Effect of the nitroimidazole Ro 03-8799 on the activity of chemotherapeutic agents against a murine tumour *in vivo*

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Summary The effect of the 2-nitroimidazole Ro 03-8799 (8799) on the activity of 11 chemotherapeutic agents against the anaplastic MT tumour in mice has been determined by soft agar cloning. The 8799, whilst producing little cytotoxicity by itself, potentiated the cytotoxic actions of the alkylating agents melphalan and cyclophosphamide, and the nitrosoureas BCNU, CCNU and MeCCNU. This potentiation was influenced by the time interval between the administration of 8799 and the chemotherapeutic agents, and also by the site of tumour implantation. However, 8799 did not potentiate the cytotoxicities of the compounds CBDCA, cisplatin, adriamycin, vincristine, 5-fluorouracil and bleomycin. A review is included of the reported *in vivo* effects of nitroimidazoles on the chemotherapeutic agents investigated here.

Following reports that both *in vitro* and *in vivo*, some chemotherapeutic agents may preferentially spare hypoxic tumour cells (Hill & Stanley, 1975; Sutherland *et al.*, 1979), many studies have been initiated to determine the benefit of combining these agents with the known hypoxic cell cytotoxins, nitroimidazoles. In 1979 Kelly *et al.* reported that the 5-nitroimidazole, metronidazole, increased methotrexate activity *in vivo*. Subsequently, many investigators have reported that the 2-nitroimidazole, misonidazole (miso) potentiates the activity of many chemotherapeutic agents against a variety of tumours (see review by McNally, 1982).

We recently reported potentiation by many nitroimidazoles of melphalan activity against intramuscular anaplastic MT tumours in mice (Sheldon *et al.*, 1982). Maximum potentiation occurred when the nitroimidazoles were given 0–30 min before the melphalan. The most effective compound, Ro 03-8799, was about twice as effective as miso, and at a dose of 0.72 mg g^{-1} i.p. enhanced melphalan activity by a factor of 2.2.

We report here on the ability of 8799 to potentiate another ten anti-cancer drugs. The importance of the interval between administration of 8799 and the drugs has been determined. Full dose response survival curves have been obtained when the 8799 was given 15 min before the chemotherapeutic agent and also at the optimum interval (if any) determined from the time course studies. As the present work was performed using s.c. implanted tumours, whereas i.m. implanted tumours were used in the previous studies, the influence of tumour site has also been investigated.

Materials and methods

Mice and tumours

The anaplastic MT tumour was implanted by injection s.c. over the sacral region of the back of 8–10 week old male inbred WHT/Cbi mice. The mice were treated when the tumours attained a mean diameter of 6–7 mm (8–11 days after inoculation).

Cytotoxic agents

All agents were administered i.p. at 0.5 ml per 25 g body wt. The agents were always freshly prepared as follows:

8799 Ro 03-8799, a 2-nitroimidazole supplied by Dr C.E. Smithen of Roche Products Ltd, was reconstituted as required in isotonic saline and administered at a dose of 0.72 mg g^{-1} .

8800 Ro 03-8800; as for 8799 except that the dose was 0.73 mg g^{-1} . 8800, used here only to supplement 8799 data in 2 time course experiments when further 8799 supply was unavailable, has previously been shown to be of similar effectiveness as 8799 at potentiating melphalan (Sheldon *et al.*, 1982).

CYC Cyclophosphamide 100 by Farmitalia Carlo Erba Ltd; 100 mg vials were reconstituted with 5 ml distilled water injection and diluted as required with isotonic saline.

BCNU Carmustine by National Institutes of Health; 100 mg vials were reconstituted with 3 ml ethanol, 27 ml distilled water injection added, and diluted as required with isotonic saline.

CCNU Lomustine by National Institutes of

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Health; 40 mg capsules were dissolved in 1 ml DMSO and stored frozen in 0.1 ml aliquots. When required the aliquots were thawed at room temperature and diluted as required with 5% Tween 80 in phosphate buffered saline.

MeCCNU Methyl CCNU by National Institutes of Health; 50 mg capsules were treated as per CCNU.

