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In vitro cytotoxicity of the epothilone analog, BMS 247550, in pediatric malignacies

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ABSTRACT

BMS247550 is an epothilone-B analog that binds tubulin and stabilizes microtubules. Its mechanism of action is similar to taxanes (paclitaxel and docetaxel) and like taxanes, BMS247550 is cytotoxic in solid tumor cell lines including ovarian, colon, breast, prostate, and lung carcinoma with IC_{50}s ranging from 1-25nM. BMS247550 has activity in paclitaxel-resistant ovarian carcinoma cell lines and cell lines expressing multidrug resistance phenotype. Phase 1 trial and pharmacokinetic study of BMS247550 in adults with refractory solid tumors is underway at the NCI. Serum BMS247550 trough concentrations are approximately 8nM in patients receiving 6-8 mg/m^2 IV daily. I examined cytotoxicity of BMS247550 in pediatric solid tumor cell lines and compared it to other agents that inhibit microtubule function. Cell lines studied were osteosarcoma (HOS), Ewing's sarcoma (LD-EWS), and rhabdomyosarcoma (RD). Cells were plated into 96-well microtiter-plates and exposed to drug, vehicle, or no drug for 72 hours. Sulforhodamine-B assay was used to determine percent cell survival. Dose-response curves were constructed by plotting percent cell survival vs. drug concentration. IC_{50}, maximal effect (ME) and slope of the dose-response curve (h) were estimated using MLAB mathematical modeling software. IC_{50} of BMS247550 was 8.6nM, 8.2nM, and 17nM in HOS, LD, and RD cells, respectively. IC_{50} of paclitaxel in HOS, LD, and RD cells was 0.4nM, 2.0nM, and 0.6nM, respectively. IC_{50} of vincristine in HOS, LD, and RD cells was 45nM, 5.0nM, and 38nM, respectively. IC_{50} of vinorelbine in HOS, LD, and RD cells was 11nM, 4.9nM, and 18nM, respectively. I conclude that BMS247550 is cytotoxic in pediatric tumor cell lines at concentrations achievable in patients. The IC_{50} of BMS247550 is comparable to the IC_{50} of other agents that inhibit tubulin which are currently used to treat childhood solid tumors. Therefore, phase 1 trial of BMS245770 in pediatric patients with refractory solid tumors should be undertaken.

Keywords: *BMS 247550, cytotoxicity, epothilone, potency, tubulin*

INTRODUCTION

Cancer is a disease of unregulated proliferation of cells that have transformed from normal body cells. There are over 100 types of cancer that arise from almost every tissue or organ in the body. Since World War II, research efforts have focused on identifying new anti-cancer drugs that control or cure some of these cancers. Although over 60 drugs have been approved by the FDA for the treatment of cancer and significant progress has been made in prolonging the survival and quality of life of cancer patients, more effective and less toxic drugs are still needed. Childhood cancers are more responsive to current anticancer drugs than cancers in adults, but 25% of children still do not survive 5 years and those that do survive are at risk for serious long term effects of the treatment (www.cancer.org).

Anti-cancer drugs are discovered by several methods including random screening (testing whether a compound kills cancer cells in vitro without knowing how it works), molecularly-targeted screening (testing compounds for their ability to block a specific protein or pathway that is responsible for transforming a normal cell into a cancer cell), or rational drug design (chemically synthesizing a drug to inhibit a critical protein, such as an enzyme). At the National Institutes of Health, Bethesda, MD, drugs that are identified as potential new anticancer drugs from these screening procedures then undergo a preclinical drug development process, which includes 1) activity screening against a variety of tumor types in vitro and in animal tumor models, 2) synthesis and bulk production, 3) preclinical toxicology and pharmacology, and 4) formulation and production for human use (Nathan *et al.* 1998).

