Genz-644282, a Novel Non-Camptothecin Topoisomerase I Inhibitor for Cancer Treatment

Leslie S. Kurtzberg¹, Stephanie Roth², Roy Krumbholz², Jennifer Crawford², Christy Bormann², Sarah Dunham², Min Yao¹, Cecile Rouleau¹, Rebecca G. Bagley¹, Xian-Jie Yu¹, Fei Wang¹, Steven M. Schmid², Edmond J. LaVoie³ and Beverly A. Teicher¹

¹Genzyme Corporation, Framingham, MA 01701
²Genzyme Corporation, San Antonio, TX 78229
³Rutgers University, Piscataway, NJ 08855

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Corresponding Author: Leslie S. Kurtzberg

Genzyme Corporation
49 New York Avenue
Framingham, MA 01701
Phone: 508-271-3717
FAX: 508-872-4091
Email: Leslie.Kurtzberg@Genzyme.com
STATEMENT OF RELEVANCE

Genz-644282 is a novel, non-camptothecin Topoisomerase I (Top1) inhibitor. The value of Top1 inhibitors in cancer treatment is well-documented. The currently approved Top1 inhibitors are the camptothecins, topotecan and irinotecan. We demonstrate a potentially improved therapeutic index for Genz-644282 versus topotecan and SN-38 (active metabolite of irinotecan) by comparing toxicity in bone marrow colony formation assays (CFU) performed with both murine and human cells, and efficacy against growth of a variety of human cancer lines. Unlike the camptothecins, Genz-644282 is not a substrate for the MDR1 and BCRP efflux pumps. Further, we tested Genz-644282 vs. standard drugs in a variety of human tumor xenograft models, and found superior or equal efficacy in the models. Based on these and other results, Genz-644282 has entered in Phase I clinical trial.
ABSTRACT

Purpose: Genz-644282, 8, 9-dimethoxy-5-(2-N-methylaminoethyl)-2,3-methylenedioxy-5H-dibenzo[c,h][1,6]naphthyridin-6-one, emerged as a promising candidate. This report describes the bone marrow CFU-GM and tumor cell CFU activity of Top1 inhibitors, Genz-644282, topotecan, irinotecan/SN-38 and ARC-111, and examines their activity in several human tumor xenograft models.

Experimental Design: Colony-forming assays were performed with mouse and human bone marrow and eight human tumor cell lines. In addition, 29 human tumor cell lines representing a range of histology and potential resistance mechanisms were assayed for sensitivity to Genz-644282 in a 72-hr exposure assay. The efficacy of Genz-644282 was compared with standard anticancer drugs (irinotecan, docetaxel, and dacarbazine) in human tumor xenografts of colon cancer, renal cell carcinoma, non-small cell lung cancer and melanoma.

Results: Human bone marrow CFU-GM was more sensitive to the Top1 inhibitors than was mouse bone marrow CFU-GM. The ratio of mouse to human IC90s was >10 for the camptothecins and <10 for Genz-644282. Genz-644282 was a more potent cytotoxic agent toward human tumor cells in culture than the camptothecins in the colony formation assay and the 72-hr proliferation assay. Genz-644282 has superior or equal antitumor activity in the human tumor xenografts than the standard drug comparators.

Conclusions: Based upon preclinical activity and safety, Genz-644282 was selected for development and is currently undergoing Phase 1 clinical trial.
INTRODUCTION

Topoisomerase 1 (Top1) is an essential enzyme in mammalian cells (1-4). Topoisomerase activity is crucial for initiation and elongation during DNA synthesis, for the proper separation of sister chromatids during mitosis, for RNA transcription and for illegitimate recombination events (5-8). Top1 associates preferentially with transcriptionally active genes and is thought to be involved in relaxing supercoils introduced by RNA polymerase during RNA transcription (9, 10).

Top1 produces a single strand break in DNA allowing relaxation of DNA for replication. The single strand break is then religated restoring the DNA double strands. The Top1 reaction intermediate consists of the enzyme covalently linked to a nicked DNA molecule. This assembly is known as a “cleavable complex” (12, 13). The Top1-targeted drugs topotecan and irinotecan and the Top2-targeted drugs doxorubicin, amsacrine, etoposide and teniposide, stabilize the covalent enzyme-DNA complex preventing religation (11). The cytotoxicity of Top1 inhibitors is due to trapping Top1-DNA cleavage complex as opposed to inhibition of Top1 catalytic activity. The Top1-DNA cleavage complex causes DNA damage during DNA replication and transcription. Repair of Top1-mediated DNA damage has been reviewed (2, 14).

Camptothecin analogs topotecan and irinotecan are FDA-approved Top1-targeted drugs. In vivo, there is a chemical equilibrium between the lactone form and the E ring-opened form of the camptothecins. The E ring-opened carboxylate form has less than 10% the potency of the lactone as a Top1 inhibitor and is inactive in cell culture, perhaps due to inability to cross the cell membrane (15). Both topotecan and irinotecan are
substrates for P-gp efflux pumps and for the pump known as breast cancer resistant protein (BCRP) (16, 17).

