Preclinical antitumor activity, pharmacokinetics and pharmacodynamics of imexon in mice

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Imexon, a novel pro-oxidant, thiol-binding agent, is currently in phase I/II clinical trials in patients with advanced solid tumors. The aim of this study was to characterize the preclinical pharmacology of imexon *in vivo*. We investigated the anticancer activity of imexon in several cancer cell lines grown as xenografts in severe combined immunodeficient mice. Imexon was active against both hematologic and solid tumor types. The maximally tolerated dose, at the selected dosing schedule, was 150 mg/kg. Using the maximally tolerated dose of imexon, we sought to identify a potential pharmacodynamic biomarker to monitor the mechanistic effect systemically. As imexon binds cellular thiols *in vitro*, thiol depletion by imexon *in vivo* was evaluated as a potential biomarker. Following a single 150 mg/kg dose of imexon by intraperitoneal injection, glutathione levels decreased by 40% at 3 h in mouse erythrocytes. In mouse plasma, imexon treatment led to a significant decrease in cystine levels 2–4 h after drug administration. Notably, by this time, free imexon plasma levels were nondetectable. By investigating the pharmacokinetics of imexon, we also found that imexon undergoes rapid clearance from plasma in a dose-independent fashion with a half-life of 12–15 min. In summary, imexon is active against several cancer types *in vivo*. Imexon also decreases circulating thiols and exhibits dose-independent pharmacokinetics in mice. Plasma cystine levels may represent a biomarker of imexon activity *in vivo*. *Anti-Cancer Drugs* 17:1179–1184

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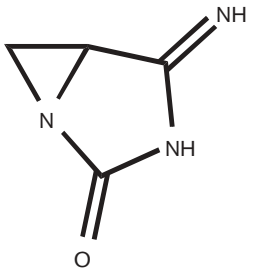
Introduction

Imexon (4-imino-1,3-diazabicyclo-[3.1.0]hexan-2-one) has been studied for its activity as an immunomodulator and as an anticancer agent (Fig. 1) [1–5]. Mechanistically, imexon has been shown to induce apoptotic cell death by causing oxidative stress and mitochondrial changes in leukemia, myeloma and pancreatic cancer cell lines *in vitro* [6–9]. In-vitro anticancer activity of imexon in human cancer cell lines has been extensively studied, but imexon activity *in vivo* has only been reported in the MiaPaCa human pancreatic cancer cell line [6–9].

Fig. 1



Chemical structure of imexon (4-imino-1,3-diazabicyclo-[3.1.0]hexan-2-one).

Incubation of imexon with cysteine, *N*-acetylcysteine and glutathione *in vitro* demonstrated that imexon formed thiol conjugates [9,10]. In addition, imexon has been shown to deplete cysteine and glutathione in the RPMI 8226 multiple myeloma cell line, and imexon-induced cytotoxicity was blocked by simultaneous treatment with *N*-acetylcysteine [9]. Recently, we demonstrated that among seven myeloma cell lines, those most sensitive to imexon demonstrated the greatest degree of glutathione depletion following a 48-h exposure to imexon [8]. These data suggest that thiol conjugation and subsequent depletion may play a role in imexon's pharmacologic mechanism.

We investigated the in-vivo antitumor activity of imexon in several human cancer types. In addition to antitumor activity, we hypothesized that imexon would decrease thiol levels *in vivo*, similar to that observed in cancer cell lines *in vitro*. The results show that imexon consistently

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alters erythrocyte and plasma thiols *in vivo*, suggesting that thiol depletion may comprise a biomarker for the drug's mechanism of action. The effect of imexon treatment on thiol levels in mouse erythrocytes and plasma was analyzed, and correlated with imexon pharmacokinetics to determine the utility of thiol depletion as a potential clinical biomarker.