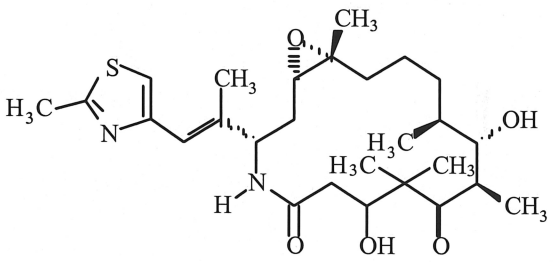


Figure 1. Chemical structure of BMS 247550. This chemotherapy is an analog of the natural product epothilone B.

After a drug completes this preclinical screening and is proven effective *in vitro* and in animal models, a clinical phase 1 trial is performed to determine the optimal dose, the toxicity spectrum and the clinical pharmacology of the new drug in humans. Phase 2 trials then identify the cancers in which the drug is

active, and phase 3 trials compare the new drug to standard therapy.

Clinical drug development is somewhat different for childhood cancers. Because of the low number of pediatric cases relative to adult cancers and concerns about the safety of new drugs in children, pediatric clinical trials will often not begin until some results from the parallel adult trial have been analyzed. Because of differences in the response to treatment in childhood cancers relative to adult cancers, it is also necessary to study on pediatric tumor cell lines separately.

BMS 247550, [1S-[1R*, 3R*(E), 7R*, 10S* 12R*, 16S*]]-7,11-dihydroxy-8,8,10,12,16-pentamethyl-3-[1-methyl-2-(2-methyl-4-thiazolyl) ethenyl]-17-oxa-4-azabicylo [14.1.0] heptadecane-5 ,9-dione (Figure 1), is an investigational anti-cancer drug that is cytotoxic to cancer cells. BMS 247550 is a semi-synthetic analog of the natural product, epothilone B, which was extracted from the fermentation broth of the cellulose degrading bacteria, *Sorangium cellulosum*. The epothilones, which contain 16-membered ring macrolides, were discovered by the random screening process. The main difference between BMS 247500 and epothilone B is the replacement of the macrolide ring oxygen atom with a nitrogen atom to give the corresponding macrolactam (Widemann *et al.* 2001). BMS 247550 (NSC 710428) has a molecular formula of $C_{27}H_{42}N_2O_5S$ and a molecular weight of 506.7 grams/mole.

Preclinical studies demonstrated that the epothilones (including BMS 247550) have a similar mechanism of action as other anticancer drugs of natural product origin, the taxanes and vinca alkaloids. BMS 247550 act in eukaryotic cells by binding to tubulin, the subunit of microtubules. Microtubules (MT) are made up of 13 strands of repeating tubulin dimers, which consist of alternating α- and β-tubulin (~50 kD each). MT exist in a dynamic state, constantly adding, or polymerizing, tubulin at one end (the + end) and depolymerizing at the negative end.

Similar to the taxanes, BMS 247550 binds to the tubulin in microtubules and stabilizes the polymer (blocks depolymerization). In this static state, the MT is no longer functional to the cell. MT are used for cell transport, cell motility, mitosis, and signal transduction. MT are especially important through their role in the formation of spindle fibers, which function during mitosis for the correct migration of chromosomes. Therefore, by binding the β-tubulin, BMS 247550 is able to interrupt the cell cycle and cause apoptosis. The exact binding site of BMS 247550 is unknown (Dumontet *et al.* 1999) (Giannakakou *et al.* 2000) (Vaishampayan *et al.* 1999).

BMS 247550 is cytotoxic against a broad range of tumor cell lines in vitro at nanomolar concentrations. It also exhibits several advantages over the taxanes.

- The simpler chemical structure of BMS 247550 lends itself more easily to large scale synthetic production than taxanes (Bollag et.al 1995).
- BMS 247550 is more potent at stabilizing microtubules *in vitro* (Lee *et al.* 2001).
- BMS 247550 is effective in taxane insensitive and resistant tumor cells. P-glycoprotein (PGP) is a cell surface drug efflux pump that is responsible for pumping "unwanted" molecules, including some drugs, out of the cell as a first line of defense. Tumor cells that over-express PGP are classified as multi-drug resistant (MDR). BMS 247550 retains greater cytotoxicity against PGP-expressing multiple drug resistant cells than taxanes (Bollag et.al 1995).
- Although chemotherapy is often administered parentally, oral administration is preferable because of patient convenience and cost. Unlike the taxanes, antitumor activity produced by BMS 247550 after oral administration is comparable to that produced with parenteral administration (Lee *et al.* 2001).

