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BMN 673, a novel and highly potent PARP1/2 inhibitor for the treatment of human cancers with DNA repair deficiency

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Abstract

Purpose—PARP1/2 inhibitors are a class of anticancer agents that target tumor-specific defects in DNA repair. Here, we describe BMN 673, a novel, highly potent PARP1/2 inhibitor with favorable metabolic stability, oral bioavailability and pharmacokinetic (PK) properties.

Experimental Design—Potency and selectivity of BMN 673 was determined by biochemical assays. Anticancer activity either as a single-agent or in combination with other anti-tumor agents was evaluated both *in vitro* and in xenograft cancer models.

Results—BMN 673 is a potent PARP1/2 inhibitor (PARP1 IC_{50} = 0.57 nM), but does not inhibit other enzymes we have tested. BMN 673 exhibits selective anti-tumor cytotoxicity and elicits DNA repair biomarkers at much lower concentrations than earlier generation PARP1/2 inhibitors (such as olaparib, rucaparib and veliparib). *In vitro*, BMN 673 selectively targeted tumor cells with *BRCA1, BRCA2* or *PTEN* gene defects with 20- to >200-fold greater potency than existing PARP1/2 inhibitors. BMN 673 is readily orally bioavailable, with >40% absolute oral bioavailability in rats when dosed in carboxylmethyl cellulose. Oral administration of BMN 673 elicited remarkable anti-tumor activity *in vivo*; xenografted tumors that carry defects in DNA repair due to *BRCA* mutations or PTEN deficiency were profoundly sensitive to oral BMN 673 treatment at well tolerated doses in mice. Synergistic or additive anti-tumor effects were also found when BMN 673 was combined with temozolomide, SN38 or platinum drugs.

Conclusion—BMN 673 is currently in early phase clinical development and represents a promising PARP1/2 inhibitor with potentially advantageous features in its drug class.

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Conflict of interest statement: YS, YF, BW and LEP are employees of BioMarin Pharmaceutical Inc. CJL and AA are named inventors on patents describing the use of PARP inhibitors, and stand to gain as part of the ICR "Rewards to Inventors" scheme.

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Introduction

DNA is constantly exposed to a range of environmental and endogenous factors that result in DNA damage (1). The repair of the resultant DNA lesions is mediated by a variety of mechanisms, including base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), nonhomologous end joining (NHEJ), and direct reversal (1). Cancer cells often display deficiencies in one or more of these DNA repair pathways and these DNA repair defects can render tumor cells more susceptible to pharmaceutical intervention of the remaining DNA repair pathways than normal cells (2).

Poly (ADP-ribose) polymerase-1 (PARP1) and the similar enzyme PARP2 play important roles in DNA repair (3). DNA strand breaks result in the recruitment and binding of PARP1/2 to DNA at the site of damage. DNA-bound PARP1/2 catalyzes the synthesis of poly (ADP-ribose) (PAR) onto a range of DNA associated proteins that mediate DNA repair. PARP1 also undergoes auto-PARsylation, a molecular change that ultimately leads to its release from DNA. Small molecule inhibitors of PARP1/2 represent a class of anticancer agents that exert their cytotoxic effect by modulating the PARsylation activity of PARP1 and PARP2 (4). Inhibition of PARP1/2 is synthetically lethal with loss of function of either the *BRCA1* or *BRCA2* tumor suppressor genes, an effect that enables tumor cells with *BRCA* gene defects to be selectively targeted with PARP 1/2 inhibitors (5). It is believed that loss of *BRCA1/2* gene function causes a deficiency in HR-mediated double strand DNA break repair (DSBR), making these cells highly susceptible to DNA lesions caused by PARP inhibition (4)(6)(7). In the clinic, Phase I and II studies have shown that the PARP1/2 inhibitor olaparib (AstraZeneca/KuDOS) can elicit significant and sustained anti-tumor responses as a single agent in cancer patients with *BRCA1 or BRCA2* mutant tumors, whilst still achieving a favorable toxicity profile (4, 8-10). Furthermore, in a Phase II study in high-grade serous ovarian cancer, olaparib reduced the risk of recurrence when used as a maintenance therapy after chemotherapy (11).