Materials and methods

Materials

Pharmaceutical grade imexon (lot no. 15106355) was provided by the Pharmaceutical Resources Branch of the National Cancer Institute (Bethesda, Maryland, USA), as part of a Rapid Access to Intervention Development grant to R.T.D. The drug was produced under Good Manufacturing Practice conditions at Seres Laboratories (Santa Rosa, California, USA). When diluted to 0.12 mg/ml with 0.9% sodium chloride, the drug has 95% imexon remaining after 6.5 h at room temperature [11]. Imexon was solubilized immediately before injections and kept on ice in between injections. The dipeptide dimer γ-glutamyl glutamate (γ-Glu-Glu) was purchased from ICN Biomedicals (Irvine, California, USA) and Sigma-Aldrich (St Louis, Missouri, USA). All other chemicals and reagents were purchased from Sigma-Aldrich unless otherwise noted.

Animals

Female severe combined immunodeficient (SCID) mice (5–6 weeks old) were purchased from a breeding colony maintained by the University of Arizona Animal Care facility (Tucson, Arizona, USA). Female BALB/c mice (5–6 weeks old) were purchased from the National Cancer Institute Animal Production Program (Frederick, Maryland, USA). Mice were housed according to guidelines of the American Association for Laboratory Animal Care, under protocols approved by the University of Arizona Institutional Animal Care and Use Committee. Mice were housed in standard microisolator caging with wood chip bedding. Mice received standard sterilized rodent chow (Harlan/Teklad, Madison, Wisconsin, USA) and water *ad libitum*. Mice were maintained on a 12-h light/dark schedule.

Invivo tumor studies

SCID mice ($n = 8$/group) were implanted with $1–10 \times 10^6$ human tumor cells subcutaneously. Imexon treatments were administered by the intraperitoneal route once tumor sizes averaged 100–200 mg. This varied from 25 to 70 days after the tumor implantation (Table 1). The mice were then pooled and randomized to obtain an average tumor size of 100–200 mg/group, and imexon treatment was started the following day. As imexon cytotoxicity was schedule-dependent, a 9-day continuous dosing schedule was used at either 100 or 150 mg/kg [9,12].

Table 1 Tumor growth delay for imexon in severe combined immunodeficient mice bearing human tumor xenografts

Human tumor type	Days to reach 100–200 mg tumor size[a]	Imexon dose (mg/kg) × 9 days	Tumor growth inhibition (*T/C*)%	Tumor growth delay (*T* − *C*)[b]
Hematologic				
KG-1 (myeloid leukemia)	27	150	59	7
RPMI 8226 (myeloma)	28	150	33	31
RPMI 8226/DOX40 (myeloma)	28	150	27	37
HL60 (promyelocytic leukemia)	25	150	76	6
LCL-B (lymphoma)	35	150	43	27
Solid tumors				
MDA MB-231 (breast)	53	150	76	12
HEY (ovarian)	50	150	42	14
A549 (non-small-cell lung)	47	100	58	9
A375 (melanoma)	40	100	28	24
SW 684 (fibrosarcoma)	70	150	23	26
PC-3 (prostate)	56	150	72	12
DU 145 (prostate)	35	150	61	19
Panc-1 (pancreas)	40	150	49	13

[a]Time after tumor implantation for tumors to reach 100–200 mg. Dosing began 24 h later.
[b]Difference in median days for treated (*T*) versus control (*C*) tumors to reach 750 mg size.

Tumors were measured with a caliper 3 times per week and mice were weighed once weekly. Tumor weights were estimated from two perpendicular dimensions in millimeters according to the formula where tumor weight = (length × width^2)/2. Tumor growth inhibition (*T/C* value) was used as a measure of efficacy for treating early-stage disease. The tumor weight was determined simultaneously for the treated (*T*) and control (*C*) group. When the median tumor weights of the control group reached 750–1000 mg, the median tumor weights for each group were determined. The *T/C* value was then calculated as follows: *T/C* (%) = (median tumor weight of treated group (*T*)/median tumor weight of control (*C*)) × 100. As per National Cancer Institute standards, *T/C* (%) values $\leq 42\%$ are considered to signify an active compound and *T/C* (%) values $\leq 10\%$ are considered highly active [13]. Tumor growth delay was calculated by determining the median difference (in days) required for the treatment group and control group to reach 750 mg.

