Pharmacology and Antitumor Activity of a Quinolinedione Cdc25 Phosphatase Inhibitor DA3003-1 (NSC 663284)

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Abstract. *Cdc25 protein phosphatases are regulators of cyclin-dependent kinases and are often highly expressed in human malignancies. Few small molecule inhibitors of the Cdc25 phosphatase family have been identified and little is known about their disposition, metabolism or efficacy in xenograft models. In this study, the efficacy, pharmacokinetics, and metabolism of a potent quinolinedione Cdc25 phosphatase inhibitor, DA3003-1, in mice was examined. DA3003-1 inhibited the growth of subcutaneous human colon HT29 xenografts in SCID mice. After a single i.v. dose of 5 mg/kg, DA3003-1 was not detectable in plasma or tissues beyond 5 min. In vitro studies showed that DA3003-1 was rapidly dechlorinated and conjugated to glutathione. Following DA3003-1 treatment of tumor-bearing SCID mice, reduced glutathione concentrations in HT29 tumor were decreased to a greater extent and remained decreased for longer than the reduced glutathione concentrations in liver and kidneys. These studies suggest that the minimal antitumor activity of DA3003-1 in mice may be due to its rapid metabolism.*

DA3003-1 is the first of a series of quinolinediones that were identified as potent *in vitro* inhibitors of the Cdc25 family of phosphatases (1). Cdc25 phosphatases regulate cell cycle checkpoints, making them an attractive molecular target for possible antineoplastic agents (1). Overexpression of Cdc25A and Cdc25B has been observed in cultured human tumor cells and many human tumor types (2-5).

Several small molecule inhibitors of Cdc25 phosphatases have been identified, and additional more potent and selective inhibitors are likely to follow (1, 6-9). Although Cdc25 inhibitors have been shown to have cytotoxicity against tumor cell lines *in vitro*, their activity against human xenografts *in vivo* has been marginal, and tumor growth continues once treatment has been stopped (6, 7). Pharmacokinetic studies of Cdc25 inhibitors in animals are lacking. DA3003-1 (NSC 663284), a prototype phosphatase inhibitor, has considerable antiproliferative activity *in vitro* against a number of murine and human tumor lines (10-12). In this study, we describe the toxicity, antitumor efficacy, pharmacokinetics and metabolism of DA3003-1 in tumor-bearing mice.

Abbreviations: Cdc2, cell division cycle 2, G1 to S and G2 to M; Cdk1, cyclin-dependent kinase; ER, endoplasmic reticulum; GSH, reduced glutathione; GST, glutathione *S*-transferase; RBCs, red blood cells.

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Materials and Methods

Reagents. Acetonitrile (HPLC-grade) and water (HPLC-grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Nitrogen gas and liquid nitrogen were purchased from Valley National Gases Inc. (Pittsburgh, PA, USA). Formic Acid (99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). DA3003-1 (NSC 663284) and JRoxime were synthesized as previously described (10, 13). Dextrose (5%) for injection, saline (0.154 M NaCl) and sterile water were purchased from Baxter Healthcare Corporation (Deerfield, IL, USA).

Mice. Specific-pathogen-free, adult, female Balb/C and C.B.-17 SCID mice (5-6 weeks of age) were obtained from the Animal Program administered by the Biological Testing Branch of the National Cancer Institute (Frederick, MD, USA). Mice were allowed to acclimate to the University of Pittsburgh Cancer Institute Animal Facility for at least 1 week before studies were initiated. To minimize exogenous infection, mice were maintained in microisolator cages and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and on a

protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Ventilation and airflow in the animal facility were set to 12 changes per hour. Room temperature was regulated at 22.2±1.1˚C, and the rooms were kept on automatic 12-hour light/dark cycles. The mice received Prolab ISOPRO RMH 3000, Irradiated Lab Diet (PMI Nutrition International, Brentwood, MO, USA) and water *ad libitium* except on the evening prior to dosing for the pharmacokinetic studies, when all food was removed and withheld until 4 hours after dosing.

Tumor. HT-29 human colon carcinoma cells were obtained from the NCI Tumor Repository (Frederick, MD, USA) and MDA-MB-435 breast carcinoma cells were obtained from ATCC (Manassas, VA, USA) and were MAP (murine antibody profile) test negative. The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (Invitrogen) and 10 μg/ml gentamicin (Invitrogen) in an humidified atmosphere of 5% CO_2. Cells in logarithmic phase growth were harvested, and $5x10^6$ cells in 0.1 ml of medium were implanted subcutaneously on the right flanks of passage C.B.-17 SCID mice. When the tumors reached approximately 100 mm^3, the passage mice were euthanized with CO_2 and the tumors harvested aseptically. The harvested tumors were cut into approximately 25 mm^3 fragments and implanted on the right flanks of study mice. When the tumors in the study mice were approximately 300 mm^3, the animals were stratified into treatment groups of 9-10 animals each, such that there were no differences in mean body weights and tumor volumes among the treatment groups.

