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Initial Testing (Stage 1) of the mTOR Kinase Inhibitor AZD8055 by the Pediatric Preclinical Testing Program

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Abstract

Background—AZD8055 is a small molecule ATP-competitive inhibitor of the serine/threonine kinase mTOR that regulates cap-dependent translation through the mTORC1 complex and Akt activation through the mTORC2 complex.

Procedures—AZD8055 was tested against the PPTP in vitro panel at concentrations ranging from 1.0 nM to 10 μ M and against the PPTP in vivo panels at a dose of 20 mg/kg administered orally daily $\times$ 7 for 4 weeks.

Results—In vitro the median relative IC₅₀ for AZD8055 against the PPTP cell lines was 24.7 nM. Relative I/O values >0% (consistent with a cytostatic effect) were observed in 8 cell lines and 15 cell lines showed Relative I/O values ranging from −4.7 to −92.2% (consistent with varying degrees of cytotoxic activity). In vivo AZD8055 induced significant differences in EFS distribution compared to controls in 23 of 36 (64%) evaluable solid tumor xenografts, and 1 of 6 evaluable ALL xenografts. Intermediate activity for the time to event activity measure (EFS T/C

>2) was observed in 5 of 32 (16%) solid tumor xenografts evaluable. The best response was stable disease. PD2 (progressive disease with growth delay) was observed in 20 of 36 (55.6%) evaluable solid tumor xenografts. AZD8055 significantly inhibited 4E-BP1, S6, and Akt phosphorylation following day 1 and day 4 dosing, but suppression of mTORC1 or mTORC2 signaling did not predict tumor sensitivity.

Conclusions—AZD8055 demonstrated broad activity in vitro, but at the dose and schedule studied demonstrated limited activity in vivo against the PPTP solid tumor and ALL panels.

Keywords

developmental therapeutics; mTOR inhibitor; preclinical testing

INTRODUCTION

In mammalian cells the serine/threonine kinase mTOR (target of rapamycin), a member of the PI3 kinase like kinase (PIKK) superfamily, exists in two complexes; mTORC1 comprising mTOR, raptor, PRAS40, and mLst8 (GβL), and mTORC2 containing mTOR, rictor, Sin1, and mLst8 [1,2]. Increasing evidence has implicated mTORC1 as a sensor that integrates extracellular and intracellular events, coordinating cell size (growth), proliferation, and survival. mTORC1 directly or indirectly regulates cap-dependent translation initiation, membrane trafficking, protein degradation, ribosome biogenesis, and tRNA synthesis, as well as transcription [1,3]. mTORC1 signaling is negatively regulated by amino acid deficiency, hypoxia, DNA damage and increased AMP levels, suppressing cap-dependent protein synthesis. Thus, mTORC1 in concert with the tuberous sclerosis complex (TSC) complex and Rheb, appears to sense nutrient and growth factor status, as well as energy charge, and regulates progression from G1 to S phase. The mTORC2 complex phosphorylates Akt (Ser473) leading to its full activation, and regulates F-actin and the cytoskeleton [4]. Over the past few years the role of mTOR complexes in tumorigenesis and survival has become apparent [1,3]. Hence inhibition of mTOR has become a focus for drug development [2].

Rapamycin, a selective inhibitor of mTORC1 signaling, inhibits the proliferation of many tumor cell lines in vitro including cell lines derived from childhood cancers [5,6], and it showed significant antitumor activity against syngeneic tumor models in the NCI in vivo screening program [7]. In our previous study, rapamycin induced significant differences in event free survival (EFS) distribution in 28 of 36 solid tumor xenografts and in 5 of 8 ALL xenografts with objective responses being observed in several panels [6]. The rapamycin analogs (rapalogs) temsirolimus (CCI-779) and everolimus (RAD001) have been approved for treatment of refractory renal cell carcinoma [8,9], and temsirolimus demonstrates a high response rate against mantle cell lymphoma at relapse [10]. Both temsirolimus and everolimus have completed phase I trials in pediatric patients [11]. However, both in non-clinical models and patient tumors, inhibition of mTORC1 signaling frequently induces hyperphosphorylation of Akt (Ser473), potentially activating this survival pathway [12]. Activation of Akt occurs due to inhibition of S6K1, downstream of mTORC1, and reduced phosphorylation of the S6K1 substrate insulin receptor substrate-1 (IRS-1) leading to its stabilization. In many cell lines the activation of Akt by rapamycin can be inhibited by

