Hypoxia-Mediated Drugs for Radiation and Chemotherapy

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THE LIKELIHOOD that hypoxic cells probably present in most solid tumors may limit, in some cases, the successful local control of these tumors by radiotherapy, has been recognized by radiation oncologists for more than a quarter of a century. However, the possibility that such cells may be an important factor also in the treatment of cancer by chemotherapy has received relatively little attention.

Hypoxic cells arise as a result of tumor growth outstripping the blood supply to the tumor. Tumor cells close to a microcapillary are fairly well oxygenated and, provided the oxygen supply remains available, cell division can occur. However, the oxygen tension decreases with distance from the capillary because of cell respiration and gradually falls to a level insufficient to sustain cell division. Eventually, cell death occurs because of this oxygen deprivation and is responsible for the areas of necrosis which usually develop about 150–200 μm from the nearest blood vessel.[1] There is evidence that the hypoxic cells that occur in the interface region between the well-oxygenated tissue and the necrotic areas, although dormant, can remain potentially viable for a considerable period.

In general, hypoxic cells are radiation-resistant relative to oxic cells and it is now well established, certainly in experimental murine tumor systems, that their radiation resistance is the largest factor influencing local tumor control by radiation. Although in an untreated tumor, hypoxic cells will eventually die, in the event of tumor regression, *i.e.* during or after treatment, some of these hypoxic cells may be reoxygenated, enter cycle and cause tumor regrowth.

In regard to the response of tumors to cytotoxic chemotherapy, hypoxic cells may influence drug action in several ways. Drug accessibility, for example, may be a problem since these cells are usually located some distance from the vascular system in the tumor. Further, cells with a low oxygen status may progress only slowly through the cell cycle, or indeed they may be arrested altogether. Clearly, they would be less susceptible, therefore, to the action of cycle-specific drugs.

This paper discusses firstly some of the advances made in the development of drugs aimed at overcoming the radiation resistance of hypoxic cells in the treatment of human tumors, and secondly, considers the wider implication that hypoxic cells may be a significant factor in the response of solid tumors to cytotoxic chemotherapy.

Hypoxic Cells in Radiotherapy

The original work of Thomlinson and Gray[1] posed two fundamental questions. Firstly, do hypoxic cells occur in human tumors? And, secondly, are they perhaps unimportant—either because the oxygen enhancement ratio is small at the low radiation doses used in conventional fractionated radiotherapy, or because reoxygenation occurring between fractions eliminates the hypoxic cells anyway?

We do, indeed, have some evidence from radiotherapy that hypoxic cells may influence the response of human tumors to radiation. Bush[2] found in an analysis of survival data from patients with Stage IIb and III carcinoma of the cervix treated with radiotherapy that those patients whose hemoglobin level at the time of treatment was greater than 12 mg/dl was significantly better than those patients with a level less than this value. This was confirmed in a prospective study.

Several methods aimed at overcoming the hypoxia problem have been investigated in clinical radiotherapy, including various studies using unconventional fractionation regimes. While some of these have been empirical in design, any influence of radiation scheduling on the proportion of hypoxic cells could, in principle, affect tumor radiation sensitivity. Another approach—treatment in high-pressure oxygen chambers—has been under study for many years. The rationale behind this approach is that the concentration of free oxygen in the blood should increase in patients breathing oxygen at high pressure and thereby extend the diffusion gradient of oxygen to an extent sufficient to reoxygenate hypoxic cells. A few trials have shown

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This review is based on the annual Milpo Schulz Lecture, delivered in the Massachusetts General Hospital, Harvard Medical School, Boston, on June 18th, 1979. The material has been updated in order to represent more appropriately the current position in this rapidly developing field.

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Accepted for publication July 15, 1980.

0008-543X/81/0801/0696 $1.15

some limited benefit,[3,4] but practical problems limit the usefulness of this technique.

Part of the rationale behind the third approach—heavy particle radiotherapy, including neutrons—is that the oxygen enhancement ratio decreases with increasing ionization density of the radiation. This means that the adverse protective effect of tumor hypoxia is reduced. Trials are in progress but the problem remains that this approach requires specialized, expensive hardware and while the oxygen effect is reduced, it is not completely eliminated.

Another method—certainly the preferred approach on the grounds of economy and simplicity—is to use drugs which specifically sensitize the response of hypoxic cells to radiation. The historical background of these drugs is discussed below.

Historical Development of Hypoxic Cell Radiation Sensitizers

Early Studies

The development of chemical compounds that possess the ability to increase the cellular radiation sensitivity is very much tied up with the study of the oxygen effect itself. As many molecular and cellular radiation studies have shown, oxygen is not unique as a radiation sensitizer. It is but one member of a very large class of chemical sensitizers that are able to selectively increase the radiation sensitivity of hypoxic cells of various types without affecting the radiation response of oxygenated cells. Most of these sensitizers belong to the electron-affinic group, so called because of the strong correlation between sensitizing efficiency and the redox properties.

Early discoveries that the radiation response of bean roots (vicia faba) was dependent upon the state of oxygenation, was followed up by many similar observations in irradiated bacteria, bacterial spores and mammalian cells. It was the apparent universality of the oxygen effect that led to the pioneering clinical studies of hyperbaric oxygen by Churchill-Davidson and collaborators.[5]

Around this time, Mitchell[6] introduced the drug Synkavit (synthetic vitamin K) into radiotherapy. However, laboratory studies showed, on the whole, little sensitizing activity by this drug in experimental systems and Synkavit did not enter widespread use. However, it is now believed that the active principle of Synkavit is its dephosphorylated oxidation product, 2-methylnaphthoquinone (menadione), a compound since found to be an active radiation sensitizer for hypoxic bacteria.[7] It is intriguing that, to this day, menadione remains one of the most potent (although highly toxic) sensitizers ever tested in mammalian cell systems *in vitro*.

In 1960, Bridges[8] found that the sulphydryl reagent N-ethylmaleimide, used for many years in the rubber industry as an antoxidant, sensitized the response of irradiated bacteria. In view of current interest at that time in the radiation protective ability of some SH compounds, it was argued that NEM might increase radiation sensitivity by oxidising SH compounds endogenous to the cell. These and other studies showed that sensitization occurred usually (although not always) in the absence of oxygen and brought about a renewed interest in sensitizers suitable for clinical use.

