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Effect of a hypoxic tumor cell cytotoxic disulfide on the membrane and DNA of tumor cells in culture

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A disulfide, *n*-butyl 2-imidazolyl disulfide (III-2), recently reported to be more toxic to EMT6 tumor cells under hypoxia has also been shown to be preferentially toxic to KHT/iv cells under hypoxia with an IC_{90} value 3-fold lower versus that measured in air. The IC_{90} values for both cell lines were markedly affected when the pH_e was decreased. Uptake studies revealed that the disulfide is metabolized at the cell membrane with only one of the metabolites, *n*-butanethiol, being taken up into the cell. There were no differences in the amount of uptake under oxia or hypoxia. Investigation of potential membrane damage revealed that III-2 was a mixed inhibitor of the enzyme Na^+,K^+-ATPase, from isolated plasma membrane. DNA damage, when examined using plasmid DNA in the absence or presence of glutathione, was absent. Minor single-strand breaks to EMT6 DNA could only be observed following exposure to III-2 at the highest concentrations tested, with no differences observed between treatments in air or under hypoxia.

Key words: Disulfide, DNA, hypoxic tumor cell, membrane, Na^+,K^+-ATPase, pH.

Introduction

We recently reported the syntheses and *in vitro* biological activity of two series of imidazolyl disulfides, of which two agents displayed selective toxicity to hypoxic EMT6 tumor cells in culture.[1] In addition to their cytotoxicity, the ability of the agents to deplete cellular glutathione (GSH) was examined and found to be more pronounced under aerobic conditions. These studies suggested that GSH conjugation was a detoxifying measure of the cells, with the greater conjugation resulting in less cytotoxicity under aerobic conditions. It is possible that GSH depletion was restricted under hypoxia due to the lack of NADPH which is required to replenish GSH pools.[2] Why only two of the disulfides displayed selective hypoxic cytotoxicity may be related to the rate of their reaction with the tripeptide and has been investigated by the kinetic analysis of the reaction.

The mechanism of the toxicity of select agents has been further examined in order to determine why one of the disulfides, specifically *n*-butyl 2-imidazolyl disulfide (III-2), was more toxic under hypoxic conditions. It was felt that these studies would allow the design of more potent hypoxic cytotoxic agents.

In the present investigation, additional cytotoxicity studies using the KHT/iv cell line have also shown a lower IC_{90} value for III-2 under hypoxic versus aerobic conditions. Uptake of III-2 into EMT6 cells under hypoxic and aerobic conditions was measured to determine if the differences in survival were due solely to an uptake differential. In addition, both membrane and DNA damage have been assessed following exposure of EMT6 cells to this agent. Initial observations suggested that the disulfide may be membrane active, as cells in culture blebbed and sloughed from the surface of flasks upon exposure to the agent. Because of the thiol reactivity of the disulfides, and the reports of four critical thiol residues on Na^+,K^+-ATPase,[3] membrane fractions containing the enzyme were isolated and inhibition studies were performed. DNA damage was measured in both plasmid DNA and that of EMT6 cells to determine if the cytotoxicity of the disulfide might be due to interaction with DNA and if there were differences

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under hypoxic and aerobic conditions. Furthermore, the cytotoxicity of the disulfide against EMT6 and KHT/iv cells was examined under conditions of decreased extracellular pH. The IC_{90} (EMT6) was found to be 2- to 3-fold lower at pH 6.5 than at pH 7.4 while the IC_{90} (KHT/iv cells) at pH 6.8 was 100-fold lower than that at pH 7.4.

Materials and methods

All chemicals were of reagent grade purchased from Sigma Chemicals, BDH Chemical Co., Aldrich or Fisher Scientific. The solvents used for HPLC analysis were glass-distilled HPLC grade or were made from ultrapure water. All were filtered prior to use. Inorganic phosphate was measured using the Fiske and Subbarow Method kit from Sigma Chemicals.

The HPLC analyses were performed on a system which included a Waters Automated Gradient Controller, two Waters 510 HPLC pumps and a Waters U6K injector. The detector was a Spectroflow 757 UV absorbance detector (Kratos Analytical Instruments) with a 12 μl flow cell. The column (150 × 2 mm inside diameter) was packed with 4 μm Novapak C_{18} stationary phase (Waters Associates). The chromatograms were integrated using a Shimadzu CR501 Chromatopac Integrator.

