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Inhibition of Doxorubicin-induced Apoptosis *in Vivo* by 2-Deoxy-D-glucose[1]

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ABSTRACT

Previous studies have shown that DNA cleavage by mammalian topoisomerase II is ATP dependent and can be inhibited by metabolic inhibitors. Furthermore, it has been shown that metabolic inhibitors also have a cytoprotective effect *in vitro* against topoisomerase II-targeting antitumor drugs. However, the nature of the ATP-dependent process is not known. We have previously shown that doxorubicin induces apoptosis (programmed cell death) in the murine small intestine which can be inhibited by the protein synthesis inhibitor cycloheximide. In the present study, we have demonstrated that 2-deoxy-D-glucose reduces the incidence of doxorubicin-induced apoptosis *in vivo* if administered within 45 min of the doxorubicin. Maximum reduction was observed at 2 h after treatment (~66%); however, significant reduction was still observable at 9 h after treatment (~33%). Significant positive correlation was observed between protein synthesis inhibition and apoptosis inhibition. Other possible mechanisms of action of the inhibitor do not appear to be important in cytoprotection. The inhibitor did not reduce the uptake of doxorubicin into the intestinal epithelium; however, it caused a significant increase in retention of the drug. The kinetics of inhibition suggest that alteration of cell cycle kinetics, inhibition of formation of doxorubicin-topoisomerase II complex or induction of glucose-regulated proteins are not significant factors in cytoprotection. These studies indicate that at least in the mouse small intestinal epithelium, the ATP-dependent process in cell killing by doxorubicin may involve protein synthesis.

INTRODUCTION

Topoisomerase II has recently been identified as a target for many antineoplastic agents (reviewed in Ref. 1). These include the intercalators (*e.g.*, doxorubicin) and epipodophyllotoxins. These drugs affect the breakage-reunion process of the DNA topoisomerase II by stabilizing the topoisomerase II-DNA cleavable complex (1). However, the mechanism whereby such interaction leads to cell killing is unclear. The interaction of nalidixic acid with bacterial DNA topoisomerase II in bacterial cell killing is analogous to the action of the antineoplastic agents with mammalian topoisomerase II. Nalidixic acid also stabilizes the enzyme-DNA cleavable complex. The cytocidal action of nalidixic acid appears to be ATP dependent since it is inhibited by the metabolic inhibitor, dinitrophenol (2). Previous studies suggest that DNA cleavage by mammalian topoisomerase II is also ATP dependent (3). Furthermore, DNA cleavage *in vitro* by antineoplastic agents that interact with topoisomerase II, is enhanced by the ATP (3). Recently, it has been shown that DNP[3] and other metabolic inhibitors also have a cytoprotective effect *in vitro* against topoisomerase II-targeting antitumor drugs (4). However, DNP does not affect the amount of cleavable complexes induced and it has been suggested that an ATP-dependent step which occurs after the induction of cleavable complex is involved in the cytotoxic action of the drug.

2-Deoxy-D-glucose, a glycolytic pathway inhibitor, has also been shown to confer cytoprotection against doxorubicin, a potent and widely used chemotherapeutic agent (5, 6). Similar effects are also observed following anoxia (6–9), glucose deprivation and treatment with the calcium ionophore A23187 (6), and 2,4-dinitrophenol (5). Since all these treatments induce GRP, it has been suggested that the induction of these proteins is related to the cytoprotection (6).

Our previous observations suggest that another mechanism may be important in the cytoprotective effects of these agents against topoisomerase II-targeting drugs. We have previously shown that doxorubicin induces apoptosis (programmed cell deletion) in the murine small intestine and this can be inhibited by cycloheximide, a protein synthesis inhibitor (10). Induction of apoptosis by other topoisomerase II-targeting drugs *in vitro* and inhibition of this process by cycloheximide and actinomycin D, a transcription inhibitor, has also been demonstrated recently (11).

It may be speculated that metabolic inhibitors such as 2-deoxy-D-glucose and DNP exert a cytoprotective effect in these situations since apoptosis is thought to be an ATP-dependent process (12). Alternatively, their effects may be mediated via secondary inhibition of protein or RNA synthesis following ATP depletion.