BMS 247550 is currently undergoing phase 1 clinical trials in adults with refractory solid tumor cancers. The pharmacokinetic portion of the study in adults indicates that serum BMS 274550 trough (24 hours post-dose) concentration are 8 nM in adult patients receiving 6-8 mg/m^2 IV daily x 5 days. The trough concentration is the level of drug achievable in a patient.

Prior to conducting pediatric clinical trials of BMS 247550, the cytotoxicity of BMS 247550 should be studied in cell lines derived from childhood cancers. Three cell lines were chosen because their growth characteristics reflect a reasonable doubling time, adherence to the growth surface, and behavior conducive to the assay used in this study.

Cell survival in the presence of varying concentrations of BMS 247550 was assessed with the sulforhodamine B (SRB) assay. SRB is a bright pink aminoxanthene dye with two sulfonic groups. It binds to basic amino acids, essentially tagging the cells by staining protein. The dye binds electrostatically to

Table 1: Four anti-cancer drugs. Along with BMS 247550, three common anti-cancer agents that bind to tubulin were assessed by the same methods in order to compare their IC_{50} values.

Drug	Class	Mol.Wt. (g/mol)	Vehicle	Concentration Range
BMS 247550	Epothilone	507	1% DMSO	0.5 nM – 1000 nM
Paclitaxel	Taxane	854	1% DMSO	0.1 nM – 1000 nM
Vincristine	Vinca Alkaloid	923	dH_20	0.1 nM – 1080 nM
Vinorelbine	Vinca Alkaloid	1079	dH_20	0.9 nM – 9270 nM

macromolecular counter ions in cells fixed by TCA, which allows their binding and solubilization to be controlled by changes in pH. As the pH rises, the stain is quantitatively extracted from the cells and the absorbance is measured (Skehan *et al.* 1990).

The concentration of dye, which is proportional to the number of tumor cells surviving, is measured spectrophotometrically using the Beer's Law:

$$C = \frac{A}{L \times K}$$

in which A is absorbance, C is the dye concentration in the solution, L is the depth of the solution through which the light must travel, and K is a proportionality constant. The absorbance, or optical density (OD), is a measure of the amount of light stopped, or absorbed by the solution (Campbell *et al.* 1984).

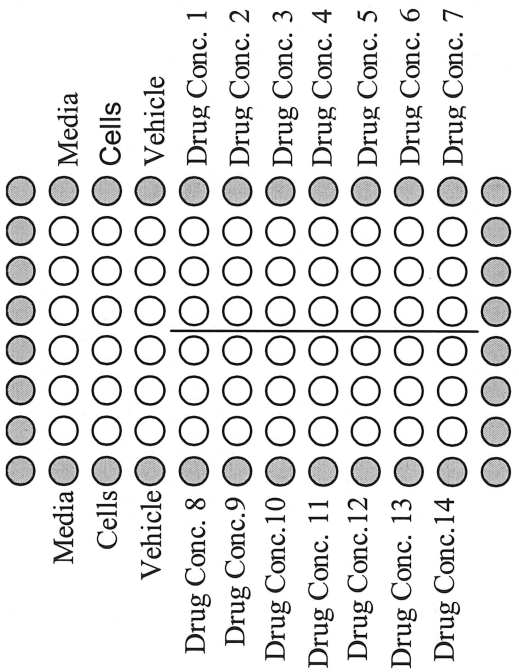
The SRB assay provides a colorimetric end point that is nondestructive, indefinitely stable and visible to the naked eye. It is preferable over other dye assays because of its effectiveness at low cell densities and sensitivity to the semi-micro dimensions of microtiter plates (Skehan *et al.* 1990). Because it is not dependent on cell metabolism like other assays, SRB is less time dependent, making it convenient for processing large batches of plates (Rubenstein *et al.* 1990).

