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Original Article

Lysosomal Oxidative Stress Cytotoxicity Induced by Dacarbazine and It's Pyridine Derivative in Hepatocytes

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ABSTRACT

Dacarbazine (DTIC) is a synthetic chemical antitumor agent which is used to treat malignant melanoma and Hodgkin's disease. DTIC is a prodrug which is converted to an active form undergoing demethylation by liver enzymes. The active form prevents the progress of disease via alkylation of DNA strand. In the structure of this drug, the imidazole ring, a triazen chain and carboxamide group exist. Based on the literature, the ring and carboxamide group do not have a key role in antitumor activity of the drug. On the other hand, imidazole ring has a unique tautomerization which may participate in the mechanism of action of DTIC and carboxamide group may determine the rich guanine pieces in DNA strand. In order to investigate the mechanistic role of imidazole group and its known tautomerization in DTIC cytotoxicity, derivative of DTIC with a pyridine ring (3-(3,3-dimethyl-1-triazenyl)pyridine, (compound I) instead of imidazole ring was synthesized. In the following, the cellular and molecular mechanism of cytotoxicity induced by DTIC and its pyridine derivative toward the isolated rat hepatocytes were studied and compared. Hepatocyte reactive oxygen species (ROS) generation was significantly increased by both DTIC and compound B before cytotoxicity ensued. In addition, DTIC and compound I induced lysosomal damage and hepatocyte protease activation. Endocytosis inhibitors, lysosomotropic agents or lysosomal protease inhibitors also prevented both DTIC and compound B induced hepatocytes cytotoxicity. Furthermore desferoxamine (a ferric chelator), antioxidants or ROS scavengers (catalase, mannitol or dimethylsulfoxide) prevented both DTIC and compound I cytotoxicity. It is concluded that H_2O_2 reacts with lysosomal Fe^{2+} to form hydroxyl radical which (Haber-Weiss reaction) causes lysosomal membrane disruption, proteases and other digestive enzymes release and finally the cell death.

Keywords: Cytochrome P450; Cytotoxicity; Dacarbazine; Lysosomes Oxidative stress; Tautomerization.
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1. Introduction

Dacarbazine ([5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide]; DTIC) is a synthetic chemical antitumor agent which is used to treat malignant melanoma and Hodgkin's disease [1-3]. Although the exact mechanism of effect of DTIC is unknown, three hypotheses have been offered: a) inhibition of DNA synthesis by acting as a purine analogue , b) action as alkylating agent, c) interaction with SH groups [4]. By the evidence of AIC in urine, it's primary mode of action appears to be alkylation of nucleic acids [5]. However, DTIC is a prodrug which becomes active by N-demethylation in liver microsomes [2,6] and MTIC is formed. Then MTIC spontaneously is metabolized to AIC and methyldiazonium which is changed to methyl carbanion that has a capability to methylate the DNA strand on of guanine [7-10].

In the following, we provide evidence that the cytotoxicity of DTIC and one of it's pyridine derivative ((3-(3,3-dimethyl-1-triazenyl)pyridine; compound I) may involve oxygen activation and reactive oxygen species (ROS) formation. The cytotoxic process that causes plasma membrane disruption is probably mediated by lysosomal membrane damage caused by the ROS formation and release of deadly proteases.

2. Materials and methods

2.1.Chemicals

DTIC was obtained from drug store. Compound B (3-(3,3-dimethyl-1-triazen) pyridine) was synthesized in our laboratory. Collagenase (from *Clostridium histolyticum*), bovine serum albumin (BSA), Hepes, trypan blue, d mannitol, dimethyl sulfoxide, catalase, superoxide dismutase, chloroquine diphosphate, methylamine HCl, 3-methyl adenine, monensin sodium, leupeptin, pepstatin, ethylene glycol bis (p-aminoethyl ether) N,NN',N' tetra acetic acid (EGT A), and heparin were obtained from Sigma (St. Louis, MO, USA). Acridine orange and dichlorofluorescin diacetate was purchased from Molecular Probes (Eugene, Ore, USA). Desferoxamine was a gift from Ciba Geigy Canada Ltd. (Toronto, ON, Canada). All chemicals were of the highest commercial grade available.

2.2. Animals

Male Sprague-Dawley rats (280-300g), fed a standard chow diet and given water *ad libitum*, were used in all experiments.