Toxicity study. Dose range-finding studies were conducted in Balb/C female mice and in C.B.-17 SCID female mice using the Up/Down method (Toxicity testing for assessment of environmental agents: interim report, National Research Council, 2006). Mice were administered single *i.v.* doses of 5, 7, 10 or 20 mg/kg DA3003-1 in sterile 5% dextrose (0.01 ml/g body weight). Mice were followed for 14 days after dosing and clinical observations and body weights were monitored daily for the first 4 days after dosing and twice weekly thereafter. Vehicle-treated controls received a single dose of 5% dextrose. Any moribund animal was euthanized.

Efficacy studies. C.B.-17 SCID mice bearing HT29 human colon carcinoma xenografts were stratified into the following groups of 9-10 animals: Control, vehicle control, positive control (gemcitabine, 50 mg/kg/dose *i.v.*), DA3003-1 at the following doses: 2, 3 or 5 mg/kg/dose *i.v.*. The mice were dosed every 4 days for 6 doses, and body weights and tumor volumes were recorded twice weekly. Tumors were measured with calipers, and tumor volumes were calculated using the formula: $TV = L \times W^2/2$, where L is the largest diameter of the tumor and W is the smallest diameter perpendicular to L. Mice were followed for 3 weeks following the completion of the dosing to monitor tumor regrowth. In a second study, C.B.-17 SCID mice bearing MDA-MB-435 human breast cancer xenografts were stratified to the same treatment groups, except that paclitaxel at 20 mg/kg *i.v.* every 7 days was used as the positive control.

Pharmacokinetic studies. C.B.-17 SCID mice bearing HT29 xenografts (2 per time point) were fasted overnight and treated with DA3003-1 *i.v.* at a dose of 5 mg/kg. The mice were euthanazed at the following times: 2, 5, 10, 15, 30, 45, 60, 120, 240, 420 and 1440 min after DA3003-1 administration or 5 min after vehicle administration. Mice were euthanized by CO_2 inhalation and blood was collected by cardiac puncture using heparinized syringes and needles. Blood was centrifuged at 12,000 xg for 4 min to obtain plasma and RBCs. The following tissues were also collected, weighed and snap frozen in liquid nitrogen: liver, kidneys, spleen, lungs and tumor. All samples were stored at –70˚C until analysis.

Pharmacokinetic sample processing. Plasma was assayed directly. Tissues were thawed and homogenized using an Omni Tissuemizer (OMNI International, Marietta, GA, USA) in three volumes of phosphate-buffered saline (Invitrogen). To 200 μl of plasma or tissue homogenate, 10 μl of internal standard (JROxime, 100 μg/ml) was added, and the samples were vortexed for 15 s at setting 4 (Vortex Genie, Scientific Industries, Inc. Springfield, MA, USA). Acetonitrile (1 ml) was added to each sample and the samples were then vortexed for 1 min and centrifuged at 12,000 xg for 6 min. Each supernatant was transferred to a clean 12x75 mm culture tube and evaporated to dryness under a gentle stream of nitrogen gas. Each dried residue was resuspended in 150 μl of initial mobile phase, and 100 μl was injected into the HPLC or resuspended in 100 μl and 25 μl was injected onto the HPLC-MS.

HPLC. The HPLC system consisted of a Beckman Coulter System Gold with a 568 auto-sampler, 126 solvent module, and a 166 detector. Separation was performed on a Phenomenx Luna C18(2) (5 μm, id 4.6x100 mm) column (Torrence, CA, USA) and a Brownlee C18 guard column (PerkinElmer, Shelton, CT, USA). The mobile phase was a gradient of acetonitrile and water, each containing 0.1% formic acid:acetonitrile from 0-40% over 10 min, 40-80% over 5 min, hold 80% for 5 min, and then acetonitrile was reduced to 0% (the initial condition) over 2 min and re-equilibrated for 15 min. The flow rate was 1 ml/min, and the column eluate was monitored at 274 nm. The assay was linear between 0.1 and 30 μg/ml. The retention time of DA3003-1 was approximately 18 min and the major metabolite's retention time was approximately 16 min. The retention time of JRoxime (internal standard) was 32 min. The ratio of DA3003-1 to internal standard was calculated for each standard by dividing the analyte peak area by the peak area of the internal standard. Standard curves of DA3003-1 were constructed by plotting the internal standard ratio *versus* the known concentration of DA3003-1 in the sample. Duplicate standard curves containing DA3003-1 concentrations of 0, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μg/mL were included with each analytic run. Standard curves were fit by linear regression followed by back calculation of concentrations. The lower limit of DA3003-1 quantification was 0.1 μg/ml. Coefficients of variation in plasma at a low mid-range concentration (1 μg/ml) and high mid-range concentration (10 μg/ml) were 2% and 5%, respectively. Recoveries of DA3003-1 from plasma containing 3 μg/μl and 30 μg/ml were 71.7±8.6% and 70.6±4%, respectively.