Bone marrow is critically sensitive to many antineoplastic agents and contributes to toxicity. Top1 inhibitors kill rapidly dividing bone marrow progenitor cells as well as tumor cells, resulting in acute reversible neutropenia and thrombocytopenia 4 to 20 days after administration (27). Bone marrow granulocyte-macrophage-colony forming unit (CFU-GM) assays comparing the sensitivity of bone marrow cells across species are useful in predicting the blood levels of an agent that might be achieved with acceptable toxicity in patients (27-30). Generally, murine bone marrow has a lower sensitivity to Top I inhibitors than does human bone marrow. The difference in sensitivity between murine and human bone marrow progenitor cells to Top1 inhibitors may explain, in part, why curative doses/blood levels of topotecan and 9-amino-camptothecin in mice with human tumor xenografts are not achievable in patients (30). A compound with similar bone marrow progenitor sensitivity across species may have better potential for reaching the same blood level in patients as in mice. Pessina et al (27) suggested that through use of the ratio of mouse/human CFU-GM IC90 values and the mouse maximum tolerated dose that the human maximum tolerated dose of a compound could be predicted and the potential for achieving a therapeutic blood level in patients estimated.

Nitidine and fagaronine are benzo[c]phenanthridine alkaloids with good antitumor potency and are active Top1 inhibitors (31, 32). Synthetic compounds of this class are as potent as camptothecin in stimulating Top1-mediated DNA cleavage using purified human Top1 and are more potent than irinotecan in many human tumor xenografts (23, 33). An extensive structure-activity relationship was conducted around the
dibenzo[c,h][1,6]naphthyridin-6-one compound family to identify structural features associated with potent Top1-targeting and suitable pharmaceutical properties (34). Genz-644282, 8, 9-dimethoxy-5-(2-N-methylaminoethyl)-2,3-methylenedioxy-5H-dibenzo[c,h][1,6]naphthyridin-6-one, emerged as a promising candidate from the structure-activity relationship. The current report describes the bone marrow CFU-GM and tumor cell CFU activity of Top1 inhibitors, Genz-644282, topotecan, irinotecan/SN-38 and ARC-111, and examines their activity in several human tumor xenograft models. Genz-644282 is currently in Phase I clinical trial.
MATERIAL AND METHODS

Materials

The purity and chemical identity of Genz-644282 (8, 9-dimethoxy-5-(2-N-methylaminoethyl)-2,3-methylenedioxy-5H-dibenzo[c,h][1,6]naphthyridin-6-one) and ARC-111 (8,9-dimethoxy-5-(2-N,N-dimethylaminoethyl)-2,3-methylenedioxy-5H-dibenzo[c,h][1,6]naphthyridin-6-one) was confirmed (FIGURE 1A) (23, 32-35). Topotecan, irinotecan and SN-38 were used as purchased from LKT Laboratories (St. Paul, MN). Genz-644282 and ARC-111 were prepared as a 100 µM stock solution in D5W (5% Dextrose, aqueous, Injection USP, B. Braun Medical Inc., Irvin, CA). For cell culture, topotecan and SN-38 were prepared as 10 mM stock solutions in DMSO from Sigma (#D-5879) and dilutions for experiments prepared in RPMI/5% FBS.

Mouse Bone Marrow

Male Balb/c mice were purchased from Charles River Laboratories (Wilmington, MA) at 6-8 weeks of age and used for studies at 7-10 weeks of age. Procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee in accordance with the Federal Animal Welfare Act (9 CFR, 1992) and were conducted in an AAALAC accredited facility.

For sterile bone marrow collection, mouse femoral and tibial shafts were flushed with sterile RPMI 1460/5% FBS with a syringe and blunt 27G needle. The resulting cellular suspension was collected into a 50 ml conical bottom tube and kept on wet ice. Approximately 25 million cells were obtained from each mouse.
**Human Bone Marrow**

Viable human bone marrow cells were purchased as frozen vials from AllCells, LLC via StemCell Technologies, (#ABM009). They were prepared with a DNase treatment (#07900, StemCell Technologies), and washed in a protocol described by the vendor in preparation for culture.

**Bone Marrow CFU-GM Assay**

For mouse CFU-GM assays, freshly isolated mouse bone marrow cells were cultured in MethoCult, containing the cytokines rmSCF, rmIL-3, rhIL-6 (StemCell Technologies, Vancouver, BC). For human CFU-GM assays, the culture medium used was MethoCult GF, containing the cytokines hSCF, hGM-CSF, hIL-3 (StemCell Technologies).