In the present study we have investigated the effects of 2-deoxy-D-glucose on doxorubicin-induced apoptosis, in the murine intestinal tract. In addition, we have investigated the effect of 2-deoxy-D-glucose on macromolecular synthesis.

MATERIALS AND METHODS

The methods have been previously described in detail (10). Brief descriptions are given below.

Animals. Ten- to 12-week-old male BDF1 (B6D2F1) mice (C57B6 ×DBA2) were used for all experiments. Animals were kept under a 12 h dark (6 p.m. to 6 a.m.)/12 h light regimen and they were given food and water *ad libitum*.

Drugs. Doxorubicin (Adriamycin, Pharmacia, Italy), and 2-deoxy-D-glucose (Sigma, St. Louis, MO), were dissolved immediately prior to use in sterile isotonic saline to give the required concentration in 0.2 ml. All drugs were administered i.p. The inhibitor was generally administered immediately after the doxorubicin and the doses of drugs used were 20 mg/kg (doxorubicin) and 2 g/kg (2-deoxy-D-glucose), unless stated otherwise.

Reagents. The radiolabeled compounds were purchased from NEN-DuPont. The reagents for flow cytometry were purchased from Sigma. Other reagents, except where stated, were purchased from BDH Chemicals, Ltd., Poole, England.

Sample Preparation and Scoring of Apoptosis. The middle one third of the small intestine was removed, the tissues were fixed in Carnoy's solution for at least 30 min, and histological transverse 5 μm thick sections were prepared. The cells showing morphological changes consistent with apoptosis (reviewed in Ref. 12) were counted in good longitudinal crypt sections (10, 13). Briefly, these morphological changes included (*a*) cytoplasmic condensation and eosinophilia, (*b*) nuclear condensation usually typified by crescentic chromatin margination, and (*c*) nuclear and cytoplasmic fragmentation (10). Twenty-five crypt sections were scored for each mouse.

Doxorubicin Uptake. The epithelium of the small intestine was scrapped off following removal of the gut from the animals and washing with ice-cold isotonic saline. Doxorubicin was extracted from the epithelium using the procedure described by Bachur *et al.* (14). The fluorescence of the samples and doxorubicin standards was measured at an excitation wavelength of 475 nm and emission wavelength of 585 nm using a Shimadzu RF540 fluorospectrophotometer (Shimadzu Corporation, Kyoto, Japan). The doxorubicin content was determined from standard curves. The total protein in the tissues was determined as described below. The data were expressed as μg doxorubicin/mg protein.

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[3] The abbreviations used are: DNP, dinitrophenol; GRP, glucose-regulated protein(s).

Precursor Incorporation. DNA, RNA, and protein synthesis were assayed by incorporation of [^{3}H]thymidine, specific activity 247.9 GBq/mMol, [^{3}H]uridine, specific activity 1.01 TBq/mMol, [^{3}H]leucine, specific activity 2.22 TBq/mmol, respectively. The animals were injected i.p. with 370 kBq of either [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine 20 min prior to killing the animals. The total protein was determined using the Bisinchoninic acid protein assay kit BCA-1 (Sigma) following tissue removal, homogenization, trichloracetic acid precipitation, and solubilization in NaOH (1 M). Aliquotes of the solubilized proteins and trichloracetic acid supernatants were used for scintillation counting. The data were expressed as specific activity (DPM acid insoluble fraction/μg protein) and corrected for the effects of the drugs on precursor uptake (specific activity/DPM acid soluble fraction/unit volume).

Statistical Analysis. Where appropriate the statistical significance was tested by Student's t test with the accepted significance level of $P < 0.05$. Pearson correlation coefficient was used to test the correlation between apoptosis inhibition and inhibition of protein and DNA and RNA synthesis.

RESULTS

Effect of 2-Deoxy-D-glucose on the Whole Animal. The animals showed a reduction in physical activity within 15 min of 2-deoxy-D-glucose (2 g/kg) administration which persisted for 5–6 h.