antibodies that block the IGF-1 receptor [13,14]. Rapamycin and its derivatives are not inhibitors of mTOR kinase activity at pharmacologically relevant concentrations. Rather, rapamycin binds the cyclophilin FKBP12, and this complex interacts with mTOR outside of the kinase domain, probably displacing raptor, and preventing the presentation of substrates (e.g., 4E-BP1 and S6K1) to the kinase domain.

Direct kinase inhibitors should target mTOR in both complexes, preventing mTORC1 signaling as well as preventing activation of Akt, assuming that mTORC2 is the only kinase to phosphorylate Akt at Ser473 [15]. Consequently, small molecule ATP-competitive inhibitors of mTOR have been identified, and several are in clinical evaluation. AZD8055 is a potent and selective inhibitor of the mTOR kinase that is in clinical phase 1 trials [16]. mTOR kinase inhibitors have been shown to suppress phosphorylation of 4E-BP1 and global translation more effectively than rapalogs [16,17]. Importantly, cellular proliferation appears to be regulated by the 4E-BP-eIF4E branch of the mTORC1 signaling pathway [18,19]. Here we have evaluated AZD8055 against the in vitro and in vivo panels of childhood cancer models of the PPTP.

MATERIALS AND METHODS

In Vitro Testing

In vitro testing was performed using DIMSCAN, a semiautomatic fluorescence-based digital image microscopy system that quantifies viable (using fluorescein diacetate [FDA]) cell numbers in tissue culture multiwell plates [20]. Cells were incubated in the presence of AZD8055 for 96 hr at concentrations from 1 nM to 10 μ M and analyzed as previously described [21]. Absolute IC₅₀ values represent the concentration of AZD8055 that reduces cell survival to 50% of the control value, while relative IC₅₀ values represent the AZD8055 concentration that reduces cell survival by 50% of the maximum AZD8055 effect [22]. Relative in/out (I/O)% values represent the percentage difference between the Y_{min} value and the estimated starting cell number and either the control cell number (for agents with Y_{min} > starting cell number) or 0 (for agents with Y_{min} < estimated starting cell number). Relative I/O% values range between 100% (no treatment effect) to -100% (complete cytotoxic effect), with a Relative I/O% value of 0 being observed for a completely effective cytostatic agent.

In Vivo Tumor Growth Inhibition Studies

CB17SC scid^{-/-} female mice (Taconic Farms, Germantown NY), were used to propagate subcutaneously implanted kidney/rhabdoid tumors, sarcomas (Ewing, osteosarcoma, rhabdomyosarcoma), neuroblastoma, and non-glioblastoma brain tumors, while BALB/c nu/nu mice were used for glioma models, as previously described [23]. Human leukemia cells were propagated by intravenous inoculation in female non-obese diabetic (NOD)/scid^{-/-} mice as described previously [24]. Female mice were used irrespective of the patient gender from which the original tumor was derived. All mice were maintained under barrier conditions and experiments were conducted using protocols and conditions approved by the institutional animal care and use committee of the appropriate consortium member. Ten mice were used in each control or treatment group. Tumor volumes (cm³) [solid tumor

xenografts] or percentages of human CD45-positive [hCD45] cells [ALL xenografts] were determined as previously described [25] and responses were determined using three activity measures as previously described [25]. An in-depth description of the analysis methods is included in the supplemental response definitions.