For experiments, final cell concentrations of $4 \times 10^4$ cells/plate for mouse cells and $8 \times 10^4$ cells/plate for human cells were set up in duplicate 100 cm$^2$ culture dishes for each compound concentration, and then placed in a 37°C incubator for 13 days for mouse bone marrow and 15 days for human bone marrow. Human and mouse bone marrow progenitor cells were continuously exposed to a concentration range of Genz-644282, ARC-111, topotecan or SN-38 in three independent CFU-GM colony formation experiments. The compound concentrations were at half-log intervals covering 5 logs (10 concentrations). Colonies were defined as clusters containing 30 or more cells. The IC$_{50}$ and IC$_{90}$ values were expressed as the mean with 95% confidence intervals in nanomolar concentrations (27).
**Tumor Cell Survival CFU Assay**

The MDA-MB-231, HCT-116, HT-29, NCI-H460, and RPMI 8226 cell lines were purchased from ATCC (Manassas, VA) (36-39). The KB3-1 and KB-V1 cell lines were a gift from Dr. Edmond LaVoie. The MDA-MB-231 is an ER, PR and Her2/neu negative breast cancer cell line. HCT-116 and HT-29 are colorectal carcinoma cell lines. The RPMI 8226 line is a B lymphocyte plasmacytoma, myeloma cell line, and NCI-H460 is a large cell lung carcinoma (39). The KB human carcinoma cell line was originally thought to be derived from an epidermal carcinoma of the mouth but was subsequently found to have been established via HeLa cell contamination and is now designated a cervical carcinoma (41). The KB3-1 line was developed by subcloning the KB cell line. The KB-V1 subline was established from the KB3-1 line by continuous exposure to vinblastine (1 µg/ml) resulting in gene amplification of mdr1 and mdr2 (42, 43). The KB-V1 cells have more than 300-fold higher expression of mdr1 mRNA than do KB3-1 cells and are more than 100-fold less sensitive to colchicines, doxorubicin and vinblastine than are KB3-1 cells.

For human tumor cell colony formation assays, the cell lines which grow as monolayers, MDA-MB-231, HCT116, HT29, NCI-H460, KB3-1 and KBV-1, were grown in RPMI medium (Invitrogen/Gibco, Grand Island, NY) supplemented with 5% FBS (Invitrogen/Gibco). RPMI-8226 cells, which are non-adherent, were grown in 0.35% agar in DMEM-F12 medium supplemented with 10% FBS over a base layer of 0.5% agar in DMEM-F12 medium supplemented with 10% FBS. Human tumor cells were plated at 1 x 10^3 in 6-well plates in medium supplemented with 5% or 10% FBS. The compounds were tested over a concentration range from 0.01 to 100 nM in half-log intervals covering
5 logs along with vehicle controls. Each compound concentration was tested in duplicate wells. Cultures were exposed to the compounds continuously for 7 days at 37°C in a humidified atmosphere of 5% CO₂. Each experiment was performed three independent times. Colonies were defined as clusters containing 30 or more cells. For the monolayer cultures, colonies were stained with crystal violet solution containing 0.41% crystal violet, 12% ethanol and DI water (Becton Dickenson, Sparks, MD). The IC₅₀ and IC₉₀ values were expressed as the mean with 95% confidence intervals in nM concentrations.

**Growth Inhibition Assay**

Twenty-nine established human tumor cell lines were exposed to a concentration range of Genz-644282 in two-four independent experiments. Human tumor cell lines representing a range of histology and potential resistance mechanisms were purchased from ATCC (Manassas, VA), including MIA PaCa-2, AsPC-1, BxPC-3, CFPAC-1, Hs766T and Capan-1 pancreatic cancers, MEL624, C32, Hs695T and SK-MEL-3 melanomas, NCI-H1299, NCI-H292, NCI-H1915 and SW900 non-small cell lung cancers, HCC1395, HCC1937, HCC202, Hs578T, T-47D and ZR-75-1 breast cancer, ACHN, 769-P, A-498, A-704, SW156, Caki-2 and TK-10 renal cancers and OVCAR-4 and OVCAR-5 ovarian cancers. Cells were plated at 4 × 10³/well in 96-well tissue culture plates in 100 µl RPMI medium supplemented with 5% FBS and 12 concentrations of Genz-644282 from 0.1 nM to 10 µM, with each concentration tested in triplicate. Plates were incubated overnight at 37°C in humidified air with 5% CO₂. Plates were incubated with Genz-644282 at 37°C with humidified air/5% CO₂ for 72 hrs. After the
incubation period, the test plates were read utilizing Promega’s Cell Titer-Glo Luminescent Cell Viability Assay according to the manufacturer’s instructions. Luminescence was measured with a Bio-Tek (Highland Park, Winooski, VT) Synergy HT plate reader utilizing the associated Kineticalc software, Version #3.4. Luminescence data were converted to growth fraction by comparison to the luminescence for the untreated control for each cell line and IC50 and IC90 values determined from the graphical data. Each cell line was tested in at least two independent experiments.

