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SENSITIZATION OF CANCER CHEMOTHERAPEUTIC AGENTS BY NITROHETEROCYCLICS

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Potentiation of chemotherapeutic agents by nitroheterocyclic sensitizing agents was first demonstrated by Rose *et al.* [1] and Clement *et al.* [2] in 1980. These authors showed that the addition of the radiosensitizer misonidazole (MISO) to certain alkylating agents significantly increased tumor cell kill *in vivo*. These initial observations, indicating that the sensitizing potential of nitroheterocycles was not restricted to the well-established radiation sensitization phenomenon associated with these agents [3], stimulated active investigation into their possible use as modifiers of chemotherapeutic agent efficacy. The enthusiasm generated by these early investigations has been substantiated by extensive evidence demonstrating that significant potentiation could indeed be realized both *in vitro* and *in vivo* [for review see 4–8]. The earliest *in vitro* experiments utilized a pre-incubation protocol in which cells were exposed to a sensitizer under hypoxic conditions prior to treatment with the chemotherapeutic agent in air. These studies established hypoxic sensitizer exposure as a pre-requisite for drug potentiation. Several antitumor drugs have been investigated extensively in this manner; particularly the alkylating agent melphalan [9–13] and the nitrosoureas [13–16]. Under pre-incubation conditions the resultant potentiation is typified by a modification of the slope and/or reduction of the shoulder of the drug dose response curve. This is illustrated in Fig. 1. From such relationships Dose Enhancement Ratios (DEF; defined as the ratio of drug doses required to achieve some biological effect alone or in combination with MISO) of the order of 1.5–2.5 commonly have been calculated [4, 10–12, 14–16]. Comparable enhancements have been observed when tumor-bearing animals were treated with a combination of sensitizer and anti-cancer drugs [5–8]. These *in vivo* investigations further demonstrated a rigid drug- and sensitizer-specificity for chemopotentiation. The chemotherapeutic agents most effectively potentiated *in vivo* have been the alkylating agents cyclophosphamide, melphalan and several of the nitrosoureas [5–8]. In virtually every case, chemopotentiation of these agents has been demonstrated at sub-toxic sensitizer doses, suggesting that the effect is not merely due to additive toxicities but rather due to a true modification of the activity of the alkylating agent. Another outcome of the *in vivo* experiments was the recognition that administration sequences and timing of the agents greatly influenced the magnitude of the observed potentiation. In general, administering the sensitizer simultaneously with, or shortly before or after, the chemotherapeutic agent resulted in the greatest enhancement ratios. Consequently to more closely simulate the *in vivo* situation, *in vitro* investigations have been expanded to include co-incubation and post-incubation protocols. Under co-incubation conditions the cells are exposed to the drug and sensitizer simultaneously under hypoxic conditions, while for post-incubation, sensitizer exposure under hypoxia follows aerobic drug treatment. Experiments performed under either of these conditions have resulted in tumor cell kill enhancements similar to those observed in pre-incubation experiments but both techniques offer certain unique advantages with respect to mechanistic investigations [15, 16]. Thus both the cell culture and tumor investigations support the suggestion that nitroheterocyclics may have a role as adjuvants in cancer chemotherapy.

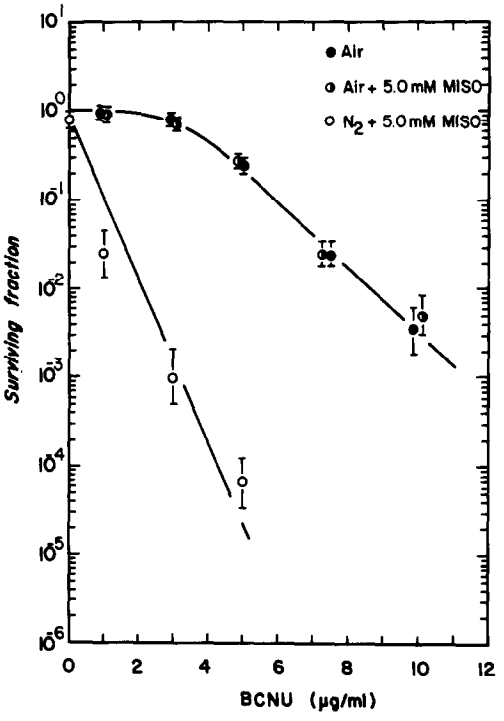

Fig. 1. Clonogenic cell survival of exponentially growing 9L rat brain tumor cells pretreated at 37° with 5.0 mM MISO for 2 hr in air (◑) or N_2 (○) prior to exposure to variable doses of BCNU for 1 hr in air. Survival after treatment in air without MISO pretreatment also is shown (●). Modified from [14].