The EMT6 mouse mammary tumor cell line was maintained in monolayer as described previously.[4] Waymouth's MB752/1 medium (Gibco) was supplemented with 15% Clex (Dextran Products), a semisynthetic serum supplement. The KHT/iv murine sarcoma cell line was grown as monolayer cultures in α-minimal essential medium supplemented with 10% fetal calf serum.[5] Cell counts were performed using a Coulter Counter ZF and Channelyzer 256 (Coulter Electronics).

Survival studies

Drug treatments on KHT/iv cells were performed in a stirred cell suspension under aerobic (95% air and 5% CO_2 at 37°C) and hypoxic (95% N_2 and 5% CO_2) conditions. III-2 was dissolved in ethanol. Following 4 h drug exposures, the cells were plated at various concentrations with lethally irradiated feeder cells in 60 mm dishes. Survival was determined based on an *in vitro* cloning assay which measured the cells' ability to form colonies containing 50 cells. For the pH studies the extracellular pH was adjusted to 6.6–6.8 prior to exposure of the cells to III-2.

Survival of EMT6 cells was measured by a colony forming assay which has been described in detail elsewhere.[4] The cells were gassed as described above and exposed to various concentrations of III-2 in Waymouth's medium at pH 7.4 or pH 6.4–6.5. Following exposure, the cells were washed, trypsinized, counted, diluted into dishes and allowed to form colonies for 10 days. The colonies were stained with crystal violet and those greater than 50 cells were counted. Survival was measured by comparing the plating efficiency of the treated cells with that of control cells.

Uptake

EMT6 tumor cells (1.5×10^6) were seeded in 150 ml flasks 3–4 days prior to the experiment. Before the flasks were gassed, the media was replaced with fresh Waymouth's. The cells were gassed with either 95% N_2/5% CO_2 or 95% air/5% CO_2 for 2 h. Vehicle or drug (1 mM) was injected through rubber septa and the cells were exposed to each for 0–4 h. Flasks containing only media and drug were also incubated for the same lengths of time to monitor for breakdown of drug by the media or extraction procedure. Identical procedures were carried out with the thiols *n*-butanethiol (BT) and 2-mercaptoimidazole (2-MI).

After incubation the media was removed and the cells were rinsed twice with phosphate-buffered saline (PBS). The media and PBS were combined and an aliquot (4 ml) was taken for drug extraction. The cells were then scraped from the backs of the flasks and resuspended in PBS (3 ml). The cells were counted and sonicated. A modified method of Gulaid *et al.*[6] was used to extract the drug or metabolites from the media or cell sonicates. After metronidazole (0.025 mg) was added as an internal standard to each sample, methyl ethyl ketone (MEK, 3 ml) was added with shaking and the samples were centrifuged at 400 *g* for 5 min. The organic layer was removed and the extraction repeated three times using 1 ml MEK. The organic layers were combined and evaporated to dryness under vacuum and residues stored at −20°C until they could be analyzed.

The media and cell residues from both drug and vehicle treated samples were dissolved in glass-distilled methanol and filtered through Corning 0.2 mm PTFE filters. The chromatographic conditions employed a mobile phase of 80% acetic acid

(0.25%, pH 3.5)/20% methanol at a flow rate of 0.4 ml/min for 1.5 min which shifted to 10% acetic acid/90% methanol at a flow rate of 0.45 ml/min over 1 min. The 10% acetic acid/90% methanol was held for 10 min. Retention times for metronidazole, III-2, and possible metabolites BT and 2-MI using these conditions were 2.30, 7.40, 1.15 and 8.09 min, respectively. The extraction efficiency for the metronidazole was found to be 99.5%, while those for III-2, BT and 2-MI ranged from 93 to 98%. The detection limits for III-2, BT and 2-MI were 12.5, 84.2 and 2.5 ng, respectively.

