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ORIGINAL ARTICLE

Biological reactive intermediates that mediate dacarbazine cytotoxicity

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Abstract
Purpose In the following, the cellular and molecular mechanism of cytotoxicity induced by prodrug dacarbazine toward the isolated rat hepatocytes was studied.
Method Accelerated cytotoxicity screening technique (ACMS) was used to perform this study.
Result Addition of dacarbazine to isolated rat hepatocytes resulted in reactive oxygen species (ROS) formation, and lysosomal membrane leakiness before hepatocyte lysis occurred. Hepatocyte ROS generation was inhibited by desferoxamine (a ferric chelator). Cytotoxicity was prevented by antioxidants or ROS scavengers (mannitol or dimethylsulfoxide), cytochorome P450 inhibitors (phenylimidazole, diphenyliodonium chloride, 4-methylpyrazole, and benzylimidazole). In addition to lysosomal damage, dacarbazine caused hepatocyte protease activation and cell proteolysis.
Conclusion Dacarbazine cytotoxicity is associated with ROS (H_2O_2, $O_2^{\bullet -}$) generation. It is suggested that H_2O_2 could cross the lysosomal membrane, react with lysosomal Fe^{2+} to form hydroxyl radical (Haber-Weiss reaction) which is the major cause of lysosomal membrane leakiness, proteases, and other digestive enzymes' release and finally the cell death.

Keywords Dacarbazine · Oxidative stress · CYP450 · Biological reactive intermediate · Cytotoxicity · Lysosome

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Abbreviations

DTIC	5-(3, 3-Dimethyl-1-triazeno)-imidazole-4-carboxamide
MTIC	5-(3-Monomethyl-1-triazeno)-imidazole-4-carboxamide
AIC	Aminoimidazole carboxamide
ROS	Reactive oxygen species
SD	Standard deviation
ANOVA	Analysis of variance
DMSO	Dimethylsulfoxide
DCF	Dichlorofluorescein
HEPES	(2-Hydroxyethyl)-1-piperazine-ethansulfonic acid)
h	Hour
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxy toluene

Introduction

5-(3,3-Dimethyl-1-triazeno)-imidazole-4-carboxamide (Dacarbazine, DTIC) is a synthetic chemical antitumor agent, which is used to treat malignant melanoma and Hodgkin's disease [1–6]. Although the exact mechanism of Dacarbazine has not yet been completely known, three hypotheses have so far been offered: (a) inhibition of DNA synthesis by acting as a purine analogue, (b) action as alkylating agent, (c) interaction with SH groups [7–9]. By detection of amino-imidazole carboxamide, AIC, (C14) in urine and separation of N-7 and O-6 from DNA strands, it's primary mode of action appears to be alkylation of nucleic acid [9–13]. However, DTIC is a prodrug, which becomes activated by N-demethylation in liver microsomes [4, 14, 15] and 5-(3-monomethyl-1-triazeno)-imidazole-4-carboxamide (MTIC)

is formed. The liver's microsomal enzymes (CYP1A1, CYP1A2, and CYP2E1) could hydroxilate and N-demethylate the dacarbazine [4, 14, 15]. However, all enzymes which are effective on DTIC metabolism have not yet been completely investigated, but with specific antibodies (CYP1A abs) and specific microsomal enzyme inhibitors (phenobarbital and lephtoflavon), the relationship between N-demethylase and CYP1A (CYP1A1 and CYP1A2) isoenzymes were found [4]. Then, MTIC spontaneously decomposes to AIC and methyldiazonium, which is subsequently changed to methyl carbanion which has a capability to methylate the DNA strand on N-7 of Guanine [10–12, 16–18; Fig. 1).

In the following, the cellular and molecular mechanism of cytotoxicity induced by dacarbazine toward the isolated rat hepatocytes was studied. Based on previous literature and also our pilot study, we postulated that the cytotoxicity of DTIC may involve metabolic activation leading to biological reactive intermediates formation which can activate cytosolic oxygen and generate reactive oxygen species.