In order to assess the possible clinical application of this combined therapy it was essential to evaluate the concomitant enhancement of toxicity in critical normal tissues. Since major side-effects associated with the chemotherapeutic agents modified by sensitizers primarily involve bone marrow and gut complications most animal studies have focused on evaluating the effect of chemopotentiation on these tissues. By and large such investigations have identified some enhancement of damage in these tissues, particularly at high sensitizer doses. Enhancement ratios typically observed range from 1.1 to 1.4 [5–8]. Compared to the enhancements reported for most tumor systems these values were significantly lower. In fact, in those studies in which tumor and normal tissue enhancements were evaluated concurrently a therapeutic benefit was usually realized [5–8]. Based on these pre-clinical studies, clinical Phase I trials of chemopotentiation have been initiated [17–21].

A number of possible mechanisms for chemopotentiation have been postulated. These include: (i) sensitizer induced alterations in chemotherapeutic agents pharmacokinetics, (ii) enhanced DNA cross-link formation, (iii) modification of DNA damage repair, and (iv) depletion of protective thiol pools. Detailed discussion of these mechanisms is beyond the scope of the current manuscript; however, such a discussion has been provided elsewhere [4, 6–8]. The relative significance of each of these various mechanisms is highly dependent upon the experimental conditions. For example, while pharmacokinetic alterations can contribute to chemopotentiation *in vivo* when high doses of sensitizers are utilized [22–26], significant enhancements also have been reported in the absence of pharmacokinetic changes [8, 27–29]. The latter situation is commonly seen when low doses of a sensitizer, such as MISO, are used in the combination or when the sensitizer itself does not modify the distribution or clearance of the chemotherapeutic drug, even at high doses. Likewise, depletion of glutathione by nitroheterocyclic sensitizers can account for some but not all of the drug potentiation *in vitro* [9, 30–32]. Even this enhancement is dependent on the experimental conditions such that glutathione depletion contributes to chemopotentiation in pre- but not post-incubation studies. So while some common features, such as enhanced DNA–DNA cross-link formation, have been demonstrated in several experimental systems no unifying mechanism for chemopotentiation has as yet been identified [8].

Despite the uncertainties relating to the mechanism(s) involved in the modification of drug activities by sensitizers, virtually all of the available evidence suggests that chemopotentiation is an oxygen-sensitive process, being greatest under hypoxic conditions [8]. It is this property which would form the basis for the selective anti-tumor effect observed when nitroheterocyclic sensitizers are used in combination with chemotherapy. This is so, since normal tissues are considered in general to be well-oxygenated, while alternatively it is widely accepted that regions of very low oxygen tension develop during the growth of a tumor as a consequence of the blood supply becoming inadequate. Evidence for the dependence of chemopotentiation on oxygenation status derives from a number of sources (Table 1). *In vitro* experiments have failed to demonstrate chemopotentiation under fully aerobic conditions [4, 6, 10, 11, 14]. Likewise, modifications of drug activity by the radiosensitizer MISO did not occur in the 9L rat brain tumor, a solid tumor lacking radiobiologically hypoxic cells [33]. Yet when these tumors were made artificially hypoxic, sensitizer-induced enhancement of the chemotherapeutic agent could be demonstrated [33]. Other studies have shown the magnitude of chemopotentiation in tumors to be a function of the size of the hypoxic cell fraction. Several investigators have shown the extent of chemotherapeutic agent activity enhancement by sensitizers in micrometastases to be reduced relative to that of the primary tumor, known to have a larger hypoxic fraction [34–36]. This difference is not solely a property of metastatic growth since investigations of chemopotentiation in metastases from the KHT sarcoma demonstrated that enhancement of cell kill in ovarian metastases was significantly larger than seen in metastatic lung nodules [37, Table 2]. The tumors growing in these sites had very similar growth kinetics but the proportion of hypoxic cells in the ovarian tumors was significantly larger. Evidence supporting the need for hypoxia in the modification of drug efficacy by sensitizers also comes from tumor cell separation studies which showed that chemopotentiation was greatest in those isolated tumor cell subpopulations containing the largest percentage of hypoxic cells [38]. Results of recent experiments further suggest that the addition

Table 1. Evidence for the requirement of hypoxia in the potentiation of chemopotherapeutic agents by nitroheterocyclics (adapted from [8])

