Preclinical selection of a novel poly(ADP-ribose) polymerase inhibitor for clinical trial

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Abstract

Poly(ADP-ribose) polymerase (PARP)-1 (EC 2.4.2.30) is a nuclear enzyme that promotes the base excision repair of DNA breaks. Inhibition of PARP-1 enhances the efficacy of DNA alkylating agents, topoisomerase I poisons, and ionizing radiation. Our aim was to identify a PARP inhibitor for clinical trial from a panel of 42 potent PARP inhibitors (K_i, 1.4–15.1 nmol/L) based on the quinazolinone, benzimidazole, tricyclic benzimidazole, tricyclic indole, and tricyclic indole-1-one core structures. We evaluated chemosensitization of temozolomide and topotecan using LoVo and SW620 human colorectal cells; *in vitro* radiosensitization was measured using LoVo cells, and the enhancement of antitumor activity of temozolomide was evaluated in mice bearing SW620 xenografts. Excellent chemopotentiation and radiopotentiation were observed *in vitro*, with 17 of the compounds causing a greater temozolomide and topotecan sensitization than the benchmark inhibitor AG14361 and 10 compounds were more potent radiosensitizers than AG14361. In tumor-bearing mice, none of the compounds were toxic when given alone, and the antitumor activity of the PARP inhibitor-temozolomide combinations was unrelated to toxicity. Compounds that were more potent chemosensitizers *in vivo* than AG14361 were also more potent *in vitro*, validating *in vitro* assays as a prescreen. These studies have identified a compound, AG14447, as a PARP inhibitor with outstanding *in vivo* chemosensitization potency at tolerable doses, which is at least 10 times more potent than the initial lead, AG14361. The phosphate salt of AG14447 (AG014699), which has improved aqueous solubility, has been selected for clinical trial. [Mol Cancer Ther 2007;6(3):945–56]

Introduction

Many anticancer therapies act by causing breaks in DNA, which, if unrepaired, can lead to cell death. The ability of tumor cells to repair their DNA can therefore compromise the efficacy of such therapies. Poly(ADP-ribose) polymerase (PARP)-1 (PARP-1; EC 2.4.2.30) is a nuclear enzyme that promotes the repair of DNA breaks and has been reported to be elevated in human cancer (1, 2). PARP-1, the founder and best characterized member of a family of PARP enzymes, is activated by binding to DNA breaks to cleave NAD^+ to form long homopolymers of ADP-ribose attached to PARP-1 itself and/or histones, causing the relaxation of chromatin (3–5). PARP-1 is a component of the DNA base excision repair multiprotein complex, along with DNA ligase III, XRCC1, and DNA polymerase β (6, 7). Inhibition of PARP-1, and hence of DNA repair, enhances the efficacy of monofunctional DNA alkylating agents, such as temozolomide and topoisomerase I poisons, such as topotecan and irinotecan (8, 9).

Since the early benzamide inhibitors synthesized in the 1980s, PARP inhibitors of increasing potency have been developed, not only for use in cancer therapy (10) but also for other therapeutic applications (11). We have developed very potent and selective PARP inhibitors (e.g., AG14361), which, in combination with temozolomide, in a 5-day dosing schedule, caused the complete regression of SW620 human tumor xenografts (9). We have reported previously the chemical synthesis and limited biological studies with PARP inhibitors based on the quinazolinone, benzimidazole, tricyclic benzimidazole, tricyclic indole, and tricyclic indole-1-one core structures (12–15). We have also reported more extensive biological evaluation of selected inhibitors representative of the first three of these classes of inhibitor (9, 16). Many of the compounds we have developed are highly potent inhibitors of human recombinant PARP-1 with K_i (inhibition constant) values <5 nmol/L. However, the biological activity of such potent inhibitors can be influenced by a complex array of factors besides K_i, such as physical chemical properties, in intact cells. In the whole animal setting, a variety of host factors, such as pharmacokinetics, can also affect the drug activity.

The aim of the current investigation was to identify a compound for clinical use by using cell-based assays to study the biological properties of potent PARP inhibitors

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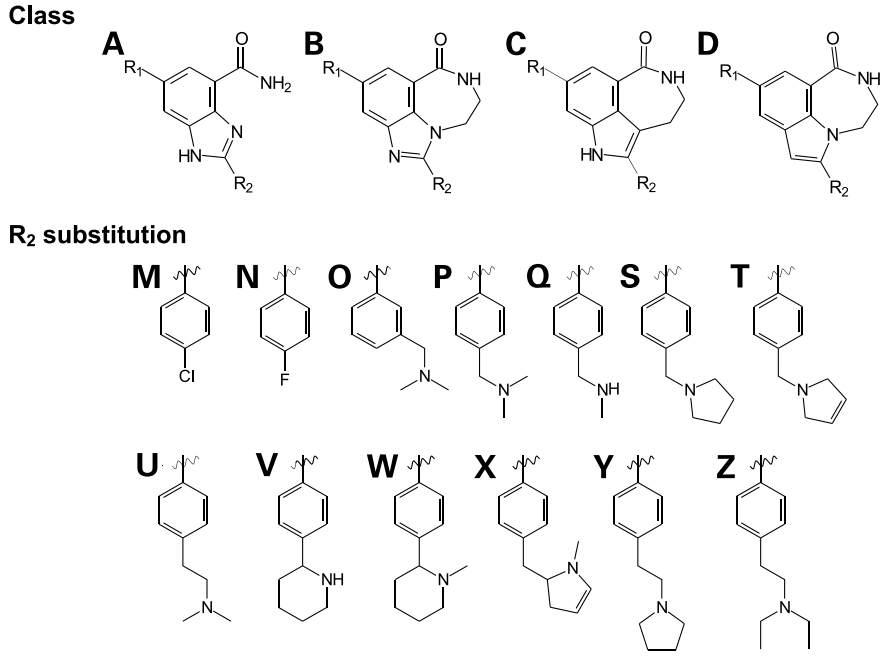


Figure 1. PARP inhibitor compound chemical structures. Compound names and corresponding core chemical structural classes (**A – D**), R_1 substituent (**H** or **F**), and R_2 substituent (**M – Z**; *bottom*).

Class A			Class B			Class C			Class D		
Compound	R_1	R_2	Compound	R_1	R_2	Compound	R_1	R_2	Compound	R_1	R_2
AG14032	H	M	AG14297	H	M	AG14328	H	M	AG14309	H	M
AG14340	H	N	AG14185	H	N	AG14167	H	N	AG14335	H	N
AG14318	H	O	AG14343	H	O	AG14155	H	O	AG14375	H	O
AG14238	H	P	AG14361	H	P	AG14154	H	P	AG14376	H	P
			AG14418	H	Q	AG14345	H	Q			
			AG14596	H	S	AG14344	H	S			
			AG14531	H	T	AG14621	H	T			
			AG14577	H	U						
			AG14584	H	V						
			AG14598	H	W						
			AG14663	H	X						
			AG14576	H	Y	AG14349	H	Y			
						AG14295	H	Z			
AG14385	F	P	AG14452	F	P	AG14644	F	P	AG14652	F	P
AG14386	F	Q	AG14450	F	Q	AG14447	F	Q			
AG14387	F	S	AG14597	F	S	AG14451	F	S			
AG14477	F	T	AG14521	F	T	AG14682	F	T			

from all of the above chemical classes and doing *in vivo* investigations on selected inhibitors. We investigated the relationships between the *in vitro* potency of the inhibitors; their ability to enhance the cell growth-inhibitory activity of the monofunctional alkylating agent, temozolomide, and the topoisomerase I poison, topotecan; their intrinsic growth-inhibitory potential; and their capacity to prevent recovery from potentially lethal ionizing radiation damage in growth arrested cells.