Electron-Affinic Sensitizers

In the early sixties rapid developments were occurring in radiation chemistry following the discovery of the absorption spectrum of the hydrated electron.[9] The new techniques of pulse radiolysis led to the intensive study of reactions of this species, including electron-attachment processes. Evidence that the cellular oxygen effect probably involved fast free-radical processes (miscellaneous early papers quoted by Michael *et al.*[10]) prompted the search for other electron-affinic compounds that might behave similarly.[11] It was found that some compounds that displayed electron attachment properties acted as radiation sensitizers for hypoxic bacteria. Many other sensitizers were found[7] although, unfortunately, most of these early compounds showed little activity in mammalian cells even *in vitro*. However, shortly after, substantial mammalian-cell radiation sensitization was found with nitro-containing aromatic compounds, including paranitroacetophenone (PNAP) and its derivatives.[12,13] It was particularly significant that some nitrofurans were also found to be active[14] since several of these were already in clinical use as antibiotics. Unfortunately, it has since been found that nitrofurans are quite toxic at the doses required for sensitization *in vivo* and generally do not possess favorable pharmacokinetic properties.

Although most sensitizers are ineffective *in vivo*, usually for these reasons, the nitroimidazoles are much more promising at the present time. In 1973, it was found that the 5-nitroimidazole, metronidazole or Flagyl, was a hypoxic cell sensitizer *in vitro*.[15] Numerous studies have since shown significant sensitization of tumor response in a variety of murine tumor models.[16] Further, some clinical evidence exists for sensitizing activity by metronidazole in human glioblastomas treated with radiotherapy.[17]

Misonidazole

The results with metronidazole led to the search for more active compounds in the nitroheterocyclic class

TABLE 1. Sensitization by Misonidazole of Tumor Response to Single Doses of Radiation. Data Collected by Fowler and Denekamp[16] (updated)

Tumor	Assay system	Time of drug administration before irradiation (minutes)	Enhancement ratio for drug dose administration (mg/g)				Reference
			0.1	0.2–0.3	0.5–0.8	1.0–1.5	
C3H Mam. Ca	Local control	30	—	1.7–1.8	—	1.8	16
C3H Mam. Ca	Local control	30	—	—	—	2.3	16
C3H Mam. Ca	Local control	30	—	>1.8	—	2.3	16
C3H Mam. Ca	Local control	30	1.5	—	1.7–1.9	—	19
C3H Mam. Ca	Local control	30	—	—	—	1.9–2.1	20
C3H Mam. Ca	Local control	30	—	—	—	2.5	21
C3H Mam. Ca	Local control	30	—	—	1.65	2.18	22
Fibro Sa	Local control	30	—	—	—	1.6	16
KHT Sa	Lung colony	60	—	—	—	1.9	16
Ca NT	Regrowth delay	15–25	1.5	1.9	2.1	2.2	16
Ca NTa	Regrowth delay	15–30	—	—	—	2.1	23
Sa F	Regrowth delay	30	—	—	—	≥2.0	16
Sa F	Regrowth delay	30	—	—	—	1.7	16
Sa FA	Regrowth delay	15–30	—	—	—	2.0	23
Sa FAa	Regrowth delay	15–30	—	—	—	1.8	23
Sa FAb	Regrowth delay	15–30	—	—	—	2.4	23
Sa F	IUdR loss	15–30	—	1.0	1.0	1.5	16
Sa F	Cell survival	15–30	—	1.3	—	2.2	16
Sa S	Regrowth delay	15–30	—	—	—	<1.1	23
Sa Sa	Regrowth delay	15–30	—	—	—	1.3	23
Sa Sb	Regrowth delay	15–30	—	—	—	1.6	23
Sa Sc	Regrowth delay	15–30	—	—	—	2.0	23
Discoid Ca	Regrowth delay	15–30	—	—	—	1.1	23
Sa FFS1	Regrowth delay	15–30	—	—	—	1.8	23
Sa FFS2	Regrowth delay	15–30	—	—	—	1.5	23
EMT 6	Cell survival	30–45	2.4	2.4–2.7	—	2.9	16
EMT 6	Cell survival	30	—	—	—	2.2	24
EMT 6	Cell survival	60	—	—	—	2.2	25
Sq. Ca G	Local control	20–30	—	1.9	—	~2.0	16
Sq. Ca D	Local Control	30	—	—	—	2.0	16
Sq. Ca D	Regrowth delay	30	—	—	—	2.2	16
MT	Regrowth delay	30	—	—	—	1.8	16
MT	Local control	30	1.5	1.6–1.7	—	2.1	16
MT	Cell survival	30	—	—	—	1.6	16
Rhod. Ca	Regrowth delay	15–20	—	—	—	1.8	23
Bone Sa 2b	Regrowth delay	15–30	1.4	—	1.7	1.8	23
Fibro Sa	Regrowth delay	15–20	—	—	1.9	1.9	23
Rat Sa 180	IUdR loss	15–90	—	—	1.5	—	16
Lewis lung	Lung colony	60	1.3	—	2.2	—	Chaplin and Sheldon, 1980*
Lewis lung	Cell survival	30	—	—	—	1.4	26
B_{16} melanoma	Cell survival	30	—	—	—	2.0	26
Fib./T	Cell survival	55–85	—	1.8	—	2.1	27
Xenografts							
Human pancreas	Cell survival	30–45	—	—	1.8	—	29
EE mal. mel.	Regrowth delay	45	—	—	1.4–1.5	—	28
Na 11 mel.	Cell survival	60	—	—	—	2.0	30

* Personal communication.

and electron-affinity considerations prompted investigation of the 2-nitroimidazoles. In these compounds, the greater interaction of the 2-nitro group with the π electron system of the ring, should confer greater sensitizing activity compared with the 5-nitroimidazoles and this was confirmed *in vitro* for a range of compounds.[18] One of these, Ro-07 0582 or misonidazole, showed a broad spectrum of activity in many different experimental tumors, including tumors growing as xenografts in immune-deprived mice. Fowler and Denekamp[16] have reviewed the literature in this area and Table 1 includes their collected data on radiosensitization by misonidazole in experimental tumor systems together with more recent information from the literature. Although the data in Table 1 may not be entirely comprehensive, they give a fair representation of the sensitizing properties of misonidazole *in vivo*.