PARP1/2 inhibitors have also been shown to sensitize tumor cells to cytotoxic drugs such as the alkylating agents temozolomide and cyclophosphamide and the topoisomerase I inhibitors irinotecan and topotecan (12, 13). This characteristic forms the basis of potential combination therapies where PARP1/2 inhibitors could be used together with DNA-damaging anticancer agents to enhance the anti-tumoral response.

There are currently at least seven PARP inhibitors at various stages of clinical development (4). Here, we report the characteristics of a new, potent and selective PARP1/2 inhibitor, BMN 673. BMN 673 exhibits many of the biochemical and cytotoxicity profiles found with earlier generation PARP1/2 inhibitors such as olaparib (AZD2281, KU0059436, AstraZeneca/KuDOS), rucaparib (AG-014699, PF-01367338, Clovis/Pfizer) and veliparib (ABT-888, Abbott). However, BMN 673 is able to achieve anti-tumor cell responses and elicit DNA repair biomarkers at much lower concentrations than these other PARP1/2 inhibitors, an effect commensurate with its enhanced biochemical potency. Moreover, the favorable metabolic stability, oral bioavailability and pharmacokinetic (PK) properties of BMN 673 suggest that it is a useful addition to existing targeted agents in oncology.

Results

BMN 673 potently and selectively inhibits PARP1 and PARP2

Through a medicinal chemistry approach (Wang *et al*, manuscript in preparation), we designed a series of drug-like small molecules that were able to inhibit the catalytic activity of PARP1. One compound, LT-00628 (Figure 1A), showed a PARP1 IC_{50} of 1.82 nM. LT-00628 contains two chiral centres and comprises a racemate that in theory consists of four isomers: L/R, R/L, L/L and R/R. Chiral separation of LT-00628 indicated that LT-00628 is primarily made of *trans* isomers (L/R and R/L) with negligible amount of *cis* isomers. The *trans* isomers were obtained with chiral purity of ≥97%. The absolute stereochemistry of BMN 673 was confirmed by single-crystal X-ray diffraction analysis (unpublished data). We found one of the *trans* isomers, LT-00673, to be highly potent with average PARP1 IC_{50} of 0.57 nM (Fig. 1A). The other *trans* isomer, LT-00674, was relatively inactive (IC_{50} against PARP1 >100 nM). As a residual amount of LT-00673 remained in LT-00674 (up to 0.8%), it was possible that the weak activity shown by LT-00674 may have been caused by contamination with LT-00673. LT-00673 was later re-named as BMN 673 and its structure, (8*S*,9*R*)-5-fluoro-8-(4-fluorophenyl)-9-(1-methyl-1*H*-1,2,4-triazol-5-yl)-8,9-dihydro-2*H*-pyrido[4,3,2-de]phthalazin-3(7*H*)-one), is shown in Figure 1A. In a side-by-side comparison we found BMN 673 to be more potent than veliparib, rucaparib and olaparib with IC_{50}s of 4.7, 2.0 and 1.9 nM, respectively (Table 1). Even though the *cis* isomers were undetectable in the LT-00628 racemate, we designed a synthetic route to make the *cis* isomers alone; analysis of either *cis*-isomer showed these to be rather inactive as PARP inhibitors (Suppl Table 1).

The kinetic characteristics of BMN 673 binding to PARP1 were assessed using Biacore T200. BMN 673 bound to PARP1 with an on-rate of 3.68×10^5 (1/Ms), an off-rate of 1.05×10^{-4} (1/s), and a dissociation constant (K_D) of 2.90×10^{-10} M (Figure 1B top). In contrast, veliparib under the same conditions displayed an on-rate of 1.74×10^6 (1/Ms), an off-rate of 4.10×10^{-3} (1/s), and a dissociation constant (K_D) of 2.39×10^{-9} M (Figure 1B bottom) suggesting BMN 673 to have a dissociation rate that is nearly 40 times slower than that of veliparib.