Observations	Reference
Chemopotentiation occurs *in vitro* only under hypoxic conditions	[for review see 4]
No potentiation of chemotherapeutic agent efficacy by radiosensitizers was seen in a tumor lacking radiobiologically hypoxic cells	[33]
Metastases (small hypoxic fraction) showed less chemopotentiation than corresponding primary tumors (large hypoxic fraction)	[34–36]
Metastases with large hypoxic fractions exhibit more chemopotentiation than those with small hypoxic fractions	[37, Table 2]
Chemopotentiation was greatest in isolated tumor cell sub-populations containing the majority of hypoxic cells	[38]
The $K_m(O_2)$ values for sensitizer cytotoxicity and chemopotentiation are very similar	[16]

Table 2. Potentiation of CCNU activity by MISO (1.0 mmol/kg) for KHT sarcoma cells growing in different sites

Tumor-site	Hypoxic fraction	DEF
Primary—leg	~15%	~1.4
Metastases—lung	~ 5%	~1.3
Metastases—ovary	~50%	~1.8

of a sensitizer to a chemotherapeutic agent prior to radiation could reduce the proportion of radiobiologically hypoxic cells in a tumor [39]. Collectively these data imply that the interaction between chemotherapeutic agents and sensitizers is critically dependent upon cellular oxygen levels. To evaluate this in detail, Mulcahy determined the effect of oxygen concentration on the enhancement of CCNU toxicity by MISO in EMT-6 cells *in vitro* [16]. Over a range of oxygen concentrations, chemopotentiation was shown to progressively decrease as the oxygen tension in solution was increased, until the effect was lost at fully aerobic conditions. It was particularly interesting that modification of CCNU activity by MISO was expressed in cells treated at intermediate oxygen tensions. This suggested not only that chemopotentiation could occur in those tumor cells at oxygen levels greater than radiobiological hypoxia but also provided a possible mechanism for some of the enhanced normal tissue damage seen in animals treated with sensitizers and chemotherapy [16].

These data clearly indicate that hypoxia plays a key role in chemopotentiation and suggest, by inference, that bioreduction of the nitro group of the sensitizer is required for the modification of chemotherapeutic agent activity by nitroheterocyclics. Direct evidence to substantiate this latter hypothesis is, however, less abundant. In contrast, the involvement of bioreduction in the cytotoxicity of nitroheterocyclic sensitizers has been well established [40, 41]. Like chemopotentiation, sensitizer cytotoxicity has been shown to be oxygen-sensitive [42, 43]. Chemical and biological studies have indicated that the parent sensitizing compound must be metabolized to its ultimate cytotoxic form via microsomal flavoprotein (probably NADPH cytochrome *c* reductase) nitroreduction [44]. Although the active toxic moiety has not been unambiguously identified, the oxygen-sensitive reaction in the metabolism of the sensitizer (RNO_2) appears to be the conversion of the nitro group to the nitro-radical anion, as shown in the following reaction scheme:

$$RNO_2 \longrightarrow NO_2^{\cdot}$$
$$O_2^{\cdot} \quad O_2$$

The oxygen-sensitivity of the cytotoxicity is attributable to a one electron transfer in the presence of oxygen resulting in the regeneration of the parent compound from the nitro-radical anion. If similar bioreduction of the nitro group is involved in chemopotentiation, then a comparison of chemopotentiation and cytoxicity as a function of oxygen concentration would be expected to result in similar oxygen-sensitivities. This was in fact observed in studies with EMT-6 cells *in vitro* [16]. As shown in Fig. 2 the two activities displayed similar oxygen-

