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Evaluation of the Cytotoxicity of Dihydroxytryptamines and 5-Hydroxytryptamine Antagonists as Cytotoxic Agents in Dimethylhydrazine-Induced Adenocarcinomata

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Summary. *The cytotoxicity of 5,6-dihydroxytryptamine (5,6-DHT), 5,7-dihydroxytryptamine (5,7-DHT), bromolysergic acid diethylamide (BOL), methysergide, and cyproheptadine, and also of 5,6-DHT together with either BOL, methysergide, or cyproheptadine in dimethylhydrazine-induced (DMH) carcinomata of rat colon was evaluated by estimating the percentage of necrotic cells in histological sections of tissues taken 15 h after injection of each of the drugs. In addition, the influence of methysergide and cyproheptadine on the tumour cell mitotic rate was estimated by means of a stathmokinetic technique. Both 5,6-DHT and 5,7-DHT were cytotoxic at each dose tested and for each of these agents the percentage of necrotic cells was directly correlated with the dose of drug used.*

BOL was not found to be cytotoxic to the colonic carcinomata, whereas both methysergide and cyproheptadine did cause detectable tumour cell necrosis. Methysergide was also found to accelerate tumour cell proliferation, whereas cyproheptadine did not. BOL competitively inhibited the cytotoxicity of 5,6-DHT and neither methysergide nor cyproheptadine potentiated the effect of 5,6 DHT.

Introduction

Abundant studies have now shown that a variety of biogenic amines are able to influence cell proliferation in a vast array of both animal and plant tissues. Amines with a well-documented ability to influence cell division include the catecholamines epinephrine [11, 12, 20, 33, 36, 37] and norepinephrine [21, 36], indolamines such as 5-hydroxytryptamine (5-HT) [22, 25, 27, 29, 35] and melatonin [8], and histamine [13, 24, 28, 30]. However, so far little therapeutic advance has been gained from this expansion in basic biological knowledge.

Recently it was observed that inhibition of monoamine oxidase stimulated cell proliferation in DMH-induced tumours of rat colon [32], and in view of the fact that monoamine oxidase is important only for the degradation of intracellular amines [26] this was taken as preliminary evidence that colonic tumour cells, unlike their non-neoplastic counterparts, take up biogenic amines. Since cell kinetic studies have shown that 5-HT is able to stimulate cell proliferation in DMH-induced colonic tumours [35] it was decided to explore the effect of 5-HT antagonists and of toxic congeners of 5-HT [5] on DMH-induced tumours. Preliminary reports on the cytotoxicity of a toxic congener of 5-HT, 5,6-DHT [34], and the 5-HT antagonists cyproheptadine and methysergide [1] have already been published. In this report the cytotoxicity of two toxic congeners of 5-HT, 5,6-DHT, and 5,7-DHT and of three 5-HT antagonists, BOL, methysergide, and cyproheptadine is evaluated. In addition, the influence of 5,6-DHT, methysergide and cyproheptadine on tumour cell mitotic rate was studied[1].

Materials and Methods

Male Sprague-Dawley rats were fed Clark Nu-pig pellets and tap water ad libitum and housed at 21–24 °C with artificial light from 07.00–21.00 h and in darkness from 21.00–07.00 h. Rats were given weekly subcutaneous (s.c.) injections of DMH (Aldwich Chemical Co. Inc., Milwaukee, Wis.) at a dose of 21 mg/kg, as previously described [17, 31]. After 20 weeks the DMH injections were discontinued. Following an interval of 2–3 weeks, the animals were used in the experiments described below.

Estimation of Mitotic Rates. Tumour-bearing rats were given vinblastine sulphate (Velbe, Eli Lilly and Co., Indianapolis, Indiana,

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[1] BOL has previously been shown to inhibit cell proliferation in DMH-induced tumours [35]

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USA) at a dose of 4 mg/kg injected i.p. at 12.00 h and were killed by decapitation at times ranging from 12.45–16.00 h. Counts of metaphase and non-metaphase cells in histological sections in colonic adenocarcinomata were made as previously described [31]. All metaphase indices were then corrected for the difference in size between metaphase and non-metaphase nuclei [19]. Details of these correction factors are described elsewhere [31].