Cytoprotection by 2-Deoxy-D-glucose. The effect of different doses (0–2 g/kg) of 2-deoxy-D-glucose on doxorubicin-induced apoptosis in the mouse small intestine is shown in Fig. 1*A*. There was a dose-dependent decrease in the doxorubicin-induced cell killing with maximum cytoprotection observed with the highest dose (2 g/kg) of 2-deoxy-D-glucose used. The yield of apoptosis in animals treated with 2 g/kg 2-deoxy-D-glucose was ~13% of that in the animals treated with doxorubicin only. Following a single dose (2 g/kg) of 2-deoxy-D-glucose administered immediately after the doxorubicin, significant inhibition ($P < 0.01$) of cell killing was observed from 2 to 9 h after treatment (Fig. 1*B*). Maximum inhibition was observed at 2 h after treatment (~66%). The incidence of cell death in both the doxorubicin and the doxorubicin plus inhibitor treatment groups were similar at later periods. The 2-deoxy-D-glucose was effective only if administered within 45–60 min after the doxorubicin; inhibition was also observed if the 2-deoxy-D-glucose was administered at least 60 min before the doxorubicin (Fig. 1*C*). As reported previously (10), doxorubicin-induced apoptosis was observed predominantly in the base of the crypt at cell positions 4–7. 2-Deoxy-D-glucose inhibited apoptosis at most cell positions although greatest differences were seen in cell positions 4–7 (data not shown).

Effects of 2-Deoxy-D-glucose on Doxorubicin Uptake/Retention. 2-Deoxy-D-glucose, when administered immediately after the doxorubicin, did not affect the uptake of doxorubicin (Fig. 2). However, there was greater retention ($P = 0.05$) of doxorubicin at 12 h after 2-deoxy-D-glucose treatment.

Effects of 2-Deoxy-D-glucose on Macromolecular Synthesis. There was a dose-dependent decrease in the incorporation of [^{3}H]leucine 30 min after treatment with significant inhibition ($P < 0.01$) at doses above 0.4 g/kg (Fig. 3*A*). Additionally, significant inhibition of [^{3}H]oridine was observed at 1 h posttreatment with the highest dose used (Fig. 3*B*). Although there was some decrease in the incorporation of [^{3}H]thymidine (Fig. 3*C*), this was not significant. The Pearson correlation coefficients between the percentage inhibition of apoptosis and macromolecular synthesis are shown in Table 1. Significant correlation was observed with only protein synthesis inhibition.