2.3. Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver as described by Pourahmad and O'Brien, 2000 [11]. Approximately 85-90% of hepatocytes excluded trypan blue. Cells were suspended at a density of 10^6 cells/ml in round bottomed flasks rotating in a water bath maintained at 37 °C in Krebs-Henseleit buffer (pH 7.4), supplemented with 12.5 mM Hepes under an atmosphere of 10% O_2, 85% N_2, 5% CO_2. Each flask contained 10 ml of hepatocyte suspension. Hepatocytes were preincubated for 30 min. prior to addition of chemicals. Stock solutions of all chemicals (× 100 concentrated for the water solutions or × 1000 concentrated for the methanolic solutions) were prepared fresh prior to use. To avoid either non toxic or very toxic conditions in this study we used EC_{50} concentration for DTIC and compound I in the isolated hepatocytes (56 µ M and 33µ M, respectively). The EC_{50} of a chemical in hepatocyte cytotoxicity assessment technique (with the total 3 h incubation period), is defined as the concentration which decreases the hepatocyte viability down to 50% following the 2 h of incubation [12]. In order to determine this value for the investigated compound dose-response, curves were plotted and then EC_{50} was determined based on a regression plot of three different concentrations (data and curves not shown). For the chemicals which dissolved in water, we added 100 µ l sample

Table 1. Preventing DTIC and compound I cytotoxicity by antioxidants, "ROS" scavengers, endocytosis inhibitors.

Addition	%Cytotoxicity at 3h
None	20 ± 2
Dacarbazine (56 μM)	76 ± 4 (1)
+Catalase (200 U/ml)	46 ± 2 (2)
+Dimethyl sulfoxide (150 μM)	44 ± 3 (2)
+Mannitol (50 mM)	48 ± 3 (2)
+Desferoxamine (200 μM)	36 ± 2 (2)
+α-Tocopherol succinate (100 μM)	41 ± 4 (2)
+Monensin (10 μM)	51 ± 2 (2)
+Methylamine (30 mM)	36 ± 4 (2)
+Chloroquine (100 μM)	40 ± 3 (2)
+3-Methyladenine (5 mM)	36 ± 4 (2)
Compound I (33 μM)	73 ± 2 (1)
+Catalase (200 U/ml)	38 ± 2 (3)
+Dimethyl sulfoxide (150 μM)	36 ± 3 (3)
+Mannitol (50 mM)	38 ± 4 (3)
+Desferoxamine (200 μM)	35 ± 3 (3)
+α-Tocopherol succinate (100 μM)	35 ± 3 (3)
+Monensin (10 μM)	51 ± 1 (3)
+Methylamine (30 mM)	31 ± 2 (3)
+Chloroquine (100 μM)	46 ± 3 (3)
+3-Methyladenine (5 mM)	48 ± 5 (3)

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37 °C for 3.0 h. following the addition of DTIC and compound I. Cytotoxicity was determined as the percentage of cells that take up trypan blue [11]. Values are expressed as means of three separate experiments (S.D.). 1: Significant difference in comparison with control hepatocytes (p <0.05). 2: Significant difference in comparison with DTIC treated hepatocytes (p<0.05). 3: Significant difference in comparison with compound I treated hepatocytes (p<0.05).

of its concentrated stock solution (× 100 concentrated) to one rotating flask containing 10 ml hepatocyte suspension. For the chemicals which dissolved in methanol we prepared methanolic stock solutions (× 1000 concentrated), and to achieve the required concentration in the hepatocytes, we added 10 μl samples of the stock solution to the 10 ml cell suspension. Ten μl of methanol did not affect the hepatocyte viability after 3 h incubation (data not shown).

2.4. Cell viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined by the trypan blue (0.2% w/v) exclusion test [11]. Aliquots of the hepatocyte incubate were taken at different time points during the 3 h of incubation period. At least 80-90% of the control cells were still viable after 3 h.

2.5. Determination of reactive oxygen species "ROS"

To determine the rate of hepatocyte "ROS" generation, dichlorofluorescin diacetate was added to the hepatocyte incubate as it penetrates hepatocytes and becomes hydrolysed to non-fluorescent dichlorofluorescin. The latter then reacts with "ROS" to form the highly fluorescent dichlorofluorescein which effluxes the cell. Hepatocytes (1×10^6 cells/ml) were suspended in 10 ml modified Hank's balanced salt solution (HBS), adjusted to pH 7.4 with 10 mM Hepes (HBSH) and were incubated with DTIC and compound I at 37 °C for 3 h. After centrifugation (50×g.1 min.), the cells were resuspended in HBS adjusted to pH 7.4 with 50 mM Tris-HCl and loaded with dichlorofluorescin by incubating with 1.6 μl dichlorofluorescin diacetate for 2 min. at 37 °C. The fluorescence intensity of the "ROS" product was measured using a Shimadzu