Statistical Methods

The exact log-rank test, as implemented using Proc StatXact for SAS[®], was used to compare event-free survival distributions between treatment and control groups. *P*-values were two-sided and were not adjusted for multiple comparisons given the exploratory nature of the studies.

Drugs and Formulation

AZD8055 was provided to the PPTP by AstraZeneca, through the Cancer Therapy Evaluation Program (NCI). AZD8055 was dissolved in 0.5% hydroxypropylmethylcellulose containing 0.1% Tween 80 in water, sonicated and stirred overnight. AZD8055 was administered P.O. daily for 28 days at 20 mg/kg per day.

Pharmacodynamic Studies

Rh10, Rh18, and Rh30 rhabdomyosarcomas were harvested between 0 and 24 hr post treatment on day 1, or 1–24 hr following treatment on day 4 (20 mg/kg/day). Samples (5 per time point) were processed for immunoblotting as previously described [26]. Phosphorylated 4E-BP (Thr37/46), Akt (Ser473), and S6 (Ser235/6) and total proteins were determined using antibodies from Cell Signaling Technologies. P-glycoprotein was detected by immunoblotting using C219 antibody (Centocore), and cleaved poly-ADP ribose polymerase (PARP) was used as a marker of apoptosis. Protein loading was normalized using GAPDH.

RESULTS

AZD8055 In Vitro Testing

AZD8055 was tested against the PPTP's in vitro cell line panel at concentrations ranging from 1 nM to 10 mM. AZD8055 potently inhibited proliferation of cells with median relative and absolute IC₅₀ values for the in vitro panel of 24.7 and 31.7 nM, respectively, Table I. Most of the PPTP cell lines showed plateau T/C values significantly greater than 0, with Relative I/O values >0% for 8 cell lines (consistent with a cytostatic effect) and with the remaining 15 cell lines showing Relative I/O values ranging from -4.7 to -92.2% (consistent with varying degrees of cytotoxic activity). The median absolute IC₅₀ ratio graph, Figure 1, shows the relative IC₅₀ values for the cell lines of the PPTP panel. Dose response curves for Ramos-RA1, a lymphoma cell line that showed the smallest Y_{min}, and NB-EBc1, a neuroblastoma cell line that showed the more typical plateau effect, are also shown.

AZD8055 In Vivo Testing

AZD8055 was evaluated in 42 xenograft models using daily dosing at 20 mg/kg shown previously to be effective against a broad range of tumor xenografts derived from adult carcinomas [16]. Twenty-one of 867 mice died during the study (2.4%), with 2 of 428 in the control arms (0.5%) and 19 of 439 in the AZD8055 treatment arms (4.3%). Two lines (KT-11 and KT-14) were excluded from analysis due to toxicity greater than 25 percent. In previous studies with rapamycin or other kinase inhibitors, no toxicity was seen in either kidney tumor line, hence this is probably not a target-class effect. One of the 7 ALL xenografts evaluated (ALL-16) was excluded from efficacy reporting because of excessive drug related toxicity. A complete summary of results is provided in Supplemental Table I, including total numbers of mice, number of mice that died (or were otherwise excluded), numbers of mice with events and average times to event, tumor growth delay, as well as numbers of responses and T/C values.