Evaluation of the *in vivo* anticancer efficacy of PARP inhibitor-cytotoxic agent combinations using the 5-day dosing schedule, as we reported previously, is labor intensive and requires significant quantities of compound. Using 10 potent inhibitors, selected on the basis of their ability to potentiate temozolomide *in vitro*, we found that a more ergonomic and economic single-dose administration protocol was a reliable and robust measure of the *in vivo* chemosensitization potency of these compounds, thereby accelerating the identification of the clinical candidate.

Materials and Methods

Materials

Temozolomide (gift from the Cancer Research Campaign UK), topotecan (Glaxo Smith Kline, Philadelphia, PA), and PARP inhibitors (Pfizer GRD, La Jolla, CA) shown in Fig. 1 were dissolved in DMSO to allow addition to cell cultures at a final DMSO concentration of 1% (v/v). [^{32}P]NAD^+ was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals and reagents were from Sigma (Poole, United Kingdom) unless otherwise stated. For

in vivo evaluation, drugs were prepared immediately before administration as follows: temozolomide was suspended in normal saline; PARP inhibitors AG14361, AG14418, AG14452, AG14477, AG14584, and AG14598 were prepared as HCl salts by dissolving in equimolar HCl in saline; MS-AG14531, MS-AG14521, MS-AG14644, and MS-AG14451 were prepared as the mesylate salt at synthesis and dissolved in saline; and AG14447 was prepared in 40% PEG300 in saline for the single-dose studies as the glucoronate salt for the 5-day studies. The mesylate salts were administered at 1.0 and 10.0 mg/kg of the salt, which is equivalent to 0.79 and 7.9 mg/kg free drug for AG14451 and AG14452 and 0.78 and 7.8 mg/kg for AG14531 and AG14644. AG14447 glucuronate salt was administered at a dose to give 0.1, 1.0, and 10 mg/kg free drug equivalent.

Cell Lines and Culture

LoVo and SW620 colorectal cancer cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 containing 10% (v/v) FCS. Cells were verified as mycoplasma-free (MycoAlert Cambrex Bioscience, Rockland, ME).

Calculation of Physical-Chemical Properties

The physical-chemical properties of the inhibitors [pKa (acid-dissociation constant) and ClogP (calculated logarithm of the octanol/water partition coefficient)] were calculated using Advanced Chemistry Development, Inc. (ACD/Labs, Toronto, Ontario, Canada)[3] software. The accuracy of the acid-base ionization constants (pKa values) calculated at 25°C and zero ionic strength in aqueous solutions is usually better than ± 0.2 pKa units except for very complex structures or poorly characterized substituents, where the accuracy is usually better than +0.5 pKa units. ClogD (calculated pH-dependent logarithm of distribution coefficient) is obtained from a ClogP and a calculated pKa and application of the appropriate Henderson-Hasselbach equation: $\log D = \log P - \log (1 + 10^{\text{pKa-pH}})$ using PhysChem Batch version 7.19 (ADC/Labs).

K_i Determination

We measured inhibition of human full-length recombinant PARP-1 by [^{32}P]NAD$^+$ incorporation as described previously (9, 17). The [^{32}P]ADP-ribose incorporated into acid insoluble material was quantified using a PhosphorImager (Molecular Dynamics, Amersham, Pharmacia Biotech). K_is were calculated by nonlinear regression analysis.

Chemopotentiation and Radiopotentiation *In vitro*

We estimated cell growth inhibition in exponentially growing LoVo and SW620 cells in 96-well plates exposed to increasing concentrations of single-agent PARP inhibitor, temozolomide or topotecan, or combinations of temozolomide or topotecan with PARP inhibitor for 5 days before staining with sulforhodamine B as described previously (18). Cell growth, determined after subtraction of time 0 values, was expressed as a percentage of the relevant DMSO, cytotoxic drug, or PARP inhibitor alone control, as appropriate. GI_{50} (concentration of drug that inhibited growth by 50%) values were calculated from the computer-generated curves (GraphPad Software, Inc. San Diego CA). The potentiation factor [PF_{50} (potentiation factor at GI_{50})] was calculated as GI_{50} of cytotoxic alone / GI_{50} of cytotoxic + PARP inhibitor. In an attempt to expedite the screening process, we exposed LoVo cells to 400 μmol/L temozolomide, the concentration of that inhibits cell growth by 20% (GI_{20}), in the presence or absence of 0.4 or 0.04 μmol/L PARP inhibitor. The enhancement factor (EF_{400}) was calculated as the growth of cells exposed to temozolomide alone / the growth of cells exposed to temozolomide + PARP inhibitor. In addition, we exposed cells to increasing concentrations of the PARP inhibition alone in the presence of a concentration of temozolomide (400 μmol/L) or topotecan (5 nmol/L) that alone had a modest effect (20% inhibition) on cell growth to determine the concentration of PARP inhibitor required to reduce cell growth by 50%, which is another indicator of the potency of inhibitor. Cell growth was expressed as a percentage of the relevant DMSO or temozolomide or topotecan alone control, as appropriate, and the data are expressed as GI_{50} + temozolomide or GI_{50} + topotecan. Potentially lethal damage (PLD) recovery (PLDR) was measured in confluent G_0-G_1–arrested LoVo cells (confirmed by flow cytometry; data not shown), exposed to 8 Gy γ-irradiation (Gammacel 1000 Elite, Nordion International, Inc. Kanata, Ontario, Canada) and seeded for colony formation immediately or maintained as confluent cultures for a 24-h recovery period before seeding for colony formation. Where indicated, PARP inhibitors (0.4 μmol/L) were added 30 min before irradiation and were present in the recovery incubation.

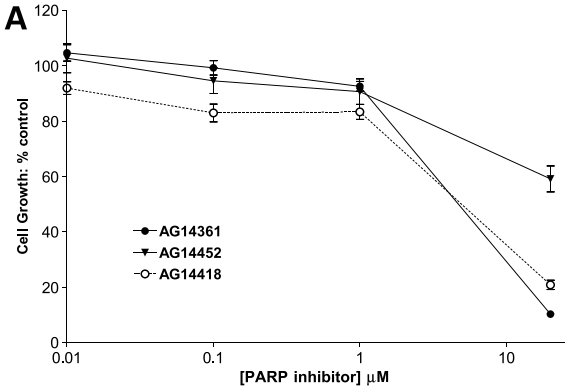
Inhibition of recovery is calculated for each individual experiment as 100 − 100 × (PLDR in PARP inhibitor–treated cells / PLDR in control) where PLDR was calculated for control cells and PARP inhibitor–treated cells as (survival at 24-h − survival at time 0) / survival at time 0 where survival is 100 × (number of colonies counted / number of cells seeded).

