Inhibition of Poly(ADP-Ribose) Polymerase-1 Enhances Temozolomide and Topotecan Activity against Childhood Neuroblastoma

Rachel A. Daniel,1 Agata L. Rozanska,1 Huw D. Thomas,1 Evan A. Mulligan,1 Yvette Drew,1 Deborah J. Castelbuono,1 Zdenek Hostomsky,2 E. Ruth Plummer,1 Alan V. Boddy,1 Deborah A. Tweddle,1 Nicola J. Curtin,1 and Steven C. Clifford1

Abstract

Purpose: High-risk neuroblastoma is characterized by poor survival rates, and the development of improved therapeutic approaches is a priority. Temozolomide and topotecan show promising clinical activity against neuroblastoma. Poly (ADP-ribose) polymerase-1 (PARP-1) promotes DNA repair and cell survival following genotoxic insult; we postulated that its inhibition may enhance the efficacy of these DNA-damaging drugs in pediatric cancers.

Experimental Design: We evaluated the chemosensitizing properties of the PARP inhibitor AG014699 (Pfizer, Inc.) in combination with temozolomide and topotecan, against human neuroblastoma cells and xenografts, alongside associated pharmacologic and toxicologic indices.

Results: Addition of PARP-inhibitory concentrations of AG014699 significantly potentiated growth inhibition by both topotecan (1.5- to 2.3-fold) and temozolomide (3- to 10-fold) in vitro, with equivalent effects confirmed in clonogenic assays. In two independent in vivo models (NB1691 and SHSY5Y xenografts), temozolomide caused a xenograft growth delay, which was enhanced by co-administration of AG014699, and resulted in complete and sustained tumor regression in the majority (6 of 10; 60%) of cases. Evidence of enhanced growth delay by topotecan/AG014699 co-administration was observed in NB1691 xenografts. AG014699 metabolites distributed rapidly into the plasma (Cmax, 1.2-1.9 nmol/L at 30 min) and accumulated in xenograft tissues (Cmax, 1.2 μmol/L at 120 min), associated with a sustained suppression of PARP-1 enzyme activity. Doses of AG014699 required for potentiation were not toxic per se.

Conclusions: These data show enhancement of temozolomide and topotecan efficacy by PARP inhibition in neuroblastoma. Coupled with the acceptable pharmacokinetic, pharmacodynamic, and toxicity profiles of AG014699, our findings provide strong rationale for investigation of PARP inhibitors in pediatric early clinical studies.

Neuroblastoma is the most common extracranial solid tumor of childhood, with around 100 new cases diagnosed annually in the United Kingdom. It is a clinically and biologically heterogeneous tumor; around half of all cases are curable or may spontaneously regress or undergo maturation (1, 2). The remaining cases exhibit unfavorable biology (e.g., MYCN oncogene amplification, metastatic disease) and require more intensive therapy. However, even with current aggressive multimodal treatment, only 30% to 40% of such high-risk patients achieve long-term cure (3, 4). New approaches to neuroblastoma therapy are therefore required to improve survival rates for this disease.

The topoisomerase I inhibitors topotecan and irinotecan, and the anticancer DNA methylating agent temozolomide, are active against neuroblastoma and are currently used clinically in the refractory and relapsed setting. Phase II studies showed efficacy of topotecan in combination with cyclophosphamide (5) or with vincristine and doxorubicin (6), and of temozolomide as monotherapy (7). Topotecan alone or in combination with cyclophosphamide showed response rates up to 76% in previously untreated patients (8) and 64% in patients with refractory or relapsed neuroblastoma, in combination with vincristine and doxorubicin (TVD; ref. 6). This TVD regimen has now been added to the current European high-risk neuroblastoma trial for patients failing to achieve an initial clinical response with first-line therapy (HRNBL-1). Similarly, combining temozolomide and irinotecan has produced
**Translational Relevance**

The cure of children with high-risk neuroblastoma represents one of the major challenges in pediatric oncology, and there is a clear need for the development of improved therapeutic strategies. The DNA methylating agent temozolomide and the topoisomerase I poison topotecan show promising clinical activity against neuroblastoma. One approach to improve the efficacy of such conventional cytotoxic agents is to combine their use with chemosensitizing agents. Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant nuclear enzyme that facilitates the repair of DNA base damage and single-strand breaks, such as those caused by temozolomide and topotecan. In this article, we present data that underscores the efficacy of temozolomide and topotecan against neuroblastoma preclinical models. Moreover, we provide the first extensive evidence establishing that inhibition of PARP-1 (using the small-molecule inhibitor, AG014699; Pfizer, Inc.) markedly potentiates the activity of temozolomide and topotecan against this disease. We further show that AG014699 displays acceptable pharmacokinetic, pharmacodynamic, and toxicity characteristics in vivo neuroblastoma models. These findings establish PARP-1 as a druggable target in neuroblastoma and provide a strong basis for the development of clinical studies of AG014699 in neuroblastoma and other pediatric solid tumors commonly treated with temozolomide and topoisomerase I inhibitors.

responses in a notable proportion of patients with refractory or progressive neuroblastoma (9).