Antitumor effects were evaluated using the PPTP activity measures for time to event (EFS T/C), tumor growth delay (tumor volume T/C), and objective response. AZD8055 induced significant differences in EFS distribution compared to controls in 23 of 36 evaluable solid tumor xenografts (64%) tested as shown (Table II). significant growth delay was observed in each of the solid tumor panels, including panels for rhabdoid tumors (1 of 2), Wilms tumor (1 of 2), rhabdomyosarcoma (4 of 6), Ewing sarcoma (4 of 5), medulloblastoma (2 of 3), glioblastoma (4 of 4), neuroblastoma (3 of 6), and osteosarcoma (3 of 6). One of the 6 evaluable ALL xenografts showed a significant difference in EFS distribution between treated and control animals. Criteria for intermediate activity for the time to event activity measure (i.e., EFS T/C >2) were met in 5 of 32 (16%) solid tumor xenografts evaluable for this measure (Table II). Intermediate activity was restricted to the rhabdomyosarcoma panel (3 of 6) and Ewing sarcoma panel (2 of 5). Four models were inevaluable for the EFS T/C activity measure due to slow tumor growth rate in control animals. No ALL xenografts met criteria for intermediate activity for the EFS T/C activity measure.

Objective responses (i.e., tumor regression) were not observed for any of the solid tumor or ALL xenografts. The best response was stable disease (SD), which was observed in 2 of 36 (5.6%) evaluable solid tumor xenografts. The stable disease observed for an ependymoma xenograft (BT-36) is largely attributable to its slow growth rate, whereas the stable disease for the medulloblastoma xenograft (BT-50) is more clearly treatment-related. PD2 (progressive disease with growth delay) was observed in 20 of 36 (55.6%) evaluable solid tumor xenografts. PD2 responses were most commonly observed in the rhabdomyosarcoma (4 of 6), Ewing sarcoma (4 of 5), glioblastoma (3 of 4), neuroblastoma (3 of 6), and rhabdoid tumor (2 of 2) panels. Two of the 6 evaluable ALL xenografts showed PD2 responses, with the remainder categorized as PD1 (progressive disease without growth delay).

The in vivo testing results for the objective response measure of activity are presented in Figure 2 in a “heat-map” format as well as a “COMPARE”-like format, based on the scoring criteria described the supplemental response definitions section. The latter analysis demonstrates relative tumor sensitivities around the midpoint score of 5 (stable disease). Examples of responses for rhabdomyosarcoma xenografts showing tumor growth inhibition

are shown in Figure 3 (Rh10, Rh18, Rh28, and Rh30). Rh10 xenografts are unresponsive to AZD8055 (PD1, T/C EFS $\frac{1}{4}$ 1.0), whereas Rh18, Rh28, and Rh30 tumors are somewhat more sensitive (PD2, T/C EFS 2.8, 2.8, and 2.4, respectively).

Pharmacodynamic Studies

Inhibition of mTORC1 was assessed by decreased phospho-4E-BP1 (Thr37/46) and phospho-S6 (Ser235/6) protein, and inhibition of mTORC2 by decreased phospho-Akt (Ser473) in Rh10, Rh18, and Rh30 xenografts following the first and fourth dose of AZD8055. As shown in Figure 4A, the phosphorylation of both 4E-BP1 and S6 was completely suppressed in Rh10 xenografts at 1 and 4 hr after first administration of AZD8055, recovering at 8 and 24 hr. In contrast phospho-Akt was completely abrogated and was detected only at 24 hr post dosing. Phospho-4E-BP1, Akt, and S6 were suppressed completely 1–24 hr following the day 4 dose in this resistant tumor. Dosing on day 1 was associated with a significant increase in cleaved PARP, whereas only a weak signal for PARP cleavage was detected after drug administration on day 4. For Rh18 tumors (Fig. 4B) AZD8055 markedly suppressed phosphorylation of 4E-BP1, Akt, and S6 after the first dose through 24 hr, but appeared to have less effect on 4E-BP1 after the day 4 dose. In this tumor there was no definitive increase in signal for cleaved PARP. For Rh30 xenografts (Fig. 4C), AZD8055 suppressed phospho-S6 and p-Akt over 24 hr following the first dose, but phospho-4E-BP1 was detected in tumors at 4 and 24 hr post dosing. Phosphorylated forms of 4E-BP1, S6, or Akt were not detected 1–24 hr post dosing on day 4, whereas cleaved PARP was detected. As other small molecule kinase inhibitors are substrates of P-glycoprotein [27-29] and as induction of ABC transporters by substrate drugs has been reported [30,31], tumor samples were probed to determine P-glycoprotein expression and whether this drug transporter was induced by treatment. P-glycoprotein was not detected in samples of Rh30 xenografts, and did not appear to be induced by treatment over 4 days in Rh10 or Rh18 xenografts.