Table 2. Preventing DTIC and compound I "ROS" formation by antioxidants, “ROS” scavengers, endocytosis inhibitors

Addition	**"ROS" 3h**
None	79 ± 4
Dacarbazine (56 μM)	230 ± 4 (1)
+Catalase (200 U/ml)	116 ± 5 (2)
+Dimethyl sulfoxide (150 μM)	121 ± 2 (2)
+Mannitol (50 mM)	136 ± 3 (2)
+Desferoxamine (200 μM)	121 ± 3 (2)
+α-Tocopherol succinate (100 μM)	11 ± 3 (2)
+Monensin (10 μM)	161 ± 2 (2)
+Methylamine (30 mM)	117 ± 3 (2)
+Chloroquine (100 μM)	128 ± 2 (2)
+3-Methyladenine (5 mM)	132 ± 4 (2)
Compound I (33 μM)	256 ± 5 (1)
+Catalase (200 U/ml)	126 ± 3 (3)
+Dimethyl sulfoxide (150 μM)	145 ± 2 (3)
+Mannitol (50 mM)	141 ± 3 (3)
+Desferoxamine (200 μM)	136 ± 3 (3)
+α-Tocopherol succinate (100 μM)	119 ± 2 (3)
+Monensin (10 μM)	164 ± 3 (3)
+Methylamine (30 mM)	141 ± 2 (3)
+Chloroquine (100 μM)	155 ± 3 (3)
+3-Methyladenine (5 mM)	151 ± 3 (3)

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37C for 3.0 h. following the addition of DTIC and compound I. DCF formation was expressed as fluorescent intensity units [13]. Values are expressed as means of three separate experiments (S.D.). 1: Significant difference in comparison with control hepatocytes (p<0.05). 2: Significant difference in comparison with DTIC treated hepatocytes (p<0.05). 3: Significant difference in comparison with compound I treated hepatocytes (p<0.05).

RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 470 nm and 540 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells [13].

2.6. Lysosomal membrane stability assay

Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange [14]. Aliquots of the cell suspension (0.5 ml) that were previously stained with acridine orange 5 μM, were separated from the incubation medium by 1 min. centrifugation at 1000 rpm. The cell pellet was then resuspended in 2 ml of fresh incubation medium. This washing process was carried out for two times to remove the fluorescent dye from the media. Acridine orange redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 470 nm excitation and 540 nm emission wavelengths.

2.7. Statistical analysis

The statistical significance of differences between the control and treatment groups in these studies was determined using a one-way analysis of variance (ANOVA) and the Bartlett’s test for homogeneity of variances. Results represent the mean ± standard error of the mean (SEM) of triplicate samples. The minimal level of significance chosen was p<0.05.

3. Results

When hepatocytes were incubated with 56 μM DTIC and 33 μM of compound I the formation of “ROS” was increased very rapidly (peak in about 60 min., curve not shown) in a concentration-dependent fashion (Table 1). The antioxidants: α-tocopheryl

Table 3. Preventing DTIC and compound I induced hepatocyte lysosomal membrane damage by inhibitors of oxidative stress, mitochondrial respiratory chain and endocytosis.

Addition	Acridine orange redistribution		
	2 min.	15 min.	30 min.
None	2 ± 1	4 ± 2	4 ± 3
Dacarbazine (56 μM)	183 ± 5 (1)	237 ± 5 (1)	250 ± 4 (1)
+Catalase (200 U/ml)	11 ± 1 (2)	14 ± 2 (2)	18 ± 2 (2)
+Dimethyl sulfoxide (150 μM)	8 ± 3 (2)	10 ± 1 (2)	12 ± 1 (2)
+Mannitol (50 mM)	8 ± 2 (2)	11 ± 1 (2)	13 ± 1 (2)
+Desferoxamine (200 μM)	8 ± 2 (2)	10 ± 2 (2)	11 ± 1 (2)
+SOD (100 U/ml)	10 ± 2 (2)	16 ± 2 (2)	20 ± 2 (2)
+Monensin (10 μM)	21 ± 2 (2)	25 ± 3 (2)	32 ± 3 (2)
+Methylamine (30 mM)	8 ± 2 (2)	11 ± 1 (2)	14 ± 1 (2)
+Chloroquine (100 μM)	12 ± 1 (2)	15 ± 2 (2)	20 ± 2 (2)
+3-Methyladenine (5 mM)	13 ± 1 (2)	18 ± 2 (2)	26 ± 3 (2)
Compound I (33 μM)	194 ± 5 (1)	240 ± 5 (1)	264 ± 5 (1)
+Catalase (200 U/ml)	12 ± 1 (3)	16 ± 2 (3)	18 ± 2 (3)
+Dimethyl sulfoxide (150 μM)	14 ± 2 (3)	18 ± 2 (3)	21 ± 2 (3)
+Mannitol (50 mM)	12 ± 1 (3)	16 ± 1 (3)	19 ± 2 (3)
+Desferoxamine (200 μM)	10 ± 1 (3)	12 ± 1 (3)	16 ± 2 (3)
+SOD (100 U/ml)	10 ± 2 (3)	15 ± 2 (3)	21 ± 2 (3)
+Monensin (10 μM)	24 ± 3 (3)	28 ± 3 (3)	34 ± 3 (3)
+Methylamine (30 mM)	12 ± 1 (3)	14 ± 1 (3)	17 ± 2 (3)
+Chloroquine (100 μM)	16 ± 2 (3)	18 ± 2 (3)	25 ± 2 (3)
+3-Methyladenine (5 mM)	16 ± 2 (3)	18 ± 2 (3)	26 ± 3 (3)