**Human Tumor Xenografts**

The efficacy of Genz-644282 was compared with standard anticancer drug therapy in human tumor xenograft studies. All procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee in accordance with the Federal Animal Welfare Act (9 CFR, 1992) and were conducted in an AAALAC accredited facility. Nu/nu mice from Harlan were implanted subcutaneously with a 4 mm³ tumor fragment, and treatments were initiated when tumors reached 200 mm³. Animals were assigned to treatment (n = 8-10) or control groups (n = 8-10). Compounds were prepared freshly prior to injection, with Genz-644282 was formulated in M/6 lactate, irinotecan in D5W, gemcitabine in saline, and docetaxel in ethanol, Cremophor EL and saline. The doses, schedules and routes for each compound were determined in earlier MTD studies. Genz-644282 was tested on three schedules and the most effective, non-toxic regimen was selected for further study.

Genz-644282 was compared with irinotecan in experiments with the human HCT-116, HT-29, HCT-15 and DLD-1 colon carcinoma and 786-O renal cell carcinoma xenografts. Irinotecan was administered at 60 mg/kg/day by IV injection every fourth
day for three injections. Genz-644282 was compared with docetaxel in the human NCI-H460 non-small cell lung carcinoma xenograft. Docetaxel was administered at 12, 16 or 20 mg/kg/day by IV injection on alternate days for three injections. Genz-644282 was compared with dacarbazine in the human LOX-IMVI melanoma xenograft. Dacarbazine was administered at 90 mg/kg/day by IP injection once daily for 5 days. Genz-644282 was administered at 1, 1.36, 1.7, 2.7 or 4.1 mg/kg/day by IV on alternate days 3-times per week for 2 weeks in all in vivo experiments.

**Pharmacokinetic Studies**

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to quantitate Genz-644282 in blood, plasma and tumor tissue of nude (nu/nu) mice both naïve and bearing NCI-H460 human tumor xenografts. The pharmacokinetics of Genz-644282 were evaluated in male nude/nude mice following a single IV dose of 2.0 mg/kg administered as the free base prepared in a lactic acid-sodium lactate-water formulation. Non-compartmental pharmacokinetic parameters were determined. Pharmacokinetic-pharmacodynamic modeling was carried out by the method of Simeoni et al (44).

**Statistical Analysis**

Concentration response data for bone marrow CFU-GM and human tumor cell line CFU were analyzed using a nonlinear curve fit based upon the equation \( y = a \exp(b \cdot x) \). The IC50 and IC90 values and the 95% confidence interval for each compound on human and mouse bone marrow were determined by nonlinear regression analysis using SAS version 8.2. Tumor volumes were calculated using the formula \((w^2 \times l) \times 0.52\). Mouse weight and tumor dimensions were measured twice weekly. The data are presented as mean tumor volume +/- SD. Antitumor activity of the compounds was
determined by calculating tumor growth delay (T-C) in days at a tumor volume of 1500 mm$^3$. Increase-in-lifespan was determined as a secondary endpoint with removal from study due to tumor size. Fold increase-in-lifespan was calculated from median survival in days for the treated versus control groups. Kaplan-Meier survival curves were prepared using GraphPad Prism software and used to determine the median survival times for each treatment group of mice.
RESULTS

The chemical structures of the non-camptothecin Top1 inhibitors, Genz-644282, ARC-111, are shown in Figure 1A (33-35). The compounds Genz-644282, ARC-111, topotecan and SN-38 are potent cytotoxic agents toward both mouse and human bone marrow CFU-GM. Exposure to the compounds produced killing of cells in an exponential manner. The concentration response curves are shown in FIGURE 1B. Coefficient of determination $R^2$ values provides a measure of the goodness of fit of the data to the model ranged between 0.75 and 0.97. For all compounds tested, concentrations killing 50% and 90% of the cells were readily achieved. Human and mouse bone marrow CFU-GM IC$_{50}$’s were 3- to 4-fold lower than the corresponding IC$_{90}$’s, thus the concentration response curves for these compounds are steep (FIGURE 1B). Human bone marrow CFU-GM was more sensitive to the cytotoxicity of the compounds than was mouse bone marrow CFU-GM. Genz-644282 and ARC-111 were more potent cytotoxic agents than the camptothecins, topotecan and SN-38 (irinotecan). The ratios of the mouse and human bone marrow CFU-GM at the IC$_{50}$ and IC$_{90}$ concentrations were calculated. For the camptothecins the ratios were greater than or equal to 10 and for Genz-644282 and ARC-111 the ratios were 4 to 7.

Genz-644282, ARC-111, topotecan and SN-38 were potent cytotoxic agents toward the eight human tumor cell lines included in the colony formation assay. Exposure to the compounds produced exponential killing of human tumor cells as in the bone marrow experiments (FIGURE 2). Concentrations killing 50% and 90% of the cells were
readily achieved for all compounds. Genz-644282 and ARC-111 were found to be more potent cytotoxic agents than the camptothecins, topotecan and SN-38 (active metabolite of irinotecan).

