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CYTOTOXIC AND RADIOSENSITIZING EFFECTS OF HYPOXIC CELL SENSITIZERS ON EMT6 MOUSE MAMMARY TUMOUR CELLS *IN VIVO* AND *IN VITRO*

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Summary.—The radiosensitizing activities and toxicities of misonidazole, metronidazole, 2-Methylthio-3-nitropyrrole (3NPR), 2-amino-5-nitrothiazole (ANT) and 2,5-dinitroimidazole (KA121) were studied using EMT6 tumour cells and BALB/c KaRw mice. For 10^{-4}M concentrations of the drugs, the DMF's for hypoxic cells were: Misonidazole, 1·6; 3NPR, 1·5; ANT, 1·5; KA121, 1·8. The OER was 3·2. In general, the drugs were toxic to hypoxic cells and were not toxic to aerobic cells at 10^{-3}M for 24 h. The LD_{50}'s in BALB/c KaRw mice were: misonidazole, 1500 mg/kg; 3NPR, 350 mg/kg; ANT, 420 mg/kg. *In vivo*, 1 mg/g misonidazole or metronidazole reduced the terminal slope of the tumour cell survival curve by a factor of 2·2 or 1·6 respectively.

HYPOXIC cell sensitizers are extremely promising therapeutic adjuvants, but the clinical use of existing compounds, such as misonidazole and metronidazole, is limited by undesirable effects of the drugs, such as neurotoxicity and mutagenicity. Bacterial data presented at this conference by Rupp, Mroczkowski and Agrawal (1978) show that there are compounds which sensitize hypoxic *E. coli* as well as, or better than misonidazole, but have other characteristics, such as their mutagenicities and toxicities to bacteria, which are quite different from those of metronidazole and misonidazole. Such compounds might have clinical advantages over the drugs now in clinical trials. This paper presents preliminary data defining the effects of 3 of these compounds on EMT6 tumour cells and on BALB/c mice and also presents data on the effects of misonidazole and metronidazole on the same tumour/host system.

MATERIALS AND METHODS

Animals and tumours.—Three month old BALB/c KaRw mice were used in these studies. The survival of cells from EMT6 tumours were measured by preparing single cell suspensions from the tumours and assaying the ability of the cells to form colonies in cell culture (Rockwell, Kallman and Fajardo, 1972; Rockwell, 1977). EMT6 cells were grown in cell culture as described previously (Rockwell *et al.*, 1972) and used for experiments during exponential growth. Cells were made hypoxic either by placing concentrated cell suspensions in small ampules, gassing them with 5% CO_2 in N_2, sealing them, and allowing the cells to metabolize the residual oxygen or by gassing TPX Petri dishes containing exponentially growing monolayers with 5% CO_2 in N_2 for at least 1 h. Cell survival was assayed by colony formation *in vitro* as described previously (Rockwell *et al.*, 1972; Rockwell, 1977).

Drugs.—2,5-dinitroimidazole was synthesized by Dr K. Agrawal at Tulane University. 2-Methylthio-3-nitropyrrole was synthesized by Dr H. Hamberger at Sandoz Forschunges Institut and obtained through the courtesy of Dr A. Sartorelli. Misonidazole was a gift from Dr W. E. Scott at Hoffman-LaRoche. Metronidazole was a gift from E. Roman at Searle. 2-amino-5-nitrothiazole was purchased from Aldrich. The structures of ANT, KA 121 and 3NPR are shown in figure 3.

Irradiation.—Animals were irradiated whole body without anesthesia using 250 kV X-rays at a dose rate of approximately 100 rad/min. Cells were irradiated with 120 kV or 250 kV X-rays at a dose rate of approximately 200 rad/min. LD_{50}'*s.*—The LD_{50}'s were

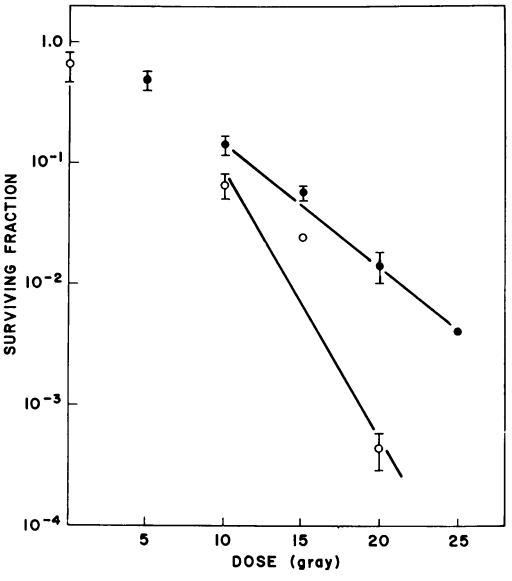

FIG. 1.—Effect of misonidazole on the survival of tumour cells irradiated *in vivo*. ●: irradiation only; ○: 1 mg/g misonidazole 1 h before irradiation.