HPLC/MS. The HPLC/MS system consisted of a Waters Alliance 2695 system with a Micromass Quattromicro triple-stage, bench-top mass spectrometer operated in single quadrupole mode. The column and gradient mobile phase were as described for the HPLC/UV but the flow rate was 0.5 ml/min. The mass spectrometer had the following parameters; positive electrospray ionization, capillary voltage of 3.0 kV, cone voltage of 20 V,

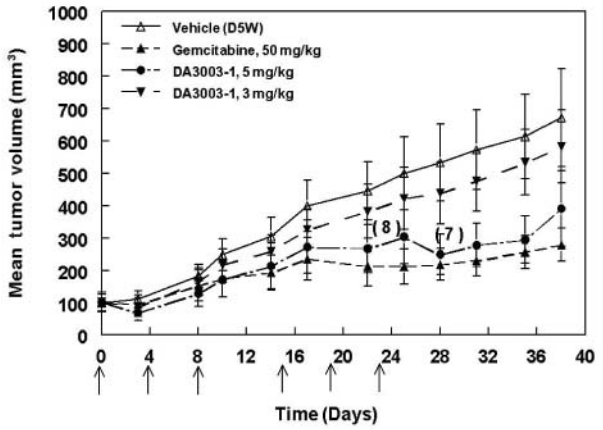

Figure 1. *Antitumor activity of DA3003-1 in mice bearing human HT29 tumor xenografts. Results are the mean±SD tumor volumes for 9 animals in each treatment group and 10 animals in the control group. Two animals in the 5 mg/kg DA3003-1 treatment group were moribund and euthanized on days 25 and day 28 of study. The days of treatment are indicated by the arrows.*

source temperature of 120˚C, desolvation temperature of 450˚C, cone and desolvation gas flows of 110 and 550 l/h, respectively. The eluate was monitored with full scan from 200-600 m/z. The system was controlled and the data were acquired using Masslynx 4.0 software.

In vitro metabolism. Livers were removed from untreated C.B.-17 SCID mice and were homogenized in 3 volumes of 0.1 M Na_2HPO_4/KH_2PO_4 buffer, pH 7.4. The S-9 supernatants, microsomes and 105,000 xg cytosol were prepared using standard methods (14) and analyzed for the disappearance of DA3003-1 by HPLC. Subcellular fractions were incubated in the presence of either NADH or NADPH as cofactors. The incubation volume (200 µl) contained 160 µl 0.1 M Na_2HPO_4/KH_2PO_4 buffer, pH 7.4, 20 µl of 1 mM DA3003-1, 10 µl of S-9 supernatant, microsomes or cytosol, and 10 µl of 30 mM NADPH or NADH. The incubations were carried out for up to 15 min in a shaking water bath at 37˚C. Reaction blanks were kept on ice for 15 min. The samples were extracted with 1 ml of acetonitrile and processed as described above. DA3003-1 remaining in the samples was measured by HPLC. The formation of the metabolite was also quantified as DA3003-1 equivalents formed/mg protein/min.

Protein assay. Protein content of the subcellular fractions was measured using the Bio-Rad Protein Assay (Hercules, CA, USA) and following the manufacturer's directions.

GST metabolism in vitro. To evaluate the role of glutathione S-transferase in the cytosolic metabolism of DA3003-1, 20 µl of a 1 mM solution of DA3003-1 was incubated for times between 0 and 15 min at 37˚C in the presence of GSH alone (10 µl of 50 mM solution) or in the presence of both GSH and GST from rat liver (20 µl of 5 mg/ml glutathione *S*-transferase, G8386, Sigma-Aldrich, St. Louis, MO, USA) and 0.1 M Na_2HPO_4/KH_2PO_4 (pH 7.4) in a total volume of 200 µl.

Table I. *Time in days for HT29 tumor xenografts to complete two doublings.*

Treatment Groups	Days to 2 doubling time±SEM
Vehicle Control	16.4±3.4
2 mg/kg DA3003-1	21.7±3.7
3 mg/kg DA3003-1	20.9±3.7
5 mg/kg DA3003-1	29.1±3.9*
50 mg/kg Gemcitabine	33.6±3.5*

Values are the mean±standard errors for each treatment group. *Values are different from vehicle control, ANOVA with pairwise comparisons using Dunnett's test ($p \leq 0.05$).

GSH concentrations. The concentrations of reduced glutathione were measured in liver, kidney, HT29 tumor xenografts and red blood cells using the colorimetric assay for glutathione (GT10, Oxford Biomedical Research, Oxford, MI, USA) following the manufacturer's instructions.

Statistics. The data were analyzed using ANOVA with pairwise comparisons made using Dunnett's *t*-test. Non-parametric analysis used Kruskall-Wallis with pairwise comparisons using the Mann-Whitney test. Significance was set at $p \leq 0.05$. The statistical package used was Minitab (State College, PA, USA).

Results

Maximum tolerated dose. The maximum tolerated *i.v.* dose of DA3003-1 in both Balb/c and C.B.-17 SCID mice was 5 mg/kg. Mice that received 10 or 20 mg/kg DA3003-1 as single *i.v.* injections were moribund by 4 days after the injection. Mice receiving 7 mg/kg DA3003-1 *i.v.* lost more than 10% of their body weight within the first 4 days after administration, while the mice receiving 5 mg/kg DA3003-1 lost less than 10% body weight and began gaining weight by 4 days after treatment.