Several points emerge. Sensitization appears to be a fairly general phenomenon in experimental tumors at least when given with large single doses of radiation.

This surely indicates that hypoxic cells are present in most experimental solid tumors (Denekamp and Fowler point out that the absence of sensitization for the slow-growing sarcoma S tumor in Table 1 is to be expected since it is known that hypoxic cells are not present in this tumor). The sensitizing efficiency of misonidazole also appears to show little variability. The tumors for which data are given in Table 1 vary considerably in histologic type, growth kinetics and, indeed, in the absolute proportion of hypoxic cells in the tumors (at least in those cases where the hypoxic fractions have been measured).

It would be expected, however, that the overall sensitization effect of misonidazole would be diminished when the irradiation is given in multiple fractions because of the phenomenon of reoxygenation of hypoxic cells occurring during the overall treatment period. This will have the effect of reducing the influence of hypoxic cells in the overall tumor radiation sensitivity.

The effect of fractionation has been studied by several authors.[31,32] A reduced level of sensitization is found but the observed enhancement ratios (ERs) depend critically on fraction size, number and overall treatment time. Suit and Brown[33] found for 8-mm MDAH/Mca IV tumors treated with 0.3 mg/g misonidazole that the ER fell from >1.8 for single doses of radiation to 1.39 when given with each of 10 daily fractions of radiation. However, a similar fractionated treatment with oxygen respired at 30 psi showed much less effect of fractionation.

Sheldon and Fowler[34] have shown how critical the choice of fractionation regimes can be in influencing misonidazole sensitization in the local control of the MT tumor in WH mice treated with single or multiple fractions of x-rays over variable periods. Local control values were measured for the x-ray doses that would produce a constant level of skin damage.[34] For x-rays alone, 20 fractions were clearly better than 5 fractions provided overall treatment time did not exceed 19 days. However, misonidazole improved both schedules to a uniformly high level. This has implications for future clinical application of sensitizers. By taking some of the criticality out of the choice of fractionation, optimum therapy might be achievable with simplified and more economic fractionation schedules that otherwise would not give optimum results.

Clinical Studies with Misonidazole

Following the clinical studies with metronidazole, misonidazole was first administered to patients in 1974. The tolerance to large single doses was good and clinical prospects were further encouraged by the direct observation of sensitization by misonidazole in

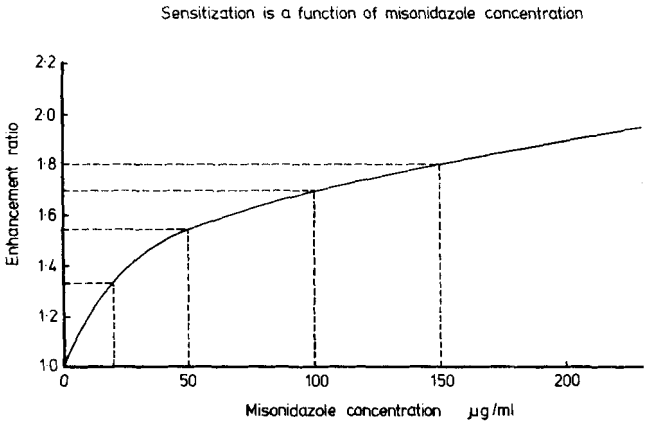


FIG. 1. Concentration dependence of the enhancement ratio for radiation sensitization by misonidazole in hypoxic Chinese hamster cells *in vitro*.[18]

subcutaneous metastatic deposits[35] and artificially-hypoxic human skin treated with single doses of radiation.[36] Further, several pharmacological studies showed that tumor penetration by this drug is generally good in most cases.[37] However, early reports that in multiple dose regimens misonidazole is neurotoxic were soon substantiated,[31,32] and it is now clear that it will not be possible to use misonidazole with fractionated radiotherapy in doses necessary to give the maximum degree of tumor sensitization.

Figure 1 shows an enhancement ratio for misonidazole in hypoxic mammalian cells irradiated *in vitro*. On the assumption that the sensitizing efficiency of misonidazole *in vivo* is not very different from that *in vitro* (an assumption that is examined later), one can interpolate the sensitization that might be obtainable in human tumors. Such an interpolation assumes also that the concentration of the drug in hypoxic tumor cells is the same as, or near to, the serum levels measured at the time of treatment and, of course, neglects any effect of reoxygenation.

The incidence of peripheral neuropathy in patients given multiple doses of misonidazole is dose-related and can be very high in patients with advanced head and neck cancer. Most studies limit dosage for the entire treatment to a total of 12 g/m^2. This means that the greater the number of drug fractions, the smaller will be the dose of drug per radiation fraction and the smaller will be any element of dose gained by sensitization.

With the dosage limitation, the maximum dose of drug that could be administered with each fraction of conventional 30-fraction radiotherapy will be 0.4 g/m^2. From the curve in Figure 1 the serum levels of 20–30 μg/g observed for this treatment schedule, would give theoretically a *maximum* ER of only about 1.3 ignoring

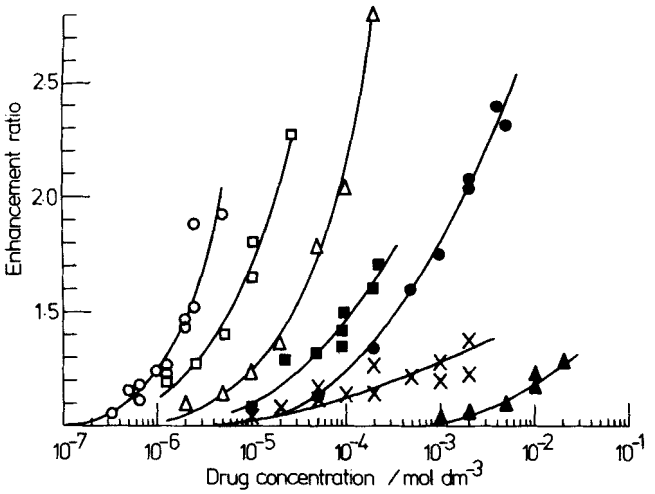


FIG. 2. Radiation sensitization by several substituted 2-, 4- and 5-nitroimidazoles. Concentration dependence of the enhancement ratios showing a wide range of sensitizing efficiency.[41-43]

any effects of reoxygenation. A smaller number of fractions permitting a larger individual dose of misonidazole would give larger ER values. Understandably, the majority of current clinical trials employ more unconventional fractionation, permitting larger individual drug doses. These include studies where the drug is administered with only some of the radiation treatments.