One approach to improve the efficacy of existing conventional cytotoxic agents is to combine their use with chemosensitizing agents. For cytotoxic agents that damage DNA, inhibitors of DNA repair can act as chemosensitizers. Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant nuclear enzyme that is activated by and facilitates the repair of DNA base damage and single-strand breaks (10). Through the poly(ADP-ribose)ylation of itself and of histones, PARP-1 loosens chromatin and recruits the other components of the base excision repair/single strand break repair pathway (11). DNA single-strand breaks resulting from removal of damaged bases following exposure to ionizing radiation or DNA methylating agents, as well as those induced by poisoning topoisomerase I-DNA cleavable complexes, require PARP-1 activity for efficient repair (12). PARP inhibitors of increasing potency have been synthesized to increase the efficacy of DNA-damaging chemotherapy and radiotherapy (12, 13). The potent PARP inhibitor AG014361 (K_{i}, 5 nmol/L) enhanced the cytotoxicity and antitumor activity of the DNA methylating agent, temozolomide and topoisomerase I poisons in in vitro and in vivo models of human colon cancer (14). Subsequent screening of structurally related compounds identified AG014447 as having superior potency (K_{i}, 1.4 nmol/L; ref. 15) and the phosphate salt of this compound, AG014699, was the first PARP inhibitor to enter clinical trial for the treatment of cancer. Phase I trials with AG014699 showed that a profound and sustained PARP inhibition can be achieved in tumors and surrogate normal tissues at tolerable doses after a single i.v. infusion (16). Phase II trials of the same combination in metastatic melanoma patients suggested an improvement in the response rate in comparison with historical data for temozolomide alone (17). At least four other pharmaceutical companies are now undertaking clinical trials of PARP inhibitors for the treatment of cancer (18); however, any efficacy for the therapy of pediatric tumors remains to be examined.

We hypothesized that the application of PARP inhibitors would sensitize neuroblastoma cells to the cytotoxic effects of topotecan or temozolomide. Here, we report the preclinical assessment of PARP inhibitors in in vitro and in vivo models of neuroblastoma. Our data show the clear potential of PARP inhibitors in combination with temozolomide or topotecan for the improved therapy of neuroblastoma and strongly support the evaluation of these drug combinations in early clinical trials in children.

**Materials and Methods**

**Materials.** Temozolomide was a gift from Cancer Research UK, London, United Kingdom; topotecan was from SmithKline Beecham Pharmaceuticals; and AG014699 (PO₄ salt of AG014447) was a gift from Pfizer, Inc. All drugs were dissolved in DMSO before addition to cell cultures at a final concentration of 0.5% (v/v) DMSO. For in vivo evaluation, drugs were dissolved in saline immediately before administration. Other chemicals and reagents were obtained from Sigma, unless otherwise stated.

**Cell lines and culture.** NB-1691, a MYCN-amplified, MDM2-amplified, chemoresistant cell line established from a neuroblastoma following chemotherapy (19), was a gift from Dr. Clinton Stewart (St Jude Children’s Research Hospital, Memphis, TN). SH-SY-5Y, a non–MYCN-amplified, p53 wild-type cell line of intermediate chemosensitivity, established following chemotherapy (20, 21) was obtained from Dr. June Biedler via Dr. Penney Lovat (Newcastle University, Newcastle upon Tyne, United Kingdom). SKNBE(2c), a MYCN-amplified, p53 mutant, multidrug-resistant cell line (20, 22, 23), was a gift from Dr. Barbara Spengler (Fordham University, New York, NY). Karyotypic analysis of all cell lines was done before the study to confirm their identity. All three neuroblastoma cancer cell lines were maintained using standard methods, in RPMI 1640 containing 10% FCS (Life Technologies) and were confirmed to be Mycoplasma-free.

**In vitro growth inhibition and cytotoxicity assays.** Cell growth inhibition was estimated in exponentially growing NB-1691, SH-SY-5Y, and SKNBE(2c) cells in 96-well plates. Seeding densities of $3 \times 10^5$, $3 \times 10^4$, and $1 \times 10^3$ cells, respectively, ensured exponential growth for the duration of the experiment. Twenty-four hours after seeding, cells were exposed to varying concentrations of topotecan or temozolomide (as shown in Fig. 1A and B) in the presence or absence of 0.4 μmol/L AG014699, a concentration previously shown to enhance temozolomide and topotecan cytotoxicity in adult tumor cell lines (14, 15, 24). After 6 d of culture (equivalent to approximately three cell divisions), the viability of these cells was quantified using an XTT cell proliferation kit assay (Roche), according to the manufacturer’s instructions. The concentration of topotecan or temozolomide alone or in combination with AG014699 that inhibited growth by 50% (GI₅₀) was calculated from computer-generated curves (GraphPad Software). Cell viability was expressed as a percentage in relation to the controls indicated (see Results). All data were derived from at least three independent experiments.