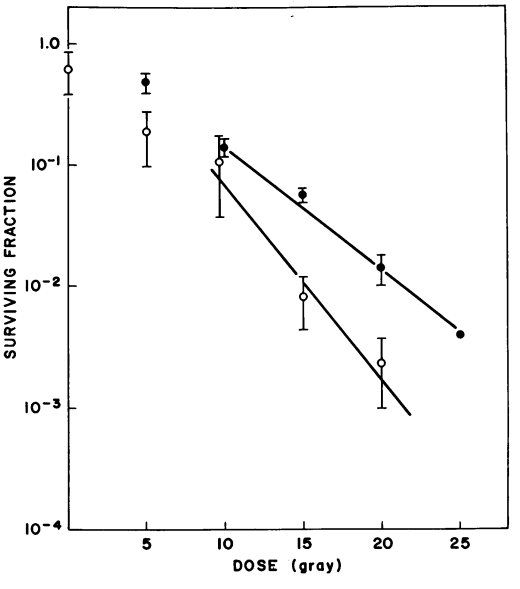

FIG. 2.—Effect of metronidazole on the survival of tumour cells irradiated *in vivo*. ●: irradiation only; ○: 1 mg/g metronidazole 1 h before irradiation.

determined by injecting mice i.p. with graded doses of drugs in solutions (misonidazole) or saline slurries (3NPR, ANT) and observing deaths among the animals for 30 days.

RESULTS

Misonidazole

Misonidazole was an effective radiosensitizer of hypoxic cells *in vitro*, reducing the D_0 of the hypoxic cell survival curve by a factor of 1·6 at 10^{-4}M (Table). *In vitro*, 10^{-3}M misonidazole was not toxic to aerobic cells with incubation times of up to 24 h and did not alter the radiosensitivity of aerobic cells. Misonidazole was slightly toxic to hypoxic cells: 10^{-4}M misonidazole reduced survival to 0·94 after 2 h and 0·85 after 5 h of incubation; 10^{-3}M misonidazole reduced survival to 0·94 after 2 h and 0·61 after 5 h of incubation.

In vivo, injection (i.p.) of 1 mg/g of misonidazole 1 h before the end of irradiation resulted in significant sensitization of the tumour cells, as shown in Fig. 1. The D_0 of the terminal portion of the cell survival curve, which reflects the survival of the 35% of the tumour cells which are hypoxic (Rockwell and Kallman, 1973), was 4·3 Gy without metronidazole and 2·0 Gy with misonidazole. The dose modifying factor (DMF) calculated from the ratio of the D_0's was 2·2. misonidazole at this concentration was slightly toxic to the tumour cells (Fig. 1). The toxicity was not sufficient to perturb the growth of the tumours: neither a single injection of 1 mg/g of misonidazole nor 5 daily injections of 0·5 mg/g of misonidazole significantly perturbed the tumour growth curve. mice injected with 1 mg/g of misonidazole showed signs of toxicity, including loss of coordination and drowsiness. The LD_{50} for misonidazole in BALB/c KaRw mice was 1·5 mg/g. Mice killed by the drug died between 2 h and 8 days after treatment.

Metronidazole

In vivo, injection (i.p.) of 1 mg/g metronidazole 1 h before the end of irradiation, reduced the D_0 of the tumour cell survival curve from 4·3 Gy to 2·65 Gy (DMF 1·6) (Fig. 2). metronidazole was

TABLE.—*Radiosensitization of Hypoxic Cells by the Experimental Drugs*

Treatment	D_0 (Gy)	DMF*
Hypoxic cells	5·4	—
10^{-4}M Misonidazole	3·4	1·6
10^{-4}M 3NPR	3·7	1·5
10^{-4}M ANT	3·65	1·5
10^{-4}M KA121	2·9	1·8
Aerobic cells	1·65	3·2

*The DMF = ratio of the D_0's of the survival curves for hypoxic cells without and with the drug.

slightly toxic to EMT6 tumour cells *in vivo* (Fig. 2), but neither a single injection of 1 mg/g of metronidazole nor 5 daily injections of 0·5 mg/g of Metronidazole perturbed the growth of the tumours.