Biomarker analysis

The effect of imexon on murine erythrocytes and plasma thiol levels was analyzed by the following methods. For both studies, mice were given a single intraperitoneal injection of 150 mg/kg imexon in sterile 0.9% sodium chloride. The control group represents mice in which only the vehicle was injected. After imexon injection,

mice were anesthetized with diethyl ether and exsanguinated via cardiac puncture with a heparinized syringe at specified times. Animals were not fasted in this study.

For erythrocyte measurement of thiols, the erythrocytes were separated from the plasma by centrifugation and counted using a hemacytometer. For each sample, an equal volume of erythrocytes was lysed by three freeze/thaw cycles from 4 to −72°C and then stored at −72°C before analysis. Samples were lysed and stored under nitrogen to prevent oxidation. Thiols from the erythrocyte lysates were measured by high-pressure liquid chromatography (HPLC) using a modification [14] of the method of Fariss and Reed [15]. Both reduced and oxidized glutathione levels were measured using a Hewlett-Packard 1050 Series HPLC (Palo Alto, California, USA) with an Adsorbosphere NH_2 250 mm × 4.6 mm, 5-μm column (Alltech Associates, Deerfield, Illinois, USA) at 365 nm. Chromatographic peaks were analyzed using the Chemstation (Agilent, Palo Alto, California, USA) integration program. Glutathione concentrations are presented as a ratio of the glutathione peak to the γ-Glu-Glu internal standard and final concentrations were normalized to the number of erythrocytes in each sample. Data are reported as μmol/l/10^9 cells.

Murine plasma thiols were analyzed by fluorescent detection of dansyl derivatives following a modification of the method of Jones *et al.* [16]. Thiol levels were measured using a Perkin Elmer Series 200 pump (Perkin Elmer Life and Analytical Sciences, Boston, Massachusetts, USA), a Perkin Elmer ISS 100 autosampler and a BASi FL-45 fluorescent detector (Bioanalytical Systems, West Lafayette, Indiana, USA) with an Adsorbosphere NH_2 150 mm × 4.6 mm, 5-μm, column at 325 nm excitation and 550 nm emission. Chromatographic peaks were analyzed using TotalChrom 6.2 software (Perkin Elmer, Wellesley, Massachusetts, USA). Thiol concentrations are presented as a ratio of the thiol peak to the γ-Glu-Glu internal standard and are reported in μmol/l.

Pharmacokinetic analysis

The pharmacokinetics of imexon in the mouse were determined after intraperitoneal injection of a single dose of 50, 100 or 150 mg/kg. Imexon was kept on ice in between injections to prevent degradation. Once plasma samples were harvested, an equal volume of tetrahydrofuran was added to the plasma to stabilize the imexon. Samples were incubated on ice for 10 min and cleared by centrifugation at 13 500 *g* for 5 min. The supernatant was then immediately analyzed by HPLC. Plasma imexon levels were measured using a Shimadzu LC-10ATVP HPLC pump (Columbia, Maryland, USA), a Hitachi AS-2000 auto-injector (San Jose, California, USA) and a Hewlett-Packard 1100 series diode-array detector with a Adsorbosphere CN 250 mm × 4.6 mm, 5-μm column (Alltech Associates) at 230 nm. A mobile phase of 0.1 mol/l ammonium acetate (pH 6.0) was used. The mobile phase was kept on ice throughout the analysis. Chromatographic peaks were analyzed using the Chemstation (Agilent) integration program. All values were interpolated within the standard curve, which was created before the analysis of the unknowns. Samples for the standard curve were prepared by spiking the blank mouse plasma with imexon in a range from 0.5 to 25 μg. A typical standard curve linear regression is described by $y = 2474.32x + 373.35$, $R^2 = 0.99$. Areas above the highest standard were diluted so that the areas would fall within the standard curve and areas below the lowest standard were rejected. The accuracy of the assay was 97.6% as determined by percent recovery and the precision was 7.5% as determined by the percent relative standard deviation. Pharmacokinetic data are reported as μg/ml.