Clonogenic cell survival assays were done using NB-1691 cells. Exponentially growing cells ($4 \times 10^5$) were seeded in standard medium.
AG014699 in vivo.

**Establishment of NB-1691 and SH-SY-5Y tumor xenografts.** NB-1691 (1 × 10^7 exponentially growing cells per mouse, harvested, and implanted in growth medium) and SH-SY-5Y [1 × 10^7 cells per mouse, implanted in a 1:1 mix of growth factor reduced, basement membrane Matrigel matrix (BD Biosciences), and medium] xenografts were established by s.c. implantation into CD-1 nude mice. Before use in the experiments, xenograft establishment was defined as when two-dimensional caliper measurements of tumors reached ~5 × 5 mm.

**AG014699 pharmacokinetics and pharmacodynamics in tumor xenografts and plasma.** One or four daily doses of the PARP inhibitor AG014699 (1 mg/kg i.p.) were given to CD-1 nude mice bearing established NB-1691 xenografts. At 0.5, 2, 6, and 24 h after the initial or fourth daily dose of AG014699, three animals per time point were bled by cardiac puncture under general anesthesia, then killed. The xenografted tumors were removed, snap frozen in liquid nitrogen, and stored at -80°C before analysis. Plasma was derived from the blood samples using standard methods and stored at -80°C. Three untreated control animals were processed equivalently.

**AG014699 distribution in tumor xenograft and plasma samples.** Plasma and tumor concentrations of AG014447, the active form of the prodrug AG014699, were determined after protein precipitation with acetonitrile by liquid chromatography/mass spectrometry/mass spectrometry using a turbo ion spray interface and multiple reaction monitoring in the positive ion mode (API 4000, Applied Biosystems) and a deuterated internal standard. This method measures total AG014447, bound and free; the percentage protein bound was not determined and the metabolites of AG014447 were not detected. The lower limit of quantitation was 1 ng/mL. Plasma pharmacokinetics of AG014447 were analyzed using a noncompartmental approach in WinNonlin version 1.3.

**PARP-1 activity assays.** PARP activity was determined in exponentially growing NB-1691 and SH-SY-5Y cells following permeabilization, as well as in s.c. NB-1691 xenografts (see above), using a validated assay (25). Maximaly stimulated PARP activity was measured in replicate samples (n ≥ 3) of 5,000 permeabilized cells or 0.5 μg/mL protein (tumor samples). Inhibition of PARP activity in permeabilized cells was measured following treatment with a range of AG014699 concentrations (0.1, 0.4, and 1 μmol/L in DMSO), in comparison with DMSO-only controls. The mean PARP activity in xenograft samples taken at each time point (n = 3; see above) was expressed as a percentage of the mean PARP activity of control xenografts from untreated mice (n = 3). The SD was calculated for each time point using the following equation: (100 × T/C) × (√(C/T) + (t/T)), where C is the SD of control samples, T is the mean of control samples, t is the SD of treated samples, and T is the mean of treated samples.

**Tumor growth inhibition in vivo.** CD-1 nude mice bearing palpable, established s.c. NB-1691 or SH-SY-5Y xenografts were treated i.p. with normal saline (control animals) or AG014699 (at 1 mg/kg) alone, daily for 5 d (five mice per group). Temozolomide was administered orally daily ×5 at a dose of 68 mg/kg, which corresponds to the murine equivalent of the standard clinical dose of 200 mg/m² and schedule and is a dosing regimen we have used in our previous studies (14, 15). Initial dose-finding experiments established that administration of
topotecan orally daily $\times 5$ at a dose of 1 mg/kg in combination with AG014699 (1 mg/kg i.p.) was well tolerated (data not shown), and this dose was used in all subsequent experiments. For drug combinations, AG014699 was administered daily for 5 days immediately after administering the cytotoxic drug (temozolomide at 68 mg/kg or topotecan at 1 mg/kg, both orally). Tumor volumes, determined from two-dimensional caliper measurements and the equation $a^2 \times b/2$ (where $a$ is the length and $b$ is the width of the tumor), were monitored for 100 days posttreatment or until tumor dimensions reached 10 mm $\times$ 10 mm or until one dimension reached 15 mm (whichever occurred the earliest), and are presented for each group of mice as median relative tumor volume (RTV) values. We aimed to commence treatment at tumor volumes in the range of 50 to 150 mm$^2$; actual mean tumor volumes reached at dosing were 74.7 mm$^2$ (SH-SY-5Y xenografts; 95% confidence interval, 63.5-85.9 mm$^2$) and 111.4 mm$^2$ (NB-1691 xenografts; 95% confidence interval, 64.2-158.7 mm$^2$). A weighted randomization was used to ensure that a range of initial xenograft volumes was used in each treatment group. RTV1 is the tumor volume on the initial day of treatment (day 0) and RTV4 is the tumor volume four times that on the initial day of treatment. Calculation of RTV values is therefore a convenient means of pooling data from individual mice bearing tumors of different starting volumes that we have used in our previous studies (14, 15, 24). Tumor growth delay is defined as the time to RTV4 in drug-treated mice compared with the time to RTV4 in control (vehicle alone) mice. No relationships between initial xenograft volume and time taken to reach RTV4 were apparent (data not shown). Median tumor volume is shown rather than the mean, as this is generally accepted as the most statistically reliable representation of the average growth rate of tumors in a small group of mice, where a normal distribution of tumor volumes cannot be assumed. Tumor growth delay (TGD) was calculated as (median time to RTV4 in treated group - median time to RTV4 in control group). Percentage enhancement was calculated as [((TGD temozolomide + PARP inhibitor) - (TGD temozolomide alone)) / (TGD temozolomide alone)] $\times 100$. Complete tumor regression was defined as reduction of tumor to an undetectable size, when tumors regrew after a (prolonged) period the regression was classed as “temporary,” when no tumor could be detected at the end of the experiment, the regression was classed as “permanent.”