Most PARP1 inhibitors are known to also inhibit the homologous enzyme PARP2 due to the sequence similarity of PARP1 and PARP2 catalytic domains (14). We found that BMN 673 inhibited PARP1 and PARP2 to a similar extent, with K_i of 1.20 and 0.85 nM, respectively. PARP1 and 2 are nuclear enzymes that synthesise poly (ADP-ribose) (PAR) chains on target proteins as a form of post-translational modification. In order to assess the ability of BMN 673 to inhibit intracellular PARP activity, we exposed LoVo cells to hydrogen peroxide (H_2O_2) to induce PAR synthesis and examined the ability of BMN 673 to inhibit PAR formation. Under these conditions, BMN 673 inhibited intracellular PAR formation with an IC_{50} of 2.5 nM and was modestly more potent than veliparib, rucaparib and olaparib which had cellular PAR formation IC_{50}s of 5.9, 4.7 and 3.6 nM, respectively (Table 1).

Several assays were used to examine the inhibition specificity of BMN 673. We first assessed the effect of BMN 673 on Poly (ADP-ribose) Glycohydrolase (PARG), a protein that is structurally related to PARP1/2. PARG degrades PAR modifications on proteins and

in doing so can counter the effects of PARP1/2 signalling (15). Although BMN 673 profoundly inhibited PARP1/2, it had no effect on PARG activity at concentrations up to 1 μM (data not shown). To identify other potential off-target activities, we screened BMN 673 against two commercially available protein panels, both from MDS Pharma: the Hit Profiling Screen Panel and the Adverse Reaction Enzyme Panel. At a 10 μM concentration, BMN 673 did not have significant interaction, either inhibitory or stimulatory, with any of the receptors, ion channels, or enzymes assayed (data not shown). The effect of BMN 673 on the potassium ion channel hERG (the human Ether-à-*go-go*-Related Gene protein) was determined *in vitro*, with no significant inhibition observed at BMN 673 concentrations as high as 100 μM, suggesting that BMN 673 is unlikely to cause clinical cardiac QTc elongation.

Identification of genetic determinants of BMN 673 sensitivity

Previous work has demonstrated that PARP1/2 inhibitors selectively inhibit tumor cells with genetic defects that abrogate HR-mediated DSBR (6, 7, 16). To rapidly identify genetic determinants of BMN 673 sensitivity in an unbiased fashion, we performed a series of parallel RNA interference drug sensitization screens (17) and compared the genetic sensitization profile of BMN 673 (i.e. the list of genes that modulated BMN 673 response) with those for three earlier generation clinical PARP1/2 inhibitors – olaparib, rucaparib and veliparib. To do this, we used a short interfering (si)RNA library targeting 960 genes, encompassing kinases and kinase-related genes (17) as well as a series of tumor suppressors and DNA repair proteins (Suppl Table 2). We used a moderately PARP inhibitor-resistant breast tumor cell line (CAL51) previously used in similar studies (17) and screened each drug at a concentration required to elicit a 20% reduction in cell survival (Surviving Fraction 80, SF_{80}) so as to maximise the potential for identifying PARP1/2 inhibitor sensitization effects (17, 18). We did note that for BMN 673, SF_{80} in CAL51 was achieved at 12.5 nM, compared to the micromolar concentrations of veliparib, rucaparib or olaparib required to reach this level of cell inhibition. The effect of each siRNA on drug sensitization was quantified by the calculation of a Drug Effect (DE) Z score, with siRNAs returning DE Z scores of <-2 being considered significant sensitization effects (17).

The siRNAs that significantly sensitized tumor cells to BMN 673 are shown in Suppl Table 3. This analysis revealed that the most profound effects on BMN 673 sensitivity were caused by siRNAs targeting genes involved in HR/DSBR including *BRCA2, BRCA1, SHFM1 (*aka *DSS1), PNKP, PALB2, ATM, ATR, CHEK1, FANCM, FANCA* etc. (Figure 2A and Suppl Table 3 genes highlighted in bold text). This observation suggested that HR/DSBR defects caused by any one of a number of genes caused cellular sensitivity to BMN 673, as is the case for other PARP1/2 inhibitors. To assess whether the overall genetic sensitization profile for BMN673 was different from that of earlier generation PARP1/2 inhibitors, we compared the DE Z scores for all 960 genes from the BMN 673 screen to those derived from the other PARP1/2 inhibitor screens. The genetic sensitization profile for BMN 673 was not significantly different than the profiles generated by olaparib, rucaparib or veliparib (Suppl Table 4). These findings, collected in a relatively un-biased fashion, suggested that BMN 673 had the potential to target cells with defects in any one of a number of HR/DSBR genes.