KBV-1 cells are engineered to express much higher levels of MDR than parental control KB3-1 cells, and H460, HT-29, and RPMI- 8226 cells are very high natural expressers of BCRP (26). Cells with high expression of either MDR or BCRP were markedly resistant to killing by topotecan and SN-38 but not to killing by Genz-644282 and ARC-111.

Twenty-nine human tumor cell lines of six histological types were tested for sensitivity to growth inhibition by Genz-644282 in a 72-hr exposure study using an ATP-content readout (FIGURE 1C). Exposure to the compound produced exponential killing of cells in a manner consistent with potent inhibition of a critical molecular target. The IC_{90} concentrations for the 29 human tumor cell lines spread over a 1000-fold concentration range from 100 nM to >10 \( \mu \)M. Four of the six pancreatic cancer cell lines tested were among the lines most sensitive to Genz-644282. Three of the four melanoma cell lines tested were among the least sensitive to Genz-644282 while most of the six breast cancer cell lines tended to have moderate response to the compound. The most disparate results were obtained with the seven renal cell cancer cell lines which ranged from the most sensitive to the least sensitive responders to the compound. Two ovarian cancer lines were tested; the OVCAR-4 cell line which was from a previously untreated patient was more sensitive to the compound than was the OVCAR-5 cell line which was from a patient who had failed multiple chemotherapies. Ten of the 29 tumor cell lines were documented to have originated from metastatic disease. There is no strong trend for
these cell lines to be more or less sensitive to the compound; however, half of the metastatic cell lines had IC₅₀ exceeding 50 nM. The median IC₅₀ value for the 29 human tumor cell lines was 33 nM and the mean IC₅₀ value for the 29 cell lines was 164 nM.

Following intravenous administration of Genz-644282 (2 mg/kg) to nude/nude mice, the mean blood clearance, volume of distribution, and terminal half-life were 21.8 ml/min/kg, 5.44 L/kg and 5.14 hours. An intravenous injection of Genz-644282 (2 mg/kg) to nude/nude mice bearing NCI-H460 human tumor xenografts resulted in plasma pharmacokinetic parameters of 104 ml/min/kg mean clearance, a 49.3 L/kg volume of distribution and terminal half-life of 8.14 hours. The mean area-under-the-curve (AUC) in tumor tissues (9690 ng*h/ml) was approximately 30-fold higher than that observed in plasma (319 ng*h/ml), demonstrating Genz-644282 has a high partition into tumor tissue in the xenograft model. The Genz-644282 IC₉₀ concentration for human tumor NCI-H460 cell line in the colony formation assay was 5 nanomolar (FIGURE 2). Analysis of the PK/PD relationship of Genz-644282 compared the exponential growth of tumors in control mice with tumor growth in the Genz-644282 treated mice. The growth decreased by a factor proportional to drug concentration (44). The estimated average tumor concentration of Genz-644282 at steady state ranged from 130-519 nM or 24- to 96-fold higher than the in vitro IC₉₀ (5.4 nM), thus predicting that the tumor would be responsive to treatment.

Many schedules have been described in the literature for the administration of irinotecan in mice (45). Both Genz-644282 and irinotecan were tested on several schedules and the optimal ones for each compound were selected for doing comparisons in several human tumor xenograft models. In vivo, Genz-644282 was tolerated at doses
up to 4 mg/kg when administered intravenously on alternate days and the compound was active at doses from 1 mg/kg to 4 mg/kg. The efficacy of Genz-644282 was compared with irinotecan in four human colon carcinoma xenograft models. In the human HCT-116 colon cancer xenograft, tumor growth delay (TGD) values were 14 days for irinotecan (60 mg/kg) and 34 days for Genz-644282 (2.7 mg/kg) giving maximum T/Cs of 23.7% and 16.8%, respectively (FIGURE 3A). A similar experiment was performed with the human HT-29 colon carcinoma xenograft, and the TGD values were 11 days for irinotecan (60 mg/kg) and 27 days for Genz-644282 with maximum T/Cs of 52% and 16.6%, respectively (FIGURE 3B). In the human HCT-15 colon carcinoma xenograft the TGD, Genz-644282 was administered at 2 mg/kg resulting in a TGD of 33 days compared with a TGD of 27 days with irinotecan (60 mg/kg) giving maximum T/Cs of 16% and 8.6%, respectively (FIGURE 3C). Mice bearing the DLD-1 human colon carcinoma xenograft were treated with Genz-644282 (1 mg/kg) resulting in a TGD of 14 days compared with a TGD of 11 days with irinotecan (60 mg/kg) with maximum T/Cs of 50.2% and 51.3%, respectively (FIGURE 3D).