There is some evidence that the incidence of peripheral neuropathy is lower in patients receiving phenytoin.[38,39] Misonidazole has a shorter half-life in those patients, due possibly to the effect of phenytoin inducing faster liver metabolism of misonidazole. However, the fact remains that the neurotoxic properties of misonidazole will prevent its use at optimum dosage, and while one hopes that some of the many trials currently in progress will reveal some benefit, there is an urgent need to develop sensitizers with improved therapeutic ratios. Progress in this area is discussed in the next section.

The Development of New Radiation Sensitizers

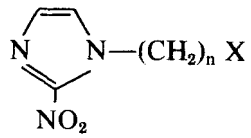
The Electron-Affinity Relationship

Many compounds now exist that sensitize hypoxic cells to radiation *in vitro*. Virtually all of these show no activity against oxic cells and this differential activity remains the sole rationale for developing suitable compounds that will sensitize tumor response to radiation without increasing the radiation morbidity of normal tissues. Sensitization of hypoxic cells *in vitro* is shown by an increase in the slopes of the exponential portions of the radiation survival curves. Under these conditions, the enhancement ratios (ER) are defined as the slope ratios. Enhancement ratio increases with sensitizer concentration, in some cases up to, or near to, the value of the oxygen enhancement ratio (OER). Some typical results are shown in Figure 2 for a number of typical nitroimidazole radiation sensitizers. Clearly the concentration ranges over which sensitization occurs, vary widely over at least three orders of magnitude.

Several factors contribute to the sensitizing efficiencies of drugs of this type, but by far the most influential are their electron-affinic properties. The proposal that the radiation sensitization properties shown by various chemical compounds was a function of their redox properties[11] was substantiated by several studies including both mammalian cells[38] and bacterial spores.[39] Relative electron-affinities for sensitizers of this type can be conveniently, and quantitatively, expressed as their "one-electron reduction potentials." These can be routinely measured by pulse radiolysis methods designed to measure one-electron transfer equilibria.[40] Figure 3A reproduces data for hypoxic mammalian cells showing the correlation of sensitizing efficiency (defined as the concentration required to give an ER of 1.6) with one-electron reduction potentials of the compounds (E_7^1).[41,42] The correlation is good, but the data points show some scatter, indicating that other factors probably contribute to the overall sensitizing efficiency of a given compound. (The hypoxic cytotoxic properties of these compounds also correlate with redox properties (Figure 3B) and this is discussed later).

An Effect of Chemical Structure on Sensitizing Efficiency

An illustration of how properties other than electron-affinity can affect sensitizing efficiency *in vitro* has been provided by studies[43] with some 2-nitroimidazole analogues of the general structure:



where n is the number of carbon atoms in the side chain and X is a terminal base (*e.g.* morpholino).

Figure 4 shows the influence of side chain length on bulk sensitizing efficiency and chronic aerobic toxicity in Chinese hamster V79 cells *in vitro*. Sensitizing efficiency is expressed as the concentration required to give an enhancement ratio of 1.6 ($C_{1.6}$). The chronic aerobic toxicity is defined as the concentration required to suppress colony-forming ability in attached cultures of *aerobic* cells by 50% when the compound is

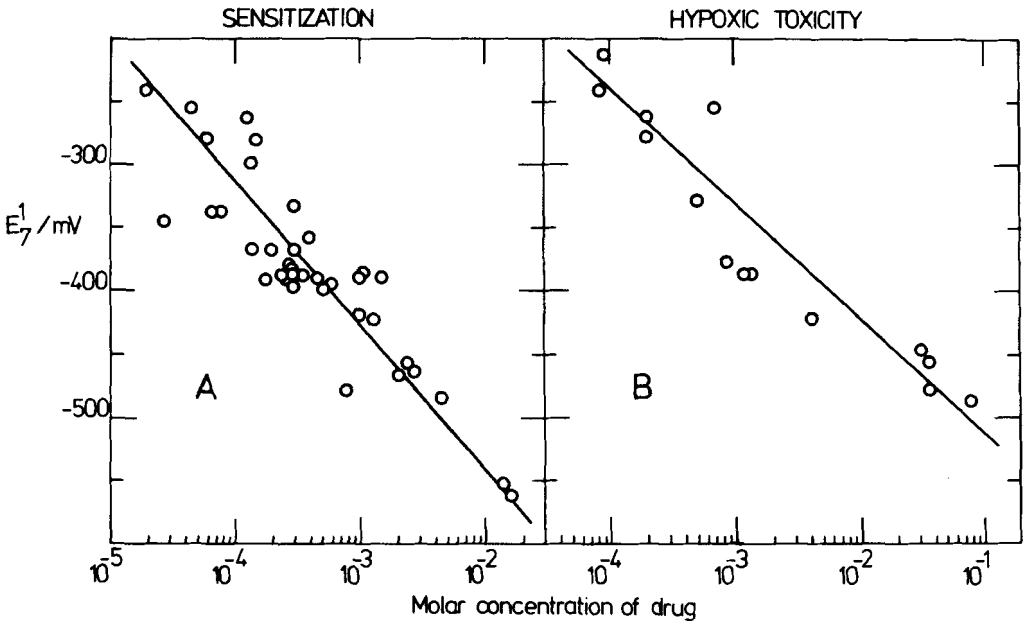


FIG. 3. Electron affinity dependence of (A) Radiation sensitization efficiency[41–43] and (B) Hypoxic cytotoxicity in Chinese hamster cells *in vitro*.[55]

Ordinate: One-electron reduction potential (mV). Abscissae: (A) Concentration of drug required to give an enhancement ratio of 1.6. (B) Concentration required to reduce surviving fraction to 10^{-2} for a contact time of 5 hours.

present throughout the incubation period (usually 7–10 days).