BLEO Bleomycin Lundbeck by Lundbeck Ltd; 15 mg vials were diluted as required with isotonic saline.

cis-Pt Cisplatin by Johnson Matthey Research Centre, supplied by Dr K.R. Harrap of Department of Biochemical Pharmacology, Institute of Cancer Research. The powder was reconstituted as required in isotonic saline.

CBDCA Cis-diamine-1, 1-cyclobutane dicarboxylate platinum (II); obtained and used as for cisplatin.

5FU 5-Fluorouracil by Roche Products Ltd; Vials (250 mg) were diluted as required with isotonic saline.

VCR Vincristine sulphate as Oncovin by Eli Lilly and Co; 1 mg vials were reconstituted as required with isotonic saline.

ADR Adriamycin doxorubicin INN by Montedison Pharmaceuticals Ltd; 10 mg vials were reconstituted with 5 ml distilled water injection and diluted as required with isotonic saline.

MEL Melphalan as Alkeran by Wellcome Foundation Ltd; 100 mg vials were reconstituted in 5 ml 2% HCl in ethanol and diluted as required in isotonic saline.

Clonogenic assay

The technique used has been described in detail previously (Sheldon *et al.*, 1982). Briefly, 2-4 tumour bearing mice were identically treated, 18 h later their tumours were excised, pooled, enzymically disaggregated into a single cell suspension, counted, and known numbers of cells seeded onto soft agar plates. Following 13-15 days incubation at 37°C the resulting colonies were counted and their plating efficiency (PE) calculated from the ratio of the number of colonies counted to the number of cells seeded.

Results

In the course of the present study, 57 groups of untreated s.c. tumours and 4 groups of untreated i.m. tumours were used as controls. Their respective

mean plating efficiencies on incubation were 0.68 (s.d. 0.15) and 0.61 (± 0.05), and their respective mean cell yields g^{-1} tumour were 8.6×10^7 ($\pm 2.1 \times 10^7$) and 1.4×10^8 ($\pm 4.8 \times 10^7$).

Relative to those for the control tumours, the cell yields g^{-1} were generally reduced after treatment with the chemotherapeutic agents (open symbols, Figure 1). This reduction in cell yield has been taken into account when expressing tumour survival. The survival has been expressed as the surviving fraction g^{-1} tumour = relative P.E. $\times$ relative cell yield g^{-1} .

The survival responses of s.c. tumours to single doses of the chemotherapeutic agents are shown in Figure 2. Data for each agent were pooled from 4 or more experiments, and include the drug alone control responses subsequently obtained during the 8799 studies. The response to each drug did not appear to differ significantly between experiments. The survival curves shown in Figure 2 were computed by least squares fit analysis using the mathematical relationship $S = 1 - (1 - \exp(D/D_0)^n)$, with the extrapolation number (n) either not set for those drugs (i.e. BCNU, CCNU, MeCCNU, BLEO) whose responses had an initial shoulder region followed by an exponential decrease in survival as a function of dose, or set at unity, for those drugs (i.e. VCR, 5FU, ADR, CisPt, CBDCA, CYC) whose responses had no shoulder region before survival decreased exponentially as a function of dose. It is evident from Figure 2 that this tumour is responsive to all the ten chemotherapeutic agents studied.

The effect of 8799 on the response to the chemotherapeutic agents was initially investigated by time course studies. A dose of chemotherapeutic agent was selected (from Figure 2) that would give about a decade of cell kill, and the 8799 was administered as a single i.p. dose from 9 h before to 8 h after the chemotherapeutic agent (Figures 3 and 4). Though there was much scatter in the time course data, the optimum interval to achieve maximum cytotoxicity was taken to be when the 8799 was administered immediately before CYC, 30 min before MeCCNU, either 15 min or 4 h before BCNU, 1 h before 5FU, CCNU and (possibly) VCR. The 8799 appeared to have little effect on the other agents and hence no optimum times could be deduced.