Materials and methods

Chemicals

5-(3,3-Dimethyl-1-triazeno)-imidazole-4-carboxamide (Dacarbazine, DTIC) was obtained from drug store. Collagenase (from *Clostridium histolyticum*), bovine serum albumin (BSA), Hepes, trypan blue, d-mannitol, dimethyl sulfoxide, butylated hydroxy toluene (BHT), butylated hydroxyanisole (BHA), chloroquine diphosphate, methylamine HCl, 3-methyl adenine, monensin sodium, leupeptin, pepstatin, ethylene glycol-bis (p-aminoethyl ether) *N*,*NN*′,*N*′-tetra acetic acid (EGTA), Gentamycin, Aurothioglucose, and heparin were obtained from Sigma (Taufkirchen, Germany). Acridine orange and dichlorofluorescin diacetate were 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.

Animals

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

Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver as described by Pourahmad and O'Brien in 2000 [19]. Approximately, 85–90% of the hepatocytes excluded trypan blue. Cells were suspended at a density of 10^6cells/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, and 5% CO_2. Each flask contained 10 ml of hepatocyte suspension. Hepatocytes were preincubated

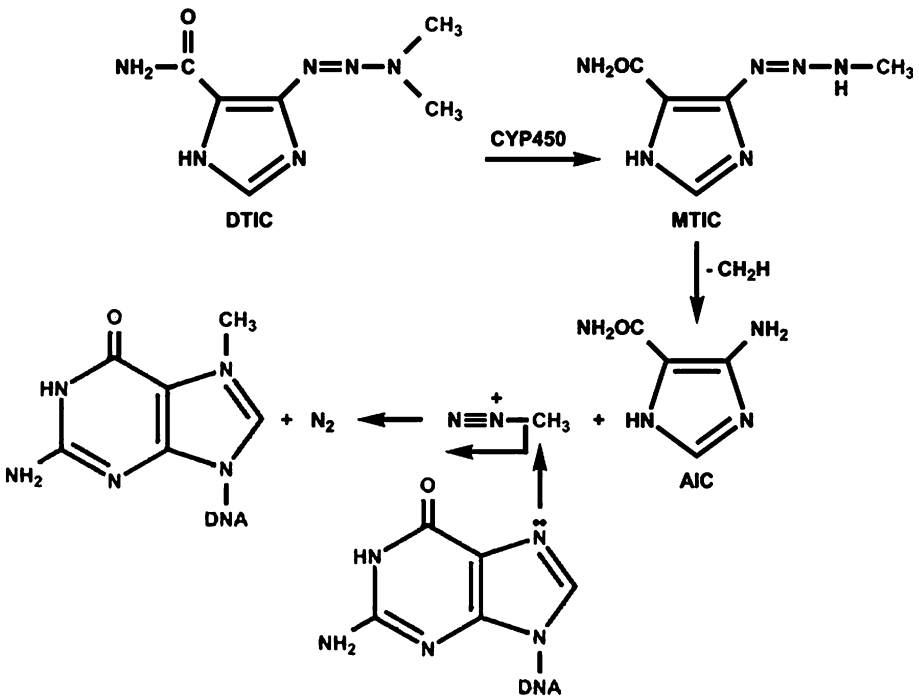

Fig. 1 DTIC becomes activated by the liver's microsomal enzymes (CYP450) and formed MTIC. Then MTIC spontaneously decomposes to AIC and methyldiazonium. The latter has a capability to methylate the DNA strand on N-7 of Guanine

for 30 min prior to addition of chemicals. Stock solutions of all chemicals (×100 concentrated for the water solutions or ×1,000 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 $EC50_{2h}$ concentration for DTIC in the isolated hepatocytes (56 μM). The EC50 of a chemical in ACMS technique (ACMS: Accelerated Cytotoxicity Mechanism Screening) (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. In order to determine this value for the investigated compound, dose–response curves were plotted and then EC50 was determined based on a regression plot of three different concentrations [20].

The $EC50_{2h}$ of DTIC 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. In order to determine this value for the investigated compound, dose–response curves were plotted, and then $EC50_{2h}$ was determined based on a regression plot of four different concentrations (10, 20, 50, and 100 μM) (data are shown below).