DISCUSSION

The rapalogs, temsirolimus, and everolimus, are approved for treatment of refractory renal cell carcinoma, and have demonstrated antitumor activity against other cancers in human trials. Exactly how these agents inhibit tumor growth, either directly by inhibiting tumor cell cycle progression, or through indirect actions on tumor angiogenesis [32] remains to be determined. Similarly, robust biomarkers for tumor response to these agents remain to be defined, as inhibition of mTORC1 signaling, as determined by changes in phosphorylation of downstream substrates, does not distinguish responding from non-responding tumors [33]. Inhibition of mTORC1 stimulates mTORC2-mediated phosphorylation of Akt at serine 473, potentially leading to its full activation, and perhaps additional downstream substrates, but does not predict tumor cell response to PI3K/mTOR inhibition [33]. The increased phosphorylation of Akt can be blocked by inhibiting the Type-1 insulin-like growth factor receptor (IGF-1R) [12-14]. This suggests that enhanced Akt activation is a consequence of reduced negative feedback on insulin receptor substrate (IRS) proteins when S6K1, a downstream substrate of mTOR1, is inhibited [13]. Thus, combining rapalogs with IGF-1R inhibitors has shown good in vivo activity against non-clinical xenograft models [14,34],

and this strategy is being evaluated in both adult and childhood clinical studies. Although the consequences of rapalog-induced Akt phosphorylation are uncertain [33], the activation of this anti-apoptotic signaling pathway potentially could self-limit the cytotoxic effect of these agents. In contrast to rapalogs, which are not inhibitors of the kinase activity of mTOR in either complex, small molecule ATP-competitive inhibitors are selective mTOR kinase inhibitors, and thus would inhibit mTOR kinase activity in both cellular complexes.

AZD8055, a selective mTOR kinase inhibitor [16], demonstrated potent activity inhibiting the proliferation of all cell lines in the PPTP panel. There was little evidence of histotype specificity. The activity of AZD8055 was consistent with a cytostatic action for a subset of PPTP cell lines, whereas other lines clearly showed some degree of cytotoxic response to AZD8055. Induction of autophagy, regulated through mTORC1 signaling, was not examined in this study.

In vivo, AZD8055 slowed the growth of most solid tumors, and significantly inhibited growth of one ALL model, but did not induce any tumor regressions in either solid tumor or leukemia models. The response of our pediatric preclinical models to AZD8055 differs from our previous results with rapamycin for which objective regressions were observed for ALL, osteosarcoma, rhabdomyosarcoma, and rhabdoid xenografts [6].

As anticipated, AZD8055 inhibited both mTORC1 and mTORC2 signaling, as determined by complete loss of phosphorylation of 4E-BP (Thr37/46) and Akt (Ser473). However, the effect was equally profound in Rh10 tumors that are non-responsive compared to Rh18 and Rh30 xenografts where AZD8055 significantly extended EFS (2.8-fold compared to control animals). Although there were somewhat different pharmacodynamic effects, following day 4 of dosing, signaling via mTORC1 and mTORC2 was essentially suppressed for 24 hr in both Rh10 and Rh30 xenografts. In contrast, both phosphorylated 4E-BP1 and Akt were detected during this period in Rh18 tumors, whereas S6 phosphorylation was not detected. Thus, similar to the situation with rapalogs, there is no clear relationship between suppression of phosphorylation of either mTORC1 (4E-BP1 and S6) or mTORC2 (Akt(Ser473)) substrates and tumor response to AZD8055. We also examined whether there was any relationship between expression of the P-glycoprotein drug transport protein and response. Both Rh18 and Rh30 have similar responses to AZD8055, but P-glycoprotein was detected only in Rh18 tumors. Conversely, Rh10, the most resistant tumor line, demonstrated only very low levels of P-glycoprotein, and it was not induced during treatment with AZD8055.