Results

AG014699 and PARP-1 inhibition in permeabilized neuroblastoma cells. AG014699 is a potent inhibitor of purified full-length human PARP-1 (15). To determine its effect on PARP-1 activity in neuroblastoma cells, we assayed the PARP-1 activity in permeabilized NB-1691 and SH-SY-5Y cells after treatment with AG014699. At concentrations of 0.1, 0.4, and 1 mM/L, AG014699 inhibited PARP-1 activity by 92%, 98%, and 98% in NB-1691 cells and by 83%, 97%, and 100% in SH-SY-5Y cells, respectively. All previous cellular chemosensitization studies with this class of PARP inhibitor, including AG014699, have utilized a concentration of 0.4 mM/L (14, 15, 24). Because this concentration produced almost total inhibition of the cellular enzyme and to allow comparison with previous data, we used a concentration of 0.4 mM/L AG014699 to investigate chemosensitization of temozolomide and topotecan in the neuroblastoma cell lines.

Potentiation of topotecan- and temozolomide-induced growth inhibition by AG014699. Temozolomide sensitivity depends on MGMT and MMR status and we have previously observed that the GI50 varies from <10 mM/L in SW620 cells (low MGMT, MMR functional) to 750 mM/L in LoVo cells (high MGMT, MMR dysfunctional; ref. 14). All three cell lines selected for study expressed equivalent levels of MGMT and the MMR proteins MLH1, MSH2, MSH3, MSH6, and PMS2 by Western blotting (see Supplementary Fig. S1). We measured the growth of cells exposed to increasing concentrations of temozolomide or topotecan alone or in combination with 0.4 mM/L AG014699 continuously, over a period of three cell doublings. Representative growth inhibition curves of NB-1691 cells are shown in Fig. 1A (topotecan) and B (temozolomide). Pooled IC50 data for topotecan and temozolomide with and without AG014699 from at least three independent experiments for each of the three cell lines are shown in Fig. 1C and D. AG014699 alone was not growth inhibitory at the concentration used (0.4 mM/L; data not shown).

Both topotecan and temozolomide alone caused concentration-dependent inhibition of growth in all three cell lines. All cell lines exhibited similar levels of sensitivity to topotecan (GI50, 3.5-5.54 mM/L) and temozolomide alone (GI50, 162-210 mM/L), which were in the range of sensitivities we have observed in adult cancer cell lines (14). AG014699 caused a significant sensitization of topotecan and temozolomide in all cell lines (all P $\leq$0.05, t test). We observed a 2- to 2.5-fold potentiation of topotecan consistent with our previous observations in studies of PARP inhibited and PARP-1-/- cells (14, 15, 26). There was a more variable 3- to 10-fold potentiation of temozolomide by AG014699, with the highest potentiation factor achieved for NB-1691, comparable with preclinical studies in adult malignancies (15).

To confirm that the growth inhibition data reflected enhanced cytotoxicity, we performed clonogenic survival assays in NB-1691 cells following a 24-hour exposure to topotecan or temozolomide in the presence or absence of 0.4 mM/L AG014699 (Fig. 2A and B). AG014699 caused a greater enhancement of temozolomide cytotoxicity (3.2-fold at LD50) compared with the enhancement of topotecan cytotoxicity (1.4-fold at LD50), reflecting the trend observed in the growth inhibition studies; however, there was a less marked potentiation of cell death than growth inhibition.