Cytotoxicity of DTIC at different concentration following the 2 h of incubation in rat hepatocyte

Addition	Cytotoxicity (2 h)			
	10 μM	20 μM	50 μM	100 μM
Dacarbazine	35 ± 2	40 ± 2	48 ± 2	64 ± 3

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C

Values are expressed as means of three separate experiments (SD)

For the chemicals which dissolved in water, we added 100 μl sample 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 (×1,000 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 microliters of methanol did not affect the hepatocyte viability after 4 h incubation (data not shown). All the inhibitors were preincubated 30 min prior to DTIC addition.

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 [19]. Aliquots of the hepatocyte incubate were taken at different time points during the 3 h incubation period. At least 80–90% of the control cells were still viable after 3 h.

Determination of ROS

To determine the rate of hepatocyte "ROS" generation, dichlorofluorescin diacetate was added to the hepatocyte incubate as it penetrates hepatocytes and becomes hydrolyzed 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 at 37°C for 30 min. 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 μM dichlorofluorescin diacetate for 2 min at 37°C. The fluorescence intensity of the "ROS" product was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 nm and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells [21].

Lysosomal membrane stability assay

Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange [22]. Aliquots of the cell suspension (0.5 ml) that were previously stained with acridine orange 5 μM were separated from the incubation medium by centrifugation (50*g*, 1 min). The cell pellet was then resuspended in 2 ml of fresh incubation medium. This washing process was carried out twice 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.

Lipid peroxidation assay

Hepatocyte lipid peroxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU®-7 spectrophotometer after treating 1.0 ml aliquots of hepatocyte suspension (10^6 cells/ml) with trichloroacetic acid [70% (w/v)] and oiling the suspension with thiobarbituric acid [0.8% (w/v)] for 20 min [19].

Statistical analysis

Levene's test was used for homogeneity of variances. Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey Post-test. Results represent the mean ± standard deviation of the mean (SD) of triplicate samples. The minimal level of significance chosen was $P \leq 0.05$.

Results

When hepatocytes were incubated with 56 μM DTIC, the formation of "ROS" was increased very rapidly (peak in about 30 min, curve not shown) in a concentration dependent fashion (Table 2). The antioxidants: α-tocopherol succinate, BHT, BHA, and "ROS" scavengers [24], mannitol and dimethylsulfoxide (DMSO) protected the hepatocytes against DTIC-induced cytotoxicity, as well as, "ROS" generation. None of these antioxidants alone affected hepatocytes viability at the concentrations used (data not shown).

However, the CYP2E1 inhibitors phenylimidazole, 4-methylpyrazole, benzylimidazole and NADPH P450 reductase inhibitor diphenyliodonium chloride (DPI) [24–27] showed significant effect on DTIC-induced cell lysis and "ROS" formation and protected the hepatocytes against dacarbazine. Lysosomotropic agents including chloroquine [28], methylamine [29], monensin (a Na^+ ionophore that inhibits hepatocyte endosomal acidification) [30], and 3-methyladenine (an inhibitor of hepatocyte autophagy) [31] also protected the hepatocytes against DTIC-induced cell lysis and "ROS" formation (Tables 1, 2). 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

Table 1 Preventing DTIC cytotoxicity by antioxidants, "ROS" scavengers, lysosomal protease inhibitors, and lysosomotropic agents

