was obtained from Orion Pharmaceutical Co. (Espoo, Finland). [1,4-^{14}C]Putrescine (sp. radioactivity 109 Ci/mol) was obtained from Amersham International (Amersham, Bucks., U.K.), and [1,5-^{14}C]cadaverine (sp. radioactivity 106 Ci/mol) from New England Nuclear Corp. (Dreieich, West Germany).

Analytical methods

Polyamines present in the $HClO_4$-soluble fraction were determined after dansylation (5-dimethylaminonaphthalene-1-sulphonylation) by the method of Seiler (1970), with the solvent system of Hölttä *et al.* (1979) in the t.l.c. Diamine oxidase (from dialysed small-intestine cytosol fraction) activity was assayed by the method of Tryding & Willert (1968). Protein was determined by the method of Lowry *et al.* (1951).

Results

Very little radioactivity was transferred into intraperitoneally growing tumour cells when radioactive putrescine was administered through a stomach tube to untreated Ehrlich-ascites-carcinoma-bearing mice (Table 1). Although difluoromethylornithine only marginally influenced the incorporation of putrescine-derived radioactivity into the carcinoma cells, treatment of the tumour-bearing mice with methylglyoxal bis(guanylhydrazone) resulted in about 20-fold increase in the radioactivity of the tumour cells (Table 1). A combination of the drugs likewise stimulated the transfer of labelled putrescine from gastro-intestinal tract into the tumour cells, although less effectively than with methylglyoxal bis(guanylhydrazone) alone (Table 1).

When labelled cadaverine was administered intragastrically, substantially more radioactivity (216 ± 32 c.p.m./μg of protein), in comparison with putrescine, was found in tumour cells of untreated animals, and, as with putrescine, the transfer of cadaverine was greatly enhanced (1470 ± 79 c.p.m./μg of protein) by the combined treatment of tumour-bearing mice with the two polyamine anti-metabolites.

The distribution of putrescine-derived radioactivity among tumour-cell polyamines is depicted in Table 1. The specific radioactivity of putrescine was highest in cells obtained from methylglyoxal bis(guanylhydrazone)-treated animals, yet definite conversion into spermidine also occurred (Table 1). The highest specific radioactivity of total polyamines was found in tumour cells of mice treated with the combination; now the major labelled polyamine was spermidine (Table 1).

Table 2 shows the specific activity of putrescine in whole blood at 2 h after the intragastric administration of the diamine. The specific activity of blood putrescine, detectable only in animals treated with methylglyoxal bis(guanylhydrazone)-containing regimens, was almost identical with that found in the tumour cells at 5 h after the administration of the label (Table 1). This may indicate that putrescine found in the tumour cells was exclusively derived from the circulation and not from endogenous biosynthesis. Only traces of labelled spermidine were found in the blood at any time point, and also the putrescine content decreased to an almost undetectable value at 5 h after the administration.

In agreement with our earlier results (Kallio & Jänne, 1983), intestinal diamine oxidase activity was almost totally inhibited in animals receiving

Table 1. *Transfer and distribution of putrescine-derived radioactivity among polyamines in Ehrlich ascites-carcinoma cells of tumour-bearing mice treated with polyamine anti-metabolites*

The animals (five or six in each group) received 1 μCi of labelled putrescine by stomach tube 5 h before death. Results are means $\pm$ S.D.; in comparison with untreated controls: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Abbreviations used: DFMO, difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone).

Treatment	Total radioactivity in tumour cells (c.p.m./μg of protein)	Distribution of radioactivity (c.p.m./nmol)		
		Putrescine	Spermidine	Spermine
None	68 ± 6	0	1.2 ± 0.7	0
DFMO	$88 \pm 13^{*}$	0	2.3 ± 0.8	0.3 ± 0.1
MGBG	$1280 \pm 139^{***}$	$58 \pm 8^{***}$	$9.2 \pm 3.8^{**}$	0
DFMO + MGBG	$752 \pm 78^{***}$	$32 \pm 11^{***}$	$42 \pm 6^{***}$	0.8 ± 1

Table 2. *Radioactive putrescine in whole blood after its intragastric administration to tumour-bearing mice treated with polyamine anti-metabolites*

Experimental details are as in Table 1, except that the blood sample was taken from the retro-orbital plexus 2 h after the administration of the radioactive diamine. Results are means ± S.D.; in comparison with untreated controls, ***$P < 0.001$. Abbreviations used: DFMO, difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone).

Treatment	Putrescine (c.p.m./nmol)
None	0
DFMO	0
MGBG	57 ± 11***
DFMO + MGBG	46 ± 14***

any drug regimens containing methylglyoxal bis(guanylhydrazone) (results not shown).

Discussion

The present results, supported by earlier experimental data, show directly that the profound inhibition of diamine oxidase (especially intestinal) exerted by methylglyoxal bis(guanylhydrazone) or its derivatives is a major complicating factor associated with the combined use of inhibitors of ornithine decarboxylase and diguanidines. Under conditions of depressed intestinal diamine oxidase activity, intestinal (bacteria-derived) diamines (and polyamines) appear to enter the general circulation at strikingly enhanced rates and finally find their way into the tumour cells. This mechanism undoubtedly explains earlier observations of the paradoxical restoration of normal polyamine patterns and enhanced cadaverine accumulation when methylglyoxal bis(guanylhydrazone) (or its derivatives) is combined with difluoromethylornithine (Prakash *et al.*, 1980; Alhonen-Hongisto *et al.*, 1982; Kallio & Jänne, 1983).

It is also noteworthy that, although methylglyoxal bis(guanylhydrazone) effectively inhibits the accumulation of spermidine and spermine in cultured animal cells (for references see Heby & Jänne, 1981), this is not necessarily true when the drug is administered to experimental animals. Using doses close to the LD_{50} in rats, Hölttä *et al.* (1973) found that it is remarkably difficult to achieve any kind of polyamine depletion in regenerating liver with the drug. In tissues such as thymus, drug treatment even increased the concentration of spermidine, which is expectable, since thymus contains very high diamine oxidase activity (Hölttä *et al.*, 1973).

All these experimental results strengthen the view that the profound cytotoxicity exerted by methylglyoxal bis(guanylhydrazone) in several animal tumour screens (for references see Mihich, 1975; Porter *et al.*, 1981) is probably not related to an inhibition of accumulation of spermidine and spermine. The same conclusion holds true to the combined use of the drug with inhibitors of ornithine decarboxylase, such as difluoromethylornithine. However, this does not mean that the above-mentioned combination would be useless as an anti-cancer regimen, since (i) difluoromethylornithine (or polyamine depletion in general) rather selectively enhances the accumulation of methylglyoxal bis(guanylhydrazone) in tumour cells (Seppänen *et al.*, 1983), (ii) the combination lengthens the life-span of animals bearing L1210 leukaemia (Burchenal *et al.*, 1981; Sunkara *et al.*, 1983) and (iii) the combination shows clinical activity in human leukaemia (Siimes *et al.*, 1981). However, it means that polyamine-depletion-induced growth inhibition in many experimental animal models is totally or partly lost when diguanidines are combined with inhibitors of ornithine decarboxylase.

Methylglyoxal bis(guanylhydrazone) and difluoromethylornithine still remain as a combination of interest, since difluoromethylornithine serves as an excellent vector for methylglyoxal bis(guanylhydrazone) and the combination is strongly cytotoxic in tumours sensitive to the latter drug.

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