AG014699 levels in plasma and NB-1691 tumor xenografts. A dose of 1 mg/kg AG014699 daily $\times 5$ has previously been shown to be nontoxic and sufficient for profound chemosensitization of human colon cancer xenografts (15). Therefore, this dose was used to investigate the chemosensitization of neuroblastoma xenografts. Before undertaking these studies, we determined the distribution of the PARP inhibitor in the plasma and to tumor tissue following a single dose and four daily doses of 1 mg AG014699/kg i.p. to mice bearing NB-1691 s.c. xenografts. AG014699 is the phosphate salt of AG014447 and rapidly liberates the parent drug following injection. For this reason, we measured concentrations of AG014447 in NB-1691 xenografts and plasma samples (taken at 0.5, 2, 6, or 24 hours after dosing; three mice per time point) from mice that had been given either one or four daily doses of AG014699 (1 mg/kg i.p.). AG014447 was rapidly detected in mouse plasma (Fig. 3A), with maximal levels recorded at 30 minutes after the first dose (mean 192 nmol/L $\pm$ 13.8, 95% confidence interval) and after the fourth dose (mean 121 nmol/L $\pm$ 18.8, 95% confidence interval), after which there was a gradual elimination of AG014447 from plasma over time (Fig. 3A). Plasma concentrations of AG014447 after the first dose were always higher than after the fourth dose. AUIC0-24h values for the first and fourth doses were 57.9 and 20.3 mmol/L, respectively.
indicating an increase in clearance with repeated doses, although the half-life did not decrease (436 and 539 minutes, respectively).

AG014447 accumulated more slowly in the NB-1691 xenograft tissue, reaching a peak at 2 hours following administration. Peak concentrations detected in the tumor tissue were much higher (7-12 × 10^6 M) than those detected in the plasma. In contrast to the lower plasma concentrations after the fourth dose, concentrations of AG014447 in the tumor were similar to, or higher than, after the first dose (Fig. 3B). The concentration of AG014447 detected in the tumor tissue remained above that used for in vitro chemosensitization studies (0.4 μmol/L) for the entire dosing period.

PARP-1 inhibition in tumor xenografts by AG014699. The marked accumulation of AG014447 and prolonged retention within the tumor suggested that PARP-1 activity would be suppressed for the duration of the dosing period. This was confirmed by determining PARP-1 activity in the tumor homogenates used for AG014447 analysis in comparison with tumor homogenates from untreated mice (Fig. 3C). After the first dose, PARP-1 activity was suppressed by ∼ 50% for the first 6 hours but had recovered by 24 hours. After the fourth dose, there was a more profound and sustained inhibition of tumor PARP-1 activity, which was inhibited by at least 50% for 24 hours, with a 70% to 75% suppression for the first 2 hours, commensurate with the higher drug levels in these samples.

Combination of topotecan or temozolomide with AG014699 in human tumor xenografts. We examined the effect of AG014699 on the antitumor activity of temozolomide and topotecan in mice bearing established s.c. NB-1691 or SH-SY-5Y xenografts. Both xenografts grew rapidly, with time to RTV4 being 3 to 17 days for NB-1691 and 11 to 23 days for SH-SY-5Y xenografts. At 1 mg/kg daily × 5, AG014699 alone did not cause any marked toxicity or affect tumor growth compared with vehicle-only controls; however, a modest increase was observed in topotecan toxicity in NB-1691-bearing mice and temozolomide toxicity in SH-SY-5Y-bearing mice, when administered in combination with AG014699 (Table 1A and B). No treatment-related deaths or morbidity was observed.

Treatment of mice bearing NB-1691 xenografts with temozolomide alone (68 mg/kg, daily × 5) caused a transient regression followed by regrowth and an overall tumor growth delay of 19 days (Fig. 4A). Co-administration of AG014699 with temozolomide increased the tumor growth delay by >300% (to 59 days) and increased the number of complete regressions from 1 of 5 to 3 of 5, two of which persisted until the end of the experiment (Table 1A). Treatment of NB-1691 xenografts with topotecan alone (1 mg/kg, daily × 5) similarly resulted in transient regression followed by regrowth and an overall tumor growth delay of 10 days. The median tumor

![Fig. 2. Chemosensitization to topotecan and temozolomide by AG014699 reduces neuroblastoma cell line survival. A and B, clonogenic cell survival determined for NB-1691 cells following 24-h exposure to topotecan or temozolomide either alone (△) or with 0.4 μmol/L AG014699 (○). Data are shown normalized to 0.5% DMSO or 0.4 μmol/L AG014699 controls, respectively. Each data point represents the mean of three replicate determinations (± SD).](link)