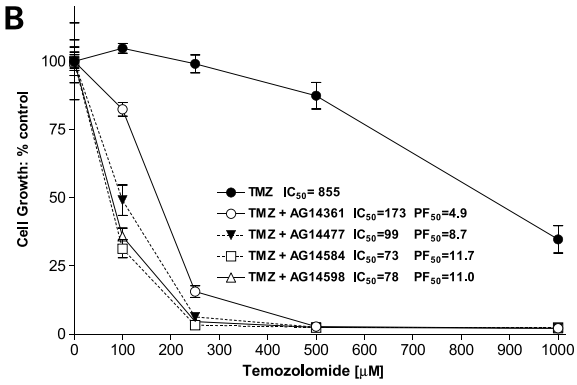
In all cell-based assays, each compound was assayed at least thrice for its intrinsic growth inhibition (GI_{50}), chemosensitization (PF_{50}, GI_{50} + temozolomide, GI_{50} + topotecan) activity, and radiosensitization (PLDR).

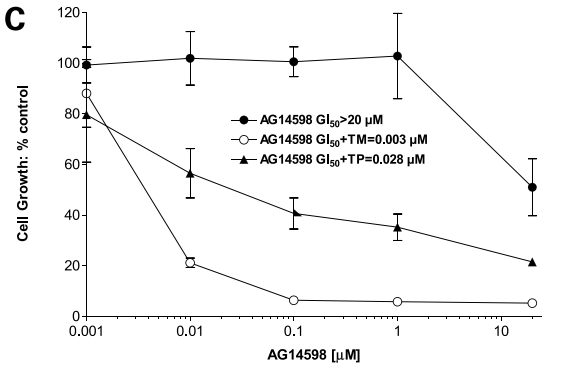
Determination of Antitumor Activity *In vivo*

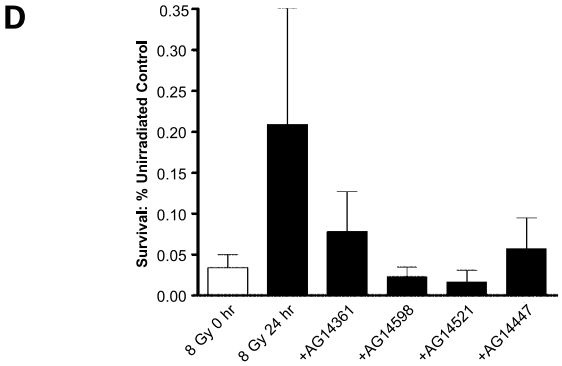
All of the *in vivo* experiments were reviewed and approved by the relevant Institutional Animal Welfare Committees and done according to national law. Female athymic nude mice (CD1 *nu/nu*, Charles River, Margate, United Kingdom) used for antitumor studies were maintained and handled in isolators under specific pathogen-free conditions. We implanted SW620 colorectal tumor cells (1×10^7 cells per animal) s.c. into one flank of each mouse, treated the mice (five animals per group) when tumors were palpable (10–12 days after implantation), and monitored tumor growth using two-dimensional caliper measurements. Tumor volume was calculated using the equation $a^2 \times b$ / 2, where a is the smallest measurement

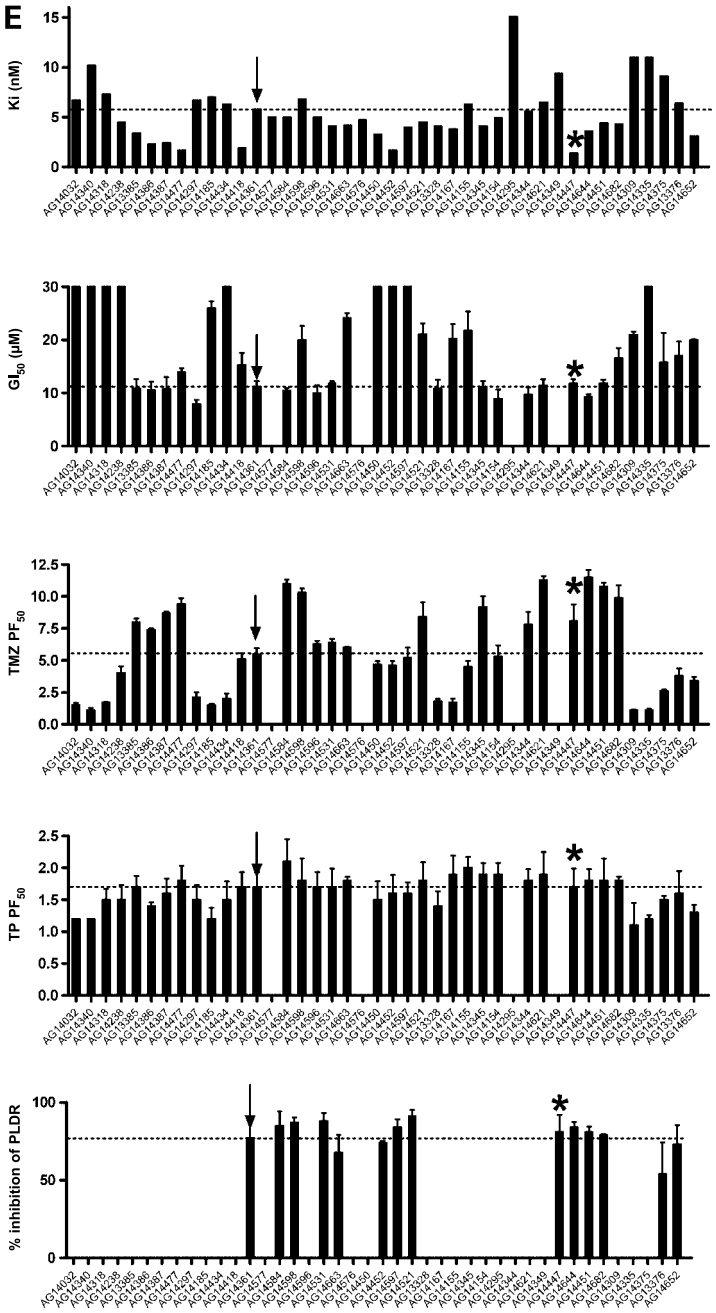
[3] http://www.acdlabs.com/











and b is the largest. Data are presented as median relative tumor volumes (RTV), defined as the calculated tumor volume divided by the calculated tumor volume on the initial day of treatment (day 0). Thus, on day 0, the RTV value is 1 and RTV4 is when the tumor is four times as large as its initial value.

Single-Dose Studies. We administered a single dose of temozolomide p.o. as a suspension in saline at 200 mg/kg either alone or in combination with a single i.p. administration of PARP inhibitor administered at 0.1 [AG14447 and MS-AG14644 (equivalent to 0.078 mg/kg free AG14644 only)], 1.0, and 10 mg/kg (for the mesylate salts equivalent to 0.79 and 7.9 mg/kg free AG14451 and AG14452 and 0.78 and 7.8 free AG14531 and AG14644). Control animals were treated with either normal saline p.o. and i.p or normal saline p.o and PARP inhibitor 10 mg/kg i.p.

