Interaction of Dacarbazine and Imexon, *In Vitro* and *In Vivo*, in Human A375 Melanoma Cells

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Abstract. *Aim: We evaluated mechanisms of interaction between the alkyating agent dacarbazine (DTIC) and the pro-oxidant, imexon, in the human A375 melanoma cell line. Materials and Methods: The effect of DTIC and imexon, alone and in combination, was evaluated for growth inhibition (MTT), radiolabeled drug uptake, cellular thiol content (HPLC), and DNA strand breaks (Comet assay). Pharmacokinetic and antitumor effects were evaluated in mice. Results: Growth inhibition in vitro was additive with the two drugs. There was no effect on drug uptake or on the number of DNA strand breaks. There was a >75% reduction in cellular glutathione and cysteine with imexon but not DTIC. Co-administration of the two drugs in mice caused an increase in the area under the curve of both drugs, but the combination was not effective in reducing human A375 melanoma tumors in vivo. Conclusion: Imexon and dacarbazine show additive effects in vitro but not in vivo in human A375 melanoma cells.*

An estimated 68,130 new cases of melanoma were diagnosed in 2010, with an estimated 8,700 deaths attributed to this disease in the United States (1). Dacarbazine (DTIC) is a commonly used chemotherapeutic agent for the treatment of advanced stage metastatic melanoma (2), although response rates are low. Many combination therapies with DTIC have been tried and gave either no improvement or only marginal improvement over DTIC alone (3-5).

Imexon is an aziridine-based small molecule that exhibits antitumor activity in a variety of cell types, including multiple myeloma, melanoma, and pancreatic cell lines *in vitro* (6-8). The drug is a pro-oxidant which binds to reduced sulfhydryls (9) causing an accumulation of reactive oxygen species (10), and a loss of the mitochondrial membrane potential (11). This leads to apoptosis mediated primarily by caspases 3 and 9 (12). The depletion of sulfhydryls by imexon suggested possible synergy with DTIC, since glutathione has been shown to block DTIC cytotoxicity *in vitro* (13).

The purpose of the current research was to investigate whether there was a pharmacologic interaction between DTIC and imexon *in vitro* and *in vivo*. In addition, the effect of imexon on the pharmacokinetics (PK) of DTIC was evaluated since it is well established that combining therapies can sometimes alter the plasma PK of combined agents.

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Materials and Methods

Cell culture. Human A375 melanoma cells (CRL-1619) and HepG2 hepatocellular carcinoma cells (HB-8065) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in a humidified incubator at 37˚C, 5% CO_2, in CellGro® RPMI 1640 (Mediatech, Herndon, VA, USA) supplemented with 10% heat-inactivated bovine calf serum, 2 mM L-glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

Drugs and reagents. Imexon (4-imino-1,3-diazabicyclo-(3.1.0)-hexanone) was provided by AmpliMed Corporation (Tucson, AZ, USA) and DTIC was obtained from Bayer Corp (West Haven, CT, USA). 3-(4,5-Dimethylthiozol-3-yl)-2,5-diphenyltetrozolium bromide (MTT), DMSO, and RNase A were obtained from Sigma Chemical Company (St. Louis, MO, USA). ^{14}C-DTIC was synthesized by ABC Laboratories (Columbia, MO, USA) and ^{14}C-imexon was provided by Aptuit Ltd (Edinburgh, Scotland, UK). Proteinase K was obtained from Boehringer Mannheim-Roche (Pleasanton, CA, USA), and γ-glutamyl-glutamate (γ-glu-glu) from ICN Biomedical (Irvine, CA, USA). Human liver microsomes were purchased from XenoTech, LLC (Lenexa, KS, USA). The comet assay for DNA strand breaks was performed following the manufacturer's instruction (Trevigen, Inc., Gaithersburg, MD, USA).