BMN 673 targets tumor cells with defects in homologous recombination

Although siRNA screens are a rapid and useful means of identifying multiple genetic determinants of drug sensitivity in an unbiased fashion, and in this case confirmed that a number of different HR/DSBR genes modulated the response to BMN673, the variable extent and stability of gene silencing achieved by siRNA often limits their ability to accurately define the scale of sensitization caused by a particular gene-drug combination. To formally assess the scale of selectivity of BMN 673 for tumor cells with HR gene defects, we measured the ability of BMN 673 to inhibit cells with either *BRCA1* or *BRCA2* gene mutations. We first tested the inhibitory effects of BMN 673 and other clinical PARP1/2 inhibitors in a panel of human tumor cell lines (Table 2). Models such as SW620 and MDA-MB-231 that do not have *BRCA* gene mutations or HR/DSBR defects were relatively resistant to BMN 673 with SF_{50}s of 0.13 μM and 1.85 μM respectively (Table 2). Likewise, non-transformed MRC-5 cells were also resistant to BMN 673. In contrast, tumor models that were either BRCA1 deficient (MX-1, SUM149) or BRCA2 deficient (Capan-1) were profoundly sensitive to BMN 673 (Table 2). PTEN deficiency (for example caused by N-terminal *PTEN* truncating mutations) has previously been shown to cause a defect in HR and sensitivity to other PARP1/2 inhibitors (19). We found BMN 673 to be highly potent in inhibiting human tumor cells with PTEN deficiency (Table 2). For example, the SF_{50} values for BMN 673 in the PTEN-null models MDA-MB-468, LNCap and PC-3 were 6, 3 and 4 nM respectively, values comparable with SF_{50}s in BRCA deficient models.

It was notable that compared to other clinical PARP1/2 inhibitors, BMN 673 was at least 18-fold more potent in BRCA-deficient tumor cells. In the *BRCA1* mutant, triple negative (ER, PR, HER2 –ve) breast tumor cell line model SUM149, BMN 673 delivered a SF_{50} of 8×10^{-12} M, 1×10^5 fold lower than that of veliparib (SF_{50} = 0.8 uM) and 922 and 231 times lower than that of rucaparib and olaparib respectively (Table 2). In contrast, the differences in PARP1/2 inhibitor SF_{50} between different PARP1/2 inhibitors in cells without HR/DSBR defects was significantly less (Table 2). To confirm the BRCA selectivity of BMN 673, we also assessed cell growth inhibition in isogenic models of BRCA deficiency, namely mouse ES cells carrying *BRCA1* gene defects (20) as well as human DLD1 tumor cells carrying homozygous *BRCA2* gene knockout (21). In both model systems, BMN 673 selectivity inhibited BRCA deficient cells and delivered a therapeutic window between BRCA proficient and BRCA deficient models at much reduced concentrations when compared to veliparib, rucaparib or olaparib (Figure 2B and 2C).

Compared with other PARP1/2 inhibitors, BMN 673 is about 3- to 8-fold more potent at inhibiting PARP1/2 enzymatic activities, but has a much greater potency advantage in inhibiting BRCA-deficient cells when used as a single agent (Table 2 and Figure 2). This raised the possibility that the ability of BMN 673 to inhibit these cells might be partially due to activities that are unrelated to PARP1/2 inhibition. To investigate this possibility, we compared the *in vitro* activities of BMN 673 and its *trans* isomer LT-00674, which, despite the structural similarity, had a greatly reduced PARP1/2 inhibitory activity when compared to BMN 673. There was a strikingly tight correlation between PARP1 enzyme inhibition and the ability to inhibit the BRCA2 deficient cancer cell line Capan-1 as well as the ability to sensitize tumor cells to temozolomide, another well-established property of PARP1/2

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inhibitors (Suppl Table 5). The correlation of the chiral selectivity strongly suggested that the potent inhibition of cancer cells is a direct effect of BMN 673 PARP inhibition.