Efficacy. Based on the results of the range finding toxicity study, SCID mice bearing established HT29 tumor xenografts were treated with 2, 3 or 5 mg/kg of DA3003-1 or a therapeutic dose of 50 mg/kg of gemcitabine *i.v.* every 4 days for 6 treatments. Significant tumor growth inhibition was seen with 5 mg/kg DA3003-1 that was slightly less than that seen with 50 mg/kg gemcitabine (Figure 1; Table I), but not significantly different. The maximum mean %TC (%treated tumor volume/control tumor volume) was 50% on day 25 after 5 mg/kg/dose of DA3003-1 compared to 30% for gemcitabine at 50 mg/kg on the same schedule. The mean time for the tumors to double was also significantly prolonged in the mice that were treated with 5 mg/kg DA3003-1 (Table I). No significant decrease in body weight was seen with any of these treatments. However, two mice

Table II. *Pharmacokinetics of DA3003-1 in mice after i.v. injection. Average concentration of DA3003-1 in plasma and tissues of C.B.-17 SCID mice bearing HT29 tumors after a 5 mg/kg i.v. injection.*

Time after injection (min)	Plasma (μg/ml)	Red blood cells (μg/ml)	Liver (μg/g)	Kidney (μg/g)	Lung (μg/g)	Tumor (μg/g)
2	0.44, 0.49	0.14, 0.18	0.12, 0.14	0.23, 0.25	1.70, 1.72	ND
5	0.21, 0.21	0.24, 0.26	ND[a]	ND	0.36, 0.38	ND

[a]ND=not detected.

treated with DA3003-1 were euthanized due to toxicity. The first was euthanized on day 25 after the measurements were taken and the second was euthanized on day 28. In contrast, in an additional study, no significant tumor growth inhibition or toxicity was observed in SCID mice bearing MDA-MB-435 tumors using similar DA3003-1 doses and schedule (data not shown).

Pharmacokinetics. At 2 min after an *i.v.* injection of 5 mg/kg of DA3003-1 into a C.B.-17 SCID mouse bearing an established HT29 tumor, DA3003-1 was detected in plasma, red blood cells, liver, kidneys and lungs, but was below the detection limit in tumor (Table II). Within 5 min after treatment, the DA3003-1 concentration in the plasma was reduced to half that seen at 2 min (0.21 μg/ml) while the red blood concentration was slightly higher (0.25 μg/ml). Parent compound could not be detected in the liver or kidney while its concentration in the lung was 0.37 μg/g. DA3003-1 was not detectable in plasma or other normal tissues at 15 min and all later time points.

Metabolism. The short plasma half-life of DA3003-1 prompted us to examine the metabolic fate of DA3003-1. DA3003-1 was metabolized by the 9000 xg supernatant of mouse liver and by both the cytosolic and microsomal fractions with NADPH being preferred slightly over NADH in both fractions (Figure 2). Cytosolic metabolism of DA3003-1 occurred much more rapidly than did microsomal metabolism. Incubation of DA3003-1 with mouse liver cytosol for 2 to 15 min resulted in a loss of the parent DA3003-1 and the appearance of a more polar metabolite that eluted approximately 2 min earlier (Figures 3A and 3B). Because of the almost complete metabolism of DA3003-1 by cytosol, the addition of GSH to the cytosol did not increase the metabolism of DA3003-1 (Figure 3C). Both DA3003-1 (*m/z* 322) and a metabolite, a double-charged positive ion [m +2H] of 297.5 that corresponded to a *m/z* of 595 were detected in the LC/MS ESI positive spectrum (*m/z* 200 to m/z 600 scan) chromatogram of plasma obtained 2 min after mice were administered 5 mg/kg DA3003-1 *i.v.* The same metabolite ion spectrum was seen when DA3003-1 was added to liver cytosol fractions in the *in vitro* studies (Figure 4 A and B). The mass of the metabolite was 595,

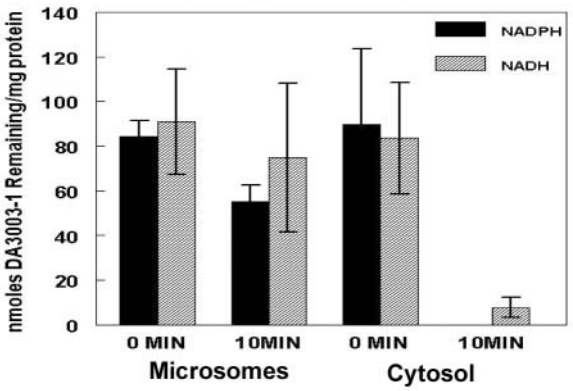

Figure 2. *Disappearance of DA3003-1 by subcellular fractions obtained from mouse liver. DA3003-1 was incubated with microsomes or cytosol with NADPH or NADH for 10 min at 37˚C. The reaction products were extracted with 1 ml acetonitrile and DA3003-1 remaining in the reaction was measured by HPLC as described in Materials and Methods.*

consistent with the loss of chlorine and the formation of a glutathione conjugate. When DA3003-1 was incubated with GST and GSH *in vitro* for 2 to 5 min, DA3003-1 was almost quantitatively converted to a similar metabolite (Figure 4C). Over the time intervals examined, no significant conjugation of DA3003-1 was detected when GST was omitted from the incubation mixture.