Addition	%Cytotoxicity		
	1 h	2 h	3 h
None	15 ± 2	17 ± 2	20 ± 2
Dacarbazine (56 μM)	38 ± 3(1)	55 ± 3(1)	76 ± 4(1)
+BHA (50 μM)	26 ± 3(2)	32 ± 3(2)	43 ± 4(2)
+BHT(50 μM)	27 ± 2(2)	33 ± 2(2)	42 ± 3(2)
+Dimethyl sulfoxide (150 μM)	26 ± 2(2)	30 ± 3(2)	44 ± 3(2)
+Mannitol (50 mM)	28 ± 3(2)	33 ± 3(2)	48 ± 3(2)
+Desferoxamine (200 μM)	24 ± 3(2)	28 ± 2(2)	36 ± 2(2)
+α-Tocopherol succinate (100 μM)	26 ± 2(2)	33 ± 3(2)	41 ± 4(2)
+4-Methylpyrazole (500 μM)	35 ± 3(2)	42 ± 3(2)	50 ± 2(2)
+Benzylimidazole (100 μM)	38 ± 2(2)	44 ± 5(2)	56 ± 2(2)
+Phenylimidazole (300 μM)	36 ± 3(2)	41 ± 4(2)	53 ± 3(2)
+Diphenyliodoniumchloride (50 μM)	37 ± 4(2)	42 ± 4(2)	48 ± 5(2)
+Leupeptine (50 μM)	22 ± 2(2)	28 ± 3(2)	42 ± 4(2)
+Pepstatine (50 μM)	20 ± 2(2)	26 ± 3(2)	40 ± 4(2)
+Monensin (10 μM)	27 ± 2(2)	39 ± 3(2)	51 ± 2(2)
+Methylamine (30 mM)	18 ± 2(2)	25 ± 2(2)	36 ± 4(2)
+Chloroquine (100 μM)	17 ± 2(2)	24 ± 2(2)	40 ± 3(2)
+3-Methyladenine (5 mM)	18 ± 1(2)	25 ± 2(2)	36 ± 4(2)
+Gentamycin (1.5 mM)	56 ± 3(2)	72 ± 3(2)	93 ± 4(2)
+Aurothioglucose (1.5 mM)	57 ± 2(2)	73 ± 2(2)	92 ± 3(2)

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C for 3.0 hrs

Following the addition of DTIC. Cytotoxicity was determined as the percentage of cells that take up trypan blue [17]

Values are expressed as means of three separate experiments (SD)

(1) Significant difference in comparison with control hepatocytes ($P < 0.05$)

(2) Significant difference in comparison with DTIC treated hepatocytes ($P < 0.05$)

Table 2 Preventing DTIC "ROS" formation by antioxidants, "ROS" scavengers, endocytosis inhibitors, and lysosomotropic agents

Addition	"ROS"		
	2 min	15 min	30 min
None	66 ± 2	73 ± 4	79 ± 4
Dacarbazine (56 μM)	150 ± 7(1)	194 ± 8(1)	230 ± 4(1)
+BHA (50 μM)	81 ± 4(2)	98 ± 3(2)	121 ± 4(2)
+BHT(50 μM)	77 ± 3(2)	84 ± 3(2)	118 ± 4(2)
+Dimethyl sulfoxide (150 μM)	81 ± 3(2)	93 ± 2(2)	121 ± 2(2)
+Mannitol (50 mM)	85 ± 4(2)	103 ± 4(2)	136 ± 3(2)
+Desferoxamine (200 μM)	108 ± 5(2)	111 ± 4(2)	121 ± 3(2)
+α-Tocopherol succinate (100 μM)	77 ± 2(2)	88 ± 4(2)	11 ± 3(2)
+4-Methylpyrazole (500 μM)	116 ± 5(2)	117 ± 3(2)	167 ± 4(2)
+Benzylimidazole (100 μM)	110 ± 3(2)	119 ± 4(2)	165 ± 3(2)
+Phenylimidazole (300 μM)	108 ± 4(2)	115 ± 4(2)	161 ± 3(2)
+Diphenyliodoniumchloride (50 μM)	111 ± 5(2)	122 ± 5(2)	166 ± 3(2)
+Monensin (10 μM)	125 ± 5(2)	131 ± 7(2)	161 ± 2(2)
+Methylamine (30 mM)	107 ± 4(2)	111 ± 6(2)	117 ± 3(2)
+Chloroquine (100 μM)	111 ± 5(2)	115 ± 6(2)	128 ± 2(2)
+3-Methyladenine (5 mM)	118 ± 5(2)	125 ± 4(2)	132 ± 4(2)

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C for 30 min

DCF formation was expressed as fluorescent intensity units [18]

Values are expressed as means of three separate experiments (SD)

(1) Significant difference in comparison with control hepatocytes ($P < 0.05$)