Clearly, both properties depend markedly on side-chain length in these compounds, even though the compounds show little variation in their electron-affinities. Figure 4A shows that as n increases, the value of $C_{1.6}$ decreases to a minimum at n = 5, *i.e.* sensitizing efficiency is at a maximum. In contrast, as n increases, the compounds become progressively more toxic to aerobic cells in that lower concentrations are required to produce inhibition of colony-forming ability.

The ratio of the toxicity to the sensitizing efficiency is a measure of the sensitizer "effectiveness" and is a term analogous to the therapeutic ratio concept applicable *in vivo*. Figure 5 shows the sensitizer effectiveness of these morphilino compounds plotted as a function of n. The smooth parabola shows that effectiveness varies overall by about a factor of 20 in these compounds and is at a maximum at n = 5.

The reasons for this non-redox effect on sensitizer efficiency are still obscure. The lipophilic properties of the compounds which might be thought to be significant appear not to be important in this series. Indeed *in vitro* studies of sensitizers have failed to show any correlation of sensitization efficiency with octanol/water partition coefficient[42] over a broad range of *P* values. However, Brown *et al.*[44] have observed that compounds with partition coefficients less than 0.04 tend to be less effective.

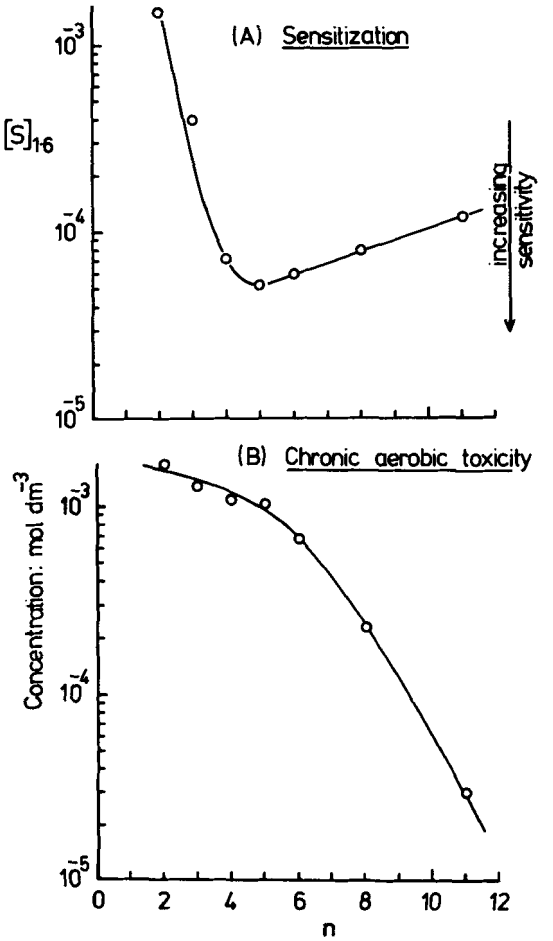


FIG. 4. Dependence of sensitizing efficiency (concentration required for an enhancement ratio of 1.6) and chronic aerobic toxicity (concentration required for 50% inhibition) on the length of the N1 side chain for a range of substituted 2-nitroimidazoles. Terminal base group: morpholino.[43]

Neurotoxic Properties of Radiation Sensitizers

The main objective in investigating the sensitizing properties of new compounds is to identify drugs that

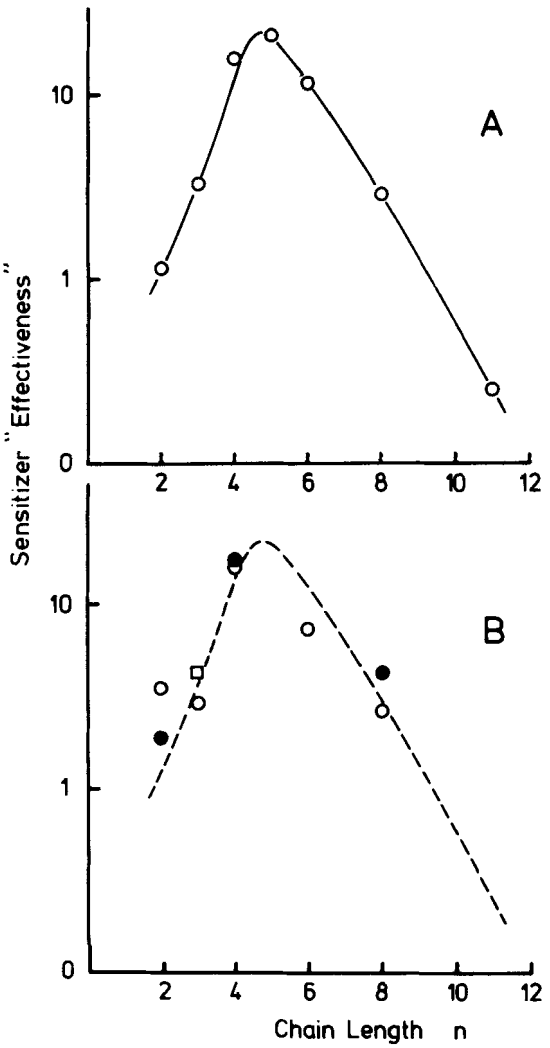


FIG. 5. Sensitizer "effectiveness" as a function of chain length for (A) the morpholino series (data from Fig. 4). (B) Similar series with piperidine, (○) and pyrrolidine, (●) as terminal base group, □ misonidazole (dotted line: curve from Fig. 5A).[43]

will be superior to misonidazole in the clinic. Compounds already exist that sensitize *in vitro* at lower concentration than misonidazole and one hopes that some will prove to be efficient *in vivo* also. However, knowledge of their toxicology must be obtained before any conclusion can be drawn as to their prospects for clinical use. Hence, the general study of the neurotoxic properties of these drugs is essential if new agents are to be identified on a non-empirical basis.