Sigmoidal dose response curves are created in order to find the inhibitory concentrations at which 50% of the cells survive (IC_{50}) as a measure of drug potency. In order to determine more accurate IC_{50} values, Modeling LABoratory (MLAB), interactive mathematical modeling software is used (Civilized Software, Inc.; Gary Knott; Revision Date: Oct. 8, 1996). MLAB is an iterative curve fitting procedure that will adjust model parameters to minimize the difference between the model simulated value (x_i) and the measured value (y_i).

$$\sum_{i=1}^{n} (f(x_i) - y_i)^2$$

The objectives of this study are 1) to determine the *in vitro* growth characteristics of a panel of pediatric tumor cell lines, 2) to assess the *in vitro* cytotoxicity of BMS 247550 in cell lines derived from common pediatric solid tumors, and 3) to compare the IC_{50} of BMS 247550 to commonly used anticancer agents that interfere with microtubule assembly and depolymerization.

Figure 2. Diagram of the experimental design in a 96-well microtiter plate. Grayed wells were not used.



MATERIALS AND METHODS

Pediatric Tumor Cell Lines. RD and HOS cell lines were obtained from the American Type Culture Collection (ATCC). LD-EWS was established at the Pediatric Oncology Branch of the NCI from an 18 year old female with Ewing's sarcoma of the scapula. All cell lines were maintained in RPMI 1640 with 10% heat inactivated fetal bovine serum (FBS, Mediatech; Herndon, VA) at 37°C/ 5% CO_2. Cell lines were negative for *Mycoplasma species* by Hoechst Staining and culture. Experiments were performed when cells were between the 20-50 passage. Basic methods were taken from Cell Culture (Jakoby *et al.* 1979) and Cell Biology (Celis *et al.* 1994).

The doubling time for each cell line was determined by serial hemocytometer counts. In brief explanation, 5000-10000 cells/well were plated into a 96 well microtiter plate. Each day cells were detached using 0.05% trypsin (Trypsin, Mediatech; Herndon, VA) and 0.1% trypan blue stain (Bio-Whittaker; Walkersville, MD) was added. Viable cells which exclude trypan

Table 2: The pediatric tumor cell lines selected for testing with BMS 247550. Lines represent a broad range of childhood sarcomas.

Cell Line	Tumor Type	Doubling Time	Patient Characteristics
HOS	Osteosarcoma	24 hr	13 yo female, metastatic osteosarcoma
LD-EWS	Ewing's Sarcoma	20 hr	18 yo female, scapula primary
RD	Rhabdomyosarcoma	58 hr	7 yo female, primary pelvic tumor

blue stain were counted using a Hausser scientific hemocytometer. Counts were done in triplicate. Doubling time was calculated from the growth curve, a semilogarithmic sigmoidal plot of mean cell density plotted as a function of time.

Drug Preparation. BMS247550 (Brystol-Myers Squibb, Wallingford, CT) was dissolved in 100 % dimethylsulfoxide (DMSO, SIGMA, St.Louis , MO) to make a 1 mM stock solution. Stock was stored at 4°C, protected from light. Prior to being added to cells, the BMS247550 was serially diluted to a concentration of 5-10,000 nM in 1% DMSO. The final 1:10 dilution was made when 20 µL of drug was added to 180 µL of cells and media in each well. Final BMS247550 concentration on the cells was 0.5-1000 nM in 0.1% DMSO. Similarly, paclitaxel (SIGMA, St. Louis, MO) was dissolved and serially diluted in DMSO. The final paclitaxel concentration on the cells ranged from 0.1-1000 nM in 0.1 % DMSO. Vincristine (Eli Lily, Indianapolis, IN) and vinorelbine (GlaxoWelcome, Research Triangle Park, NC) are water-soluble and were serially diluted in de-ionized H_2O. The final drug concentration on the cells ranged from 0.1-1080 nM and 0.9-9270 nM for vincristine and vinorelbine, respectively. Table 1 summarizes the agents and concentrations used.