Cytotoxicity assays. For single drug cytotoxicity assays (14), cells were exposed to either DTIC or imexon alone for 4 days. For combination studies, drugs were used at a fixed molar ratio of concentrations previously determined to be the IC_{25} to IC_{75} of each agent alone, which was determined to be 25 and 75 μM,

respectively, for each drug. Five drug concentrations were evaluated for combination studies. Both simultaneous exposure to both drugs, and pre-exposure to one drug prior to the addition of the second drug were evaluated. Combination effects were analyzed using the median effect analysis method described by Chou and Talalay (15).

^{14}C-DTIC and ^{14}C-imexon uptake. ^{14}C-DTIC uptake in human A375 melanoma cells was measured in the absence and presence of imexon and *vice versa*. A concentration of 500 μM of imexon and 500 μM of DTIC was used. ^{14}C-DTIC was added to cells and total cellular ^{14}C-DTIC uptake or DNA-specific uptake was measured (for 0-240 min). DNA-specific uptake was measured by sequentially digesting RNA and protein (using RNase A and Proteinase K, respectively). Results are the mean±SEM (n=3). Because DTIC requires metabolic activation by hepatic cytochrome P450 enzymes, these studies were repeated after adding 100 μg/ml of purified microsomes in an NADPH-generating system. We also performed these studies using metabolically competent HepG2 hepatocellular carcinoma cells.

Cellular thiol analysis. Glutathione (GSH) and cysteine (Cys) content in human A375 melanoma cells was measured by HPLC after 24 h treatment with imexon, DTIC, and their combination, using a modification of the method of Jones *et al*. (16).

Comet assay. The comet assay was used to evaluate single-strand DNA breaks in human A375 melanoma cells treated with 3 or 5 mM DTIC, imexon, and a combination of agents, after a 30- or 60- min exposure. Comets were visualized with SyberGreen I nucleic acid stain (Invitrogen). Results are the mean±SEM (n=50).

Mouse plasma PK. Non-tumor bearing Balb/c mice (25-30 g male, Taconic, Hudson, NY, USA) were administered 70 mg/kg DTIC intravenously (*i.v.*) *via* tail vein, 150 mg/kg imexon by interperitoneal (*i.p.*) injection, or the two drug combination, and plasma collected 0, 15, 30 and 60 min post dose. Mouse plasma imexon concentrations were measured by our published HPLC assay (17). Mouse plasma DTIC and the major DTIC metabolite, 5-amino imidazole-4-carboxamide (AIC), were measured by HPLC tandem mass spectrometry (18). The area under the plasma concentration time curve (AUC) and half-life were estimated by WinNonlin (Pharsight Corporation, Mountain View, CA, USA), using a noncompartmental approach. Results are the mean±SEM (n=4-6).

Antitumor efficacy in vivo. The effect of the combination on tumor growth *in vivo* was evaluated in 25-30 g male SCID mice (Arizona Cancer Center Experimental Mouse Shared Service (EMSS), Tucson, AZ, USA) (n=8/group). Mice received 5×10^6 A375 cells subcutaneously and were pair matched on day 30, when the average tumor burden was approximately 100 mm^3. Treatment began the following day, as follows: (i) saline vehicle control; (ii) 80 mg/kg/day DTIC; (iii) 100 mg/kg/day imexon; (iv) a combination of both drugs at the same doses. Drugs were administered (*i.p.*) for nine consecutive days and imexon was administered 15 min before DTIC when combined. Measurement of tumor burden and body weights were made every 3-4 days. Tumor burden (mm^3) was calculated as (length × width^2)/2.

Statistical considerations. Combination index (CI) results for median effect analyses were analyzed using 95% confidence intervals. CI<1.0 indicated synergistic interaction and CI>1.0 indicated antagonistic interaction. Comparison of effects on drug uptake and DNA strand breaks were evaluated by ANOVA, with $p<0.05$ constituting a statistically significant effect. Antitumor efficacy *in vivo* was evaluated by comparing the mean tumor burden for each mouse using ANOVA with $p\leq0.05$ for significance. The estimation of variance for the AUC calculations in the mouse PK studies was performed as described by Yuan (19).