(2) Significant difference in comparison with DTIC treated hepatocytes ($P < 0.05$)

Table 3 Preventing DTIC-induced hepatocyte lysosomal membrane damage by inhibitors of oxidative stress, "ROS" scavengers and lysosomotropic agents

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)]
+BHA(50 μM)	10 ± 1[(2)]	14 ± 2[(2)]	23 ± 2[(2)]
+BHT(50 μM)	14 ± 1[(2)]	19 ± 2[(2)]	25 ± 3[(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)]
+4-Methylpyrazole (500 μM)	18 ± 2[(2)]	24 ± 3[(2)]	31 ± 2[(2)]
+Benzylimidazole (100 μM)	17 ± 2[(2)]	23 ± 3[(2)]	36 ± 2[(2)]
+Phenylimidazole (300 μM)	16 ± 2[(2)]	22 ± 1[(2)]	30 ± 3[(2)]
+Diphenyliodoniumchloride (50 μM)	18 ± 3[(2)]	26 ± 3[(2)]	33 ± 3[(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)]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C

Lysosomal membrane damage was determined as intensity unit of diffuse cytosolic green fluorescence induced by acridine orange following the release from lysosome [19]

Values are expressed as means of three separate experiments (SD)

[(1)] Significant difference in comparison with control hepatocytes ($P < 0.05$)

[(2)] Significant difference in comparison with DTIC treated hepatocytes ($P < 0.05$)

Table 4 Preventing DTIC-Induced hepatocyte proteolysis with inhibitors of oxidative stress lysosomotropic agents and lysosomal protease inhibitors

Addition	Hepatocyte tyrosine release (μM)		
	30 min	60 min	120 min
None	10 ± 1	12 ± 1	21 ± 2
Dacarbazine (56 μM)	57 ± 3[(1)]	89 ± 2[(1)]	120 ± 3[(1)]
+Dimethyl sulfoxide (150 μM)	14 ± 2[(2)]	21 ± 3[(2)]	32 ± 2[(2)]
+Mannitol (50 mM)	15 ± 3[(2)]	27 ± 2[(2)]	39 ± 2[(2)]
+BHT(50 μM)	14 ± 2[(2)]	24 ± 2[(2)]	33 ± 3[(2)]
+BHA(50 μM)	12 ± 3[(2)]	20 ± 5[(2)]	36 ± 2[(2)]
+Desferoxamine (200 μM)	15 ± 3[(2)]	31 ± 2[(2)]	46 ± 3[(2)]
+Monensin (10 μM)	16 ± 2[(2)]	27 ± 3[(2)]	38 ± 4[(2)]
+Methylamine (30 mM)	13 ± 3[(2)]	31 ± 4[(2)]	45 ± 2[(2)]
+Chloroquine (100 μM)	15 ± 4[(2)]	29 ± 3[(2)]	37 ± 4[(2)]
+3-Methyladenine (5 mM)	18 ± 5[(2)]	33 ± 2[(2)]	43 ± 4[(2)]
+Leupeptine (50 μM)	14 ± 3[(2)]	30 ± 3[(2)]	41 ± 2[(2)]
+Pepstatine (50 μM)	15 ± 4[(2)]	28 ± 4[(2)]	45 ± 3[(2)]
+Gentamycin (1.5 mM)	103 ± 2[(2)]	202 ± 4[(2)]	308 ± 4[(2)]
+Aurothioglucose (1.5 mM)	113 ± 3[(2)]	94 ± 5[(2)]	332 ± 3[(2)]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C

Lysosomal induced proteolysis was determined by measuring the cellular release of tyrosine into the media [23]

Values are expressed as means of three separate experiments (SD)

[(1)] Significant difference in comparison with control hepatocytes ($P < 0.05$)