The antitumor efficacy of Genz-644282 was compared with the antitumor activity of dacarbazine in the human LOX-IMVI melanoma xenograft (FIGURE 4A). Administration of Genz-644282 (2 mg/kg) produced a TGD of 28 days in mice bearing LOX-IMVI melanoma compared with a TGD of 14 days after administration of dacarbazine (90 mg/kg) giving maximum T/Cs of 66.2% and 80.6%, respectively. The antitumor efficacy of Genz-644282 was compared with the antitumor activity of irinotecan in the human 786-O renal cell carcinoma xenograft (FIGURE 4B). Administration of Genz-644282 (1.7 mg/kg) produced a TGD of 23 days in mice bearing
786-O tumors compared with a TGD of 16 days after administration of irinotecan (60 mg/kg) with maximum T/Cs of 8.7% and 20.7%, respectively.

The antitumor activity of Genz-644282 was compared with the antitumor activity of docetaxel in NCI-H460 and NCI-H1299 human non-small cell lung carcinoma xenografts. Administration of Genz-644282 (2.7 mg/kg) to mice bearing the NCI-H460 human non-small cell lung carcinoma produced at TGD of 27 days compared with a TGD of 21 days from administration of docetaxel (20 mg/kg) giving maximum T/Cs of 4.6% and 19.5%, respectively (FIGURE 4C). In the NCI-H1299 human non-small cell lung carcinoma xenograft, treatment with Genz-644282 (1.7 mg/kg) resulted in a TGD of 33 days while treatment with docetaxel (20 mg/kg) produced a TGD of 15 days with maximum T/Cs of 8.7% and 20.7%, respectively (FIGURE 4D). As an initial combination chemotherapy regimen, administration of Genz-644282 (1.36 mg/kg) along with docetaxel (12 mg/kg) was tested in mice bearing NCI-H460 human non-small cell lung carcinoma xenografts (FIGURE 5). The TGD with docetaxel (12 mg/kg) alone was 12 days and the TGD obtained following administration of Genz-644282 (1.36 mg/kg) was 24 days and the TGD achieved with the combination regimen was 27 days.
DISCUSSION

Topoisomerase 1 is a fully validated target in cancer with two drugs specifically targeted to the enzyme in clinical use. Top1 inhibition became an important target in cancer with the discovery that camptothecin, a very potent anticancer plant alkaloid, specifically targets Top1 (46). Top1 remains a target of active interest in the development of new anticancer agents because Top1 inhibitors are clearly active and effective anticancer drugs and because the current Top1 inhibitors are molecules that can be improved upon. There are camptothecin-derivatives and non-camptothecin Top1 inhibitors in preclinical and clinical development. Each of these investigational molecules may have properties that lead to improved therapeutic benefit to patients. There are also very active preclinical efforts based upon protein phosphorylation levels and mRNA levels in tumor and blood samples to define biomarkers that can select patients most likely to benefit from treatment with specific Top1 inhibitors and to guide clinical investigators toward definition of the lowest effective dose and optimal schedule for administration of these agents (21).

Bone marrow granulocyte-macrophage-colony forming (CFU-GM) assays comparing the toxic effects on bone marrow by investigational agents across species have been useful in predicting the blood levels that might be reached in patients compared with blood levels in preclinical efficacy and safety species (27). Frequently, mouse bone marrow is less sensitive than human bone marrow to investigational agents allowing blood levels to be achieved in mouse preclinical efficacy testing that can not be reached in patients. The bone marrow toxicity of four Top1 inhibitors was examined (FIGURE 1B) (26-30). Murine bone marrow is 4- to 28-fold less sensitive to the Top1 inhibitors
than human bone marrow. The differential sensitivity between mouse and human bone marrow to Top1 inhibitors may explain, in part, why curative doses/blood levels of topotecan and irinotecan/SN-38 in mice with human tumor xenografts are not achievable in patients (29, 30). Compounds with little differential in bone marrow sensitivity across species may have greater potential for reaching similar blood levels in patients as in mice. From these data, Genz-644282 and ARC-111 may have promise as development candidates.