In summary, AZD8055 slowed tumor progression in the majority of solid tumor models but did not induce objective regressions. Future studies of interest include evaluating AZD8055 with standard cytotoxic agents as well as with inhibitors of other signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. AZD8055 in vitro activity. **Top panel:** The median IC₅₀ ratio graph shows the relative IC₅₀ values for the cell lines of the PTP panel. Each bar represents the ratio of the panel IC₅₀ to the IC₅₀ value of the indicated cell line. Bars to the right represent cell lines with higher sensitivity, while bars to the left indicate cell lines with lesser sensitivity. **Bottom panels:** Representative dose response curves for Ramos-RA1 leukemia and NB-EBc1 neuroblastoma cell lines.

Fig. 2. AZD8055 in vivo objective response activity, **left:** The colored heat map depicts group response scores. A high level of activity is indicated by a score of 6 or more, intermediate activity by a score of >2 but <6, and low activity by a score of <2. **Right:** Representation of tumor sensitivity based on the difference of individual tumor lines from the midpoint response (stable disease). Bars to the right of the median represent lines that are more sensitive, and to the left are tumor models that are less sensitive. Red bars indicate lines with a significant difference in EFS distribution between treatment and control groups, while blue bars indicate lines for which the EFS distributions were not significantly different.

Fig. 3.

AZD8055 activity against individual rhabdomyosarcoma xenografts. Kaplan–Meier curves for EFS, median relative tumor volume graphs, and individual tumor volume graphs are shown for selected lines, Rh10, Rh18, Rh28, and Rh30 sarcoma xenografts. Controls (gray lines); Treated (black lines). [correction made to figure after initial online publication].

Fig. 4. Pharmacodynamic evaluation. Western blot analyses were performed as previously described with minor modifications [26]. Tumors were harvested prior to treatment (Controls at day 1 and day 4) or at the indicated time points after the day 1 (**left panels**) or day 4 dose (**right panels**). Each lane represents an individual tumor, five tumors per time point were assessed. GAPDH was used as a loading control. **A:** Rh10; **B:** Rh18; **C:** Rh30.