Graphs of true metaphase index versus duration of vinblastine treatment were then constructed for each experimental group of tissues having mitoses blocked for periods of 0.75–4.0 h. The regression coefficient for each of the graphs was then calculated; this calculated value represents the rate at which cells enter metaphase and has the units of mitoses per cell per h. The statistical significance of apparent differences between the values of the regression coefficient for different experimental groups of tissue was then estimated by analysis of variance [10].

Initially cell proliferation was studied in five DMH-induced adenocarcinomata taken from rats not treated with any 5-HT antagonist; results from these tumours served as control values. The mitotic rates were also measured in four rats injected with methysergide (Sandoz, Sydney, Australia, Pty., Ltd.) at a dose of 0.1 mg/kg and in four rats with cyproheptadine (Merck Sharp and Dohme, Sydney, Australia) at a dose of 1 mg/kg, and an attempt was made to measure the mitotic rate in four rats treated with 5,6-DHT at a dose of 40 mg/kg.

Evaluation of Cytotoxicity. Rats were given the agents whose cytotoxicity was being evaluated by i.p. injection, and were killed by decapitation 15 h later. Tumours of the transverse or descending colon were then prepared for histological examination. Estimation of the numbers of necrotic and of non-necrotic cells in histological sections of tumours were made in the following way. Sections of tumours were examined at 400× magnification and the number of necrotic and non-necrotic cells per visual field were counted with the aid of an eyepiece with a rectangular graticule. Each successive visual field at an interval of 0.3 mm along two mutually perpendicular axes extending from edge to edge of the histological section was examined in this way and the mean percentage of necrotic cells per tumour calculated. Between 1,000 and 2,000 cells were scored per tumour. Only cells with a distinctly pyknotic nucleus, that is, one lacking the normal vesicular chromatin pattern, were recorded as necrotic. Results from each group of tumours treated in a particular way were conflated and the mean standard error for percentage of necrotic cells was calculated. The statistical significance of the apparent difference between treatment means was estimated by Student's *t*-test.

Initially the percentage of necrotic cells was estimated in tumours taken from untreated animals and from animals injected with either 5-HT, at a dose of 0.1 mg/kg, or the monoamine inhibitor iproniazide at a dose of 50 mg/kg. The possible cytotoxic effects of 5-HT and of iproniazide were assessed because 5,6-DHT has been shown to be both a 5-HT receptor agonist, with about one tenth of the activity of 5-HT itself [3, 6, 14], and a monoamine oxidase inhibitor [16]. The cytotoxicity of 5,6-DHT at doses ranging from 0.1–100 mg/kg and 5,7-DHT at doses ranging from 1–400 mg/kg was then evaluated. Both 5,6-DHT and 5,7-DHT were stored at 0–4 °C under dry nitrogen and dissolved in 3 ml aqueous sodium ascorbate solution (1 mg/ml) immediately prior to i.p. injection. The upper dose limits for 5,6-DHT and 5,7-DHT were based on their reported general toxicity [15]. The cytotoxicity of BOL at an i.p. dose of 1 mg/kg, methysergide at i.p. doses ranging from 0.1–100 mg/kg, and cyproheptadine at i.p. doses ranging from 0.1–50 mg/kg was also assessed. The upper dose limits for BOL and methysergide were chosen because of the low or declining toxicity of the doses used (see results) and the upper dose level for cyproheptadine was based on its reported general toxicity [2].

In the next series of experiments the cytotoxicity of 5,6-DHT administered with 5-HT antagonists was evaluated: BOL (1 mg/kg), methysergide (0.1 mg/kg) and cyproheptadine (1 mg/kg) being used. Finally, the percentage of necrotic cells in tumours taken from animals treated concurrently with 5,7-DHT (100 mg/kg) and vincristine (Oncovin, Eli Lilly and Co., Indianapolis, Indiana, USA) at a dose of 0.1 mg/kg was estimated. This experiment was undertaken because a number of disubstituted tryptamines have been shown to activate 5-HT receptors [3, 14, 16] and 5-HT has been shown to stimulate cell division in DMH-induced tumours [34]; thus unless 5,7-DHT is cytotoxic in the presence of a metaphase-blocking drug its cell killing effect may be outweighed by its proliferogenic effect.