Membrane enzyme inhibition

Plasma membrane isolation. EMT6 cells were grown in suspension in a spinner flask. Approximately 5×10^6 cells were seeded in the flask in Waymouth's media and allowed to grow for up to 7 days. The cells were harvested by removal from the flask, centrification, washing and resuspension in cold Tris–HCl (50 mM, pH 7.5) to yield 2.5×10^7 cells/ml. Stabilization of the membranes was accomplished by the addition of $MgCl_2$ (50 mM, 5% of final volume).[7] After standing on ice for 8 min the cells were sonicated, layered over Histopaque 1077 (Sigma) and centrifuged at 400 *g* for 30 min. The bottom layer containing the membrane fraction was resuspended in Tris–HCl (10 mM, pH 7.5) and placed on ice until the enzyme assay was performed. The protein content of the membrane fraction was determined by the method of Lowry *et al.*[8]

Na^+,K^+-ATPase inhibition. A modified method of Baxter *et al.*[3] and Dornand *et al.*[9] was used to study Na^+,K^+-ATPase activity. Membrane homogenate (0.20 mg protein) in 495 µl was incubated at 37°C for 5 min before MLJ III-2, IV-2 or *N*-ethylmaleimide in 5 µl were added into duplicate tubes. The final concentrations of the compounds were 1.0 and 0.5 mM. The samples were incubated for 1 h at 37°C. Following incubation the tubes were divided into two sets and either 500 µl Tris–HCl (10 mM, pH 7.5) or 400 µl Tris–HCl and 100 µl ouabain (2.5 mM) was added to bring the volume to 1 ml. Ion solutions (0.9 ml) were added to each set of samples. These consisted of 1.5 mM $MgCl_2$ in Tris–HCl (10 mM, pH 7.5) or 1.5 mM $MgCl_2$, 120 mM NaCl and 15 mM KCl in Tris–HCl (10 mM, pH 7.5).[10] ATP (1 mM, 100 µl) was added to all samples and they were incubated with shaking at 37°C for 30 min. The reaction was terminated by the addition of 100 µl of trichloroacetic acid (50%). The samples were then centrifuged at 14 000 *g* for 20 min and aliquots of the supernatants were assayed for inorganic phosphate. One set of the samples represented the activity of Na^+,K^+, Mg^{2+}-ATPase while the other set represented the activity of Mg^{2+}-ATPase.

Inorganic phosphate (Pi) determination. Pi was measured according to the method of Fiske and Subbarow.[11] Sample or standard Pi (1 ml) was added to a mixture of acid molybdate and Fiske and Subbarow reducer, and allowed to stand at room temperature for 10 min. The absorbance was then read at 660 nm. The Na^+,K^+ activity was calculated as Mg^{2+},Na^+,K^+-ATPase minus (Mg^{2+}-ATPase + ouabain). The activity was reported as µM Pi/mg protein/h.

Na^+,K^+-ATPase inhibition kinetics. Purified Na^+,K^+ ATPase (ouabain sensitive) from Sigma was solubilized in Tris–HCl (10 mM, pH 7.5). Vehicle, drug (20–50 µM) and 0.25 units of enzyme in a total of 500 µl were incubated at 37°C for 1 h. The samples were then assayed for enzyme activity using various concentrations of ATP (20–50 µM) as described above.

DNA Damage

pBR322. Basic procedures for plasmid DNA isolation, agarose gel electrophoresis and transformation of bacterial cells have been described.[12] pBR322 plasmid, isolated from *Escherichia coli* KUR1260C as a large scale rapid lysate and purified by cesium chloride ultracentrifugation, was exposed to different concentrations of III-2 (100–500 µM) or to III-2 following preincubation with GSH at ratios of 1:1, 1:2, 1:5 and 1:10 (III-2:GSH). Incubation times ranged from 0 to 24 h under both hypoxic and oxic conditions. The sample was divided to allow both physical and biological integrity to be studied. To study the physical integrity, DNA was precipitated by the addition of potassium acetate to 30 mM and 3 volumes of ethanol (95%, −20°C). Following centrifugation, the DNA pellet was washed with 70% ethanol and dried. The DNA was redissolved in 10 mM Tris–HCl, 1 mM EDTA (pH 8.0) buffer, electrophoresed on a 1% agarose gel and stained with ethidium bromide. To study the biological integrity of the DNA, *E. coli* KUR1260C was transformed with treated and untreated plasmid DNA and plated on LB-ampicillin medium. Plates were

incubated overnight at 37°C, the resulting colonies were counted and the frequency of transformation was calculated.[12]