The qualitative effects of 8799 described above were subsequently quantified by derivation of full dose response curves. The 8799 was administered either at 15 min or at the optimum time, before the chemotherapeutic agents. The resulting survival curves are shown in Figures 5 and 6. The responses to the chemotherapeutic agents alone have been redrawn from Figure 2 (omitting data points for clarity). This was done as the responses to the

Figure 1 The cell yield g^{-1} of s.c. tumour obtained after treatment with the chemotherapeutic agents relative to that for untreated tumours. Saline ($\circ$) or 8799 ($\bullet$) given 15 min before, or 8799 given at "optimum time" before ($\blacksquare$), chemotherapeutic agent. The drug dose for each agent has been scaled as shown by the factor "x".

chemotherapeutic agents did not differ here between experiments and as these curves represented more data than those available as concurrent drug alone controls (these data having been included in Figure 2). The survival curves for the 8799 plus chemotherapeutic agents in Figures 5 and 6 were computed as described above for the chemotherapeutic agents alone except that, to allow for the cytotoxicity of 8799 itself, n was correspondingly reduced. However, as the 8799 alone toxicity was relatively small (the geometric mean survival after 8799 alone was 0.73), the application or otherwise of this correction made no substantial difference to the conclusions reached.

These were that 8799 did not increase the toxicity of CBDCA, did give an additive increase with *cis*Pt, ADR, VCR, BLEO and 5FU, and potentiated the toxicities of CYC, MeCCNU, CCNU and BCNU.

As this potentiation was relatively small compared to that reported previously for 8799 in combination with melphalan (Sheldon *et al.*, 1982), and as that study employed i.m. tumours, this combination has been retested here both in s.c. and i.m. tumours. The responses, shown in Figure 7, were computed as described above for CYC. The drug enhancement ratios (DER), calculated from the ratio of the computed D_{05} s for 8799 plus melphalan response to melphalan-alone response,

Figure 2 Survival responses of s.c. tumours treated with the chemotherapeutic agents (given 15 min after a dose of saline).

were 1.5 and 2.0 for s.c. and i.m. tumours respectively. This difference in DER reflects the poorer response of i.m. tumours to melphalan since the responses of 8799 in combination with melphalan are similar for both tumour sites.

Discussion

The effect of the 2-nitroimidazole 8799 on the activity of chemotherapeutic agents against the anaplastic MT tumour is summarised in Table I. Bearing in mind that many drugs do have more than one mode of action, it can be seen that 8799 did not (according to the sem. of the dose enhancement ratios) significantly potentiate the

activity of the cross linking CBDCA and *cisPt*, the intercalating ADR, the DNA fragmenting BLEO, the mitotic inhibitor VCR, the anti-metabolite 5FU, but did significantly potentiate MEL, CYC, BCNU, CCNU and MeCCNU which possess alkylating activity and, in the case of the nitrosoureas, also carbamoylating activity.

The failure to observe chemopotential by 8799 of the nonalkylating agents is unlikely to be because of the use of suboptimal intervals since extensive time course studies were done. Though these data showed much scatter, there was clearly no optimum time for the administration of 8799 relative to CBDCA, *cisPt*, ADR and BLEO, and although an interval of 60 min between 8799 and 5FU and VCR appeared optimal, this is thought to reflect only their additive toxicities. Of the agents

Figure 3 The effect of a single dose of 8799 given at various times either before or after a dose of CYC (25 mg kg^{-1}), MeCCNU (15 mg kg^{-1}), BCNU (30 mg kg^{-1}), 5FU (200 mg kg^{-1}) or CCNU (10 mg kg^{-1}). —, shows the response to the chemotherapeutic agent alone derived from Figure 2.

Figure 4 The effect of a single dose of 8799 (●) or 8800 (■) given at various times either before or after a dose of *cis*Pt (4 mg kg^{-1}), CBDCA (100 mg kg^{-1}), BLEO (500 mg kg^{-1}), ADR (20 mg kg^{-1}) or VCR (2 mg kg^{-1}). —, shows the response to the chemotherapeutic agent alone derived from Figure 2.

Figure 5 The effect of 8799 on the survival response of s.c. tumours to 4 chemotherapeutic agents. Drug alone responses redrawn from Figure 2 (—), and 8799 given either 15 min (●) or at optimum time (○) before chemotherapeutic agent.