TABLE I

Activity of AZD8055 Against the PPTP In Vitro Panel

Cell line	Histology	Relative IC ₅₀ (nM) ^a	Absolute IC ₅₀ (nM) ^b	Relative median IC ₅₀ ratio ^c	Actual Y _{min} ^d	Hill eqn Y _{min}	Relative I/O ^e (%)
RD	Alveolar RMS	33.3	45.0	0.74	8.2	9.6	2.8
Rh41	Alveolar RMS	30.7	39.8	0.81	9.9	11.3	-55.6
Rh18	Embryonal RMS	41.4	51.3	0.60	10.2	11.2	-77.1
Rh30	Alveolar RMS	14.0	15.1	1.77	6.5	5.9	-60.9
BT-12	Rhabdoid	24.7	31.7	1.00	12.1	12.3	4.3
CHLA-266	Rhabdoid	1.1	25.3	22.08	27.1	18.3	1.4
TC-71	Ewing sarcoma	46.5	48.5	0.53	3.0	3.2	1.8
CHLA-9	Ewing sarcoma	15.3	14.9	1.62	3.4	3.5	-4.7
CHLA-10	Ewing sarcoma	3.9	4.6	6.36	2.9	1.0	-54.7
CHLA-258	Ewing sarcoma	10.4	12.6	2.38	6.2	6.6	-84.1
GBM2	Glioblastoma	40.7	40.3	0.61	5.4	2.8	-45.4
NB-1643	Neuroblastoma	33.8	70.6	0.73	22.9	20.1	2.3
NB-EBc1	Neuroblastoma	27.1	45.1	0.91	20.0	18.2	-12.3
CHLA-90	Neuroblastoma	34.5	37.7	0.72	5.0	4.6	-82.2
CHLA-136	Neuroblastoma	162.6	>10,000	<0.01	59.9	61.4	43.8
NALM-6	ALL	32.9	33.9	0.75	4.2	2.6	1.3
COG-LL-317	ALL	1.3	2.6	19.31	4.1	1.8	-8.5
RS4;11	ALL	35.0	38.6	0.71	7.9	6.0	-47.6
MOLT-4	ALL	8.6	13.6	2.89	4.6	0.0	-53.7
CCRF-CEM	ALL	4.1	8.1	5.97	9.8	8.1	3.7
Kasumi-1	AML	1.4	5.1	17.18	5.1	0.2	-82.2
Karpas-299	ALCL	21.7	19.9	1.14	3.5	1.8	-54.5
Ramos-RA1	NHL	8.2	8.2	3.03	0.1	1.2	-92.2
Median	—	24.7	31.7	1.00	6.2	5.9	-45.4
Minimum	—	1.1	2.6	<0.01	0.1	0.0	-92.2
Maximum	—	162.6	110.0	22.08	59.9	61.4	43.8

^aRelative IC₅₀ is the concentration of agent that gives a response half way between bottom and top.^bAbsolute IC₅₀ values represent the concentration at which the agent reduces cell survival to 50% of the control value.^cTo compare activity between cell lines, the ratio of the median relative IC₅₀ to individual cell line's relative IC₅₀ value is used (larger values connote greater sensitivity).^dThe lowest T/C% value is the Y_{min}.^eRelative in/out (I/O)% values represent the percentage difference between the Y_{min} value and the estimated starting cell number and either the control cell number (for agents with Y_{min} > starting cell number) or 0 (for agents with Y_{min} < estimated starting cell number); Relative I/O% values range between 100% (no treatment effect) to -100% (complete cytotoxic effect), with a Relative I/O% value of 0 being observed for a completely effective cytostatic agent.