SRB Cytotoxicity Assay. The sulforhodamine B *in vitro* cytotoxicity assay originally described by Skehan (Skehan *et al.* 1990) was modified for use in this study. Cells were grown to confluency (approximately 6 X 10^6 cell/ml) in a 75 cm^2 (250 mL) tissue culture flask. Adherent cells were detached and counted using trypan blue exclusion as previously described. Cells were then diluted in RPMI 1640 and 10% FBS so that 180µL of media contained 5000 HOS cells or 10000 cells/180µL for RD and LD cells. Using an automated pipettor (SerialMate; Matrix Technologies; Hudson, NH) 180 µL of cell suspension was dispensed into the wells of 96 well microtiter plates. For HOS cells 5000 cells/well and for LD and RD 10000 cells/well were plated in five microtiter plates. All plates were incubated 24 hours at 37°C/ 5% CO_2.

After the initial 24 hour incubation one plate (P1) was fixed (see below) and drug was applied to three plates (P2-4) and incubated 72 more hours at 37°C/ 5% CO_2. The final plate (P5) acted as a growth control (no drug added) and was incubated 72 hours.

Each plate contained control wells and drug exposed wells (Figure 2). The exterior wells of the 96 well plate were excluded from analysis due to inconsistent cell growth. Six wells had only RPMI 1640/10% FBS added and were the media control wells for determination of background SRB staining. Six wells contained only media and cells with no drug and served as the cell growth control. Six wells had media plus cells plus vehicle (0.1% DMSO or dH_2O) to act as a vehicle control to account for any nonspecific cell death due to the vehicle in which the drug was dissolved. Fourteen drug concentrations were added to wells in triplicate. Cells were exposed to concentrations ranging from 0.5– 1000 nM, 0.1-1000nM, 0.1 – 1080 nM, and 0.9- 9270 nM for BMS247550, paclitaxel, vincristine, and vinorelbine, respectively. The highest concentration of each drug served as a positive control for cytotoxicity, since it was in excess of the expected cytotoxic concentration of the drug.

After 72 hours of drug exposure cells were fixed by the addition of cold tricholoracetic acid (TCA, Sigma; St. Louis, MO). 50 µL of 50% TCA was added to each well for a final concentration of 14% TCA/well. Plates were stored at 4°C for one hour and then washed in dH_2O (DYNEX Ultrawash Plus; Microtiter Company; Chantilly, VA). During fixation, all dead cells were washed from the plate and viable cells were fixed to the bottom of the well. Plates were air dried for approximately one hour, or until completely dry.

Fixed cells were stained for 30 minutes with 100µL/well of 0.4% SRB (Sigma; St. Louis, MO) in 1% acetic acid (MG Scientific, Inc.; Pleasant Prarie, WI) then washed with 1% acetic acid (DYNEX Ultrawash Plus) and air dried.

SRB stain was solubilized from the cellular protein using 10mM unbuffered Trizma base (pH 10.7) (Sigma, St. Louis, MO) by adding 100µL/well. All plates were shaken for 5 minutes on a gyratory shaker (Titer plate shaker; Lab-Line Instruments, Inc.; Melrose Park, Ill.) to assure all SRB dye was soluabilized from the cells.

Dual wavelength (540nm/405nm) endpoint optical density (OD) was measured using the EL-340 microplate reader (Bio-tek; Winooski, VT) and analyzed using DeltaSoft3 software (BioMetallics, Inc.; Princton, NJ).