A hallmark of malignant cells is the capacity for unlimited renewal or proliferation. The cancer stem cell hypothesis postulates that malignant disease is maintained by a rare subset of cells with stem cell properties (47, 48). The colony formation assay is the gold-standard for the determination of the cytotoxicity of compounds toward malignant cells because it measures the survival/killing of cancer cell capable of proliferating to form colonies or clones. Each colony originates from a single cell capable of self-renew with the ability to generate malignant disease. Genz-644282 cytotoxicity was assessed in eight human tumor cell lines of varied histology and resistance mechanisms by colony formation. Compared with topotecan and SN38, Genz-644282 and ARC-111 were more potent cytotoxic agents. Genz-644282 and ARC-111 remained highly effective in cells expressing efflux pumps. Meng et al. (49) reported that, like other Top1 inhibitors, the antiproliferative effects of ARC-111 were oxygen-independent, which is distinguishable from inhibition of hypoxia-inducible factor-1α accumulation by ARC-111, only observed under hypoxia and supporting the potential anticancer efficacy of ARC-111 and similar compounds toward hypoxic tumors.
In earlier reports, ARC-111 was shown to be an effective antitumor agent against several standard human tumor xenografts. ARC-111 was as active as irinotecan in the HCT-8 colon carcinoma and as active as topotecan or irinotecan in the SKNEP anaplastic Wilm’s xenograft (23). ARC-111 was also a very effective antitumor agent in animals bearing the SJ-BT45 medulloblastoma (34). In subsequent studies, ARC-111 was as or more effective as an anticancer agent compared with irinotecan in the HCT116 and HT29 human colon carcinoma xenografts, docetaxel in the NCI-H460 human non-small lung carcinoma xenograft and gemcitabine in the MiaPaCa2 human pancreatic carcinoma xenograft (26). In the current study, Genz-644282 demonstrated greater or equal antitumor efficacy as irinotecan in four human colon carcinoma xenografts, greater antitumor efficacy than dacarbazine in the human LOX IMVI melanoma xenograft, greater antitumor efficacy than irinotecan in the human 786-O renal cell carcinoma xenograft, and greater than or equal antitumor efficacy than docetaxel in two human non-small lung carcinoma xenografts. In an initial combination chemotherapy regimen, Genz-644282 and docetaxel together showed a modest increase in tumor response in the NCI-H460 non-small cell lung carcinoma xenograft compared with Genz-644282 as a single agent.

Top1 inhibitors are active and effective anticancer drugs. The FDA approved Top1 inhibitors are molecules that have flaws which newer compounds can improve upon. For this reason, Top1 remains a target of active interest in the development of new anticancer agents. Investigational molecules, both camptothecin and non-camptothecin Top1 inhibitors in preclinical and clinical development, have properties that lead to improved therapeutic benefit to patients. There is an active search in the Top1 inhibitor
field for biomarkers such as gene signatures or markers based upon protein 
phosphorylation levels and mRNA levels in tumor or blood samples that can identify 
patients most likely to benefit from treatment with a specific Top1 inhibitor.

Genz-644282 is a promising non-camptothecin Top1 inhibitor that based upon 
preclinical activity and safety was selected for development and is currently undergoing a 
Phase 1 clinical trial.
FIGURE LEGENDS

FIGURE 1. A. Chemical structures of the non-camptothecin Top1 inhibitors, Genz-644282 and ARC-111 are shown. B. Concentration response curves for each of the four Top1 inhibitors in mouse (Δ) and human (▲) bone marrow CFU-GM showing an exponential curve fit (dotted lines). C. Tumor cell IC₉₀ values for 29 human tumor cell lines upon exposure to Genz-644282 in a 72 hr growth inhibition assay in micromolar concentrations are shown. The IC₅₀ and IC₉₀ concentrations determined from the concentration response curves for the 29 tumor lines are shown in Supplemental TABLE 1.

FIGURE 2. Concentration response curves in a colony formation assay for each of the four Top1 inhibitors, Genz-644282 (♦), ARC-111 (☐), SN-38 (■) and topotecan (□), in human HCT116 and HT29 colon carcinoma cells, NCI-H460 non-small cell lung carcinoma cells, MDA-MB-231 breast carcinoma cells, RPMI8226 multiple myeloma cells and KB3-1, KBV-1 and KBH5.0 cervical carcinoma cells as representative human tumor cell lines. The red dot curves fit the nonlinear exponential model for the Genz-644282 data.

FIGURE 3. Growth of subcutaneously implanted human tumor xenografts in nude mice controls, treated with Genz-644282 or irinotecan. A. HCT-116 colon carcinoma untreated control (♦), Genz-644282 vehicle treated (■), irinotecan vehicle treated (▲), Genz-644282 (1.36 mg/kg, iv, alternate days for 2 wks) (☐), Genz-644282 (2.7 mg/kg, iv, alternate days for 2 wks) (Δ), and irinotecan (60 mg/kg, iv each fourth day for 3
injections) (●). B. HT-29 colon carcinoma untreated controls (●), Genz-644282 vehicle treated (■), irinotecan vehicle treated (▲), Genz-644282 (1.36 mg/kg, iv, alternate days for 2 wks) (□), Genz-644282 (2.7 mg/kg, iv, alternate days for 2 wks) (Δ), Genz-644282 (4.1 mg/kg, iv, alternate days for 2 wks) (◇), and irinotecan (60 mg/kg, iv each fourth day for 3 injections) (●). C. HCT-15 colon carcinoma untreated controls (♦), Genz-644282 vehicle treated (■), irinotecan vehicle treated (▲), Genz-644282 (1 mg/kg, iv, alternate days for 2 wks) (○), Genz-644282 (2 mg/kg, iv, alternate days for 2 wks) (□), and irinotecan (60 mg/kg, iv each fourth day for 3 injections) (●). D. DLD-1 colon carcinoma untreated controls (♦), Genz-644282 vehicle treated (■), irinotecan vehicle treated (▲), Genz-644282 (1 mg/kg, iv, alternate days for 2 wks) (○), and irinotecan (60 mg/kg, iv each fourth day for 3 injections) (●). The data are the means +/- SD for groups of 8-10 mice.