Various studies are in progress on the behavioral changes induced in experimental animals treated with misonidazole and other electron-affinic agents. Conroy and co-workers[45] have used the rotor-rod technique in attempts to rank the relative neurotoxicity of different sensitizers including misonidazole. Similarly, Sheldon and Chaplin (quoted in Clarke *et al.*[51]) are using a narrowing bridge technique to assess the performance status of treated mice. Although differences are observed in the neurotoxic potential of various sensitizers, it is too early to assess whether or not such studies will prove to be sufficiently quantitative to permit structure-activity relationships to be derived.

The pathology of misonidazole-induced nerve damage is also under investigation in several centers. Nerve-conduction velocities are reduced in drug-treated mice immediately after treatment and also several weeks after chronic drug dosage.[46] It has been found, however, that the *acute* effect is probably an artefact attributable to the significant drop in body temperature after a high dose of misonidazole.[47]

De-myelination and axonal degeneration induced by misonidazole has been observed by electromicroscopy in preparations of both human[48] and mouse neural tissue.[47,49] The effect is not peculiar to misonidazole and has been observed with other nitroheterocyclic compounds that behave as radiation sensitizers.

Biochemical changes associated with the neurotoxicity of sensitizers offer perhaps the best prospects at the present time for quantitative intercomparison of the neurotoxic properties of these agents. Chemically-induced neuropathies have been shown to be accompanied by substantial increases in lysosomal enzymes in the affected nerves and this is also true for misonidazole in rats.[50] Peak levels appear to occur about three to four weeks after administration of the drug. Clarke[51] has developed a cytochemical technique for measuring these changes *in situ* and some of the data are shown in Figure 6.

Mice were given seven daily doses of 0.03 mg/g misonidazole and the increased levels of β-glucoronidase measured weekly for up to eight weeks in both the proximal and distal portions of peripheral nerve sections. Peak levels in both regions were again observed at about three to four weeks after treatment with misonidazole which fell to normal levels by about six weeks. The method is clearly highly sensitive since this dosage is of the same order as that given clinically.

Changes in lysosomal enzyme levels have been observed with other nitroheterocyclic sensitizers, although the drug doses required vary considerably. The high sensitivity of the technique coupled with its quantitative potential offer real prospects for assessing the chemical, structural and other characteristics of these drugs (*e.g.* lipophilicity) that may be conducive to their neurotoxic properties. With such information, meaningful structure-activity relationships should be-

come available and this will expedite the development of clinical radiation sensitizers with therapeutic ratios significantly greater than misonidazole.

The Differential Cytotoxic Properties of Nitro-Containing Sensitizers

Background

In 1974, Sutherland[52] noted in his studies with the multicellular spheroid system that metronidazole was considerably more cytotoxic to hypoxic cells compared with oxic cells. This was subsequently confirmed in experiments with single-cell mammalian cultures[53,54] for a range of nitroheterocyclic compounds including misonidazole. Figure 7 shows this differential toxic effect in Chinese hamster cells exposed to 5 mM misonidazole. In hypoxia, cell survival is reduced to 10^{-3} after 5 hours, whereas oxic cells retain full viability after several days' exposure to this concentration.

Studies with a wide range of compounds of this type have shown that, similar to their radiation sensitizing efficiencies, their hypoxic–cytotoxic properties also correlate closely with electron-affinity.[55] Figure 3B shows the correlation with these compounds with the highest one-electron redox potentials showing the greatest cytotoxic efficiencies. The similar electron-af-

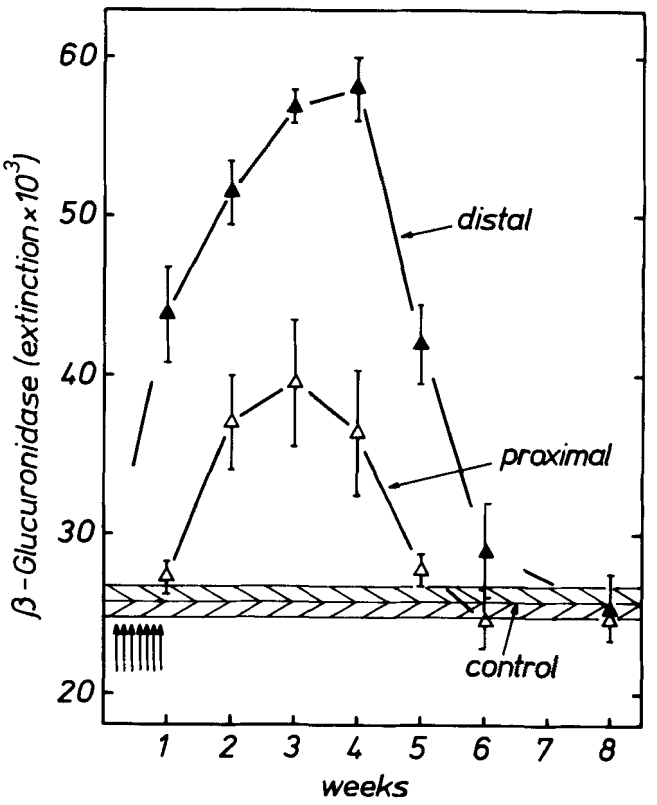


FIG. 6. Activity of β-glucoronidase activity in the sciatic nerves of C57BL mice following 0.5 mg/g misonidazole daily for 7 days.[51]