BMN 673 induces DNA damage at pM concentrations

One working hypothesis that could explain the HR-selectivity of PARP1/2 inhibitors centres on their ability to cause a DNA lesion or lesions that inhibit the normal function of the replication fork (22). The frequency of stalled and damaged replication forks caused by PARP1/2 inhibitors can be monitored by estimating the formation of nuclear γH2AX foci (6, 23). We assessed the ability of BMN 673 to induce nuclear γH2AX foci formation, as measured by immunofluorescence and confocal microscopic imaging. We found that BMN 673 induced nuclear γH2AX foci at concentrations as low as 100 pM (Suppl Figure 1). In contrast, 100 nM of olaparib was required to elicit a similar γH2AX response, suggesting that the increased intrinsic potency of BMN 673 also resulted in an increased ability to elicit a DNA response biomarker.

Metabolism and PK properties of BMN 673

One of the objectives of our PARP inhibitor discovery program was to improve metabolic stability, PK properties and oral bioavailability over existing PARP1/2 inhibitors. *In vitro* metabolism studies of BMN 673 in liver microsomes from rats, dogs and humans demonstrated that BMN 673 had excellent liver microsome stability; more than 90% of BMN 673 remained after two hours of incubation at 1 μM concentration (Suppl Table 6). In rat PK studies, BMN 673 demonstrated oral bioavailability of >40% when dosed in 0.5% carboxylmethyl cellulose, and PK properties that would predict a human half-life that is sufficient to support a regimen of once daily administration (manuscript in preparation). *In vitro* studies assessing the potential for inhibition of human cytochrome P450 enzymes (CYP450s) showed that at concentrations up to 10 μM, BMN 673 did not inhibit any of the five major human hepatic CYP450 enzymes (CYP1A2, 2C9, 2C19, 2D6 and 3A4) (data not shown). Overall, BMN 673 demonstrated excellent metabolic stability, oral bioavailability and PK properties.

Anti-tumor effect of BMN 673 oral administration in xenograft tumor models

To assess the *in vivo* anti-tumor effects of BMN 673, when used as a single agent, we treated nude mice bearing established subcutaneous MX-1 tumor xenografts with BMN 673. MX-1 is a human mammary carcinoma cell line that harbors *BRCA1* deletion events and is BRCA1 deficient (24). Oral administration of BMN 673 for 28 days (once-a-day dose of 0.33 mg/kg), significantly inhibited the growth of MX-1 xenografts in mice, with four out of six mice achieving a complete response (CR, tumor impalpable) (Figure 3A). At the lower dose of 0.1 mg/kg, oral BMN 673 only had a small effect on tumor growth after extended treatment (>21 days, Figure 3A), but was still more effective than olaparib dosed orally at 100 mg/kg once daily. BMN 673 at these doses (0.33 mg/kg and 0.1 mg/kg) was well tolerated, with no animal lethality or significant weight loss observed after 28 consecutive, once daily, oral doses.

To assess the *in vivo* pharmacodynamics of BMN 673 (PARP inhibition), we gave MX-1 xenograft-bearing mice a single oral administration of 1 mg/kg BMN 673. Tumors were

harvested at 2, 8 and 24 hours post drug dosing, and intra-tumor PAR levels were determined using an anti-PAR ELISA. BMN 673 treatment drastically decreased intratumoral PAR levels at 2 and 8 hours following drug administration. Partial recovery of basal PAR levels was observed at 24 hours after dosing (Figure 3B), an effect probably due to the clearance of BMN 673. In comparison, a single oral administration of olaparib at 100 mg/kg produced a significant decrease of intratumoral PAR level at 2-hr post dosing, with partial recovery at 8 hours and complete recovery at 24 hours.