Figure 6 The effect of 8799 on the survival response of s.c. tumours to 6 chemotherapeutic agents. Drug alone responses redrawn from Figure 2 (—), and 8799 given either 15 min (●) or at optimum time (○) before chemotherapeutic agent. (N.B. the optimum time for BCNU being taken as 4 h.).

Figure 7 Potentiation by 8799 of melphalan activity against s.c. and i.m. tumours. Saline (○) or 8799 (●) given 15 min before melphalan. DERs are shown.

that were potentiated, the probable optimum time to administer 8799 was 4 h before BCNU, 60 min before CCNU, 30 min before MeCCNU and immediately before CYC. For CCNU and MeCCNU at least, these timings appear critical. Little, if any, potentiation occurred when the 8799 was given after any of the chemotherapeutic agents.

It can be seen in Table I that (on the basis of $DER \pm \text{sem}$) of those chemotherapeutic agents significantly potentiated against s.c. tumours by 8799, MEL and CCNU were more responsive than MeCCNU which in turn was more responsive than either CYC or BCNU. However, the DERs of 1.5 for even the most responsive agents MEL and CCNU are relatively small compared to those reported by McNally (1982) in his review of nitroimidazole potentiation of chemotherapeutic activity. An explanation for the relatively low DERs observed in the present studies could be the routine use of s.c. tumours, for we have also shown in these studies that for MEL at least, the potentiation by 8799 is less in s.c. than i.m. tumours. Another possible explanation, though not investigated, is that although 8799 has previously been reported to be the most effective nitroimidazole examined by us at potentiating melphalan activity against the MT tumour (Sheldon *et al.*, 1982), it may not be the most effective for the other chemotherapeutic agents used here.

The mechanism of nitroimidazole potentiation of chemotherapeutic drug activity is currently receiving much attention. We have previously shown that for MEL, potentiation of the MT

tumour does not result from nitroimidazole-induced hypothermia or elimination of the ability to recover from potentially lethal melphalan damage. Further, the degree of potentiation is influenced by the lipophilicity and electron-affinity of the nitroimidazoles (Sheldon & Batten, 1982). The present study shows that the increase in toxicity when 8799 is combined with chemotherapeutic agents must occur as a reduction in plating efficiency at cloning since no reduction in cell yield at tumour disaggregation is indicated in Figure 1. This differs from the report by Stephens *et al.* (1981) that in the Lewis Lung carcinoma, the additive toxicity that occurred when the nitroimidazole miso was combined with various chemotherapeutic agents was expressed as a reduced cell yield rather than reduced plating efficiency.

Data from the literature on the *in vivo* effects of nitroimidazoles on the chemotherapeutic agents investigated here are reviewed in Table II. The objective of this table is to show those chemotherapeutic agents most frequently potentiated by nitroimidazoles. For each tumour at any laboratory only the first reported potentiation has been cited, irrespective of considerations such as nitroimidazole, drug dose, or time interval employed. It can be seen from the lowest row of the table that there are no reported cases of potentiation of CBDCA, *cis*Pt or BLEO activities, potentiation of 5FU, VCR or ADR activities occurred in a third of the tumours examined, and potentiation of BCNU, MeCCNU, CCNU, MEL

Table I Summary of the effect of 8799 given either 15 minutes or at the optimum time before the chemotherapeutic agents

<i>Agent</i>	$D_0^a (\pm sem)$			$DER^b (\pm sem)$	
	<i>No.</i> 8799	8799 at 15 min	8799 at Opt. time	8799 at 15 min	8799 at Opt. time
CBDCA	50.9 (6.6)	64.5 (5.8)	—	0.8 (0.1)	—
<i>cis</i> Pt	1.85 (0.03)	1.83 (0.05)	—	1.0 (0.0)	—
ADR	18.6 (2.2)	16.2 (1.3)	—	1.1 (0.2)	—
BLEO	309 (36)	283 (39)	—	1.1 (0.2)	—
VCR	1.66 0.14	1.56 0.15	1.45 ^e 0.24	1.1 (0.1)	1.1 (0.2)
5FU	1.03 (0.08)	0.93 (0.04)	0.87 ^e (0.10)	1.1 (0.1)	1.2 (0.2)
CYC	13.0 (0.5)	10.8 (0.7)	9.8 ^e (0.9)	1.2 (0.1)	1.3 (0.1)
BCNU	9.16 (1.20)	7.61 (0.33)	7.32 ^f (0.53)	1.2 (0.2)	1.3 (0.2)
CCNU	3.44 (0.61)	2.78 (0.12)	2.25 ^e (0.14)	1.2 (0.2)	1.5 (0.3)
MeCCNU	6.85 (0.48)	5.02 (0.20)	4.84 ^d (0.21)	1.4 (0.1)	1.4 (0.1)
MEL (sc)	1.66 (0.14)	1.08 (0.11)	—	1.5 (0.2)	—
MEL (im)	2.34 (0.19)	1.16 (0.12)	—	2.0 (0.3)	—