Results

Cytotoxicity assays. The effect of combining DTIC and imexon *in vitro* was evaluated by adding the two drugs simultaneously and by pretreating with one drug for 1 h prior to adding the second drug (Figure 1). When cells were treated simultaneously with both drugs, the mean (SD) CI was 0.96 (0.26) with a 95% confidence interval of 0.86 to 1.06 (Figure 1A). When cells were exposed to DTIC before imexon, the mean (SD) CI was 0.98 (0.26) with a 95% confidence interval of 0.88 to 1.08 (Figure 1B). The reverse sequence with imexon exposure first yielded a mean (SD) CI of 0.94 (0.31) with a 95% confidence interval of 0.82 to 1.06. Thus, the effect of all of the sequences were additive.

^{14}C-DTIC and ^{14}C-imexon uptake. Imexon did not change either the total cellular uptake or DNA-specific ^{14}C-DTIC uptake (data not shown). Because DTIC is not metabolically active until it is metabolized, we repeated these studies after adding purified human liver microsomes to the culture so that the conversion of DTIC to metabolically active species could occur (20). We also repeated these studies with metabolically competent HepG2 hepatocellular carcinoma cells. In all cases, there was no alteration in the uptake of ^{14}C-DTIC. Similarly, the presence of DTIC did not alter the uptake of ^{14}C-imexon (data not shown). These studies demonstrate that the cellular uptake of one drug was not affected by the presence of the other drug.

Cellular thiol analysis. In the human A375 melanoma cells, there was a >75% reduction in cellular GSH (Figure 2A) and CYS (Figure 2B) when imexon, but not DTIC, was present in culture media of the cells for 24 h. There was no further loss of thiols when DTIC was combined with imexon. Other cellular thiols which were measured but remained unchanged included oxidized glutathione (GSSG), cystine, Cys-Gly, Glu-Cys, and GSH-Cys.

Comet assay. Since DTIC is an alkylating agent, but imexon does not alkylate DNA (9), studies were performed to examine how adding both drugs would affect DNA strand breaks. Both imexon and DTIC alone induced single-stranded breaks in the human A375 melanoma cells but only significantly at the highest concentrations for each agent compared to controls (Figure 3). The combination of the two drugs produced slightly higher levels of single-stranded breaks, but the increase was only additive (Figure 3).

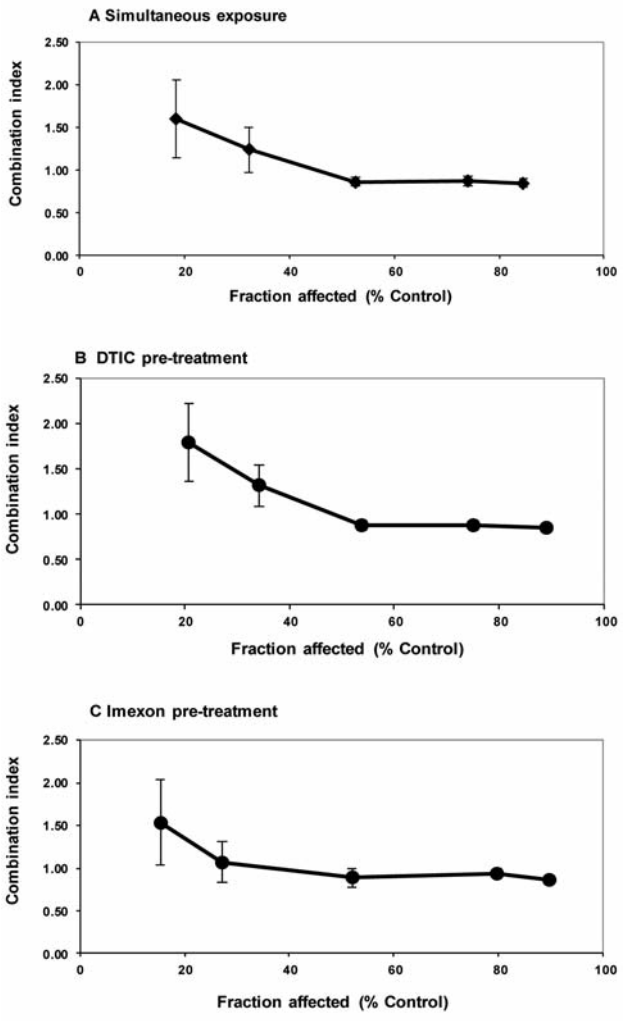

Figure 1. *DTIC plus imexon cytotoxicity is additive. Human A375 melanoma cells were treated for 4 days, either simultaneously with DTIC and imexon, or after 1 h pre-treatment with one drug, and cell growth inhibition was measured by MTT. Drugs were used at a fixed ratio of concentrations previously determined to be approximately the IC_{25} to IC_{75} of each agent alone. The mean combination index ± SD is shown (n=5-8).*