Five Daily Dosing Studies. We treated animals with five daily doses of temozolomide administered p.o. as a suspension in saline at 68 mg/kg either alone or in combination with a five daily i.p. administrations of PARP inhibitor at 0.05, 0.15, and 0.5 mg/kg AG14447; 0.15 and 0.5 mg/kg MS-AG14644 (equivalent to 0.12 and 0.39 mg/kg free AG14644); 1.5, 5, and 15 mg/kg AG14361; and 5 mg/kg AG14452. Control animals were treated with either normal saline p.o. and i.p. or normal saline p.o and PARP inhibitor at the higher dose (0.5, 5, or 15 mg/kg, depending on the compound studied) i.p.

$$\text{Tumour growth delay (TGD)} = \text{Time to RTV4 in treated group} - \text{time to RTV4 control}$$

$$\%\ \text{Enhancement} = 100 \times \frac{\text{TGD temozolomide} + \text{PARP inhibitor} - \text{TGD temozolomide alone}}{\text{TGD temozolomide alone}}$$

Tissue Distribution

We administered AG14361, AG14452, or AG14447 (10 mg/kg i.p.) to mice (three animals per group) bearing SW620 xenografts (~10 × 10 mm). After 120 min, the animals were bled by cardiac puncture, under general anesthesia, the tumor was removed and snap frozen on liquid nitrogen. Plasma was removed and stored at −20°C. The concentrations of PARP inhibitor in acetonitrile-treated plasma and homogenized tumor were measured using reverse-phase high-pressure liquid chromatography (isocratic mobile phase: 40% acetonitrile in 0.1% ammonium formate, Hypersil BDS 3 μm 4.6 × 250 mm column, Waters Alliance 2690 high-pressure liquid chromatography; Waters, Elstree, Herts, United Kingdom) by the method of addition (19).

Statistical Analysis

In vitro correlations were analyzed by linear regression and Spearman's rank correlation analysis (significance values are given as less than the higher of the two values) and *in vivo* data were analyzed by Mann-Whitney using Graphpad Prism software.

Results

Structure-Activity Relationships *In vitro*

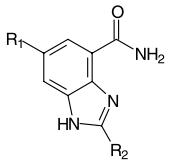
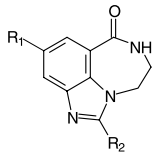
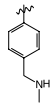
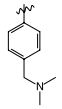
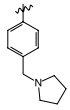
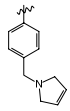
The compounds selected for biological investigation were all very potent inhibitors of PARP-1 with K_i values in the range 1.4 to 15.1 nmol/L (Supplementary Table S1, column 8)[4] and, with such a narrow range of potencies, clear structure-activity relationship based solely on K_i values was difficult to define. Nevertheless, for all the series (A–D), there was a clear trend of enhanced potency with analogues that contain a basic amine. As a general trend, nonbasic and less basic analogues (calculated pKa, <4.5) with high lipophilicity (ClogD, >2.0) were less active in the PARP inhibition and cellular assays. In each of the series, the analogues with basic amines seemed to be generally more active in all of the assays (K_i, PF_{50}, and GI_{50}). As noted in the published cocrystal structures, the flexible ASP-766 residue may participate in hydrogen bonding with the basic amine functionality and this may contribute to the enhanced potency of the amino analogues.

Relationship of Cellular Activity to K_i and Physical-Chemical Properties

We investigated the cytostatic and chemopotentiation effects of the compounds in LoVo cells and SW620 cells. Topotecan chemosensitization was investigated in both LoVo and SW620 cells. Potentiation of temozolomide was investigated in LoVo cells only because our previous studies had shown that it was not possible to enhance temozolomide cytotoxicity in SW620 cells *in vitro* by PARP inhibition (9). Examples of representative growth inhibition

Figure 2. *In vitro* chemosensitization and radiosensitization. **A,** growth inhibition determined in LoVo cells after 5-d exposure to increasing concentrations of PARP inhibitor alone: AG14361 (●), AG14418 (○), and AG14452 (▼). Cell growth as a percentage control was calculated as 100 × (OD day 5 of treated sample − OD time 0) / (OD day 5 of control − OD time 0). *Points,* mean of six replicates in a single representative assay; *bars,* SD. **B,** growth inhibition determined in LoVo cells after 5-d exposure to increasing concentrations of temozolomide alone (●) or in combination with 0.4 μmol/L AG14361 (○), AG14477 (▼), AG14584 (□), or AG14598 (△). *Points,* mean of six replicates in a single representative assay; *bars,* SD. **C,** growth inhibition determined in LoVo cells after 5-d exposure to increasing concentrations of AG14598 alone (●) or in combination with 400 μmol/L temozolomide (○) or 5 nmol/L topotecan (▲). *Points,* mean of six replicates in a single representative assay; *bars,* SD. **D,** recovery from 8 Gy γ-irradiation – induced PLD. Cells were seeded immediately (*white column*) or after a 24-h recovery in control medium (*black column*) or medium containing 4 μmol/L PARP inhibitor (*black columns*, compound number indicated on *X* axis label). *Columns,* mean of three independent experiments; *bars,* SD. **E,** pooled data for all compounds: K_i against purified recombinant human PARP and GI_{50}, temozolomide PF_{50}, and topotecan PF_{50} determined in LoVo cells. *Arrow* and *horizontal dotted line,* benchmark compound AG14361 and its level of activity. *, AG14447, the compound selected for clinical trial. *Columns,* mean of at least three independent experiments.

Table 1. Structure-activity relationships *in vitro*

Core structure								
R_2 substituent	R_1 = H		R_1 = F		R_1 = H		R_1 = F	
	GI_{50}, μmol/L	TMZ PF_{50}	GI_{50}, μmol/L	TMZ PF_{50}	GI_{50}, μmol/L	TMZ PF_{50}	GI_{50}, μmol/L	TMZ PF_{50}
			10.6 ± 2.7	7.4 ± 0.2	15.3 ± 3.9	5.1 ± 0.8	>20	4.7 ± 0.4
	41.3 ± 4.6	4 ± 0.9	10.9 ± 3	8 ± 0.5	11.2 ± 1.8	5.5 ± 0.8	>20	4.6 ± 0.6
			10.8 ± 3.9	8.7 ± 0.2	10 ± 2.6	6.3 ± 2.8	>20	5.2 ± 1.4
			13.9 ± 1.3	9.4 ± 0.8	11.8 ± 0.7	6.4 ± 3.5	21.1 ± 3.4	8.4 ± 2

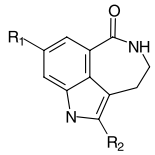
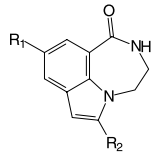
curves (used to calculate GI_{50}) obtained from LoVo exposed to PARP inhibitor alone are shown in Fig. 2A, and examples of representative potentiation curves of temozolomide by a fixed concentration (0.4 μmol/L) of PARP inhibitor (used to calculate temozolomide PF_{50}) are given in Fig. 2B. A PARP inhibitor concentration of 0.4 μmol/L was selected for chemopotentiation and radiopotentiation studies because at this concentration none of the compounds caused significant growth inhibition or cytotoxicity. The effect of increasing concentrations of PARP inhibitor alone and in combination with a modestly inhibitory concentration of temozolomide or topotecan (to calculate GI_{50} + temozolomide and GI_{50} + topotecan) is shown in Fig. 2C. K_i values and calculated LoVo growth inhibition data for all compounds (pooled from at least three independent experiments) are given in bar chart format in Fig. 2E. Pooled growth inhibition and chemopotentiation data in both cell lines are given in Supplementary Table S2.[4]

In general, the cytostatic effects of the compounds alone were similar in both cell lines (Supplementary Table S2,[4] columns 3 and 11) with GI_{50} values in the range 8 to 75 μmol/L (LoVo) and 7 to 24 μmol/L (SW620) and there was a significant relationship between the GI_{50} values in SW620 cells and those in LoVo cells ($P < 0.002$). All compounds, at concentrations that were >15 times lower than the GI_{50}, caused >3-fold potentiation of temozolomide. In LoVo cells, but not SW620 cells, the potency of the inhibitors against the isolated enzyme (K_i) was related to their intrinsic growth-inhibitory GI_{50} activity ($P < 0.05$). In LoVo cells, the intrinsic growth-inhibitory (GI_{50}) activity of the compound was related to the potentiation of temozolomide (temozolomide PF_{50}) and, to a lesser extent, topotecan (topotecan PF_{50}; $P < 0.0003$ and $P < 0.014$, respectively), which may reflect cellular accumulation or an effect on other cellular targets important for proliferation.