Results

Mitotic Rates. In rats treated with 5,6-DHT the presence of large numbers of necrotic tumour cells and disrupted tumour acini made the counting of metaphase and non-metaphase cells unreliable, and hence no estimate of the tumour cell mitotic rate was possible. Mitotic rates for tumour cells in control rats and in rats treated with methysergide or cyproheptadine are shown in Table 1. In addition the previously reported tumour cell mitotic rate for BOL-treated rats [35] is included for comparison.

Cytotoxicity. In DMH-induced tumours taken from control rats an average of 9% of cells were judged necrotic. In tumours taken from rats treated 15 h earlier with 5-HT (0.1 mg/kg) a statistically similar fraction of the cells was found to necrotic (Table 2). In animals treated with iproniazed 12% of the tumour cells were necrotic, an increase from control values which is of borderline statistical significance. However, treatment with 5,6-DHT (Table 2) or 5,7-DHT (Table 3) resulted in a highly significant increase in the percentage of necrotic cells at each dose tested. In each case, the level of cytotoxicity showed a direct correlation with the dose of di-

Table 1. Mitotic rates for DMH-induced carcinomata treated with the 5-HT antagonists BOL (1 ml/kg), methysergide (0.1 mg/kg), or cyproheptadine (1 mg/kg)

Treatment	Mitotic rate (mitoses/cell/h)		p[a]
	Mean	SE	
Nil	0.025	0.007	–
BOL	0.005[b]	0.002	< 0.025
Methysergide	0.054	0.011	< 0.025
Cyproheptadine	0.039	0.007	0.1–0.05

[a] Probability values relate to differences from untreated animals
[b] This result has been published previously [35] and is included here only for comparison

Table 2. Cytotoxicity of 5,6-DHT-induced adenocarcinomata

AATreatment	*n*	% of necrotic cells 15 h after injection		p^a
		Mean	SE	
Nil (control)	4	9	1	–
5-HT (0.1 mg/kg)	4	10	3	> 0.2
Iproniazide (50 mg/kg)	3	12	2	< 0.05
5,6-DHT (0.1 mg/kg)	3	21	4	< 0.001
5,6-DHT (1.0 mg/kg)	3	26	5	< 0.001
5,6-DHT (10 mg/kg)	5	23	2	< 0.001
5,6-DHT (20 mg/kg)	2	31	5	< 0.001
5,6-DHT (40 mg/kg)	3	36	5	< 0.001
5,6-DHT (80 mg/kg)	4	60	10	< 0.001
5,6---DHT (100 mg/kg)	4	43	7	< 0.001

[a] Probability values relate to the differences from untreated animals

Table 3. Cytotoxicity of 5,7-DHT in DMH-induced adenocarcinomata

Dose of 5,7-DHT (mg/kg)	*n*	% of necrotic cells 15 h after injection		p^a
		Mean	SE	
Nil (control)	4	9	1	–
1	4	26	7	< 0.001
10	2	34	1	< 0.001
100	2	53	8	< 0.001
400	2	79	19	< 0.01

[a] Probability values relate to the differences from untreated animals

Table 4. Cytotoxicity of 5-HT antagonists. Drugs given i.p. 15 h before sacrifice of animals

5-HT Antagonist	Dose mg/kg	No of tumours	% of necrotic cells 15 h after injection		p^a
			Mean	SE	
Nil	–	4	9	1	–
BOL	1	3	9	3	> 0.9
Methysergide	0.1	3	30	7	< 0.001
	1	3	27	9	< 0.01
	5	3	27	5	< 0.001
	100	3	15	3	< 0.01
Cyproheptadine	0.1	3	24	9	< 0.01
	1	3	25	4	< 0.001
	10	3	19	3	< 0.001
	50	3	24	2	< 0.001