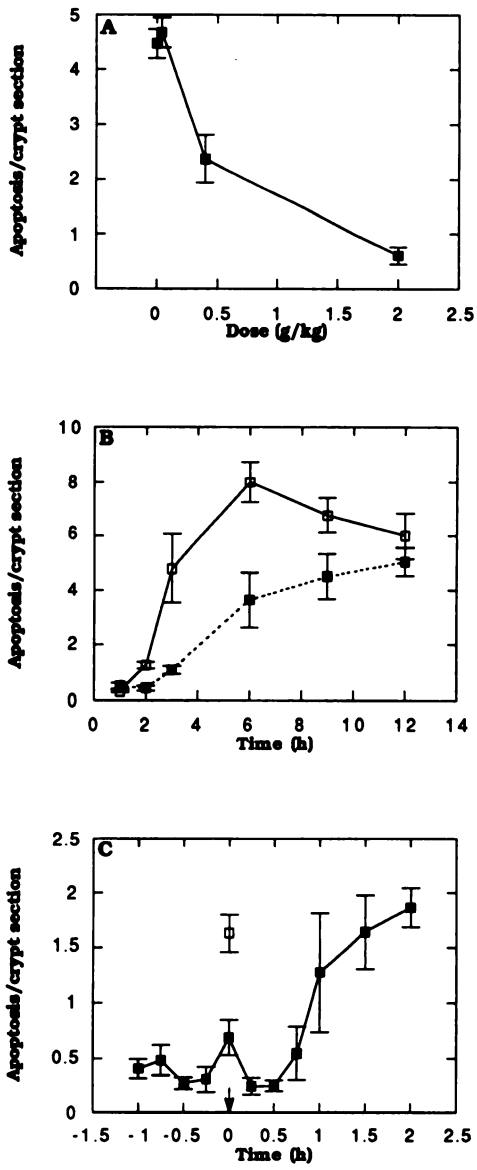

Fig. 1. Effect of 2-deoxy-D-glucose on the incidence of doxorubicin-induced apoptosis. *A*, 3 h after treatment with doxorubicin and different doses of 2-deoxy-D-glucose (0–2 g/kg); *B*, at times after treatment with 20 mg/kg doxorubicin alone (□) or together with 2 g/kg 2-deoxy-D-glucose (■); *C*, 3 h after treatment with doxorubicin (20 mg/kg). 2-Deoxy-D-glucose was administered either immediately after or at times before or after the doxorubicin; doxorubicin was administered at time 0 (*arrow*). □, mean ± 1 SE of apoptosis in animals treated with doxorubicin only. *Points*, mean ± 1 SE of data from at least 4 animals.

DISCUSSION

It has been previously shown that DNA cleavage *in vitro* by antineoplastic agents that interact with topoisomerase II is enhanced by ATP (3) and cytoprotection against such agents *in vitro* is conferred by metabolic inhibitors (4–6). In the present study, we have demonstrated that 2-deoxy-D-glucose, a glycolytic pathway inhibitor, confers cytoprotection against doxorubicin *in vivo*.

Previous studies have assessed cytotoxicity of antineoplastic agents both *in vivo* and *in vitro* using clonogenic assays (4–9). These assays measure the loss of reproductive potential rather than cell killing. We have used morphological criteria to assay the cell death. Such criteria may be more correctly correlated with cell killing. With regards to the biochemical assays used in the present study, these were done on isolated whole epithelium and may not correctly reflect the events occurring in the few cells (<2%) undergoing cell death. However, it has previously been shown that that there does not appear to be a preferential uptake or retention of doxorubicin by any cells in the murine intestinal epithelium (15). Similarly, 2-deoxy-D-glucose has been shown to act as metabolic inhibitor in a variety of tissues (16) and it is unlikely that its effects on the macromolecular synthesis will vary in the different cells of the intestinal epithelium.

It is unclear from the present study whether 2-deoxy-D-glucose causes an irreversible inhibition or a temporary inhibition (*i.e.*, a delay) of the apoptotic process. A gradual increase in the apoptosis incidence with time is observed in the inhibitor-treated animals with the levels at 12 h posttreatment being similar to those in the control animals. This is similar to the inhibition by cycloheximide of doxorubicin-induced apoptosis in this tissue (10). However, the incidence of apoptosis in the cycloheximide-treated animals does not increase at any time to the peak levels observed in the cytotoxic-only treated

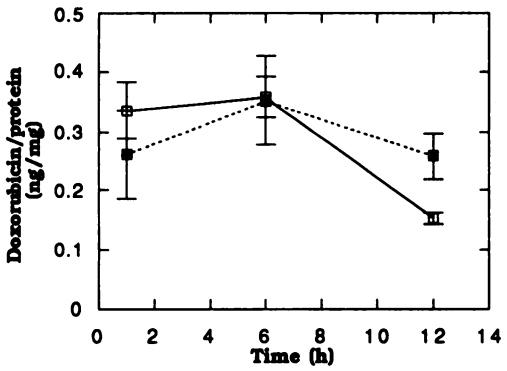

Fig. 2. Effect of 2-deoxy-D-glucose on the uptake and retention of doxorubicin in the mouse small intestine (□, 20 mg/kg doxorubicin; ■, 20 mg/kg doxorubicin and 2 g/kg 2-deoxy-D-glucose). The data are expressed as amount of doxorubicin/acid insoluble protein. *Points*, mean ± 1 SE of data from at least 4 animals.

group even when assayed up to 18 h posttreatment (10). This suggests that protein synthesis inhibition may irreversibly inhibit doxorubicin-induced apoptosis in some cells. 2-Deoxy-D-glucose, like cycloheximide, appears to inhibit apoptosis by inhibiting protein synthesis as discussed below.