DNA precipitation

The DNA precipitation assay developed by Olive[13] was used to assay single- and double-strand breaks imposed by III-2 on EMT6 tumor cells in culture. A parallel study was carried out with SR4233, a bioreductive agent known to cause DNA damage.[14] EMT6 cells growing in monolayer were labelled for 20 h with [^{14}C]thymidine (2-^{14}C; 1.85 GBq/mmol). Two hours prior to exposing to III-2, the medium was changed to fresh and the cells were gassed with either 95% N_2/5% CO_2 or 95% air/5% CO_2. Various concentrations of III-2 in 100 μl or vehicle (100 μl) were injected into the medium through a rubber septum and the cells were treated for 1–4 h. The cells were then trypsinized, resuspended in medium (3×10^6 cells/ml) and 0.1 ml was lysed with 0.5 ml 2% sodium dodecyl sulfate detergent at pH 8.3 for neutral lysis or 12.4 for alkaline lysis. This was followed by the addition of 0.12 M KCl (0.5 ml) and heating at 65°C for 10 min. After cooling on ice for 5 min, the precipitate which formed was collected by low speed centrifugation. The supernatant was decanted into a liquid scintillation vial and 1 ml water or 1 ml of 0.05 M HCl was added to neutral or alkaline lysis samples, respectively. The remaining pellet was resolubilized with mixing with 2×1 ml water (65°C) and then poured into a scintillation vial. Scintillation fluid (8 ml of Scinti Verse II) was added to all vials and the radioactivity of the two fractions was measured. The percent DNA precipitation was calculated by dividing the radioactivity in the pellet by that of the pellet plus supernatant multiplied by 100.[13]

Results

The selective toxicity of III-2 under hypoxic conditions, which has been reported for the EMT6 cells,[1] has been reproduced using the KHT/iv cell line in culture. The survival of KHT/iv cells, exposed to varying concentrations of III-2 for 4 h, was measured under both aerobic and hypoxic conditions. A 3-fold difference in the IC_{90} values was measured with the cells being more sensitive under hypoxic conditions (aerobic $IC_{90} = 15$ μM; hypoxic $IC_{90} = 45$ μM). This difference was similar to that observed for the EMT6 cells in culture.[1]

Table 1. The effect of extracellular pH on survival on EMT6 and KHT/iv cells following exposure to III-2

Cell line	IC_{90} (mM) at pH_e					
	7.2–7.4		6.6–6.8		6.4–6.5	
	O[a]	H	O	H	O	H
EMT6[b]	550	400	—	—	80	80
KHT/iv[c]	45	15	0.1	0.1	—	—

[a] O, oxic conditions (95% air/5% CO_2); H, hypoxic conditions (95% N_2/5% CO_2).
[b] Exposure = 2 h.
[c] Exposure = 4 h.

When the extracellular pH was reduced to 6.5 for the EMT6 cells or 6.8 for the KHT/iv cell the sensitivity of the cells was increased as shown in Table 1. In addition, at the lower pH_e equivalent cell killing was achieved for each cell line under aerobic and hypoxic conditions.

Prior to performing the uptake studies the stability of III-2 was monitored in the absence of EMT6 cells to determine if components in the media or CLEX caused the decomposition of the disulfide. After a 4 h incubation at 37°C, 100% of the III-2 was recovered unchanged.

The uptake studies involved the analysis of cell sonicate extracts as well as extracts of the remaining media after exposure to III-2 under both aerobic and hypoxic conditions. Within 15 min of the addition of III-2 to the medium, a 22% decrease was found in the total amount added. Over the next 3.75 h there was only an additional 8% decrease in total III-2 remaining in the medium. This initial loss of III-2 was accompanied by a corresponding increase in the amount of 2-MI in the medium. Following a rise in concentration of 2-MI over the first 15 min, there was a small but consistent decrease in its concentration in the medium at 30 min and an increase over the remaining 3.25 h (Figure 1A). There was no differences observed between the levels of either III-2 or 2-MI measured under aerobic and hypoxic conditions at any of the time points examined.

Only BT was detected in the extract from the cell sonicates. The quantity of BT in the cells increased to a maximum of approximately 13 pg/cell at 2 h, following which it decreased over the next 2 h of exposure (Figure 1B). Again no difference was observed between cells treated under aerobic and hypoxic conditions. This metabolite was not detected in the extracts from the media.