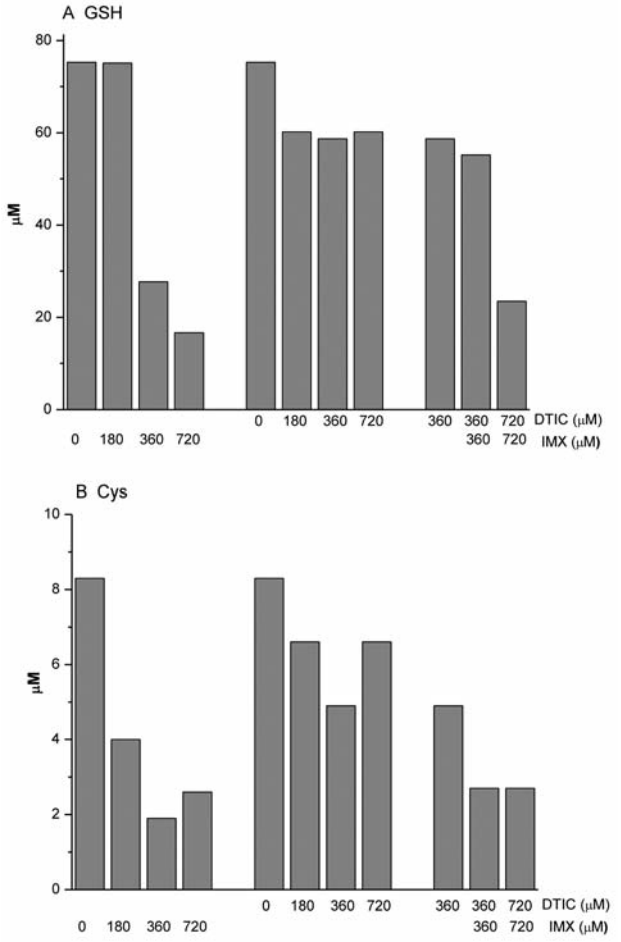

Figure 2. *Imexon (IMX) causes a loss of glutathione (GSH) and cysteine (Cys). GSH, Cys, oxidized glutathione (GSSG), cysteine, Cys-Gly, Glu-Cys, and GSH-Cys in human A375 melanoma cells were measured by HPLC after 24 h treatment with IMX, DTIC, or their combination. GSH (A) and Cys (B) levels after 24 h are shown. No change in GSSG, cysteine, Cys-Gly, Glu-Cys, or GSH-Cys was seen (data not shown).*

Mouse plasma PK. Imexon: The co-administration of DTIC and imexon in non-tumor-bearing mice resulted in an increase in the peak plasma imexon level when DTIC was added. The combination of both drugs increased plasma imexon AUC by 22% (p=0.026). This was associated with a decrease in plasma imexon clearance, and an extension of the half life of imexon by 34% from 44 to 59 min (Table I).

DTIC: In a similar manner, co-administration of DTIC and imexon increased peak plasma DTIC and AIC levels. It also increased the plasma DTIC AUC by 71% (p<0.001). As with imexon, the increased AUC was accompanied by decreased plasma DTIC clearance by almost one-half, from 97.3 to 56.6 ml/min/kg (Table II).

Antitumor effects in vivo. The effect of the imexon/DTIC combination was evaluated in SCID mice bearing human A375 melanoma tumors. There were no treatment-related deaths, however, the average body weights decreased by 6% in the DTIC group and 9% in the combination group during the 9-day treatment period. There was no significant difference in tumor growth for single agent imexon, DTIC, or the combination (p=0.58 by ANOVA).