[(2)] Significant difference in comparison with DTIC treated hepatocytes ($P < 0.05$)

hepatocytes with 56 μM of DTIC (Table 3). The DTIC-induced acridine orange release, which is a marker of lysosomal membrane damage, was prevented by ROS scavengers including dimethylsulfoxide, mannitol, BHT, BHA, or desferoxamine (Table 3). Phenylimidazole, 4-methylpyrazole, benzylimidazole, and NADPH P450 reductase inhibitor diphenyliodonium chloride (DPI) also inhibited dacarbazine acridine orange release (Table 3). The dacarbazine-induced acridine orange redistribution was prevented by 3-methyladenine, chloroquine, methylamine, monensin (Table 3), and dacarbazine-induced hepatocytes cytotoxicity was prevented by the lysosomal protease inhibitors [32–34; Table 1). None of these agents alone at the concentrations used show any significant effect on acridine orange release in acridine orange-loaded hepatocytes (data not shown). Hepatocyte proteolysis as determined by the release of the amino acid tyrosine into the extracellular medium over 120 min was next measured. Hepatocyte proteolysis was markedly increased, when hepatocytes were incubated with DTIC and was completely prevented by the lysosomal protease inhibitors leupeptin and pepstatin or the ROS scavengers dimethylsulfoxide, mannitol, BHT, BHA and desferoxamine (Table 4). However, the lysosomal protease inhibitors (leupeptin and pepstatin) did not affect DTIC-induced hepatocyte acridine orange release (data not shown). The DTIC-induced proteolysis was also inhibited by 3-methyladenine, chloroquine, and methylamine. On the other hand, gentamysin and aurothioglucose which can release lysosomal proteases [35, 36] significantly increased DTIC-induced cell lysis and hepatocyte proteolysis (Tables 1, 4). None of these agents alone show any toxic effect on hepatocytes at concentrations used (data not shown).

Discussion

We have shown that dacarbazine was cytotoxic toward isolated hepatocytes, and the antioxidants and "ROS" scavengers prevented DTIC-induced "ROS" formation and cytotoxicity suggesting that ROS formation contributes to DTIC-induced cell lysis (Tables 1, 2).