Because the inhibitors identified by K_i determinations were all very potent, structure-activity relationships were rather subtle (Table 1; Supplementary Tables S1 and S2).[4] Nevertheless, in all the series, analogues containing a basic amino functionality effected the highest level of chemosensitization (temozolomide PF_{50} values) in LoVo cells. A similar activity correlation was seen between basicity and growth inhibition GI_{50}. Excellent temozolomide chemopotentiation was observed with a large number of the compounds, with 16 of the compounds causing a greater sensitization than the benchmark inhibitor AG14361 (i.e., a temozolomide PF_{50} value of at least 5.5 and a topotecan PF_{50} of at least 1.7 in LoVo cells and a topotecan PF_{50} of at

[4] Supplementary material for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Table 1. Structure-activity relationships *in vitro* (Cont'd)

							
R_1 = H		R_1 = F		R_1 = H		R_1 = F	
GI_{50}, μmol/L	TMZ PF_{50}	GI_{50}, μmol/L	TMZ PF_{50}	GI_{50}, μmol/L	TMZ PF_{50}	GI_{50}, μmol/L	TMZ PF_{50}
11.1 ± 2	9.2 ± 1.4	11.8 ± 1.4	8.1 ± 2.2				
8.9 ± 3	5.3 ± 1.5	9.3 ± 0.9	11.5 ± 1.0	17 ± 4.7	3.8 ± 0.8	20 ± 0.3	3.4 ± 0.5
9.7 ± 2.3	7.8 ± 1.7	11.8 ± 1.2	10.8 ± 0.5				
11.4 ± 2.0	11.3 ± 0.5	16.6 ± 3.2	9.9 ± 1.7				

least 1.6 in SW620 cells), 5 of which caused >10-fold potentiation of temozolomide (AG14451, AG14584, AG14598, AG14621, and AG14644). PARP inhibitors enhanced the growth-inhibitory activity of topotecan in both LoVo and SW620 cells. Potentiation of topotecan was generally ≤2-fold in the LoVo cells and 2 ± 0.3–fold in the SW620 cells.

Overall, the potency of the inhibitors in a cell-free system (K_i) was significantly related to their ability to enhance temozolomide-induced growth inhibition (temozolomide PF_{50}; $P < 0.005$), confirming the strong association between PARP inhibitory potency and chemosensitization activity, and this correlation was highest in the benzimidazole series. In LoVo cells, there was a significant correlation of topotecan potentiation with temozolomide potentiation ($P < 0.0001$). In the benzimidazole series, there was also a correlation between K_i and GI_{50} values ($P < 0.025$). However, the other series (B, C, and D) did not show an obvious correlation between K_i value and cellular activity. In general the tricyclic indoles (C) were more potent chemosensitizers (temozolomide PF_{50}, 5.3–11.5) than the tricyclic benzimidazoles (B; temozolomide PF_{50}, 4.6–8.4) and the 4-pyrrolidin-1ylmethylphenyl and 2,5-dihydropyrrol-1ylmethylphenyl substitution at the R_2 position improved activity (Table 1). There was no consistent effect of a fluorine substitution at the R_1 position on activity, but in general, these compounds were marginally less growth inhibitory. Correlations and trends of the experiments in SW620 cells were not obvious and this may be due to the narrow range of experimental values. The analysis of the composite profiles suggests that, within these series, compounds with optimum PARP inhibitory and cellular activity have the following properties: molecular weight, 290 to 364; calculated pKa, 8.5 to 10; and ClogD, 0.0 to 2.0.

The cellular potency of the compounds was also shown by determining the concentration of PARP inhibitor needed to cause a 50% inhibition of the growth of cells treated with the temozolomide GI_{20} or topotecan GI_{20} (Fig. 2C; Supplementary Table S2).[4] In LoVo cells, there was a strong negative correlation of the temozolomide PF_{50} values with both GI_{50} + temozolomide and GI_{50} + topotecan and between topotecan PF_{50} and GI_{50} + topotecan ($P < 0.008$, $P = 0.0001$, and $P < 0.005$, respectively), that is the compounds that had higher PF_{50} values were able to enhance cytotoxic drug-induced growth inhibition at lower concentrations, as expected. In general, the PARP inhibitors that were the most potent chemosensitizers in LoVo cells were also the most potent in SW620 cells. However, overall, there was no significant correlation, possibly because all the inhibitors had similar chemosensitization activity in both cell lines (<2-fold difference between the most potent and

the least potent: PF_{50}, 1.4–2.3). However, some compounds (e.g., AG14477, AG14584, and AG14598) were most potent enhancers of temozolomide- and topotecan-induced cell growth inhibition in both cell lines.

In an effort to expedite the *in vitro* screening process, we adapted an initial screen in LoVo cells where the ratio of cell growth after exposure to 400 μmol/L temozolomide (GI_{20}) alone to the growth after exposure to temozolomide in combination with 0.4 or 0.04 μmol/L PARP inhibitor gives the enhancement factor (EF_{400}). We determined the EF_{400} value of 19 of the PARP inhibitors (Supplementary Table S2, columns 4 and 5)[4]; these values correlated very strongly with both the temozolomide PF_{50} ($P < 0.0001$) and the topotecan PF_{50} ($P < 0.001$) such that a further four compounds that had EF_{400} values <3 (AG14577, AG14576, AG14295, and AG14349) were not subject to further evaluation.

Radiosensitization by Selected Compounds

We also investigated radiosensitization by 14 of the PARP inhibitors in LoVo cells. A major factor contributing to radiation resistance *in vivo* is the ability of quiescent cells to repair PLD (20, 21). *In vitro* models of PLDR measure the increase in survival of irradiated, growth-arrested cells following delayed plating for colony formation. We investigated the ability of the PARP inhibitors to inhibit PLDR in G_0-G_1–arrested LoVo cells. Cell survival was increased 6.3 ± 4.9–fold (nine independent experiments) following a 24-h recovery in control medium. Incubation with PARP inhibitor during the recovery period inhibited PLDR by between 54% and 91%. Survival data are given in Fig. 2D for a limited number of compounds and percentage inhibition of PLDR for all compounds evaluated are given in Fig. 2E and Supplementary Table S2.[4] With the exception of AG14376, which was less potent than the other compounds evaluated, there was no significant difference in the ability of the PARP inhibitors to prevent PLDR.