The imexon concentration–time data were analyzed by the noncompartmental approach using the WINNONLIN version 4.0.1 program (Pharsight, Mountain View, California, USA). The following pharmacokinetic parameters were obtained: area under the plasma concentration–time profile (AUC), terminal dispositional rate constant (λ_Z), terminal half-life ($T_{1/2}$), systemic clearance/bioavailability (CL/*F*) and apparent volume of distribution/bioavailability (V_β/F).

Statistical analysis

Data shown represent the mean ± SEM. Statistical analysis was performed by one-way ANOVA with a Bonferroni multiple comparisons test. A probability (*P*) level below 0.05 was considered statistically significant.

Results

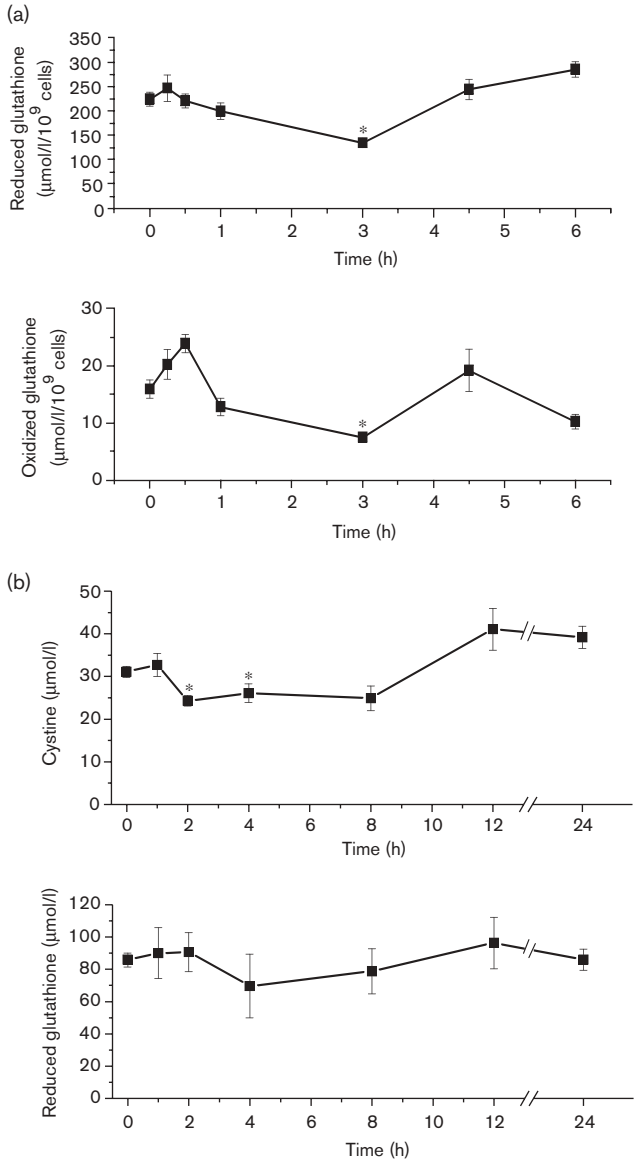
Antitumor activity

Imexon's antitumor activity was investigated using the xenograft tumor regression model and we determined that imexon was active against a number of different human tumor types grown in SCID mice. The maximally tolerated dose of imexon was determined to be 150 mg/kg. Sensitive tumors, those showing $\leq 42\%$ *T/C* values, included ovarian cancer, melanoma, fibrosarcoma and myeloma (Table 1). The LCL-B lymphoma (*T/C* = 43%) and Panc-1 cell lines (*T/C* = 49%) were near the 42% cut-off for defining an active agent using National Cancer Institute guidelines [13]. These data show that imexon leads to significant growth inhibition in several human tumor types in tumor-bearing mice. Imexon also produces a tumor growth delay of 6–37 days, depending on the tumor type.