**FIGURE 4.** Growth of subcutaneously implanted human tumor xenografts in nude mice controls, treated with Genz-644282 or dacarbazine or irinotecan or docetaxel. A. LOX-IMVI melanoma untreated control (♦), Genz-644282 vehicle treated (■), Genz-644282 (1 mg/kg, iv, alternate days for 2 wks) (○), Genz-644282 (2 mg/kg, iv, alternate days for 2 wks) (□), dacarbazine (90 mg/kg, ip, daily for 5 days) (●). B. 786-O renal carcinoma untreated controls (♦), Genz-644282 vehicle treated (■), Genz-644282 (1 mg/kg, iv, alternate days for 2 wks) (○), Genz-644282 (1.36 mg/kg, iv, alternate days for 2 wks) (◇), Genz-644282 (1.7 mg/kg, iv, alternate days for 2 wks) (□), and irinotecan (60 mg/kg, iv each fourth day for 3 injections) (●). The data are the means +/- SD for groups of 8-10 mice. C. NCI-H460 non-small cell lung carcinoma untreated control (♦), Genz-644282 vehicle treated (■), docetaxel vehicle treated (▲), Genz-644282 (1 mg/kg, iv, alternate
days for 2 wks) (○), Genz-644282 (1.36 mg/kg, iv, alternate days for 2 wks) (□), Genz-644282 (2.7 mg/kg, iv, alternate days for 2 wks) (Δ) and docetaxel (20 mg/kg, iv, alternate days for 3 injections. D. NCI-H1299 non-small lung carcinoma untreated controls (♦), vehicle treated (■), Genz-644282 (1 mg/kg, iv, alternate days for 2 wks) (○), Genz-644282 (1.36 mg/kg, iv, alternate days for 2 wks) (□), Genz-644282 (1.7 mg/kg, iv, alternate days for 2 wks) (Δ), docetaxel (16 mg/kg, iv, alternate days for 3 injections) (●) and docetaxel (20 mg/kg, iv (▲)).

**FIGURE 5.** Growth of subcutaneously implanted human tumor xenografts in nude mice controls, treated with Genz-644282 in combination with docetaxel. NCI-H460 non-small cell lung carcinoma untreated controls (♦), Genz-644282 vehicle treated (■), Genz-644282 (1.36 mg/kg, iv, alternate days for 2 wks) (○), Genz-644282 (1.36 mg/kg, iv, alternate days for 2 wks) + docetaxel (12 mg/kg, iv, alternate days for 3 injections) (■), and docetaxel (12 mg/kg, iv, alternate days for 3 injections) (●). The data are the means +/- SD for groups of 8-10 mice.
REFERENCES


**Supplemental TABLE 1.** Twenty-nine human tumor cell lines of six histological types were tested for sensitivity to growth inhibition by Genz-644282 in a 72-hr exposure study using an ATP-content readout. The IC\textsubscript{90} concentrations for the 29 human tumor cell lines spread over a 1000-fold concentration range from 100 nM to >10 \(\mu\)M. This data is represented in **FIGURE 1C**.

<table>
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<th>DISEASE</th>
<th>CELL LINE</th>
<th>IC\textsubscript{50}, NAMOMOLAR</th>
<th>IC\textsubscript{90}, MICROMOLAR</th>
<th>RATIO IC\textsubscript{90}/IC\textsubscript{50}</th>
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<td>&gt;10</td>
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</table>
Figure 1

A

ARC111: $R^1 = \text{CH}_3$, $R^2 = \text{CH}_3$
Genz644282: $R^1 = \text{H}$, $R^2 = \text{CH}_3$

B

C

Surviving Fraction

Compound Concentration, nM

Figure 1
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<tr>
<th>Tumor Type</th>
<th>Compound Concentration, nM</th>
<th>Genz: IC50, IC90</th>
<th>ARC111: IC50, IC90</th>
<th>SN-38: IC50, IC90</th>
<th>Topo: IC50, IC90</th>
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</table>
Figure 3
Figure 4
Human NCI-H460 Non-Small Cell Lung Carcinoma

- Untreated Control
- Genz Vehicle
- Genz644282 1.36 mg/kg
- Genz644282 1.36 mg/kg + Docetaxel 12 mg/kg
- Docetaxel 12 mg/kg

**Mean Tumor Volume, mm³**

Days post tumor implantation

Figure 5