In vivo Evaluation of the Inhibitors

To minimize the number of *in vivo* studies carried out, we selected 11 PARP inhibitors for *in vivo* evaluation of antitumor chemosensitization, based on their *in vitro* potency and chemical structure. From the 16 compounds that had equivalent or greater potency than that of the lead compound AG14361, we selected the four most potent

Table 2. *In vivo* toxicity and efficacy data

Treatment	Nadir body weight (% day 0)	Toxicity	Median time to RTV4 (range), days	Tumor growth delay	% Enhancement
Saline control	97	0/45	8 (6.5–11)		
TMZ	96	0/45	24 (22–27)	16	
TMZ + AG14477 (1 mg/kg)	99	0/5	30 (26–32)	22	37.5 ns
TMZ + AG14477 (10 mg/kg)	95	0/5	31 (25–80)	23	43.8 na
TMZ + AG14418 (1 mg/kg)	98	0/5	25 (15–33)	17	6.3 ns
TMZ + AG14418 (10 mg/kg)	95	0/5	32 (25–36)	24	50
TMZ + AG14361 (1 mg/kg)	96	0/20	32 (27–34)	24	50
TMZ + AG14361 (10 mg/kg)	94	3/45	36 (35–38.5)	28	75
TMZ + AG14584 (1 mg/kg)	91	0/5	34 (20–40)	26	62.5 ns
TMZ + AG14584 (10 mg/kg)	87	2/5	21 (21–25)	13	−18.8 ns
TMZ + AG14598 (1 mg/kg)	95	0/5	35 (29–39)	27	68.8
TMZ + AG14598 (10 mg/kg)	96	0/5	38 (36–42)	30	87.5
TMZ + MS AG14531 (1 mg/kg)*	98	0/5	33 (31–34)	25	56.3
TMZ + MS AG14531 (10 mg/kg)†	95	1/5	33.5 (33–40)	25.5	59.4
TMZ + AG14452 (1 mg/kg)	94	0/5	30 (24–100)	22	37.5
TMZ + AG14452 (10 mg/kg)	90	0/5	34 (31–53)	26	62.5
TMZ + MS AG14521 (1 mg/kg)‡	97	0/5	36 (35–36)	28	75
TMZ + MS AG14521 (10 mg/kg)§	90	1/5	41 (36–49)	33	106.3
TMZ + MS AG14644 (1 mg/kg)*	91	2/5	33 (32–33)	25	56.3
TMZ + MS AG14644 (10 mg/kg)†	83	5/5	n/a		
TMZ + MS AG14451 (1 mg/kg)‡	96	0/5	30 (29–46)	22	37.5
TMZ + MS AG14451 (10 mg/kg)§	90	1/5	37 (36–38)	29	81.3
TMZ + MS AG14447 (0.1 mg/kg)	96	0/5	32 (26–39)	24	50
TMZ + MS AG14447 (1 mg/kg)	83	1/10	41 (30–45)	33	106.3
TMZ + MS AG14447 (10 mg/kg)	83	5/5	n/a		

NOTE: Temozolomide (200 mg/kg i.p.).

Abbreviations: TMZ, temozolomide; ns, not significant [All other % Enhancement values represent significant ($P > 0.05$) enhancement of temozolomide-induced tumour growth delay]; n/a, not analyzed (all mice died prior to RTV4).

*Mesylate salt dose equivalent parent compound = 0.78 mg/kg.

†Mesylate salt dose equivalent parent compound = 7.8 mg/kg.

‡Mesylate salt dose equivalent parent compound = 0.79 mg/kg.

§Mesylate salt dose equivalent parent compound = 7.9 mg/kg.

(AG14644, AG14584, AG14451, and AG14598). In addition, AG14477 was chosen as the most potent benzimidazole, and AG14521 and AG14531 were selected as analogues of AG14361 (fluorinated and nonfluorinated, respectively) where the amine at the R_2 position has been incorporated into a dihydropyrrole ring. We also selected AG14521, as the best compound in the EF_{400} (0.4) studies, and AG14447, as the most potent PARP inhibitor in enzyme assays (K_i, 1.4 nmol/L), and a possible *N*-demethylation metabolite of AG14644. Two compounds that failed to meet the *in vitro* potency criteria, AG14452 and AG14418, were also tested because their structural similarity to AG14361 warranted further investigation. AG14452 was selected as the fluorinated analogue of AG14361and second most potent compound based on the K_i value for PARP.

Single-Dose Studies: Toxicity

None of the 11 PARP inhibitors were found to be toxic as single agents at any of the doses used. However, when 10 mg/kg PARP inhibitor was administered in combination with temozolomide, body weight loss was observed at days 4 to 13 posttreatment with the nadir body weight ranging from 83% to 96% of the starting weight (Table 2). MS AG14644, AG14447, and AG14584 significantly ($P < 0.05$) increased temozolomide toxicity. At a dose of 1 mg/kg, these three compounds also significantly increased temozolomide-induced body weight loss ($P < 0.05$). Nadir body weights ranged from 90% to 99% of starting weight at days 3 to 8 posttreatment.

Single-Dose Studies: Efficacy

Temozolomide alone caused a 16-day delay (time to RTV4) in tumor growth, which was extended by up to 33 days by coadministration of 1 mg/kg PARP inhibitor. Examples of studies with individual compounds are shown in Fig. 3A and data on all compounds are shown in Table 2. Eight of the 11 compound tested caused a significant enhancement of activity (Table 2). The rank order of potency was AG14447*** > MS AG14521** > AG14598** > AG14584(n/s) > MS AG14531** = MS AG14644* > AG14361*** > MS AG14451** = AG14452* = AG14477(n/s) > AG14418(n/s) (***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$; n/s, not significantly different from temozolomide alone). At the 10 mg/kg dose level, three of the inhibitors, MS AG14644, AG14447, and AG14584, were too toxic in combination with temozolomide to give meaningful efficacy data. However, all of the remaining eight inhibitors caused a significant increase in the efficacy of temozolomide in a similar rank order of potency as seen at the lower dose: MS AG14521*** > AG14598*** > MS AG14451** > AG14361*** > AG14452** > MS AG14531** > AG 14418* > AG 14477* > AG 14584(n/s). There was no significant correlation between the enhancement of temozolomide efficacy and the toxicity of the combination. In fact, with the exception of AG14447, the most toxic combination was with MS AG14644, which was demonstrably less active than other compounds (e.g., AG14598) that caused negligible body weight loss.