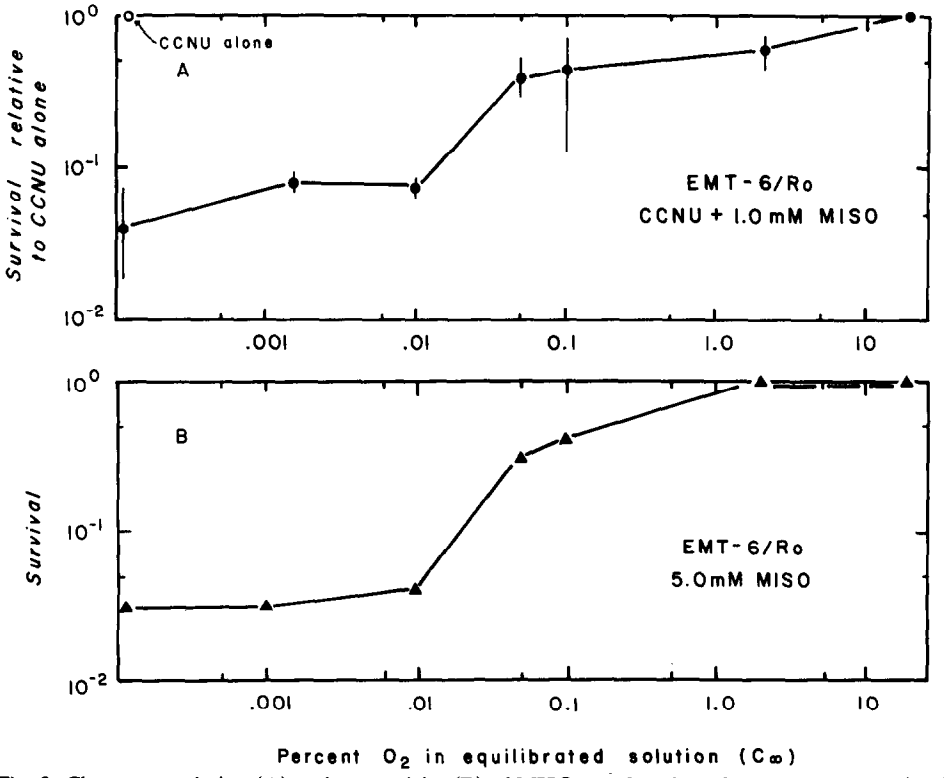

Fig. 2. Chemopotentiation (A) and cytotoxicity (B) of MISO as a function of oxygen concentration in solution. Both activities demonstrate a similar oxygen-sensitivity. Reproduced from [16].

Table 3. Relationship between cytotoxicity and chemopotentiation

Compound	D_{50} (mM)*	$C_{1.5}$ (mM)†
1. Acetohydroxamic acid-3-nitropyrazole	0.45	0.34
2. (O-Acetyl)-acetohydroxamic acid-3-nitropyrazole	0.1	0.07
3. (O-Benzoyl)-acetohydroxamic acid-3-nitropyrazole	0.01	0.01
4. Pyrazole-acetohydroxamic acid (no nitro group)	non-toxic	$\gg$2.0
5. Misonidazole	1.0	0.35

* Dose required to reduce survival to 50% of control in a 4-hr hypoxic exposure.
† Dose required to produce an enhancement ratio of 1.5 in a 4-hr co-incubation exposure with CCNU.

dependencies. From further analysis $K_m(O_2)$ values (i.e. the oxygen concentrations at which the cell inactivation by MISO or chemopotentiation are half-maximal) for cytotoxicity and chemopotentiation were found to be ~350 and 420 ppm, respectively. This observation suggests that a similar metabolic pathway, i.e. the oxygen-sensitive reduction of MISO to the nitro-radical anion by cellular nitro-reductases, is involved in the mechanism of both cytotoxicity and chemopotentiation. As such, the $K_m(O_2)$ values reported in these studies are in close agreement with that reported by Wardman and Clarke [42] from chemical analysis of this reaction as well as with that observed for MISO cytotoxicity by Taylor and Rauth [43]. The association between nitroreduction and chemopotentiation is further supported by a relationship between the cytotoxicity of a given sensitizer and its chemosensitivity potential. This is illustrated in Table 3 for MISO and a series of acetohydroxamic acid derivatives of 3-nitropyrazole. As the dose required to reduce cell survival to 50% of control decreases, a concomitant reduction in the dose required to produce a DEF of 1.5 is observed. Similar relations between cytotoxicity and chemopotentiation can be observed for other classes of sensitizers as well, and is not restricted to *in vitro* investigations. In general, the most cytotoxic agents are also the best chemopotentiators. This relationship is discussed in detail elsewhere [45]. Further evidence in support of this hypothesis is provided by preliminary data indicating that cell lines markedly resistant to the cytotoxic action of sensitizers are frequently more refractory to chemopotentiation than their more sensitive counterparts. Finally, structure–activity studies with experimental chemopotentiating agents, demonstrated that the removal of the nitro group from the ring of active compounds resulted in the complete elimination of toxicity and chemosensitizing ability (Table 3); again implicating nitroreduction in chemopotentiation.

In summary, evidence that chemopotentiation is a hypoxia-mediated phenomenon is fairly strong. More recently, nitroreduction has been suggested to be the basis of this oxygen-sensitive interaction. Specifically, the similarity between the reported $K_m(O_2)$ values for sensitizer cytotoxicity and chemopotentiation strongly implies that nitroreduction is a common metabolic step required for both activities. The importance of nitroreduction in chemopotentiation may not simply be of academic interest, since it could have profound influence on future drug development and selection. For example, selection of chemosensitizing agents on the basis of their radiosensitizing potential as has been common, may be inappropriate because nitroreduction is not involved in radiosensitization. Most importantly, the requirement for hypoxia in the potentiation of chemotherapeutic agents by nitroheterocyclic sensitizers provides a basis for possible therapeutic selectivity for this form of cancer treatment.

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