[a] Probability values relate to differences from untreated animals

Table 5. Influence of 5-HT antagonists on the cytotoxicity of 5,6-DHT

Treatment	*n*	% of necrotic cells 15 h after injection		p^a
		Mean	SE	
5,6-DHT (1 mg/kg) alone	3	26	5	–
+ BOL	6	8	2	< 0.001
+ Methysergide	6	36[b]	8	< 0.05
+ Cyproheptadine	3	35[c]	6	< 0.05
5,6-DHT (40 mg/kg) alone	3	36	5	–
+ BOL	5	19	1	< 0.001
+ Methysergide	3	44[b]	9	0.1–0.2
+ Cyproheptadine	3	22[c]	3	< 0.02
5,6-DHT (100 mg/kg) alone	4	43	7	–
+ Methysergide	4	44[b]	16	> 0.5
+ Cyproheptadine	5	29[c]	6	< 0.01

[a] Probability values relate to differences from animals treated with the corresponding dose of 5,6-DHT and no 5-HT antagonists
[b] Not significantly different from value obtained for methysergide alone
[c] Not significantly different from value obtained for cyproheptadine alone

hydroxytryptamine injected (for 5,6-DHT $r = 0.86$, $P < 0.05$; for 5,7-DHT $r = 0.96$, $P < 0.01$).

Estimates for the percentage of necrotic tumour cells in rats treated with 5-HT antagonists are shown in Table 4. It should be noted that BOL did not appear to be cytotoxic, whereas methysergide and cyproheptadine showed distinct cytotoxic effects. In the case of cyproheptadine the level of cytotoxicity was independent of the dose used ($r = 0.06$, $P > 0.9$), whereas for methysergide there was a significant negative correlation ($r = -0.98$, $P < 0.05$) between dose and cytotoxicity.

BOL was found to antagonize the cytotoxic effect of 5,6-DHT at doses of 1 mg/kg and 40 mg/kg (Table 5). Because of its intrinsic cytotoxicity it was not possible to evaluate the blocking effect of cyproheptadine against 5,6-DHT at doses of 1–40 mg/kg, but cyproheptadine was found to inhibit the effect of 5,6-DHT at 100 mg/kg. Methysergide did not antagonize the effect of 5,6-DHT in any dose tested.

Concurrent administration of vincristine with 5,7-DHT did not appear to influence the cytotoxicity of 5,7-DHT.

Discussion

The results just presented show that 5,6-DHT, 5,7-DHT, cyproheptadine, and methysergide are cytotoxic to DMH-induced colonic carcinomata, whereas 5-HT, its antagonist BOL, and the monoamine oxidase inhibitor iproniazid each have little if any toxic effect on these tumours. Monoamine oxidase inhibition has been reported for 5,6-DHT [16] and for both cyproheptadine and methysergide [18], and appears to be the only pharmacological property shared by these cytotoxic compounds. However, monoamine oxidase inhibition per se does not appear to result in extensive tumour cell necrosis, and hence it is not possible at present to define a single pharmacological property of these 5-HT related compounds which is directly correlated with their tumour cytotoxicity.

The primary site of action of these cytotoxic compounds is difficult to determine at present but could feasibly be located somewhere outside the tumour (e.g., in the autonomic nervous system or some endocrine gland), somewhere in the stroma of the tumour (e.g., in mast cells or in blood vessels), or in the tumour cells themselves. In favour of the last option are the observations that ^{14}C-labelled 5,6-DHT and 5,7-DHT are bound mainly to mitochondria [4] and that the first morphological sign of 5,6-DHT cytotoxicity in tumour cells is mitochondrial damage [34].

The biochemical mechanism for the cytotoxicity of dihydroxytryptamines has been extensively studied in neural tissues [4, 5, 7, 9, 23]. In aqueous solution at pH 7, 5,6-DHT and 5,7-DHT undergo spontaneous oxidation to form an indole-o-quinone and an indole-p-quinoneimine respectively. Both of these substances could then covalently bind to a variety of cell constituents [4]. Alternatively it has been suggested that dihydroxytryptamines may generate peroxide radicals during their antioxidation [4]. This suggested property of dihydroxytryptamines has only been confirmed for 6,7-dihydroxytryptamine [23] and the ability of 5,6-DHT and 5,7-DHT to act in this way does not appear to have been reported.

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