Subsequent studies that were designed to assess various intermittent dosing schedules of BMN 673 demonstrated little benefit in terms of tumor growth delay (data not shown), suggesting that continuous suppression of PARP might be required for a therapeutic effect. As PAR levels partially recovered in mouse xenograft tumors between 8 and 24 hours post administration of BMN 673 (Figure 3B), we reasoned that twice-a-day (BID) dosing might be more effective than a once-a-day (QD) dosing regimen. To test this hypothesis, we compared the anti-tumor effect of administration of BMN 673 at 0.33 mg/kg/dose QD for 28 days *vs.* 0.165mg/kg/dose BID for 28 days in MX-1 tumor bearing mice. The results of these studies indicated that both QD and BID dosing regimens inhibited MX-1 tumor growth with significant tumor regression (Figure 3C). QD treatment generated four CRs out of six animals, consistent with the previous experiment. However, while tumors in the QD-treated cohort did eventually re-establish after BMN 673 treatment ceased, the BID × 28 treatment schedule resulted in 6/6 CRs with no re-establishment of tumor until the end of the study, eight weeks after BMN 673 dosing ceased (Figure 3C). One of six mice treated with BID had significant weight loss (>20%), and was sacrificed on day 53. All other animals in the study tolerated the treatment well. Taken together with the results from the *in vivo* PAR inhibition study (Figure 3B), these results suggested that continuous suppression of PARP1/2 is required for maximum anti-tumor effect in the context of a single agent application, and that in mice, BID dosing of BMN 673 was necessary to achieve optimal therapeutic effect, perhaps by continuously suppressing PARP activity. It should be noted that the half-life of BMN 673 in human is expected to be much longer (due to slower metabolism) than in mice. Therefore, we anticipate that once-a-day dosing will be sufficient in human patients to continuously suppress PARP and show anticancer effect. The effectiveness of once-a-day oral dosing in human has been validated in Phase 1 clinical trials of BMN 673 (data to be published in ASCO 2013, Chicago).

The anti-tumor effect of BMN 673 on growth of PTEN null tumors was also examined *in vivo* (Suppl Figure 2). Two PTEN-null cell lines were established as subcutaneous xenograft models in nude mice, and treated with 0.33 mg/kg BMN 673 (oral, QDx28). Both tumor models responded well to BMN 673 treatment, resulting in tumor growth delay of 15.9 days (MDA-MB-468) and 22.8 days (LNCap) when compared to the vehicle-treated control cohort. In a separate study, we demonstrated that treatment with BMN 673 at 0.165 mg/kg/dose BIDx28 was slightly more effective than 0.33 mg/kg/dose QDx28 in the MDA-MB-468 xenograft model, consistent with the MX-1 tumor study results described earlier (Suppl. Figure 3).

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BMN 673 sensitizes tumor cells to DNA damaging chemotherapies

The chemosensitizing property of PARP1/2 inhibitors has been well documented(25). One of the most robust chemopotentiation effects involves the combination of temozolomide (TMZ) with PARP1/2 inhibitors (26-29). To investigate whether BMN 673 shared this characteristic, we tested its ability to potentiate the cytotoxic effect of TMZ using an *in vitro* assay. We found that single agent TMZ exposure (200 μM) resulted in approximately 15% cell growth inhibition after a five-day treatment in LoVo tumor cells (Figure 4A). Combining BMN 673 with 200 μM TMZ resulted in a significant potentiation of TMZ cytotoxicity (Figure 4A and Table 1). We also examined the effect of BMN 673 on SN-38, active metabolite of the DNA topoisomerase I inhibitor irinotecan. BMN 673 potentiated the cytotoxic effect of SN38 in MX-1 cells *in vitro* in a dose dependent manner (Figure 4B).

We observed a clearly additive effect of combining BMN 673 with platinum drugs *in vitro* (data not shown). The ability of BMN 673 to potentiate platinum drugs *in vivo* was also readily demonstrated. We first examined the effect of BMN 673 on cisplatin-induced anti-tumor effect. Growth of MX-1 tumor xenografts implanted subcutaneously in female athymic mice was significantly inhibited in a dose-dependent manner when animals were treated with BMN 673 in combination with a 6 mg/kg intraperitoneal (IP) injection of cisplatin (Figure 4C). However, we did note that the combination regimens involving cisplatin resulted in moderate body weight loss. Maximum average weight loss of 11%, 6%, 5% and 3% were observed for groups that contained BMN 673 doses of 1, 0.33, 0.1 and 0.033 mg/kg, respectively. In the same experiment, animals that were treated with cisplatin alone had maximum average body weight loss of 3%. One animal died in the BMN 673 1 mg/kg plus cisplatin group on Day 20, while most animals recovered their body weight after treatment terminated. Olaparib at 100 mg/kg also demonstrated activity in this treatment regimen, with a maximum body weight loss of 3%. In a separate study, BMN 673 showed significant potentiation of carboplatin anti-tumor effect *in vivo* (Figure 4D). No animal lethality or significant body weight loss was observed.