Following *i.v.* treatment of mice with 5 mg/kg DA3003-1, the concentrations of GSH in liver, kidney and tumor tissue fell (Figure 5). No decrease in GSH levels was detected in RBCs. In kidneys the decrease in GSH was seen at the earliest time point (10 min). It is of some note that the decrease was most sustained in HT29 tumor tissue; 4 hours after DA3003-1 treatment GSH remained lower, while hepatic and renal levels had returned to normal.

Discussion

Quinones, such as doxorubicin, mitoxantrone, and actinomycin, or drugs, such as etoposide, that can be converted to quinones by *in vivo* oxidation represent the second largest category of antineoplastic agents (15, 16). Consequently, there has been extensive interest in studying

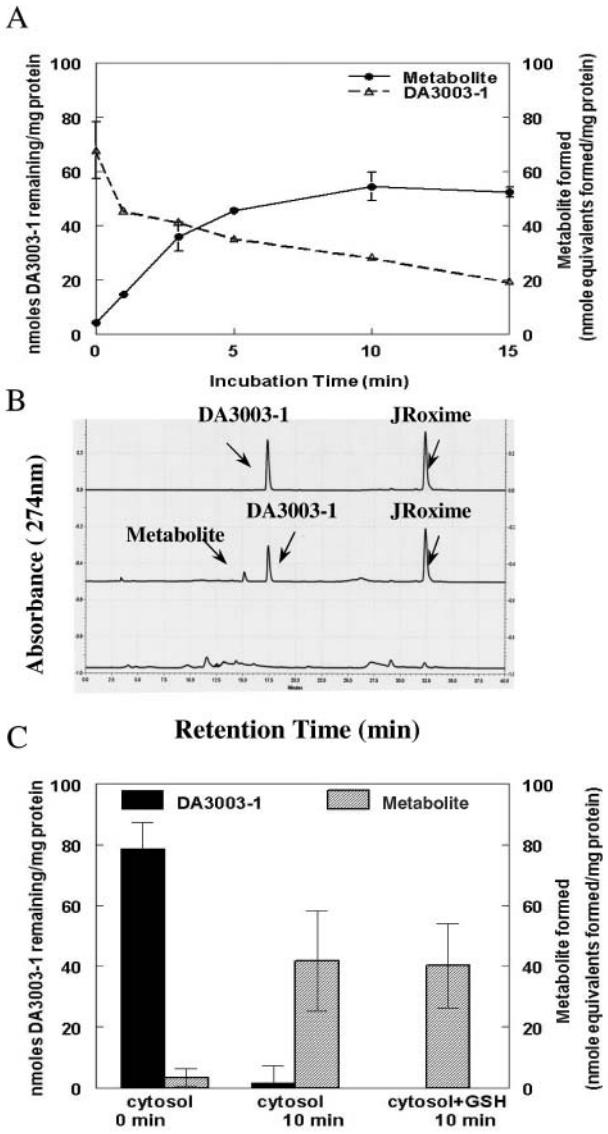

Figure 3. *In vitro metabolism of DA3003-1. DA3003-1 was incubated with liver cytosol in the presence of NADPH for 15 min at 37˚C. The reaction products were extracted and quantified by HPLC methods. A) Time course of metabolism of DA3003-1 and formation of metabolite. B) HPLC chromatograms of DA3003-1 and metabolite. In the top panel is a chromatogram of DA3003-1 added to cytosol as a quality control assay standard. In the middle panel, a chromatogram of DA3003-1 and the metabolite formed after 5 min incubation with cytosol at 37˚C. The chromatogram in the bottom panel is cytosol without the addition of DA3003-1. C) Formation of metabolite and disappearance of DA3003-1 in cytosol with or without exogenous GSH.*