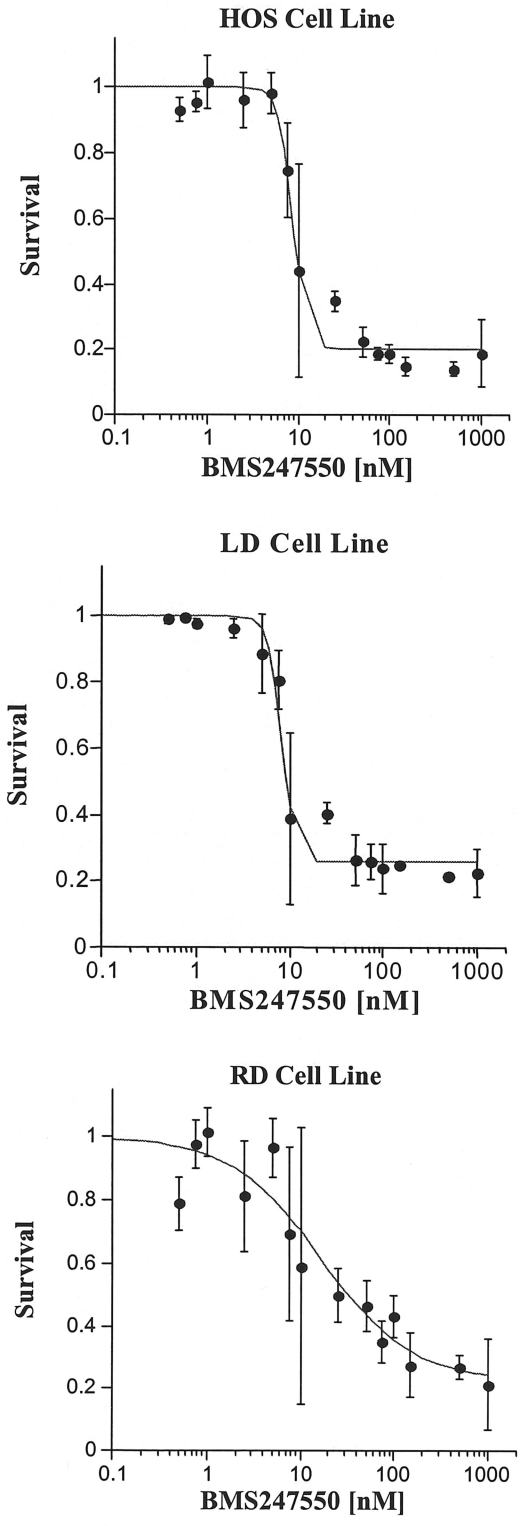
Dose Response Curves and IC_{50} Determination. Dose response curves were created by plotting the percent cell survival (percent control) versus the drug concentration on the cells. Percent control was calculated from plate OD readings with the formula:

$$\%\,\text{Control} = \frac{\text{Mean OD drug exposed well for each [drug]}}{\text{Mean OD control well (cells only)}}$$

IC_{50} values were then estimated using MLAB mathematical modeling software (Civilized Software Inc.; Silver Spring, MD) using the formula

$$C(d) = \frac{(1- ME)}{1+\left(\frac{d}{IC_{50}}\right)^h} + ME$$

Figure 3. Dose response curves for BMS 247550 in the HOS, LD-EWS, and RD pediatric tumor cell lines. Points and error bars represent the mean ± SD for each drug concentration studied and the line represents the model fit to the data.



where *C(d)* is the response to treatment with concentration d, *ME* is maximal effect, IC_{50} is the midpoint between the minimal (no drug) and maximal effect, and *h* is the slope of the sigmoidal dose response curve. For modeling purposes, 100% cell survival was assumed to occur in DMSO vehicle.

RESULTS

Pediatric Tumor Cell Lines. Characteristics and experimental doubling time for each cell line are presented in Table 2. Under the experimental conditions, all cell lines had rapid cell cycles as demonstrated by the doubling times of 20-58 hours. When plated at densities of 5,000-10,000 cells/well, all cell lines remained in the log growth phase throughout the drug exposure (data not shown).

Dose Response Curves and IC_{50} Determination. BMS247550 dose response curves for each cell line are shown in Figure 3. Each point represents the mean ± standard deviation from five experiments. For each experiment drug exposure was done in triplicate wells on three 96 well microtiter plates. Best-fit models of the dose response curves were calculated with values estimated by MLAB and plotted as the solid line.

The range of BMS 247550 concentrations was plotted as a function of the fraction of cell survival, each cell line's dose response to BMS247550 is graphically represented in Figure 3. Model parameters included the maximal effect (ME), the IC_{50} of BMS247550, and the slope (h) of the sigmoidal dose response curve for each cell line are presented in Table 3.