![Fig. 3. AG014699 pharmacokinetics and PARP-1 inhibition in an in vivo model of neuroblastoma. The concentration of the AG014699 parent compound AG014447 is shown in (A) plasma or (B) NB-1691 xenograft homogenates from tumor-bearing mice, following a single (solid symbols and lines) or the last of four daily doses (open symbols/dotted lines) of AG014699 (1 mg/kg i.p.) and harvested at the indicated times after administration. Corresponding PARP-1 activity in the NB-1691 xenograft homogenates is shown in C, following a single (filled columns) or the last of four daily doses (open columns) of AG014699. Data are the mean (± SD) from three tumor-bearing mice per time point. Where data from 3 mice were available for a given time point, mean and actual data points are shown.](link)
growth delay was approximately doubled (to 22 days) by coadministration of AG014699 (Fig. 4B; Table 1A). There were two complete tumor regressions, one of which persisted for the duration of the experiment (100 days), in each of the groups of mice treated with either topotecan alone or topotecan + AG014699. Individual tumor growth curves for all mice in all treatment groups are shown in Supplementary Fig. S2.

SH-SY-5Y xenografts were more sensitive to topotecan and temozolomide than NB-1691 xenografts, and treatment of mice with topotecan or temozolomide alone resulted in tumor growth delays of 53 and 60 days, respectively, compared with controls (Fig. 4C and D). AG014699 increased the temozolomide-induced tumor growth delay by 50% (to >89 days) and the number of mice with complete and persistent tumor regressions (≥100 days) was increased from 2 of 5 to 4 of 5 (Table 1B). Co-administration of AG014699 with topotecan did not increase the median growth delay, but the total number of complete regressions was increased from 3 of 5 to 4 of 5 (Table 1B). Individual tumor growth curves for all mice in all treatment groups are shown in Supplementary Fig. S3.

Discussion

The cure of children with high-risk neuroblastoma remains a significant challenge in pediatric oncology. In this article, we have presented data that underscore the efficacy of temozolomide and topotecan against preclinical models of neuroblastoma. Moreover, we have provided, for the first time, extensive evidence that inhibition of the DNA repair enzyme PARP-1 markedly potentiates the activity of topotecan and temozolomide against neuroblastoma and have shown that such drug combinations have clear potential for the improved therapy of neuroblastoma.

In phase I and phase II studies in adult malignancies, the PARP-1 inhibitor AG014699 (Pfizer, Inc.) causes sustained inhibition of PARP-1 activity in tumors and tissues without significant safety concerns (in combination with temozolomide; refs. 16, 17) and was therefore selected for our studies in neuroblastoma. Coadministration of AG014699 produced clear and convincing evidence of enhancement of topotecan and temozolomide activity against preclinical models of neuroblastoma. In our in vitro studies, AG014699 (at 0.4 μmol/L) caused >95% inhibition of PARP-1 activity and consistently potentiated topotecan (2.2- to 2.5-fold) and temozolomide (3- to 10-fold) activity in growth inhibition assays, across multiple neuroblastoma cell lines, but did not cause growth inhibition when used alone. These levels of PARP-1 inhibition and degrees of chemopotentiation in vitro are consistent with results previously reported for AG014699 in in vivo models of adult cancers (15).

Our in vitro data are borne out by evidence of chemopotentiation of temozolomide efficacy by AG014699, in in vivo xenograft models of neuroblastoma (SHSY5Y and NB-1691 xenografts) treated with regimens that mimic the clinical setting. Temozolomide had significant activity against both xenograft models when administered alone, consistent with previous preclinical studies in neuroblastoma (27). SHSY5Y xenografts were much more sensitive than NB1691 xenografts to both temozolomide (71- versus 19-day growth delay) and topotecan (64- versus 18-day growth delay). Because SHSY5Y cells were less sensitive to topotecan and similarly sensitive to temozolomide compared with NB1691 cells in in vitro assays, the greater in vivo sensitivity is likely to be due to differences in host factors and the tumor microenvironment, such as vascularization and drug delivery. Because of the intrinsic chemosensitivity of SHSY5Y xenografts, and the 100-day maximum length for our in vivo experiments, there was limited

Table 1. Summary of in vivo efficacy and toxicity, following daily treatment of mice bearing NB-1691 (A) and SH-SY-SY (B) neuroblastoma xenografts for 5 d with either vehicle control alone, AG014699 alone (1 mg/kg), topotecan alone (1 mg/kg), topotecan (1 mg/kg) + AG014699 (1 mg/kg), temozolomide alone (68 mg/kg) or temozolomide (68 mg/kg) + AG014699 (1 mg/kg), based on observations of five animals per group over 100 d