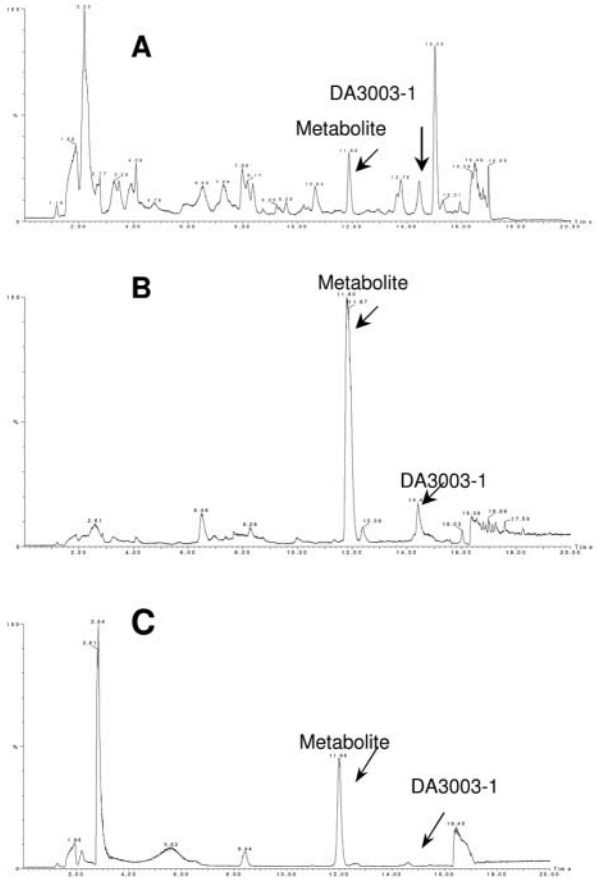

Figure 4. *Total ion current chromatographic analysis of DA3003-1 fate. A) A chromatogram of plasma obtained from a mouse 2 min after i.v. administration of 5 mg/kg DA3003-1. B) A chromatogram of liver cytosol incubated at 37˚C for 5 min with 20 μl of 1 mM DA3003-1 (final concentration 5 μM). C) A chromatogram of DA3003-1 (5 μM) incubated with GST (1.6 mM) and GSH (5 mM) at 37˚C for 5 min.*

quinones as new antineoplastic agents. As a class, quinones are highly reactive compounds that frequently generate reactive oxygen species through redox cycling and Michael adduct formation with cellular nucleophiles such as thiols on cysteine residues of proteins and GSH. The reactive oxygen species can lead to DNA damage while Michael adducts appear to promote endoplasmic reticulum (ER) stress by activating the pancreatic ER kinase signaling pathway, which leads to cell death (17). In the past several years, as part of a screening activity for small molecule inhibitors of the Cdc25 phosphatases, we have identified a number of *para*-quinones that are potent *in vitro* inhibitors of this enzyme family (1, 10, 11, 18). However, not all *para*-quinones are *in vitro* inhibitors of Cdc25. *In vitro*, DA3003-1 displays considerable selectivity for Cdc25 family members when compared with the sensitivity of other protein phosphatases (18). Moreover, DA3003-1 generates cellular reactive oxygen species (19) and participates in Michael adduction in the catalytic domain of Cdc25A (20).

With the exception of BN82002 and BN82685 (6, 7), which are not quinones, there have been no comprehensive *in vivo* evaluations of potent *in vitro* small

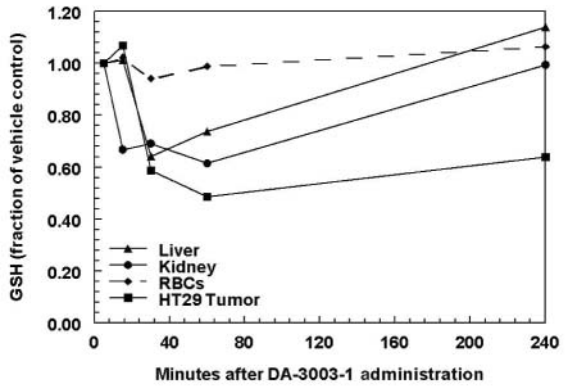

Figure 5. *Decrease in GSH in tumor and tissues after DA3003-1 injection in mice. Concentrations of GSH in liver, kidney, red blood cells (RBCs) and tumors from vehicle treated mice were respectively: 5.069±0.266; 1.859±0.194, 1.377±0.178 and 1.240±0.198 μmole/g wet weight tissue.*

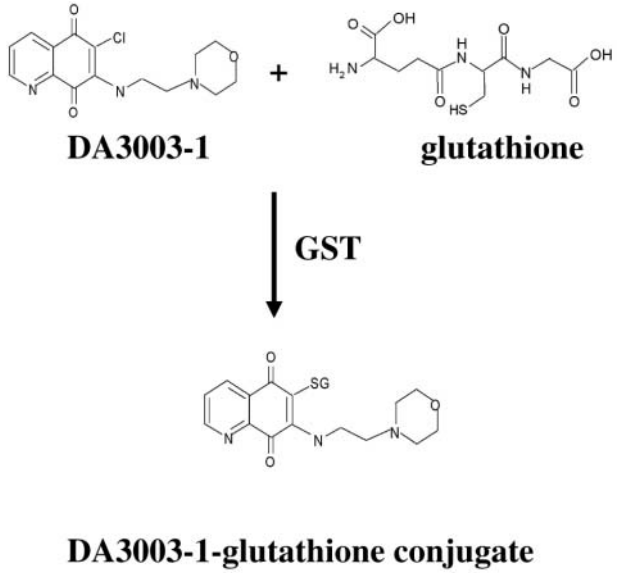

Figure 6. *Proposed metabolic fate of DA3003-1.*

molecule inhibitors of Cdc25 reported. DA3003-1 is of particular interest because it is one of the most potent Cdc25 inhibitors reported (1, 9), has been shown to block cell cycle progression of tumor cells in culture at both G1 and G2/M, and causes hyperphosphorylation of Cdk1/Cdc2, consistent with inhibition of cellular Cdc25 (18). Unlike other quinones, the ability of DA3003-1 to inhibit G2 progression and cell proliferation was not affected by cellular levels of NAD(P)H:quinone oxidoreductase-1 (21).