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37C. Lysosomal membrane damage was determined as intensity unit of diffuse cytosolic green fluorescence induced by acridine orange following the release from lysosome [14]. Values are expressed as means of three separate experiments (S.D.). 1: Significant difference in comparison with control hepatocytes ($p<0.05$). 2: Significant difference in comparison with DTIC treated hepatocytes ($p<0.05$). 3: Significant difference in comparison with compound I treated hepatocytes ($p<0.05$).

succinate, catalase, superoxide dismutase (SOD) and “ROS” scavengers [15]: mannitol and dimethylsulfoxide (DMSO) protected the hepatocytes against DTIC and compound I induced cytotoxicity as well as “ROS” generation. All of these agents did not show any toxic effect on hepatocytes at concentrations used (data not shown). However, the CYP2E1 inhibitor phenylimidazole [15,16] and P450 reductase inhibitor diphenyliodonium chloride (DPI) [15,16] showed significant effect on DTIC and compound I induced cell lysis and "ROS" formation (data not shown). Endocytosis inhibitors including lysosomotropic agents; chloroquine [17], methylamine [18], monensin a Na^+ ionophore that inhibits hepatocyte endosomal acidification [19], and 3-methyladenine, an inhibitor of hepatocyte autophagy [20] also protected the hepatocytes against DTIC and compound I induced cell lysis and "ROS" formation (Tables 1-3). All of these agents did not show any toxic effect on hepatocytes at concentrations used (data not shown).

When hepatocyte lysosomes were preloaded with acridine orange, a release of acridine orange into the cytosolic fraction ensued within 30 min. after treating the loaded hepatocytes with 56 μM of DTIC and 33 μM of compound I (Table 3). The DTIC and compound B induced acridine orange release was prevented by DMSO, mannitol, catalase, superoxide dismutase (SOD) or the ferric chelator desferoxamine (Table 3). All endocytosis inhibitors also inhibited DTIC and compound I induced acridine orange release (Table 3).

4. Discussion

ROS formation was markedly increased following the treatment of hepatocytes with

DTIC and compound I and the antioxidants and “ROS” scavengers prevented both DTIC and compound I induced "ROS" formation and cytotoxicity suggesting that ROS formation contributes to DTIC and compound I induced cell lysis (Table 1,2). It was previously suggested that methylation of the nucleic acid is the only mechanism involved in DTIC and it’s analogue. However in our study, we determined huge increase in "ROS" formation following the treatment of hepatocytes with DTIC and compound I.

Hepatocyte lysosomal disruption before toxicity ensued within 30 min. following addition of DTIC and compound I (Table 3). Hepatocyte lysosomal disruption was inhibited by the lysosomal protease inhibitors (lysosomal inactivatores), leupeptin and pepstatin (Table 3) and lysosomal disruption were also prevented by the hepatocyte endocytosis inhibitors; methylamine, chloroquine, monensin and 3-methyladenine (Table 3). Methylamine or chloroquine or the ferric chelator desferoxamine also prevented hepatocyte cytotoxicity induced by DTIC and compound I.

In conclusion, these results suggest that DTIC and compound I induced hepatocyte toxicity involves oxidative stress and formation of several reactive oxygen spices.

The ROS (H_2O_2) generated by DTIC and compound I easily diffuse inside the lysosomes and interact with lysosomal Fe^{2+}/Cu^{+} leading to hydroxyl radical formation (Haber-weiss reaction). Hydroxyl radicals cause lysosomal membrane damage and deadly protease release which is finally the ultimate cause of cell lysis.

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