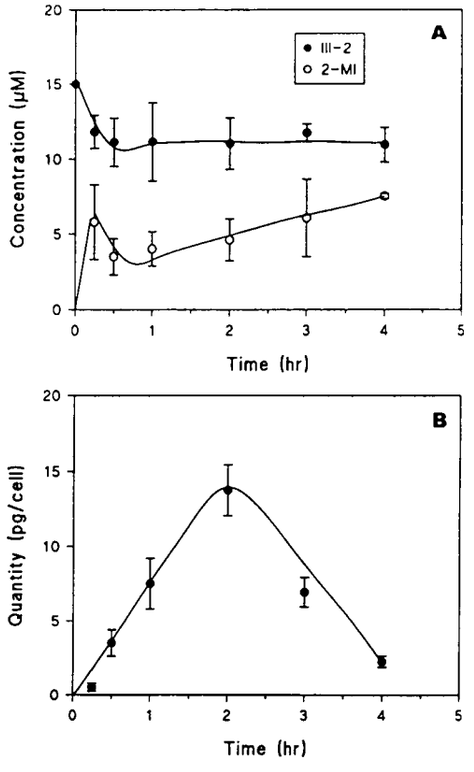

Figure 1. (A) Concentration of III-2 remaining and 2-MI appearing in the media following exposure of EMT6 cells to III-2 over time. (B) Quantity of BT detected in EMT6 cells following exposure to III-2 (1 mM) over time.

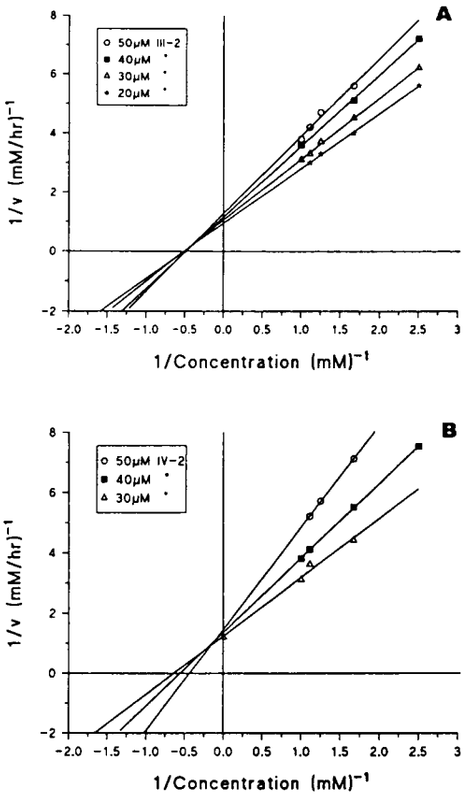

Figure 2. Lineweaver–Burk plots illustrating mixed inhibition of Na^+,K^+-ATPase by (A) III-2 and (B) IV-2.

The inhibition of Na^+,K^+-ATPase by MLJ III-2 and IV-2 (1-methylpropyl 2-imidazolyl disulfide) and *n*-ethylmaleimide (NEM) is reported in Table 2 as percent of control activity (489 ± 145 μM Pi/mg protein/h). At a concentration of 1 mM III-2 inhibited the enzyme activity to the greatest extent (93%), while IV-2 and NEM, a known thiol active agent,[15] inhibited the activity by 74 and 47%, respectively.

Table 2. Inhibition of Na^+,K^+-ATPase isolated from EMT6 cells by MLJ III-2, IV-2 and NEM after 1 h exposure

Compound	Concentration (mM)	Percent inhibition[a]
MLJ III-2	1.0	93 ± 6[b]
	0.5	74 ± 9
MLJ IV-2	1.0	74 ± 15
	0.5	62 ± 2
NEM	1.0	47 ± 2
	0.5	39 ± 4

[a] Control Na^+,K^+-ATPase activity: 489 ± 145 μM Pi/mg protein/h.
[b] Mean ± SD of triplicate values from at least three experiments.

Kinetic studies to determine the type of inhibition were performed by monitoring product formation when varying the concentration of inhibitor and substrate in the reaction mixture. Lineweaver–Burk ($1/v$ versus $1/[S]$) plots revealed the inhibition was of a mixed nature (Figure 2). Secondary plots of slope versus inhibitor concentration and $1/V_{max}$ versus inhibitor concentration were linear (data not shown) and were used to determine inhibitor constants K_1 and K_1' ($K_1 = [E][I]/[EI]$ and $K_1' = [ES][I]/[ESI]$, where [E] is the enzyme concentration, [I] is the inhibitor concentration and S is the substrate). The constants for studies involving III-2 were found to be $K_1 = 40$ M and $K_1' = 50$ M. For IV-2 they were $K_1 = 10$ M and $K_1' = 40$ M.