Interestingly, compounds that were more potent than AG14361 at 1 mg/kg *in vivo* (56–106% enhancement for AG14584, AG14598, AG14531, AG14521, AG14644, and

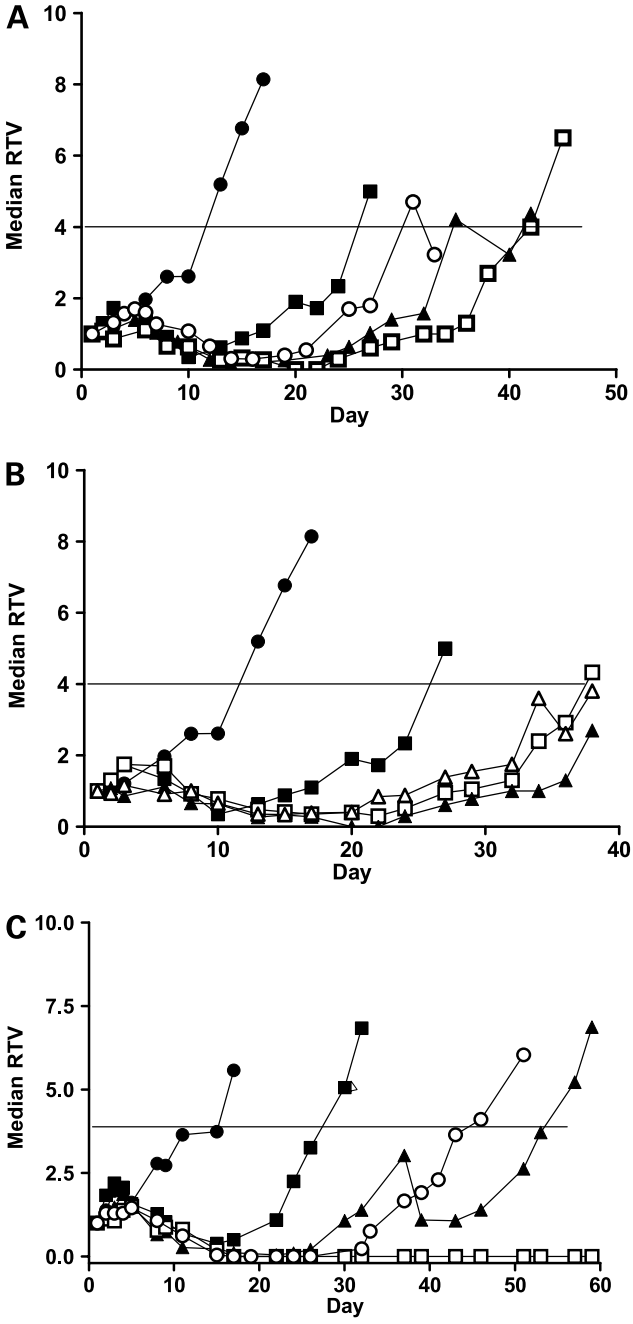


Figure 3. *In vivo* chemosensitization. **A,** single-dose study: growth of SW620 tumor xenografts (median RTV) following a single dose of vehicle alone (●), 200 mg/kg temozolomide (■), 200 mg/kg temozolomide + 1 mg/kg AG14361 (▲), 200 mg/kg temozolomide + 1 mg/kg AG14447 (□), or 200 mg/kg temozolomide + 1 mg/kg AG14452 (○). *Horizontal line,* RTV4. **B,** single-dose study: growth of SW620 tumor xenografts (median RTV) following a single dose of vehicle alone (●), 200 mg/kg temozolomide (■), 200 mg/kg temozolomide + 10 mg/kg AG14361 (□), 200 mg/kg temozolomide + 1 mg/kg AG14447 (▲), or 200 mg/kg temozolomide + 0.1 mg/kg AG14447 (△). *Horizontal line,* RTV4. **C,** 5-d schedule: growth of SW620 tumor xenografts (median RTV) following daily treatment for 5 d with vehicle alone (●), 68 mg/kg temozolomide (■), 68 mg/kg temozolomide + 1.5 mg/kg AG14361 (▲), 68 mg/kg temozolomide + 0.15 mg/kg AG14447 (□), or 200 mg/kg temozolomide + 5 mg/kg AG14452 (○). *Horizontal line,* RTV4.

Table 3. ***In vivo*** **toxicity and antitumor activity: 5-d schedule**

Treatment	Nadir body weight (% day 0)	Toxicity	Median time to RTV4 (range), d	Tumor growth delay	% Enhancement
Saline p.o., i.p.	98	0/20	9 (7–13)	—	—
TMZ (68 × 5)	95	0/20	28 (25–29.5)	19.0	—
TMZ (68) + AG14447 (0.05) (698)	89	0/5	33 (29–100)	24.0	26.32*
TMZ (68) + AG14447 (0.15) (698)	90	0/5	58 (43–100)	49.0	157.89†
TMZ (68) + AG14447 (0.5) (698)	86	1/5	100 (79–100)	91.0	378.95†
TMZ (68) + MS AG14644 (0.15)	94	0/5	32 (31–43.5)	23.0	21.05*
TMZ (68) + MS AG14644 (0.5)	89	1/5	47 (41.5–57.5)	38.0	100†
TMZ (68) + AG14361 (1.5)	91	0/5	52 (39–96.5)	43.0	126.32†
TMZ (68) + AG14361 (5)	96	0/20	51 (46.5–58)	42.0	121.05‡
TMZ (68) + AG14452 (5)	94	0/5	44 (36.5–55)	35.0	84.21†

NOTE: Insert mesylate salt as for previous tables.
*$P < 0.05$ By Mann-Whitney analysis.
†$P < 0.005$.
‡$P < 0.0005$.

AG14447 compared with 50% for AG14361) were also more potent *in vitro* (temozolomide PF_{50}, 8.1–11.5 compared with 5.5 for AG14361) and AG14418, which was marginally less effective than AG14361 *in vitro* (temozolomide PF_{50}, 5.1), was also ineffective *in vivo* (6% enhancement). However, AG14477, which was more potent than AG14361 *in vitro* (temozolomide PF_{50}, 9.4), was less effective *in vivo* (enhancement, 37%).

AG14447 was identified as the most potent compound in these studies, causing greater chemosensitization of temozolomide at a dose of 1 mg/kg than could be achieved with AG14361 at 10 mg/kg (Fig. 3B). Even a 10-fold lower dose of AG14447 (0.1 mg/kg) resulted in a 50% increase in the temozolomide-induced tumor growth delay ($P < 0.05$).

Five-Day Studies: Toxicity

Based on the rank order of potency determined in the single-dose schedule, three compounds representing the full spectrum of activity (AG14447, the most potent compound; MS AG14644, approximately equivalent to AG14361; and AG14452, the less potent) were compared with the benchmark compound, AG14361, in a 5-day combination study, similar to that used previously (9). The doses used in the 5-day studies of PARP inhibitors were predicted, based on the single-dose study data, to cause measurable enhancement of temozolomide antitumor activity without unacceptable toxicity. That is, AG14447 and MS AG14644 were administered at 0.5, 0.15, and 0.05 mg/kg/d and AG14452 at 5 mg/kg/d in comparison with AG14361 at 1.5 and 5 mg/kg/d. As expected, none of the PARP inhibitors caused any measurable toxicity as single agents on a 5-day schedule (maximum body weight loss, ≤5%) and temozolomide alone was only mildly toxic (median body weight loss, 5%). However, the combination of temozolomide with either AG14447 or MS AG14644 caused significant toxicity (Table 3). Lower doses of AG14447 and MS AG14644 and all doses of AG14361 and AG14452 were tolerated (≤11% body weight loss and no toxic deaths).

Five-Day Studies: Efficacy

In agreement with the single-dose study, AG14447 was the most potent chemosensitizer in the 5-day schedule; at a dose of 0.15 mg/kg, it caused a greater enhancement of temozolomide activity than AG14361 at a 33-fold higher dose (5 mg/kg; Table 3). MSAG14644 at 0.5 mg/kg was marginally less effective than AG14361 at 1.5 mg/kg and, as expected, AG14452 was the least active compound. Thus, the rank order of potency, AG14447 > MSAG14644 ≥ AG14361 > AG14452, was the same as seen in the single-dose study.