The plasma pharmacokinetics of DA3003-1 suggested rapid removal, similar to that of other quinones (15). This appears to be due, at least in part, to the formation of a glutathione adduct as has been seen with other not fully substituted quinones. The metabolism of DA3003-1, potentially, could be catalyzed by reductive dechlorination by cytochrome P450s such as CYP1A in liver microsomes, as has been observed with dichlorodiphenyltrichloroethane (22) followed by conjugation catalyzed by GSTs present in both the microsomes and cytosol (23). Alternatively, dechlorination and conjugation can occur nonenzymatically in both microsomes and cytosol (24, 25). Because the dechlorinated, unconjugated metabolite of DA3003-1 was not detected in either plasma or *in vitro* incubations, this rapid metabolism, particularly in the cytosol, suggests a direct role in the dechlorination and adduct formation for GSTs. *In vitro* metabolism of DA3003-1 to dechlorinated, glutathione conjugated metabolite by purified rat hepatic GST confirms the role of GST. The marked decrease of GSH in liver, kidney and tumor tissue is consistent with this conclusion (Figure 6). It is notable that the GSH concentrations remained low in tumor tissue for an extended period, which may provide preferential toxic effects to tumor tissue. Although GSH adducts are often cytoprotective products, there is increasing evidence that the GSH thioethers can be more toxic than the parent compound (26).

The inhibition of HT29 tumor growth after DA3003-1 treatment was similar to that previously reported with the Cdc25 inhibitors, BN82002 and orally administered BN82685 (6, 7); however, treatment with DA3003-1 had no significant antitumor activity against MDA-MB-435 breast cancer xenografts, even though these cells were shown to be responsive to DA3003-1 *in vitro* (11). We cannot exclude the possibility that mechanisms other than Cdc25 inhibition are responsible for HT29 tumor growth inhibition, although HT29 cells are inhibited by other Cdc25 phosphatase inhibitors in cell culture (7, 11). We are not aware of any studies with inhibitors that demonstrate direct inhibition of Cdc25 in xenografts.

The data presented in this study suggest that the rapid metabolism of DA3003-1 limits its potential as an anticancer therapeutic. Future studies will be directed at structural modifications to develop Cdc25 inhibitors with better pharmacokinetic characteristics.

Acknowledgements

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References

1 Lyon MA, Ducruet AP, Wipf P and Lazo JS: Dual-specificity phosphatases as targets for antineoplastic agents. Nature Reviews Drug Discov *1*: 961-976, 2002.

2 Wu W, Fan Y-H, Kemp BL, Walsh G and Mao L: Overexpression of *cdc25A* and *cdc25B* is frequent in primary non-small cell lung cancer but is not associated with overexpression of c-*myc*. Cancer Res *58*: 4082-4085, 1998.

3 Ito Y, Yoshida H, Nakano K, Kobayashi K, Yokozawa T, Hirai K, Matsuzuka F, Matsuura N, Kakudo K, Kuma K and Miyauchi A: Expression of cdc25A and cdc25B proteins in thyroid neoplasms. Brit J Cancer *86*: 1909-1913, 2002.

4 Takemasa I, Yamamoto H, Sekimoto M, Ohue M, Noura S, Miyake Y, Matsumoto T, Aihara T, Tomita N, Tamaki Y, Sakita I, Kikkawa N, Matsuura N, Shiozaki H and Monden M: Overexpression of CDC25B phosphatase as a novel marker of poor prognosis of human colorectal carcinoma. Cancer Res *60*: 3043-3050, 2000.

5 Prevost GP, Brezak, M-C, Goubin F, Mondesert O, Galcera M-O, Quaranta M, Alby F, Lavergne O and Ducommun B: Inhibitors of the CDC25 phosphatases. Prog Cell Cycle Res *5*: 2425-2434, 2003.

6 Brezak MC, Quaranta M, Mondesert O, Galcera MO, Lavergne O, Alby F, Cazales M, Baldin V, Thurieau C, Harnett J, Lanco C, Kasprzyk PG, Prevost GP and Ducommun B: A novel synthetic inhibitor of CDC25 phosphatases: BN82002. Cancer Res *64*: 3320-3325, 2004.

7 Brezak MC, Quaranta M, Contour-Galcera MO, Lavergne O, Mondesert O, Auvray P, Kasprzyk PG, Prevost GP and Ducommun B: Inhibition of human tumor cell growth *in vivo* by an orally bioavailable inhibitor of CDC25 phosphatases. Mol Cancer Ther *4*: 1378-1387, 2005.

8 Aoyagi Y, Masuko N, Ohkubo S, Kitade M, Nagai K, Okazaki S, Wierzba K, Terada T, Sugimoto Y and Yamada YA: Novel cinnamic acid derivative that inhibits Cdk1/Cdc2/Cdc25 dual-specificity phosphatase activity. Cancer Sci *96*: 614-619, 2005.