<table>
<thead>
<tr>
<th></th>
<th>Median maximum weight loss as % of starting weight (day occurred)</th>
<th>Median time to RTV4 (d)</th>
<th>Tumor growth delay (d)</th>
<th>% Enhancement Total complete regressions</th>
<th>Temporary complete regressions</th>
<th>Persistent complete regressions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. NB-1691</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0 (1)</td>
<td>8.5</td>
<td>N/A</td>
<td>N/A</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>AG-014699</td>
<td>0 (1)</td>
<td>4.5</td>
<td>0</td>
<td>N/A</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Topotecan</td>
<td>2 (6)</td>
<td>18.3</td>
<td>10</td>
<td>N/A</td>
<td>2/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Topotecan + AG-014699</td>
<td>15 (8)</td>
<td>30</td>
<td>22</td>
<td>121</td>
<td>2/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>4 (6)</td>
<td>19.3</td>
<td>11</td>
<td>N/A</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Temozolomide + AG-014699</td>
<td>9 (10)</td>
<td>59.5</td>
<td>51</td>
<td>372</td>
<td>3/5</td>
<td>1/5</td>
</tr>
<tr>
<td>B. SH-SY-SY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6 (9)</td>
<td>11</td>
<td>N/A</td>
<td>N/A</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>AG-014699</td>
<td>4 (6)</td>
<td>18</td>
<td>0</td>
<td>N/A</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Topotecan</td>
<td>3 (2)</td>
<td>64</td>
<td>53</td>
<td>N/A</td>
<td>3/5</td>
<td>2/5</td>
</tr>
<tr>
<td>Topotecan + AG-014699</td>
<td>5 (6)</td>
<td>63</td>
<td>52</td>
<td>0</td>
<td>4/5</td>
<td>4/5</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>6 (9)</td>
<td>71</td>
<td>60</td>
<td>N/A</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Temozolomide + AG-014699</td>
<td>12 (6)</td>
<td>100</td>
<td>&gt;89</td>
<td>48.3</td>
<td>5/5</td>
<td>1/5</td>
</tr>
</tbody>
</table>
The assessment of topotecan/AG014699 combinations has not been reported previously in in vivo preclinical models. In our evaluations, administration of topotecan alone had significant activity against both neuroblastoma xenograft models tested, in line with previous data from adult preclinical models (15). Most notably, the temozolomide/AG014699 drug combination was commonly curative in neuroblastoma preclinical models; complete and sustained tumor regressions were observed in 40% and 80% of mice bearing NB-1691 and SHSY5Y xenografts, respectively.

One theoretical concern regarding the use of chemopotentiating agents such as PARP inhibitors, in combination with cytotoxic therapies, is the potential for more severe toxicity. No evidence of toxicity was observed when AG014699 was administered alone in vitro or in vivo. The temozolomide/AG014699 and topotecan/AG014699 combinations were well tolerated in our preclinical models of neuroblastoma. Consistent effects on toxicity were not evident across the doses of chemosensitization by AG014699 using this model. Nonetheless, evidence of marked chemosensitization was observed using the AG014699/temozolomide combination in both models tested, in line with previous data from adult preclinical models (15). Most notably, the temozolomide/AG014699 drug combination was commonly curative in neuroblastoma preclinical models; complete and sustained tumor regressions were observed in 40% and 80% of mice bearing NB-1691 and SHSY5Y xenografts, respectively.

Levels of potentiation observed for NB-1691 xenografts in vivo were in line with those produced using PARP inhibitors in combination with other drugs of the topoisomerase I poison class (i.e., irinotecan) in preclinical models of adult cancers (14). Consistent with the levels of potentiation we observed using the topotecan/AG014699 combination in vitro, the degree of enhancement against NB-1691 xenografts was more modest than that observed with temozolomide/AG014699 combinations, with an increased median tumor growth delay observed, but no evidence of further complete regressions. The future assessment of the activity of AG014699/topotecan combinations in extended groups of animals will aid the definition of the statistical significance of the observed data trends.

AG014699 displayed acceptable pharmacokinetic and pharmacodynamic profiles in our neuroblastoma models. Although the plasma concentrations of AG014447 achieved in vivo (maximal levels, ~0.2 μmol/L) were below the concentrations of AG014699 used for chemosensitization in vitro (0.4 μmol/L), there was a rapid accumulation in the tumors to higher concentrations than used in cell-based studies (1-2 μmol/L at maximal levels), which was accompanied by sustained inhibition of tumor PARP-1 activity. Inhibition of tumor PARP-1 activity in xenografts was less than expected from in vitro data, and from levels of AG014447 achieved in tumors in vivo, suggesting binding of AG014447 to plasma or stromal proteins. Nevertheless, the association between 50% inhibition of tumor PARP activity and marked chemosensitization is consistent with our observations in models of adult malignancies, where the combination with temozolomide was also curative (14). Interestingly, although there was evidence of increased clearance from the plasma after 4 days of dosing with AG014699 (compared with day 1), this was not associated with reduced accumulation in the tumor. In fact, there was a modest increase in tumor levels of AG014447, which was associated with a more marked and sustained PARP inhibition. No equivalent data in animal models of adult human cancer are available for comparison, but it is noted that there was a progressive inhibition of PARP activity with each successive dose in patients enrolled on the phase I trial of AG014699 in adults (16).