^a $D_0 \pm s.e. (mg\ kg^{-1})$ determined at the computation (as described above) of the survival curves shown in **Figures 5 and 6**.

^b $DERs$ calculated from D_0 as described in text.

Optimum times: ^cimmediately; ^d30 min; ^e60 min and ^f4 h before.

Table II Review of the potentiation *in vivo* by nitroimidazoles of the activities of the chemotherapeutic agents investigated here against murine and human xenograft (HX) tumours
(+ = significant potentiation; - = no significant potentiation)

Tumour	Chemotherapeutic Agent										
	CBDCA	Cis Pt	BLEO	5FU	VCR	ADR	BCNU	MeCCNU	CCNU	MEL	CYC
MT	-1	-1	-1	-1	-1	-1	+1	+1	+1	+1	+1
Lewis Lung		-2	-2	+6	+2	-6		-4	+2	+6	+6,21
SCC		-3	-3	+3	-3	-3	-3		+14	+3	-22
KHT				-7		+7	+10,11	+13	+14,15,16	+16	+3
M5076		-4		-8				+4		+18	+10,15,23
Fab			-5			-5				+4	+4
l6c				-7		+7			+15,17	+19,27	+5
RIF									+14	-16	+10
EMT6										+5	+23,24
NTa										+5	+5
Fib/T										+20	+20
B16											-4
FSa										+25	
SaF											+25
MT-1									+15		
MAC-15				-9							
P388											
HX 32		-2						+2		+2	+21
HX 34							+12			+12	+2
HX 47							+12			+12	
No. potentiated/ No. examined	0/1	0/5	0/4	2/7	1/3	2/6	4/5	4/5	7/7	12-13/14	12-13/14
% No. potentiated	0	0	0	29	33	33	80	80	100	86-93	86-93

(1) present study; (2) Stephens *et al.* (1981); (3) Fu *et al.* (1982); (4) Clement *et al.* (1980); (5) Randhawa *et al.* (1982); (6) Rose *et al.* (1980); (7) Tannock (1980a); (8) Clement *et al.* (1982); (9) Kelly *et al.* (1979); (10) Tannock (1980b); (11) Mulcahy *et al.* (1981); (12) Clutterbuck *et al.* (1982); (13) Mulcahy (1982); (14) Hirst *et al.* (1982); (15) Siemann (1981); (16) Workman & Twentyman (1982); (17) Twentyman & Workman (1982); (18) Siemann & Mulcahy (1982); (19) Hirst & Brown (1982); (20) Martin *et al.* (1981); (21) Wodinsky *et al.* (1979); (22) Pedersen *et al.* (1982); (23) Twentyman (1981); (24) Law *et al.* (1981); (25) Murray & Meyn (1983); (26) McNally *et al.* (1983); (27) Twentyman & Workman (1983).

or CYC occurred in more than three-quarters of the tumours examined. Since greater potentiation of MEL activity has been reported in tumours than dose-limiting normal tissues (review, McNally, 1982), a therapeutic gain may be anticipated clinically. It follows from Table II that the most promising chemotherapeutic agents for consideration for clinical trial in combination with nitroimidazoles are the alkylating agents, be they the direct acting MEL or enzymically activated CYC (Sladek, 1973), or the nitrosoureas BCNU, MeCCNU or CCNU.

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