DNA studies revealed that no physical or biological damage to the plasmid pBR322 was evident following exposure to III-2 at any of the concentrations tested for up to 24 h under either hypoxic or oxic conditions. Since GSH causes III-2 to break down into the components observed in the uptake studies (unpublished data), additional trials

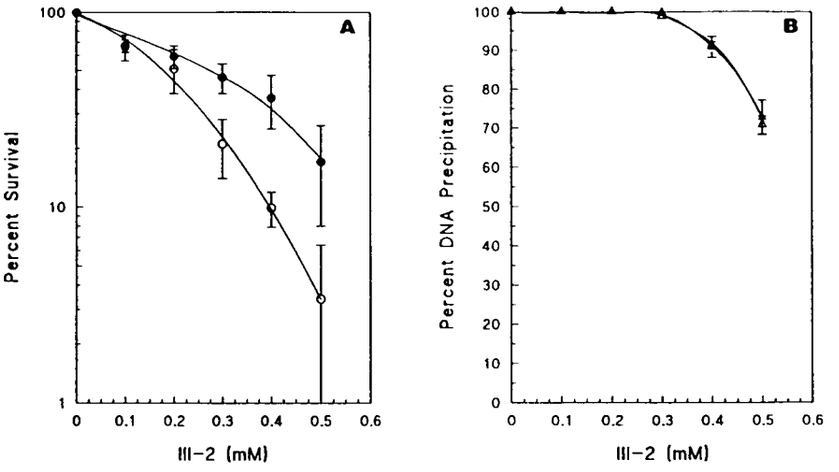

Figure 3. Effect of III-2 on (A) survival and (B) DNA single-strand breaks under oxia (●, ▲) and hypoxia (○, △).

in the presence of up to 10-fold excess GSH were performed. Again, no difference was observed between treated and control DNA when examined by electrophoresis or by transformation studies (data not shown).

Using EMT6 cells, the DNA precipitation assay was also used to measure DNA damage following exposure to III-2 under hypoxic and oxic conditions. No double-strand breaks were observed under any of the 2 h treatment conditions. Longer exposures to III-2 (4 h) at 500 μM cause only minor DNA precipitation (7.2%) under neutral conditions. Single-strand breaks could not be detected at the lower concentrations tested (100–300 μM). Concentrations of 400 and 500 μM after a 2 h exposure showed only very minor strand breaks. The percent DNA precipitate versus control increased at these concentrations when the exposure time increased to 4 h; however, the amount of damage observed did not compare with the level of survival at these concentrations. There were no differences found between those cells treated under the aerobic or hypoxic conditions (Figure 3). In addition to examining the disulfide, the thiol metabolites BT and 2-MI were studied for DNA damaging activity. Neither of these agents produced any single- or double-strand breaks following a 2 h exposure (data not shown).

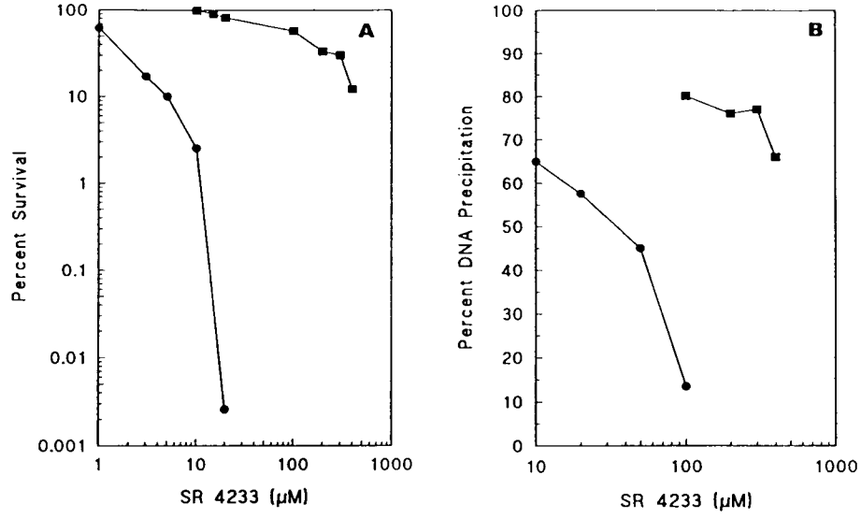

Figure 4. Effect of SR-4233 on (A) survival and (B) DNA single strand breaks under oxia (■) and hypoxia (●).

As a control, the DNA damaging bioreductive agent SR-4233 was also studied under both hypoxic and oxic conditions. In contrast to III-2, this agent produced markedly different amounts of DNA damage under the two exposure conditions (Figure 4). The percent DNA precipitation showed a close correlation to survival following a 4 h exposure to this agent.