TABLE II

Activity of AZD8055 Against the PPTP In Vivo Panel

Xenograft line	Histology	Median time to event	P-value	EFS T/C ^a	Median final RTV	T/C ^b	P-value	T/C activity ^c	EFS activity	Response activity
BT-29	Rhabdoid	>EP	<0.001	>1.3	2.10	0.61	0.009	Low	NE	Int
KT-12	Rhabdoid	10.7	0.076	1.6	>4	0.42	0.035	Int	Low	Int
KT-10	Wilms	17.2	0.001	1.3	>4	0.6	0.004	Low	Low	Low
KT-13	Wilms	29.6	0.692	1.4	>4	0.92	0.573	Low	Low	Low
SK-NEP-1	Ewing	11.1	0.054	1.6	>4	0.57	0.017	Low	Low	Int
EW5	Ewing	22.9	0.002	3.1	>4	0.45	<0.001	Low	Int	Int
EW8	Ewing	13.9	0.041	1.4	>4	0.77	0.113	Low	Low	Low
TC-71	Ewing	12.6	<0.001	2.1	>4	0.38	<0.001	Int	Int	Int
CHLA-258	Ewing	14.3	0.034	1.4	>4	0.79	0.356	Low	Low	Int
Rh10	ALV RMS	31.1	0.686	1.0	>4	0.9	1.000	Low	Low	Low
Rh28	ALV RMS	29.8	0.001	2.8	>4	0.29	0.005	Int	Int	Int
Rh30	ALV RMS	23.7	0.042	2.8	>4	0.46	0.001	Low	Int	Int
Rh30R	ALV RMS	15.8	0.004	1.7	>4	0.6	0.010	Low	Low	Int
Rh41	ALV RMS	18.3	0.112	1.4	>4	0.81	0.123	Low	Low	Low
Rh18	EMB RMS	32.9	<0.001	2.4	>4	0.39	<0.001	Int	Int	Int
BT-28	Medulloblastoma	6.2	0.089	1.2	>4	0.72	0.156	Low	Low	Low
BT-45	Medulloblastoma	22.1	<0.001	1.9	>4	0.5	0.002	Low	Low	Int
BT-50	Medulloblastoma	>EP	0.011	>1.2	1.20	0.8	0.063	Low	NE	Int
BT-36	Ependymoma	>EP	0.474	—	1.10	0.77	0.013	Low	NE	Int
BT-44	Ependymoma	11.0	0.021	1.5	>4	0.67	0.011	Low	Low	Low
GBM2	Glioblastoma	23.0	0.003	1.6	>4	0.69	0.052	Low	Low	Int
BT-39	Glioblastoma	22.0	0.006	1.6	>4	0.63	0.011	Low	Low	Int
D645	Glioblastoma	8.3	<0.001	1.6	>4	0.45	<0.001	Low	Low	Int
D456	Glioblastoma	5.5	0.028	1.1	>4	0.74	0.043	Low	Low	Low
NB-SD	Neuroblastoma	11.2	0.118	1.5	>4	0.97	0.481	Low	Low	Low
NB-1771	Neuroblastoma	5.6	0.431	1.0	>4	0.76	0.315	Low	Low	Low
NB-1691	Neuroblastoma	8.7	<0.001	1.6	>4	0.48	<0.001	Low	Low	Int
NB-EBc1	Neuroblastoma	11.0	0.066	2.1	>4	0.48	<0.001	Low	Low	Int

Xenograft line	Histology	Median time to event	P-value	EFS T/C ^a	Median final RTV	T/C ^b	P-value	T/C activity ^c	EFS activity	Response activity
NB-1643	Neuroblastoma	7.6	0.004	1.3	>4	0.71	0.006	Low	Low	Low
SK-N-AS	Neuroblastoma	9.6	<0.001	1.5	>4	0.55	<0.001	Low	Low	Int
OS-1	Osteosarcoma	>EP	0.023	>1.2	3.10	0.63	0.013	Low	NE	Int
OS-2	Osteosarcoma	30.1	0.101	1.1	>4	0.85	0.101	Low	Low	Low
OS-17	Osteosarcoma	22.9	<0.001	1.3	>4	0.7	<0.001	Low	Low	Low
OS-9	Osteosarcoma	21.0	0.162	1.1	>4	0.9	0.247	Low	Low	Low
OS-33	Osteosarcoma	22.5	0.057	1.4	>4	0.82	0.340	Low	Low	Low
OS-31	Osteosarcoma	19.2	0.002	1.3	>4	0.63	0.002	Low	Low	Low
ALL-2	ALL B-precursor	21.6	0.251	1.1	>25	—	—	—	Low	Low
ALL-4	ALL B-precursor	4.4	0.423	0.8	>25	—	—	—	Low	Low
ALL-7	ALL B-precursor	11.3	0.033	1.5	>25	—	—	—	Low	Int

^aEFS (T/C): event-free survival for treated mice (T) compared to control mice (C).

^bT/C tumor volume in treated mice (T) compared to tumor volume in control mice (C), at the last day all control mice were measured.

^cFor the EFS T/C measure, agents are considered highly active if they meet three criteria: (a) an EFS T/C >2; (b) a significant difference in EFS distributions ($P < 0.050$), and (c) a net reduction in median tumor volume for animals in the treated group at the end of treatment as compared to at treatment initiation. Agents meeting the first two criteria, but not having a net reduction in median tumor volume for treated animals at the end of the study are considered to have intermediate activity. Agents with an EFS T/C <2 are considered to have low levels of activity.