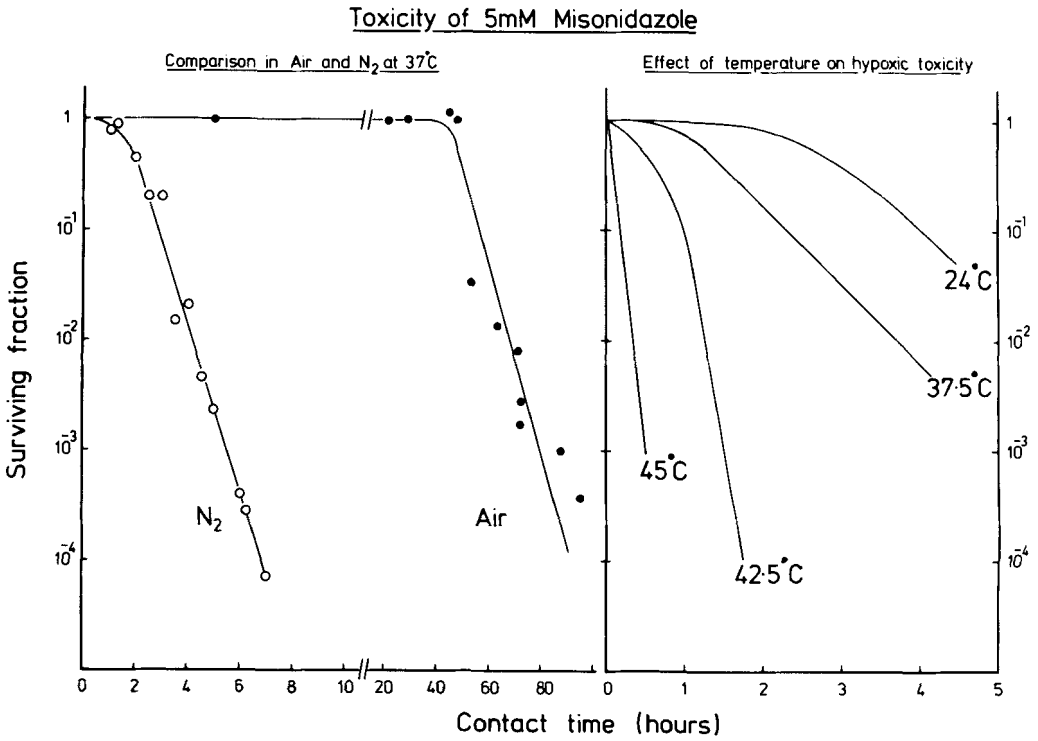


FIG. 7. Cytotoxic effect of 5 mM misonidazole on hypoxic and oxic Chinese hamster cells (Stratford and Adams[58] and Horsman, private communication) and its temperature-dependence.[59]

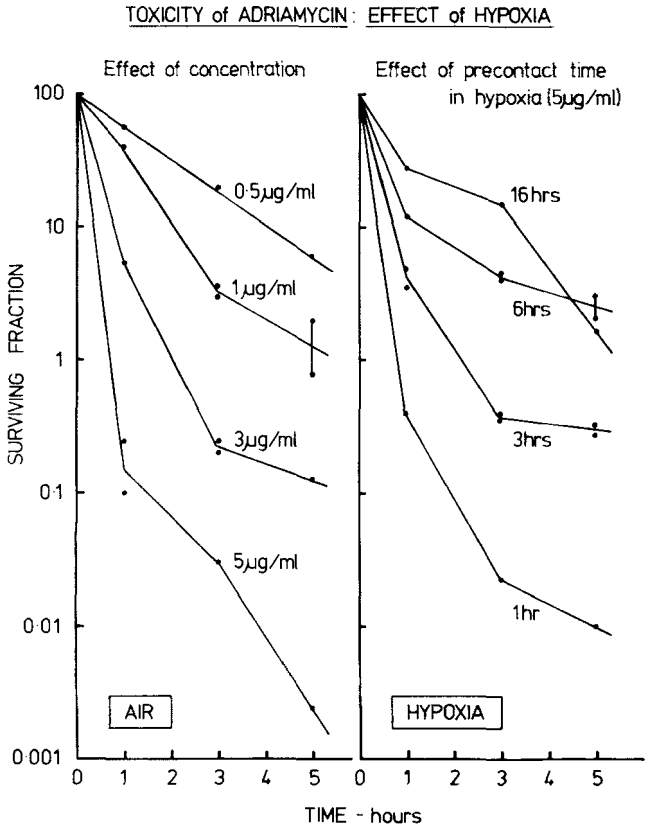


FIG. 8. Toxicity of Adriamycin in oxic and hypoxic exponential phase Chinese hamster cells. (*Left*) Effect of concentration in aerobic cells. (*Right*) Effect of *pre-contact* time in hypoxia on the toxicity of 5 μg/ml Adriamycin to hypoxic cells.[71]

finity dependence for both the sensitization and the hypoxic cytotoxicity properties might suggest that the mechanisms are similar. There is, however, good evidence that this is not so.

Firstly, the time scale of sensitization is very fast, as has been shown by rapid-mixing studies. This is to be expected since the mechanism of sensitization involves fast free-radical redox processes involving cellular DNA. On the other hand, the hypoxic cytotoxic effect requires a substantial exposure time between the cell and the drug before it becomes manifest. The cytotoxic property is believed to be a consequence of reductive metabolism of the drug in which a cytotoxin is produced,[56,57] and it is possible that the mechanism has some similarities with the mechanism by which drugs of this type are active against anaerobic bacterial infections. Secondly, the efficiency of radiation sensitization *in vitro* shows little dependence on temperature, whereas the cytotoxic effect is profoundly temperature-dependent.[58,59] Figure 7 shows data from Hall and colleagues[59] illustrating the temperature effect in hypoxic Chinese hamster cells exposed at various temperatures in the presence of 5 mM misonidazole. A large increase in the cytotoxic effect of misonidazole is observed even at 41 C,[58] and there is some interest developing on the possible use of electron-affinic agents in conjunction with hyperthermia because of this large temperature coefficient. Thirdly, the efficiency of the cytotoxic effect shows a marked cell-cycle dependence with maximum cell-killing efficiency appearing in early S phase.[60,61] Finally, it is known that although vitamin C greatly enhances the cytotoxic effect of misonidazole,[62] it has no effect on the efficiency of radiation sensitization by this drug.[63]

Are Hypoxic Cells in Tumors Resistant to Cytotoxic Chemotherapy?

There is much evidence that the response of experimental tumors to cytotoxic drugs can be size-dependent.[64] There can be several reasons for this, of course, including problems associated with drug accessibility. However, it is reasonable to speculate that hypoxic tumor cells that are in a resting state may be relatively resistant in some cases. If this were so, then appropriate drugs that are *more* active against hypoxic cells might have a role to play in the combination chemotherapy of cancer.

Evidence exists[65] that misonidazole *can*, in some cases, increase tumor response to radiation when administered *after* irradiation, an effect that cannot be due to normal "oxygen-like" sensitization. Further, misonidazole alone has been shown to produce considerable cell kill in EMT6 tumors and multicellular spheroids and to produce extensive necrosis in the central regions.[66,67] However, problems remain in studying such cytotoxic effects in experimental tumors because of the relatively short half-lives of nitroheterocyclic drugs in mice. While this property is of much less consequence for radiation sensitization studies, the prolonged contact time necessary for the hypoxic-cytotoxicity makes it difficult to optimize the effect.