Biomarker analysis

Imexon-mediated depletion of intracellular thiols has been demonstrated *in vitro* [8,9]. Therefore, in addition to examining imexon's antitumor activity *in vivo*, we also investigated whether imexon treatment leads to thiol

Fig. 2



Imexon-mediated thiol depletion in mouse erythrocytes and plasma. (a) Time course of reduced and oxidized glutathione levels in mouse erythrocytes after imexon administration. Each point represents the mean ± SEM of four mice except for control ($n=16$) and 60-min time point ($n=8$). (b) Time course of mouse plasma cystine and reduced glutathione levels after imexon administration. Each point represents the mean ± SEM of four mice except for the control group ($n=27$). For erythrocytes and plasma, the '0' time point represents the vehicle-treated control group. Thiols were analyzed from control mice at several time points throughout the day to account for overall fluctuations in thiol levels. A statistical significance level of $P<0.05$ is denoted by an asterisk (*) as determined using one-way analysis of variance with a Bonferroni multiple comparisons test.

depletion *in vivo*. In-vivo thiol depletion was examined as a potential biomarker of imexon's pharmacological mechanism. To examine imexon-mediated thiol depletion, we used the maximally tolerated 150 mg/kg dose. Of note, this dose was also active in several tumor types in the xenograft tumor model (Table 1). Thiol levels were analyzed in control mice at several time points throughout the day to account for any diurnal fluctuations in thiol levels [17]. Analysis of erythrocytes revealed that imexon treatment led to a transient decrease and subsequent recovery of reduced and oxidized glutathione levels over a 6-h time period after dosing (Fig. 2a). A statistically significant ($P < 0.05$) decrease was observed at 3 h for both reduced and oxidized glutathione levels in erythrocytes. To further investigate the effects of imexon on sulfhydryls, we evaluated thiol levels in mouse plasma following a single 150 mg/kg dose of imexon. To account for diurnal changes and the dynamic state of thiols in plasma, we once again analyzed control thiol levels throughout the day [18]. In addition to reduced and oxidized glutathione, cysteinyl-glycine, cysteine, homocysteine and the cysteine–cysteine dimer, cystine were quantitated. Of the thiols analyzed, only cystine was consistently reduced by imexon (Fig. 2b, top panel). Cystine levels were significantly lower than the control groups at 2 and 4 h post imexon injection ($P < 0.05$). By 24 h, cystine levels in the imexon treatment group had recovered to those of the vehicle-treated group. Reduced glutathione levels in plasma also decreased after imexon treatment, but they were not statistically different from the controls (Fig. 2b, bottom panel). No significant decreases were observed in any other thiols measured (data not shown).

Pharmacokinetic analysis

Pharmacokinetic analyses of imexon at several doses have not previously been reported and it was not known whether imexon demonstrated linear pharmacokinetics with dose. Figure 3(a) demonstrates the log plasma imexon concentration versus time profiles obtained after single intraperitoneal injection of three different imexon doses. Plasma imexon levels peaked within 10–15 min after imexon injection and then declined rapidly as a function of time. Peak plasma concentrations averaged 20, 39 and 53 μg/ml for the 50, 100 and 150 mg/kg doses, respectively. Figure 3(b) illustrates the dose-normalized log plasma imexon concentration versus time profiles for the three doses. No statistically significant differences were observed for dose-normalized concentrations, for each collection time, among the three doses studied.