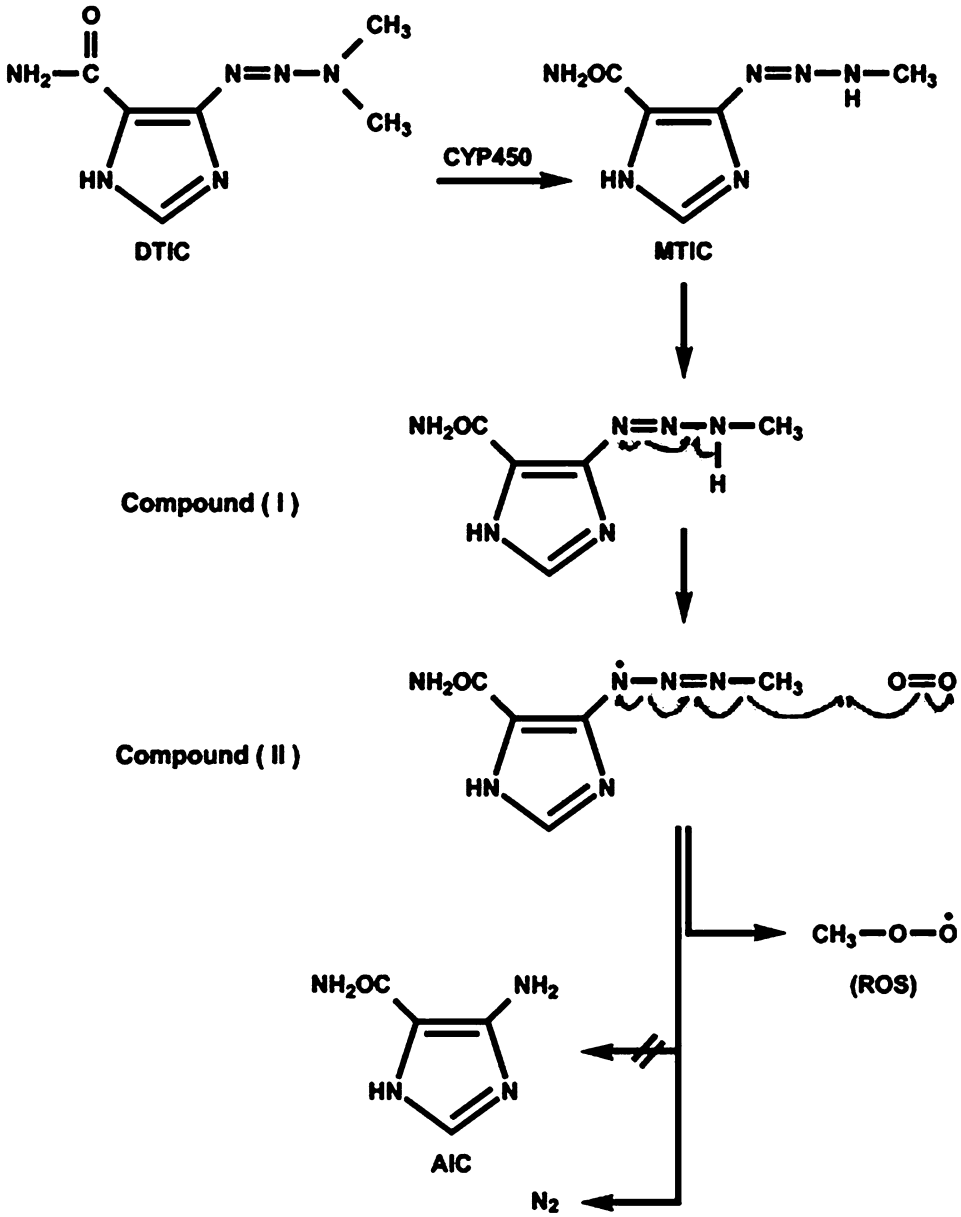

Fig. 2 The biological reactive intermediate that is shown as a compound II is formed following demethylation of DTIC by CYP450 isoenzymes which produced MTIC. Then, MTIC is spontaneously changed to its other isomers (compound I and II). When compound II reacts to cytosolic oxygen, after some electron shifts (shown in Fig 2), three substrates are formed which one of them is a reactive oxygen spices (CH_3–O–$O^{\bullet}$) that is highly reactive

It was already suggested that methylation of the nucleic acid is the only mechanism involved in DTIC cytotoxicity [9–13]. However, our results showed sharp increase in "ROS" formation following the treatment of hepatocytes with DTIC (Table 2).

Preincubation of hepatocytes with the CYP2E1 inhibitors phenylimidazole, 4-methylpyrazole, benzylimidazole, and NADPH P450 reductase inhibitor diphenyliodonium chloride (DPI) prevented both dacarbazine-induced cytotoxicity and ROS formation which suggested that reduced CYP2E1 or P450 reductase reductively activate dacarbazine.

Dacarbazine cytotoxicity, as well as, hepatocyte ROS formation were prevented by the lysosomotropic agents (lysosomal inactivators), chloroquine [28], methylamine [29], monensin (a Na^+ ionophore that inhibits hepatocyte endocytosis and endosomal acidification) [30], or 3-methyladenine (an inhibitor of hepatocyte autophagy) [31]. The hepatocyte lysosomal protease inhibitors leupeptin or pepstatin [32–34] also prevented dacarbazine-induced cytotoxicity. The ferric chelator desferoxamine also prevented hepatocyte cytotoxicity induced by DTIC.

In addition, dacarbazine induced lysosomal membrane leakiness, since acridine orange was readily redistributed from lysosome to cytosol following incubation of DTIC in the acridine orange-loaded hepatocytes. Hepatocyte lysosomal leakiness occurred within 30 min following addition of DTIC, before toxicity ensued (Table 3). Lysosomal leakiness was prevented by the hepatocyte lysosomotropic agents (lysosomal inactivatores); methylamine, chloroquine, monensin, and 3-methyladenine (Table 3).