Although cellular ATP was not assayed in this study, it is clear from the effects on the whole animal that the 2-deoxy-D-glucose was having the desired effect. Depletion of cellular ATP may be directly responsible for inhibition of cell death. DNA cleavage *in vitro* by antineoplastic agents that interact with topoisomerase II has been shown not only to be ATP dependent but also enhanced by the ATP (3). Furthermore, DNP and other metabolic inhibitors including 2-deoxy-D-glucose have a cytoprotective effect *in vitro* against topoisomerase II-targeting antitumor drugs (4–6). However, DNP does not affect the amount of cleavable complexes induced (4). This suggests that the metabolic inhibitor does not prevent the drug from reaching its cellular target (topoisomerase II) or its interaction with this target. This is consistent with the observations reported here and in previous studies (5–6) of lack of effect of 2-deoxy-D-glucose on the uptake of doxorubicin and the effectiveness of 2-deoxy-D-glucose even when administered 45 min after the doxorubicin. It is likely that an ATP-dependent step which occurs after the induction of cleavable complex is involved in cytotoxic action of the drug.

Depletion of ATP may inhibit apoptosis because this process is thought to be ATP dependent (12). However, the nature of the ATP-dependent process is unclear. Because (*a*) ATP depletion is likely to effect other metabolic activities, (*b*) our previous studies (10) indicate that doxorubicin induces apoptosis in this tissue that is abrogated by protein synthesis inhibition, and (*c*) the similarity of the kinetics of inhibition with both the cycloheximide (10) and 2-deoxy-D-glucose, we investigated the role of macromolecular synthesis in the cytoprotection. As with cycloheximide, significant positive correlation between inhibition of protein synthesis and inhibition of cell death was observed.

An obvious explanation for such an effect could be that protein synthesis inhibition affects cellular topoisomerase II levels and the formation of doxorubicin-topoisomerase II complex. Protein synthesis inhibitors such as cycloheximide have been shown to have a cytoprotective action not only against doxorubicin but also against other topoisomerase II-targeting agents (11, 17–20). However, as discussed above, the data on the kinetics of inhibition reported here and in our previous study with cycloheximide (10) support the observation that the mode of action of these agents is not through interference with the formation of the cleavable complex (4). Both 2-deoxy-D-glucose and cycloheximide were effective even when administered 45–60 min after the doxorubicin (which diffuses rapidly into the tissues). Furthermore, there does not appear to be a clear relationship between cellular topoisomerase II level and cell killing and cycloheximide may have a cytoprotective action without a significant change in the enzyme content or DNA cleavage (20).

Cellular ATP depletion could affect many processes such as protein phophorylation which may be important in apoptosis. These have not been investigated in this study and cannot be excluded. More specifically, 2-deoxy-D-glucose could inhibit cell death through induction of GRP (6–9), alteration of the kinetic state of the intestinal epithelium, or inhibition of protein glycosylation.

The role of GRP in cytoprotection against doxorubicin is unclear. Coinduction of GRP and doxorubicin resistant in Chinese hamster ovary cells following treatment with 2-deoxy-D-glucose has been demonstrated (6). However, a lack of correlation between (*a*) GRP