One theoretical concern regarding the use of chemopotentiating agents such as PARP inhibitors, in combination with cytotoxic therapies, is the potential for more severe toxicity. No evidence of toxicity was observed when AG014699 was administered alone in vitro or in vivo. The temozolomide/AG014699 and topotecan/AG014699 combinations were well tolerated in our preclinical models of neuroblastoma. Consistent effects on toxicity were not evident across the
different models tested; however, modest increases in topotecan toxicity (in NB-1691–bearing mice) and temozolomide toxicity (in SH-SY-5Y–bearing mice) were observed when administered in combination with AG014699. Previous studies that have assessed specific toxicity indices in preclinical models have suggested that, at least as far as myelotoxicity is concerned, enhanced toxicity does not occur as a result of the co-administration of the PARP inhibitor CEP-8983 (29). Moreover, in some cases, toxicity associated with the primary cytotoxicity may be reduced, as it has been reported that a combination of irinotecan and the oral PARP inhibitor GPI 15427 was able to reduce the gastrointestinal toxicity and increase the efficacy of irinotecan in colorectal cancer xenograft models (30). Toxicity data from phase I and II trials of AG014699 in combination with temozolomide in adult patients are similarly encouraging; a temozolomide dose of 200 mg/m² could be achieved in conjunction with a PARP-inhibitory dose of AG014699 (<20% PARP activity at 12 mg/m²/d AG014699) in its phase I study (16). At this dose, the AG014477 C_max was 600 ng/mL, approximately 10× the maximal level achieved in mice (~130 nmol/L) and approximately 3× the in vitro concentration (400nmol/L) used in the current study. Although modest temozolomide dose reductions were necessary in the recently reported phase II trial, increases in response rates compared with historical temozolomide-only controls were observed (17).

PARP-1 inhibitors seem to have potential for therapeutic application across the neuroblastoma clinical disease spectrum. Equivalent levels of temozolomide and topotecan chemosensitization by AG014699 were observed in all models tested, independent of their genotype (MYCN amplification and p53 pathway status) and reported chemoresistance (19–23). NB-1691, the chemoresistant MYCN- and MDM2-amplified neuroblastoma cell line investigated in the current study, has been reported to be chemosensitized in vitro to temozolomide and irinotecan by the PARP inhibitor CEP-8983, but in vivo investigations were not undertaken in this study (29). No evidence of mismatch repair or MGMT defects were apparent in any of our models, in line with findings in primary neuroblastomas (31–33). These data expand our findings in a panel of 12 human adult cancer cell lines with differing p53 status, where neither tissue of origin nor p53 status had any effect on the level of chemosensitization achieved using PARP inhibitors (34). Chemopotentiation of topotecan and temozolomide in p53 nonfunctional cell lines and tumor xenografts is of particular importance in neuroblastoma, in view of studies reporting that p53 inactivation is a frequently acquired feature of relapsed/refractory neuroblastomas associated with their chemoresistance (22, 23, 35), and offers a potential therapeutic strategy for these tumors.

Preclinical and early clinical studies of AG014699 should now be considered in other high-risk pediatric solid tumor types, in which temozolomide and topoisomerase I inhibitors show evidence of clinical efficacy, such as Ewing’s sarcoma, rhabdomyosarcoma, and medulloblastoma (36–41).

In summary, the combination of PARP-1 inhibitors with temozolomide has significant preclinical activity against neuroblastoma and evidence of enhanced efficacy when used in combination with topoisomerase I poisons (topotecan). Clinical data in adult cancer patients show that AG01699 is safe and nontoxic when used alone, causes a modest increase in temozolomide toxicity in combination, but is associated with an increased response rate to temozolomide. Coupled with its acceptable pharmacokinetic and pharmacodynamic profiles in in vivo models of neuroblastoma, these findings lead us to predict that clinical studies of AG014699 in neuroblastoma, and other pediatric solid tumors commonly treated with temozolomide and topoisomerase I inhibitors, will be equally encouraging.

Disclosure of Potential Conflicts of Interest


Acknowledgments

We thank Prof. Andy Pearson and Dr. Darren Hargrave for useful discussions regarding this work.

References

18. Ratnam K, Low JA. Current development of clinical


Inhibition of Poly(ADP-Ribose) Polymerase-1 Enhances Temozolomide and Topotecan Activity against Childhood Neuroblastoma


Updated version Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/15/4/1241

Supplementary Material Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2009/02/03/1078-0432.CCR-08-1095.DC1

Cited articles This article cites 39 articles, 25 of which you can access for free at: http://clincancerres.aacrjournals.org/content/15/4/1241.html#ref-list-1

Citing articles This article has been cited by 15 HighWire-hosted articles. Access the articles at: /content/15/4/1241.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.