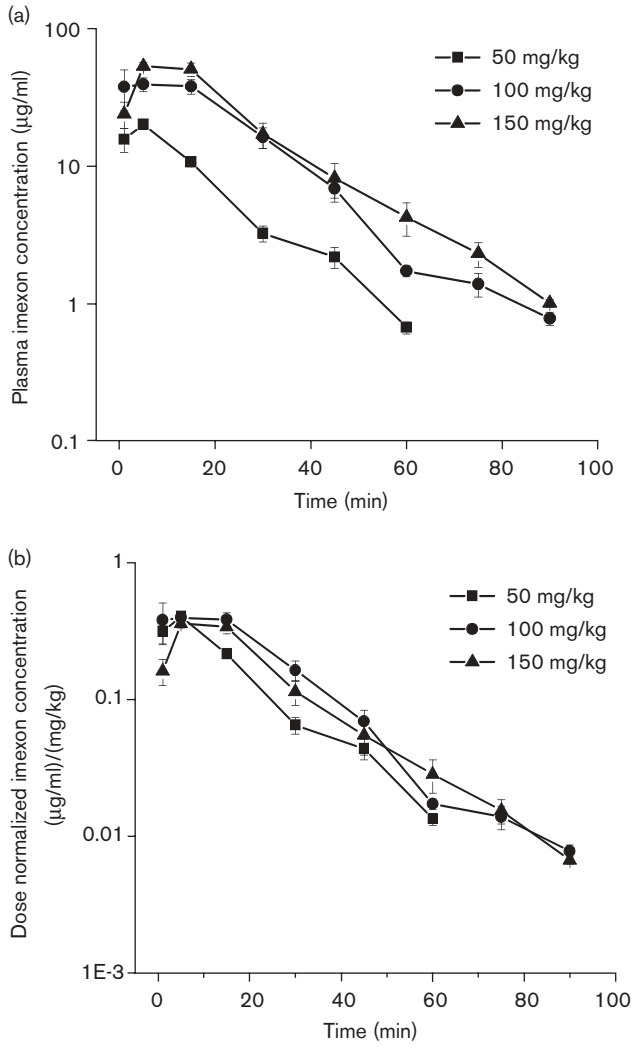
The pharmacokinetic parameters obtained by the non-compartmental analysis are summarized in Table 2. The half-life of imexon was similar for all doses with values ranging from 12 to 15 min. The AUC of imexon increased linearly ($R^2 = 0.94$) as the dose was increased, with values of 415.0, 1266.7 and 1587.9 (μg/ml)min for the 50, 100 and 150 mg/kg doses, respectively. No statistically significant dose-dependent changes were observed in the clearance and volume of distribution of imexon. Thus,

Table 2 Pharmacokinetic parameters obtained after a single intraperitoneal dose of imexon to the mouse

Dose (mg/kg)	λ_Z (min^{-1})	$T_{1/2}$ (min)	AUC (μg/ml)min	CL/*F* (ml/min/kg)	V_β/F (l/kg)
50	0.0592	12	415.0	121	2.04
100	0.0512	14	1266.7	78.9	1.54
150	0.0464	15	1587.9	94.5	2.04

The following pharmacokinetic parameters were obtained using noncompartmental analysis: area under the plasma concentration–time profile (AUC), terminal dispositional rate constant (λ_Z), terminal half-life ($T_{1/2}$), systemic clearance/bioavailability (CL/*F*) and apparent volume of distribution/bioavailability (V_β/F).

Fig. 3



(a) Plot of log imexon plasma concentrations versus time following intraperitoneal injection of three doses of imexon: 50, 100 and 150 mg/kg. Each point represents the mean ± SEM of four mice. (b) Plot of dose normalized log imexon plasma concentrations versus time following intraperitoneal injection of three different imexon doses: 50, 100 and 150 mg/kg. Each point represents the mean ± SEM of four mice. Statistical analysis was performed using one-way analysis of variance with a Bonferroni multiple comparisons test.

imexon demonstrates dose-independent (first-order) clearance in mice.