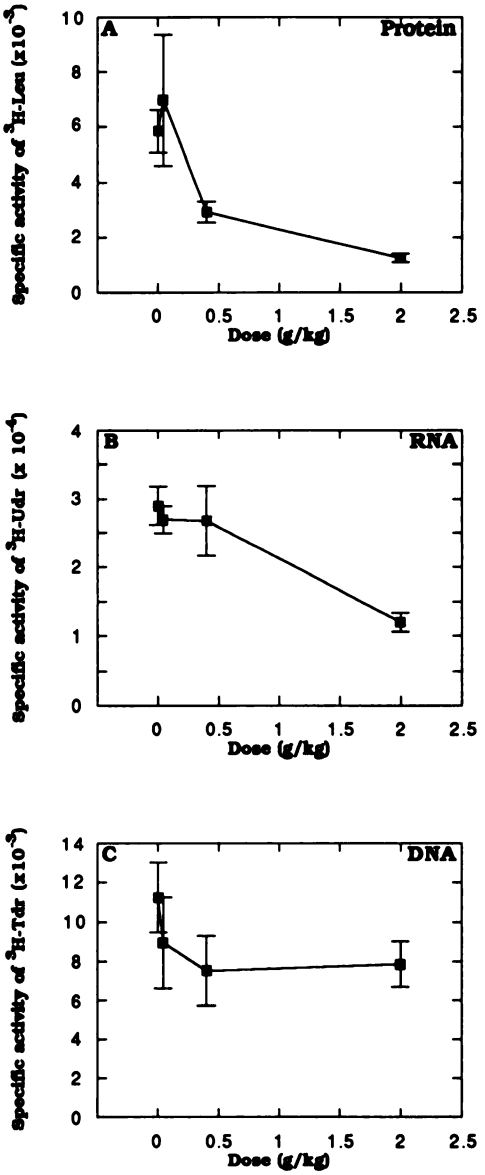

Fig. 3. Effect of 2-deoxy-D-glucose (0–2 mg/kg) on the incorporation of [³H]leucine (*A*), [³H]uridine (*B*), and [³H]thymidine (*C*) into acid-insoluble material. *Points*, the mean ± 1 SE of data from at least 4 animals.

Table 1

Synthesis	Pearson correlation coefficient (*r*)	Significance
Protein	0.97	$P < 0.02$
DNA	0.74	NS[a]
RNA	0.83	NS

[a] NS, not significant.

induction and cytoprotection following glucose deprivation and (*b*) decay of the induced GRP and repression of cytoprotection suggests that it is the inductive state rather than the GRP that is important in cytoprotection (6). It is unlikely that induction of GRP is important in the inhibition of doxorubicin-induced apoptosis in the present study because (*a*) 2-deoxy-D-glucose was effective even when administered 30–45 min after doxorubicin and (*b*) increasing the induction period by administration of 2-deoxy-D-glucose 1 h prior to the doxorubicin did not increase the level of cytoprotection. This is similar to the findings of Kupfer *et al.* (4).

2-Deoxy-D-glucose has been shown to inhibit glycosylation of *N*-linked glycoproteins (21). These glycoproteins are involved in the recognition and phagocytosis of apoptotic cells by macrophages (reviewed in Ref. 12). Although 2-deoxy-D-glucose could affect this process, it is not apparent how it could lead to a decrease in the incidence of apoptosis. *N*-linked glycoproteins may also play a role in apoptosis induction in situations where cell-cell interactions or cell surface receptors are involved, *e.g.*, in immune mediated cell killing. However, it is unlikely that such interactions are involved in doxorubicin-induced apoptosis.

Cytoprotection *in vitro* by agents such as cycloheximide and anguidine against doxorubicin (17–19) appears to be related to the induction of a frozen cell cycle state. However, we have previously demonstrated that the expression of doxorubicin-induced apoptosis is not cell cycle dependent (10). Since doxorubicin is a cell cycle active agent with preferential killing in late G_1 and S phases (reviewed in Ref. 22), the size of the most sensitive target population could be reduced by alteration of the kinetic state of the crypt cells by 2-deoxy-D-glucose. However, the effectiveness of the 2-deoxy-D-glucose and cycloheximide even when administered up to 45 min after the doxorubicin coupled with the rapid uptake of doxorubicin argues against this possibility.

2-Deoxy-D-glucose has been reported to inhibit apoptosis occurring in other situations such as palate development (23). 2-Deoxy-D-glucose has also been shown to inhibit radiation-induced apoptosis in the murine small intestinal epithelium (24). It has been suggested that this is directly due to ATP depletion. Our data suggest that secondary inhibition of protein synthesis may be important in this process. This is consistent with the hypothesis that apoptosis is an endogenous, possibly gene-dependent process requiring protein synthesis for expression (12). However, there does not appear to be an absolute requirement for protein synthesis in apoptosis in all situations (25–28). Indeed, apoptosis can be induced by protein synthesis inhibitors both *in vivo* and *in vitro* (28–29). This suggests that there is heterogeneity in mechanisms leading to apoptosis.

The importance of protein synthesis inhibition by 2-deoxy-D-glucose in cytoprotection against doxorubicin may be specific to the mode of cell death (*i.e.*, apoptosis) occurring in this tissue on exposure to doxorubicin. However, this needs to be investigated using other systems (both *in vitro* and *in vivo*) and assays. This may help in elucidating the events leading to cell killing by topoisomerase-targeting drugs subsequent to the formation of the drug-induced DNA-topoisomerase II complex.

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