2-methylthio-3-nitropyrrole (3NPR)

3NPR was an effective radiosensitizer of hypoxic cells. The dose modifying factor of 1·5 obtained with 10^{-4}M 3NPR was similar to that obtained with misonidazole at the same concentration (Table). The radiosensitivity of aerobic cells was not altered by 10^{-4} or 10^{-3}M 3NPR (the maximum concentration obtained in cell culture medium). Neither 10^{-4}M nor 10^{-3}M 3NPR was toxic to aerobic cells for incubation times of up to 24 h or to hypoxic cells for incubation times of up to 5 h.

The LD_{50} of 3NPR in BALB/c KaRw mice was approximately 350 mg/kg. All animals killed by the drug died within 1 day after treatment.

2-amino-5-nitrothiazole (ANT)

The radiosensitizing effect of 10^{-4}M ANT was similar to that of metronidazole at the same concentration (Table). At a concentration of 10^{-3}M, ANT reduced the D_0 of the hypoxic cell survival curve to approximately 2·2 Gy (DMF 2·5). Neither 10^{-4}M nor 10^{-3}M ANT was toxic to aerobic cells for incubation times of up to 24 h. ANT was toxic to hypoxic cells on long incubations: 10^{-4}M ANT reduced survival to 0·94 in 2 h, 0·4 in 5 h, and 0·008 in 24 h.

The LD_{50} of ANT in BALB/c KaRw mice was 420 mg/kg. All animals killed by the drug died within 1 day after treatment.

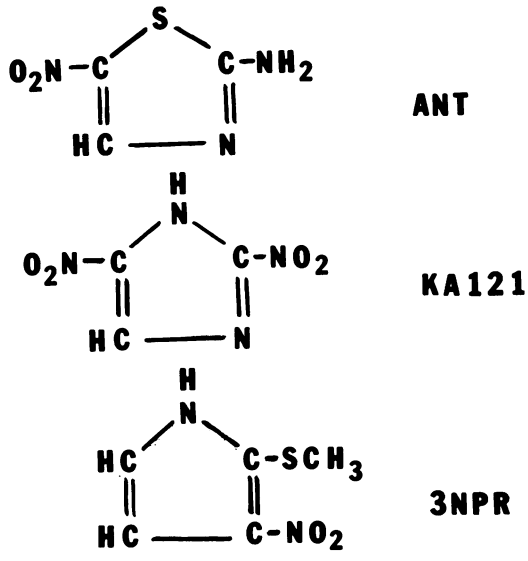

FIG. 3.—Structures of 2-amino-5-nitrothiazole (ANT), 2,5-dinitroimidazole (KA 121) and 2-methylthio-3-nitropyrrole (3NPR).

2,5-dinitroimidazole (KA121)

Preliminary experiments suggest that the radiosensitization obtained with KA121 may be greater than that obtained with misonidazole (Table). Data on the toxicity of this compound and its effects on aerobic cells are not yet available.

DISCUSSION

The results reported here for metronidazole and misonidazole are similar to those reported by others using EMT6 tumours (Brown, 1975) and other systems (Asquith *et al.*, 1974; Moore, Palcic and Skarsgard, 1975; Rauth and Kaufman, 1975). The effects of 3NPR, ANT, and KA121 in bacterial systems have been reported previously by Rupp *et al.* (1977). These drugs have radiosensitizing effects similar to (3NPR, ANT) or better than (KA121) misonidazole in *E. coli*, but differ from misonidazole in other effects, such as mutagenicity, toxicity, and induction of β-galactosidase. The preliminary data reported here show that these three compounds are effective radiosensitizers of hypoxic mammalian cells. As in bacteria, radiosensitization by

10^{-4}M 3NPR, ANT, and misonidazole were similar, and preliminary data suggest KA121 might produce greater sensitization. 3NPR appears to be less toxic to hypoxic cells than misonidazole or ANT. However, the LD_{50}'s of ANT and 3NPR are lower than that of misonidazole. The toxicities of the compounds are being studied further in BALB/c mice and the radiosensitizing effects of the drugs are being examined further using EMT6 tumour cells *in vitro* and *in vivo*.

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