In regard to the role of hypoxia in the action of other cytotoxic drugs, evidence is accumulating showing that cells with a reduced oxygen status can be resistant even *in vitro* to some of these agents. Examples include bleomycin,[68] actinomycin D,[49] and particularly Adriamycin.

It is known that Adriamycin is much less effective *in vitro* to aerobic cells in plateau phase compared with those in exponential phase,[69,70] an effect that has been attributed to reduced drug uptake. There is, however, a hypoxia factor to be considered.[67] Figure 8 shows some of our own data on the effect of chronic hypoxia on the response of V79 cells to Adriamycin.[71] Exponentially growing cells show the usual loss of viability after contact with various drug concentrations and

even when the cells are maintained in hypoxia for 1 hour and then exposed to 5 μg/ml Adriamycin in hypoxia, they are still as sensitive as they are in oxygen. However, as the precontact exposure time in hypoxia is increased, the cells become progressively more resistant. The maximum effect appears to be reached after a 6-hour precontact time. Other experiments were carried out where the cells were rendered chronically hypoxic by overnight incubation in hypoxia and then reoxygenated. Adriamycin was then added at various times after reoxygenation. It was found that these cells retain for several hours the relative resistance to Adriamycin even though drug contact only occurred under aerobic conditions. Other experiments showed that the hypoxia-induced resistance is *not* due to decreased uptake of the drug.

Interaction of Electron-Affinic Drugs and Alkylating Agents

Suggestions that the hypoxic-cytotoxic effects of electron-affinic agents might be valuable in combination chemotherapy was originally based on the assumption that they would act complementary to, but independent of, the effects of other cytotoxic drugs.[52] However, there is now evidence from studies both *in vitro* and *in vivo* that there is a *direct* interaction between electron-affinic compounds and other cytotoxic agents.

Recently, Rose *et al.*[72] investigated the response of the Lewis lung tumor in mice to the combined effects of misonidazole and other cytotoxic drugs, in particular melphalan, cyclophosphamide, 5-FU and cis-platinum. Some of their data are shown in Figure 8. Misonidazole (1 mg/g) was administered ip 30 minutes before various doses of melphalan and the response of the tumor to treatment was assayed by clonogenic cell assay. Misonidazole greatly enhances the cytotoxic effect of melphalan, giving a dose-modification factor of 2.0. Although some increase in bone-marrow and gut toxicity was also observed, the enhancement was substantially greater in the tumor compared with that in normal tissues.

Similar enhancements of tumor response have been observed in a variety of other murine tumors (Sheldon, Brown, Stephens, Spooner, Twentyman, private communications) and also in a human melanoma growing in immune-deprived CBA mice (Millar, private communication). Enhancement of melphalan response has also been observed with other electron-affinic agents (Sheldon, private communication).

The mechanism of this misonidazole-induced potentiation is, so far, unknown. However, information obtained from drug studies *in vitro* may be important in this respect. It has been shown[73] that Chinese hamster

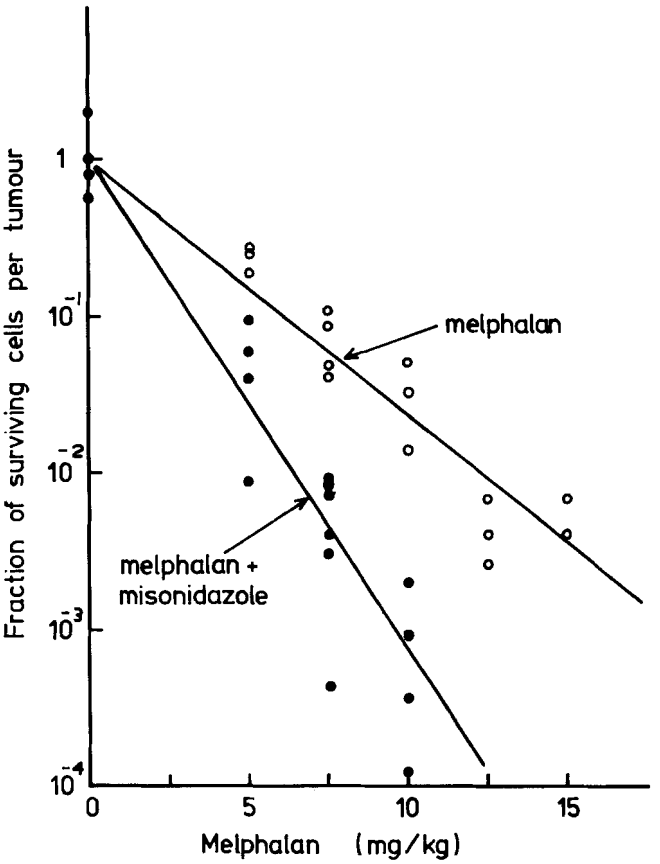


FIG. 9. Survival curves for the Lewis lung tumor in C57B1 mice treated with melphalan alone or in combination with misonidazole (1 mg/g) given 30 minutes earlier.[72]

cells exposed to misonidazole in hypoxia are subsequently more sensitive to a range of cytotoxic drugs, particularly alkylating agents. The hypoxic cells are exposed to misonidazole for a time sufficient to bring the cells just off the shoulder of the survival curve. These conditions lead to only a small degree of cell kill. However, when the cells are then exposed to some alkylating agents, *e.g.* melphalan, they are considerably more sensitive than cells exposed to melphalan alone. This potentiation is still observed, even when the cells pretreated with misonidazole are washed free of the drug and then exposed to the alkylating agent *in air*. However, *no* potentiation is observed if the pre-treatment is not carried out in hypoxia.

Although the mechanism of the *in vitro* effect appears to involve, therefore, a hypoxia-mediated process, it is too early to say whether this is relevant to the potentiation of tumor response. The possibility, however, that the antitumor effect of one agent may be enhanced by a second agent in a process requiring a physiologic property of a tumor (*i.e.* hypoxia) would indeed be an attractive prospect for chemotherapy.

Whether or not this will be achievable remains to be seen.

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