The HOS and LD dose response curves show a steep slope, which reflect their short doubling times. The RD curve displays a more gradual slope. This is expected because BMS247550 stabilizes microtubules and prevents mitosis. Cells with rapid cell cycles (shorter doubling times, like HOS and LD-EWS) will be more sensitive to the drug during a 72 hour exposure than RD cells with a longer cell cycle and less frequent mitosis.

Table 4 compares the IC_{50} of BMS247550, paclitaxel, vincristine, and vinorelbine in each cell line. Each of these anti-cancer agents interferes with microtubules and inhibits mitosis.

Table 3. MLAB modeled parameters. The maximal effect (ME), IC_{50}, and Hill constant (h) were estimated to create a best-fit sigmoidal dose-response curve for the BMS 247550 survival data. (mean ± SD)

Cell line	ME (nM)	IC_{50} (nM)	h
HOS	0.2 ± 0.02	8.6 ± 0.4	5.8 ± 1.6
LD-EWS	0.26 ± 0.02	8.2 ± 0.4	6.2 ± 1.6
RD	0.23 ± 0.09	17 ± 7.0	0.91 ± 0.26

DISCUSSION

All three dose response curves show that as the drug concentration increases, the fraction of cell survival decreases along a sigmoid dose-response curve, indicating a cytotoxic effect at nanomolar concentrations of BMS 247550 in all three pediatric solid tumor cell lines.

As shown by the IC_{50} values, the potency of BMS 247550 is similar to other tubulin-binding anticancer drugs in pediatric tumor cell lines. Previous studies have shown a similar potency of BMS 247550 in a wide range of adult cell lines, including several paclitaxel-resistant (ex. HCT116/VM46 colorectal (MDR) and paclitaxel-insensitive cell lines (ex. Pat-26 human pancreatic carcinoma) (Lee *et al.* 2001). BMS 247550 may also offer an alternative treatment for MDR or insensitive pediatric tumors.

The IC_{50} values for HOS, LD-EWS and RD range from 8.2 nM to 17 nM, which is within the range of the plasma trough concentration (8 nM) in adult patients receiving BMS 247550 at a dose of 6-8 mg/m^2 IV daily x 5 days. Therefore BMS 247550 can be cytotoxic at dosages that can be safely administered in patients.

With the assessment of the drug's IC_{50} values and the confirmation of its cytotoxicity in pediatric tumor cell lines, a phase 1 trial and pharmacokinetic study of BMS 247550 (NSC 710428) will move forward under principal investigator Brigitte Widemann, M.D., of the Pharmacology and Experimental Therapeutics Section (PETS). Sponsored by the Cancer Therapy Evaluation Program (CTEP), the trial will focus on pediatric patients with refractory solid tumors to define the maximum tolerated dose (MTD), toxicity profile, dose-limiting toxicities, pharmacokinetics, and pharmacodynamics. Results will be compared with those found in an ongoing Medicine Branch, NCI, phase I trial with BMS 247550 for adult patients with solid tumors. Both studies use the same dosing while passing through the acidic digestive tract and then withstand the first round of metabolism in the liver. Preliminary tests done on mice show that when given with a pH buffering vehicle, BMS 247550 was highly active orally against the Pat-7 human ovarian carcinoma model (Investigator's Brochure 2000). A p.o.-administered effective drug, such as BMS 247550, would offer a cost-effective alternative to the current parenteral format and "a potential therapeutic advantage" in all of the new dose-scheduling options it would offer (Rose *et al.* 2001).

Table 4: The IC_{50}s for anti-cancer agents that interfere with microtubule function.

Cell Line	BMS 247550	Paclitaxel	Vincristine	Vinorelbi
HOS	8.6 nM	0.4 nM	45 nM	11 nM
LD-EWS	8.2 nM	2.0 nM	5.0 nM	49 nM
RD	17 nM	0.6 nM	38 nM	18 nM

Further studies may include the development of oral administration (p.o.) of BMS 247550. In order to be effective, drugs administered orally must remain stable

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