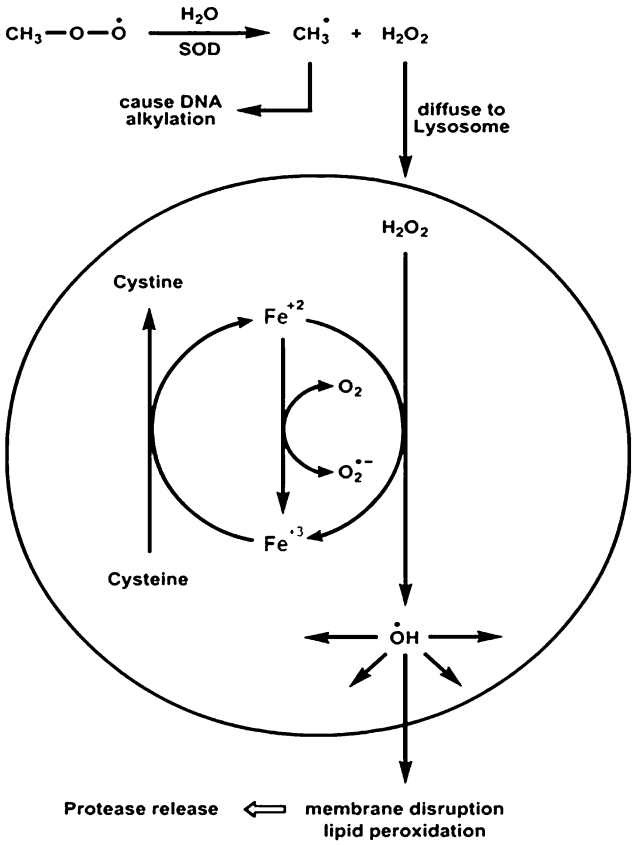

Fig. 3 CH_3–O–O$^{\bullet}$ will react with cellular water and generate H_2O_2 and methyl radical ($CH_3^{\bullet}$) which are very active radical species and probably is responsible for DNA methylation. The lipophilic H_2O_2 will easily cross the lysosomal lipid membrane and interact with lysosomal Fe^{2+}/Fe^{3+} (Haber-Weiss reaction) which leads to hydroxyl radical (OH$^{\bullet}$) formation, causing lysosomal membrane oxidative damage which is the reason for lysosomal digestive enzymes release

In general, our results suggested that dacarbazine-induced hepatocyte toxicity involves reductive activation by reduced cytochorome P450 or NADPH-P450 reductase to form a biological reactive intermediate which can activate cytosolic oxygen and induce ROS formation. On the other hand, the biological reactive intermediate,which is shown as compound II (Fig 2), is formed following demethylation of DTIC by CYP450 isoenzymes which produced MTIC. Then, MTIC is spontaneously changed to its other isomers (compound I and II) (Fig 2). When compound II reacts to cytosolic oxygen, after some electron shifts (Fig 2), three substances are formed, one of which is the free radical CH3–O–O, a highly reactive species. It will then react with cellular water to generate H_2O_2 and the methyl radical (CH3$^{\bullet}$). $CH_3^{\bullet}$ is probably responsible for DNA methylation (Fig. 3).

The lipophilic H_2O_2 will easily cross the lysosomal lipid membrane and interact with lysosomal Fe^{2+}/Fe^{3+} (Haber-Weiss reaction) which leads to hydroxyl radical (OH$^{\bullet}$) formation, causing lysosomal membrane oxidative damage and leakiness which is the reason for lysosomal digestive enzymes release into cytosol. Previously it was shown that gentamysin and aurothioglucose depleted lysosomal proteases [35, 36]. Our findings that nontoxic concentrations of gentamicine or aurothioglucose markedly increased DTIC-induced cytotoxicity and hepatocyte proteolysis (Tables 1, 4) further suggest that the lysosomal release of proteases contributes to the DTIC-induced cytotoxic mechanism.

These results also suggest that DTIC-induced hepatocyte toxicity involves oxidative stress through the formation of several reactive oxygen species. So it is concluded that monomethyl free radicals (CH3–O–O, CH3$^{\bullet}$) and hydrogen peroxide are the result of the reductive activation of DTIC by liver CYP450 isoenzyme (CYP2E1). The hydrogen peroxide generated results in the formation of the highly reactive hydroxyl radical (OH). These reactive species could attack many cellular substrates including cell membranes, suborganel membranes, and DNA strands (Fig 3).

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