Discussion

In a phase I/II clinical trial of imexon plus DTIC in metastatic melanoma patients, the median overall survival of the 68 patients

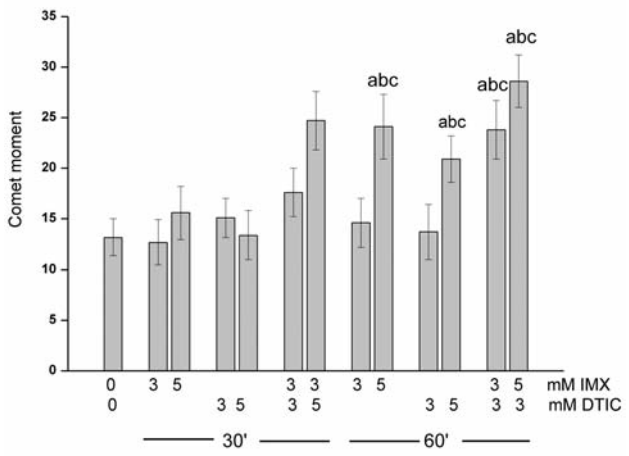

Figure 3. *Both DTIC and IMX induce single-stranded DNA breaks. Human A375 melanoma cells were treated with 3 or 5 mM IMX or DTIC, or a combination of agents, for 30 or 60 min, and DNA single-stranded breaks were measured by the Comet assay. The results are the mean±SEM (n=50). p<0.05 when compared to [a]untreated controls, [b]3 mM imexon/60 min, and [c]3 mM DTIC/60 min.*

was 11.7 months (21). This result is about twice that of historical melanoma trials which evaluated DTIC as a control arm (22-25). Despite these encouraging trends, the current preclinical results in the human A375 melanoma cell line do not show significant biologic interactions between imexon and DTIC in terms of cytotoxicity, cellular drug uptake, binding to DNA, or the induction of DNA strand breaks. Although imexon caused a reduction in cellular thiols, there was no further decrease with DTIC. The reason both DTIC and imexon exhibited altered plasma pharmacokinetics when combined is not known.

The overall conclusion is that the combination of DTIC and imexon in the human A375 melanoma cell line is largely additive *in vitro*. While there is an apparent pharmacokinetic interaction *in vivo* leading to greater systemic exposure to both drugs, this increase in drug exposure was not able to enhance the antitumor efficacy of the combination *in vivo*.

Table I. *Plasma imexon pharmacokinetics in mice co-administered dacarbazine (DTIC).*

	Imexon alone	DTIC + Imexon
Imexon		
C_{max} (μg/ml)	118.4	139.0
AUC (0-inf) (μg min/ml)	4695.9	5726.9*
$t_{½}$ (min)	44.4	59.1
CL/F (ml/min/kg)	38.0	26.2
Vβ/F (ml/kg)	2431.0	2232.3

C_{max}, Peak plasma drug concentration; AUC, area under the concentration-time curve; $t_{½}$, half life; Cl/F, apparent clearance of drug from plasma; Vβ/F, apparent volume of distribution. *p=0.026.

Table II. *Pharmacokinetics of plasma dacarbazine (DTIC) and its metabolite 5-amino imidazole-4-carboxamide (AIC) in mice co-administered imexon.*

	DTIC alone	DTIC + Imexon
DTIC		
C_{max} (μg/ml)	23.0	34.1
AUC (0-inf) (μg.min/ml)	719.7	1236.4*
$t_{½}$ (min)	50.7	49.7
CL (ml/min/kg)	97.3	56.6
V_{ss} (ml/kg)	5577.1	1924.5
AIC		
C_{max} (μg/ml)	3.7	6.5
AUC (0-inf) (μg min/ml)	312.0	345.4
$t_{½}$ (min)	71.2	41.6

C_{max}, Peak plasma drug concentration; AUC, area under the concentration-time curve; $t_{½}$, half life; CL, clearance of drug from plasma; V_{ss}, volume of distribution at steady state. *p<0.001.

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