9 Huang WH, Li J, Zhang W, Zhou Y, Xie C, Luo Y, Li Y, Wang J, Li J and Lu W: Synthesis of miltirone analogues as inhibitors of Cdc25 phosphatases. Bioorg. Med Chem Lett *16*: 1905-1908, 2006.

10 Lazo JS, Aslan DC, Souhwick EC, Cooley KA, Ducruet AP, Joo B, Vogt A and Wipf P: Discovery and biological evaluation of a new family of potent inhibitors of the dual specifity protein phosphatase Cdc25. J Med Chem *44*: 4042-4049, 2001.

11 Lazo JS, Nemoto K, Pestell KE, Cooley K, Southwick EC, Mitchell DA, Furey W, Gussio R, Zaharevitz DW, Joo B and Wipf P: Identification of a potent and selective pharmacophore for Cdc25 dual specificity phosphatase inhibitors. Molec Pharmacol *61*: 720-728, 2002.

12 Brisson M, Nguyen T, Vogt A, Yalowich J, Giorgianni A, Tobi D, Bahar I, Stephenson CR, Wipf P and Lazo JS: Discovery and characterization of novel small molecule inhibitors of human Cdc25B dual specificity phosphatase. Molec Pharmacol *66*: 824-833, 2004.

13 Wipf P, Reeves JT, Balachandran R and Day BW: Synthesis and biological evaluation of structurally highly modified analogues of the antimitotic natural product curacin A. J Med Chem *45*: 1901-1917, 2002.

14 Pan S, Akman SA, Forrest GL, Hipsher C and Johnson R: The role of NAD(P)H:quinone oxdidoreductase in mitomycin – and porfiromycin-resistant HCT 116 human colon-cancer cells. Cancer Chemother Pharmacol *31*: 23-31, 1992.

15 Powis G: Metabolism and reactions of quinoid anticancer agents. *In*: Anticancer Drugs: Reactive Metabolism and Drug Interactions. Powis G (ed.). Oxford, UK Pergamon Press Ltd., pp. 273-387, 1994.

16 Fan Y, Schreiber EM, Giorgianni A, Yalowich J and Day BW: Myeloperoxidase-catalyzed metabolism of etoposide to its quinone and glutathione adduct forms in HL60 cells. Chem Res Toxicol *19*: 937-943, 2006.

17 Wang X, Thomas B, Sachdeva R, Arterburn L, Frye L, Hatcher PG, Cornwell DG and Ma J: Mechanism of arylating quinone toxicity involving Michael adduct formation and induction of endoplasmic reticulum stress. Proc Natl Acad Sci USA *103*: 3604-3609, 2006.

18 Wipf P, Joo B, Nguyen T and Lazo JS: Synthesis and biological evaluation of caulibugulones A-E. Org Biomol Chem *2*: 2173-2174, 2004.

19 Brisson M, Nguyen T, Wipf P, Joo B, Day BW, Skoko JJ, Schreiber EM, Bansal P and Lazo JS: Redox regulation of Cdc25B by cell-active quinolinediones. Molec Pharmacol *68*: 1810-1820, 2005.

20 Pu L, Amoscato AA, Bier ME and Lazo JS: Dual G1 and G2 phase inhibition by a novel, selective Cdc25 inhibitor 7-chloro-6-(2-morpholin-4-ylethylamino)-quinoline-5,8-dione. J Biol Chem *277*: 46877-46885, 2002.

21 Han Y, Shen H, Carr BI, Wipf P, Lazo JS and Pan S-S: NAD(P)H:quinone oxidoreductase-1-dependent and -independent cytotoxicity of potent quinone Cdc25 phosphatase inhibitors. J Pharmacol Expt Therap *309*: 64-70, 2004.

22 Kutamura S, ShimizuY, ShiragaY, Yosida M, Sugihara K and Ohta S: Reductive metabolism of *p,p'*-DDT and *o,p'*-DDT by rat liver cytochrome P450. Drug Metabol Dispos *30*: 113-118, 2002.

23 Rossi B, Mancini I and Guella G: Kinetic studies of glutathione *S*-transferase-catalysed processes through on-line liquid chromatography-electrospray mass spectrometry. Philosophical Magazine *84*: 1373-1382, 2002.

24 Guenthner TM, Whalen R and Jevtovic-Todorovic V: Direct measurement of melphalan conjugation with glutathione: studies with human melanoma cells and mammalian liver. J Pharmacol Exptl Therap *260*: 1331-1336, 1992.

25 Joseph E, Ganem B, Eiseman JL and Creighton DJ: Selective inhibition of MCF-7^{piGST} breast tumors using glutathione transferase derived 2-methyene cycloalkenones. J Med Chem *48*: 6549-6452, 2005.

26 Bolton JL, Trush MA, Penning TM, Dryhurst G and Monks TJ: Role of quinones in toxicology. Chem Res Toxicol *13*: 135-160, 2000.

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