Greater temozolomide sensitization was observed in the 5-day schedule compared with the single-dose study. For example, AG14361 at a total dose of 7.5 mg/kg (1.5 mg/kg daily × 5) caused a 121% enhancement compared with 75% enhancement with a single dose of 10 mg/kg. In the 5-day schedule, AG14447 was also administered at 0.5 mg/kg (total dose of 2.5 mg/kg), which caused complete tumor regression persisting to day 100 (termination of the experiment) in three of five mice and day 16 in another mouse.

Relation of *In vivo* Efficacy to *In vitro* Chemosensitization and Tumor Drug Accumulation

Our previous studies had shown that, although the SW620 xenograft was the most sensitive *in vivo* model for temozolomide efficacy studies, it was not possible to potentiate temozolomide cytotoxicity by PARP inhibition *in vitro* (9). In the present study, temozolomide chemosensitization *in vivo* (SW620 xenografts) was not related to temozolomide chemosensitization *in vitro* (LoVo cells) or K_i value. However, there was a trend for compounds that were potent enhancers of topotecan in SW620 growth inhibition *in vitro* to be more potent enhancers (at 1 mg/kg) of temozolomide antitumor activity against SW620 xenografts. The relationship was not statistically significant ($P = 0.109$), probably due to the limited number of data points and preselection of only the most potent compounds, resulting in very little variation in chemosensitization potency.

We investigated if accumulation of the compound in the tumor contributed to the chemosensitization potency of the most potent (AG14447) and least potent (AG14452) compared with the benchmark PARP inhibitor, AG14361. In mice bearing SW620 xenografts given a dose of 10 mg/kg i.p., the tumor concentrations of AG14447, AG14361, and AG14452 2 h after administration were 3.5, 3.3, and 2.7 μmol/L, respectively. These differences in accumulation alone would not seem to be sufficient to account for the magnitude of the differences in efficacy, which was probably more a function of their cellular potency.

Discussion

Our primary goal in conducting the studies described here was to identify a compound, with improved biological activity compared with the initial lead compound AG14361, for use as a chemosensitizer in the clinic. In the process, we evaluated whether more economic (in terms of drug and time) assays are sufficiently robust for candidate selection.

Forty-two compounds representative of four compound classes, selected on the basis of PARP inhibitory potency (K_i, ≤15 nmol/L), were evaluated in growth inhibition assays in combination with temozolomide in LoVo cells. In addition to conventional chemosensitization assays with a variable cytotoxic concentration (PF_{50}), we investigated the ability of a fixed concentration of PARP inhibitor to enhance the cytotoxicity of a low fixed concentration of temozolomide (EF_{400}). The correlation between EF_{400} and PF_{50} data was so strong that we believe this more streamlined approach is a robust and reliable method to determine chemosensitization potency when screening large numbers of PARP inhibitors. The determination of the concentration of inhibitor that, in combination with a fixed low temozolomide concentration, reduced cell growth to 50% (GI_{50} + temozolomide) may be the most sensitive discriminator of potency for compounds with similar activity as there was a much greater dynamic range of values (1.6–3,900 nmol/L) compared with PF_{50} values (1.1–11.5). Despite the relatively narrow range of values, chemosensitization potency *in vitro* for both temozolomide and topotecan was related to K_i, a strong indication that inhibitory potency against the isolated enzyme is a major factor in determining cellular activity.

The intrinsic growth-inhibitory activity of the compounds was also related to K_i. Our previous studies showed that the GI_{50} of AG14361 was nearly identical in PARP wild-type and null mouse embryonic fibroblasts (9) so, by analogy, cytotoxicity of these novel compounds is likely to be due to an effect on another cellular target. Moreover, in all cases, the GI_{50} value was >15 times higher than the concentration used for chemosensitization and significant chemosensitization could be achieved at concentrations ~1,000 times lower than the GI_{50}. Therefore, although these inhibitors may be interacting with other cellular targets, they are only doing so at concentrations far in excess of those needed for chemopotentiation. Furthermore, no toxicity was observed in mice treated with PARP inhibitors alone, although there was enhancement of temozolomide-induced toxicity, as expected from data obtained in PARP-1 null mice (22–24).

Clinically, temozolomide is administered in a 5-day schedule (25) and previous preclinical investigations of chemosensitizers in combination with temozolomide have also used this schedule (9, 16, 26). To determine if data obtained using a less stressful approach could reliably identify effective compounds, we evaluated the use of a single-dose schedule. Of the 11 compounds that were evaluated in the single-dose schedule, 4 (representative of the full range of potency and sensitization) were also evaluated in a conventional 5-day schedule. The finding that the compounds ranked in the same order of potency in both schedules supports the use of single-dose studies as a primary screen. However, greater chemosensitization (including complete tumor regressions) and less toxicity (i.e., an improved therapeutic index was observed using the 5-day schedule). The finding that there was no correlation between the antitumor activity and the toxicity of the PARP inhibitor-temozolomide combinations in either single or 5-day schedules suggests that toxicity and chemosensitization are by different mechanisms. The predictive value of the subcutaneous human tumor xenograft as a model, which does not pose the technical difficulties of the orthotopic model and is less artificial than the hollow fiber technique, has been highlighted recently (27). We believe that the s.c. xenograft combined with a single-dose schedule provides reliable antitumor efficacy data on a large number of compounds, from which favorable pharmacokinetics and pharmacodynamics can be inferred, and that these data can be confirmed in selected compounds using a conventional 5-day schedule.

In general, the *in vivo* chemosensitization data did not correlate with K_i values but the compound with the lowest K_i (AG1447) was the most potent enhancer of temozolomide antitumor activity. Although not statistically significant, compounds that were more potent than AG14361 in the *in vivo* studies were also more potent *in vitro*, supporting the use of *in vitro* studies to guide selection of compounds for *in vivo* evaluation. The more complex nature of *in vivo* biological systems includes pharmacokinetics, which determines the drug level and duration of exposure in the tumor (28). Limited tissue distribution studies of three of the compounds showed that, although their accumulation within the tumor ranked in the same order as their efficacy, there was only ~30% difference in the tumor concentration. Clearly, although *in vitro* potency and tumor drug levels both contribute to the *in vivo* antitumor activity of the compounds, they are not the only determinants, highlighting the need for *in vivo* xenograft studies to select the most efficacious compounds. Indeed, the most potent inhibitor in the xenograft studies, which was ultimately selected for further preclinical evaluation before clinical trial, would not have been identified based on cell-based assays.

In conclusion, we have confirmed that *in vivo* chemosensitization potency is at least in part related to cellular

activity, which is in turn related to the PARP inhibitory potency determined in the isolated enzyme. In addition, we have identified a more streamlined approach to screening of PARP inhibitors. *In vitro* assays using a fixed concentration of temozolomide in combination with a fixed concentration of PARP inhibitor gave a good indication of potency, confirmed by conventional chemopotentiation studies. The single dose *in vivo* combination study is a less stressful and more rapid, cost-effective approach to compound selection.

These studies have culminated in the identification of a compound for clinical evaluation. AG14447 had outstanding chemosensitization potency, at least ten times more potent than the initial lead, AG14361, in both single-dose and 5-day dosing schedules. This compound caused >100% enhancement of temozolomide-induced tumor growth delay at tolerable doses. The phosphate salt of AG14447 (AG014699), which has improved aqueous solubility, has been selected for clinical trial.

Acknowledgments

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