Discussion

The current results show that imexon is active in a variety of human hematologic and solid tumors growing in SCID mice. Imexon-mediated antitumor activity was independent of the growth rate of each individual cancer type. One of the most sensitive tumor types was multiple myeloma, wherein activity was maintained in the parental line, RPMI 8226, and in the multidrug-resistant (MDR-1 expressing) cell line, 8226/Dox40. On the basis of *in vitro* experiments, the RPMI 8226 multiple myeloma cell line was one of the most sensitive myeloma cell lines [8]. Other human tumor types that were sensitive to imexon in the SCID mouse model included HEY ovarian cancer, A375 malignant melanoma and the very slow growing SW 684 fibrosarcoma cell line.

Imexon has been shown to covalently bind to thiols, thereby decreasing thiol levels *in vitro* [8–10]. We, therefore, investigated whether imexon-induced thiol depletion could be detected *in vivo* as a potential biomarker. Our results show that a single dose of imexon significantly reduces glutathione levels in mouse erythrocytes at 3 h. Plasma cystine levels also decreased between 2 and 4 h posti-mexon administration. In both erythrocytes and plasma, thiol levels in the imexon-treated groups slightly rebounded over control levels. The rebound over control levels has been previously described following thiol depletion by buthionine sulfoximine [19,20]. Cysteine, the rate-limiting amino acid for glutathione synthesis, is obtained primarily from intracellular reduction of cystine imported into cells via the cystine–glutamate antiporter [21,22]. Plasma cystine levels, therefore, represent the extracellular pool of cysteine used for intracellular glutathione biosynthesis. The drop in cystine levels, after imexon administration, may be due to increased cellular uptake, to maintain cysteine and glutathione levels, in response to imexon.

The transient effect of imexon on lowering erythrocyte and plasma thiol levels is consistent with the schedule dependence from previous studies, which demonstrated that longer exposures of imexon were consistently more effective at inhibiting cancer cell growth *in vitro* [9]. The current findings suggest that prolonged exposures to imexon *in vivo* would be similarly more efficacious and the results in SCID mice bearing human tumor xenografts clearly showed activity for repeated daily administration of the drug. The transient effect of imexon on thiol levels further suggests that the pro-oxidant effects of the drug may accumulate over time and, if directly correlated to cytotoxicity, then a drug administration schedule that matched the thiol-lowering effects might be rational, such as an every 4 h administration schedule in mice.

The lack of any dose-dependent features with imexon's clearance in the mouse suggests that this drug does not undergo saturable elimination, at least over the three-fold range of doses studied. If one assumes that the intraperitoneal dose is completely bioavailable ($F = 1$), imexon is considered to have an intermediate to large volume of distribution and can be classified as a high-clearance drug. Therefore, the robust clearance and short half-life demonstrated that imexon is a rapidly eliminated drug. The 150 mg/kg dose of imexon was associated with peak plasma levels of 53 μg/ml: levels greater than those required to reduce colony formation in fresh human tumor cell types grown in soft agar *in vitro* [23].

Interestingly, thiol levels reached their nadir at a time when free imexon levels in the plasma were almost undetectable. This suggests that the reductions in thiol levels are delayed effects of imexon and might be due to a yet-unidentified metabolite. No imexon–thiol conjugates were detected in the analysis of plasma imexon and thiol levels. Alternatively, the delay in thiol reduction may represent a cumulative effect of imexon binding to thiols intracellularly.

In summary, we have demonstrated that imexon is active in a variety of human tumor types *in vivo*. Furthermore, a single dose of imexon leads to a reduction in glutathione levels in mouse erythrocytes and cystine levels in plasma within 2–4 h after dosing. These data support the potential use of plasma cystine as a pharmacodynamic biomarker for imexon in clinical trials. Moreover, the transient reduction in the thiols and the antitumor activity observed with daily doses of imexon were used to design the administration schedules for imexon that are currently being used in the phase I and phase II clinical trials.

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