Discussion

We have been studying the potential of developing imidazolyl disulfides as hypoxic cell cytotoxic anticancer agents as alternatives to the nitroimidazoles currently undergoing clinical trials.[16,17] Two agents from the first series of disulfides we examined were found to be selectively toxic to hypoxic EMT6 cells in culture.[1] These data have been confirmed in this work using the KHT/iv cell line. The reasons for this selective toxicity are unknown. To date the only difference found after exposure of EMT6 cells to one of these agents was a significantly greater depletion of GSH when exposed under aerobic versus hypoxic conditions. The GSH content was depleted to 20% of control levels when treated in air yet only to 65% of controls when cells were treated under hypoxia. The present study has focused on the possible mechanism of cytotoxic activity of III-2 in an attempt to explain its greater toxicity toward hypoxic cells in culture.

To rule out simply a difference in uptake of the agent by the cells under the two conditions, studies were performed to measure the amount of III-2 in EMT6 cells after exposure in air or hypoxia. It was found that III-2 did not enter these cells under either condition; however, one breakdown product, BT, was detected in the cellular extract. The other metabolite, 2-MI, could only be found accumulating in the media. In general the amount of 2-MI detected in the medium could be correlated to the loss of III-2. However, the mole quantity of BT in the cell extract was less than that of 2-MI measured at each time point. It is possible that some of the BT may have complexed with cellular protein, therefore making it undetectable. This may also explain why after an initial increase in the amount of BT over the first 2 h exposure there was a drop in its detectable amount as the exposure time increased to 4 h. When the thiols BT and 2-MI were administered as single agents, neither of them were found to enter the cells. When III-2 was placed in media without cells present it did not decompose into BT and 2-MI. It was concluded that a metabolic process was occurring at the cell surface in a similar manner to that of the radioprotective drug WR2721. This agent was shown to be hydrolyzed at the plasma membrane and the free sulfhydryl metabolite produced then penetrated the cell membrane to reach intracellular sites.[18]

Due to the fact that there appeared to be a reaction taking place at the cell membrane, and that the membrane enzyme Na^+,K^+-ATPase has critical thiol groups that may be targets of thiol active agents, we examined the ability of III-2 to inhibit the activity of this enzyme. Both III-2 and another agent in this series, which was not selectively toxic to hypoxic cells IV-2, were found to be more inhibitory than NEM, which is known to complex with thiol groups.[3,15] Kinetic studies indicated that the inhibition was 'mixed' in nature for both of the disulfides, meaning that the inhibitor (disulfide) could combine with either the enzyme or enzyme–substrate complex.[19] It was apparent from these studies that the disulfides could be toxic due to the interaction with Na^+,K^+-ATPase at the cell membrane; however, they did not explain why there may be increased toxicity under hypoxia for III-2 and not IV-2.

To determine whether the cytotoxic activity was solely due to membrane interaction or whether III-2 also interacted with DNA we examined its activity on plasmid DNA as well as on that of the intact EMT6 cells. Both methods indicated that there was no DNA damage at concentrations up to 400 μM, concentrations which were able to reduce survival to 10%. Only the DNA precipitation assay detected minor amounts of single-strand breaks at 500 M, but this may be a secondary effect due to the disruption of cellular thiols by III-2.[20,21] This contrasts the results observed with the bioreductive alkylator SR-4233, known to produce both single- and double-strand breaks.[14] Figure 4 illustrates the similarities in both DNA precipitation and survival of EMT6 cells following exposure to SR-4233, and the differences detected under aerobic and hypoxic conditions.

Conclusion

These findings, although not conclusive as to the exact nature of the selective hypoxic toxicity of III-2, indicate that it is a membrane rather than DNA active agent. Under conditions of diminished pH the cytotoxicity of III-2 was potentiated under both aerobic and hypoxic conditions. Since solid

tumors are known to possess subpopulations of hypoxic cells and many have been found to have less than physiological pH_e, III-2 may prove beneficial for solid tumor therapy. The results also suggest that the membrane active agent may offer an alternative to alkylating agents, many of which have been found to produce secondary leukemias.[22] Previous studies have shown that III-2 interacts with thiols and suggest that the greater ability of GSH to protect the cells from its toxicity in air may result in the differential observed in the cytotoxicity studies.[1] This may be the only reason for the selective hypoxic toxicity of III-2.

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