Discussion

Here, we describe a novel, potent and selective PARP1/2 inhibitor BMN 673. Like other PARP1/2 inhibitors, BMN 673 is selective for tumor cells with defects in HR, as shown by a siRNA drug sensitivity profile that encompasses genes involved in HR/DSBR and by assessment in isogenic and non-isogenic models of BRCA1, BRCA2 and PTEN deficiency. These effects were also observed *in vivo* in mouse xenograft models where tumors with either *BRCA1* or *PTEN* defects showed significant tumor growth delay after oral administration of BMN 673. Furthermore, the anti-tumor effects of temozolomide, cisplatin and carboplatin were all potentiated by BMN 673. These therapeutic effects were achieved with tolerable toxicity, evidence of PAR inhibition *in vivo* in animal tumor models and favorable PK properties that allow once-a-day oral dosing in human patients.

With Ki of 1.2 nM and 0.9 nM against PARP-1 and -2, respectively, BMN 673 is the most potent PARP inhibitor reported to date. Still, we were initially surprised by the much greater cytotoxicity in HR-deficient cells compared with other PARP1/2 inhibitors that have apparently comparable potency against PARP catalytic activity. We initially suspected that

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BMN 673 might have activity other than PARP1 and -2 inhibition and, that this activity may be responsible for or contributing to the increase in tumor cell inhibition. However, chiral selectivity (Suppl. Table 5) shown by the BMN 673 / LT-00674 isomer pair strongly suggests that its remarkable cytotoxic properties in HR/DSBR deficient cells is very likely a direct result of its ability to inhibit PARP1/2.

BMN 673 demonstrated a notable preference for tumor cells harboring BRCA1, BRCA2 or PTEN dysfunction. Whereas other PARP inhibitors such as veliparib, rucaparib and olaparib showed selectivity in HR-deficient cells *vs.* HR-proficient cells when used at micromolar concentrations, the selectivity margin of BMN 673 could be achieved with nanomolar or even picomolar concentrations in isogenic models. In some non-isogenic systems (Table 2), the therapeutic window between BRCA deficient and proficient models was enhanced compared to other clinical PARP1/2 inhibitors (Figure 2). The siRNA screen also confirmed that, out of 960 genes tested, the most profound effects on BMN 673 sensitivity were caused by siRNAs targeting genes involved in HR/DSBR. Although a two-sided t-test did not show statistically significant differences between BMN 673 and other PARP inhibitors in their sensitization profiles (Suppl. Table 4), it is intriguing to note that silencing of DSBR genes BRCA1, BRCA2, PNKP, ATR, CHEK1 and *PALB2* resulted in sensitization for BMN 673 (Suppl. Table 3) at a low nM dose of the drug. This may generate a large therapeutic window for BMN 673, making it potentially more efficacious while maintaining manageable toxicity.

PARP1/2 inhibitors are an exciting new class of anticancer agents that exploit the biological concept of synthetic lethality as the basis for their anti-tumor selectivity (6, 7). However, the clinical use of PARP1/2 inhibitors is still in its infancy. Despite promising Phase I and Phase II trial results with olaparib, rucaparib and niraparib (4, 8-11) further development has been slow with problems such as toxicity when combined with chemotherapeutic agents (30, 31), difficulty in defining suitable patient populations beyond those with *BRCA1* or *BRCA2* mutations (32), or formulation issues. However, given the effectiveness of PARP1/2 inhibitors in patients with germ-line *BRCA* mutations (8-10), this area is still ripe for further study and exploration. Two phase I clinical trials are currently on-going for BMN 673 that are assessing its safety, pharmacokinetic and pharmacodynamic properties and preliminary efficacy in human patients. In January 2011, a first in human, single-arm, open-label study in patients with advanced tumors with DNA-repair pathway deficiencies was initiated followed by a two-arm, open-label study in patients with advanced hematological malignancies, which was started in June 2011. The discovery and characterisation of BMN 673, as a potent, selective, orally bioavailable PARP1/2 inhibitor and its advancement into Phase I studies thus provides a welcome addition to this field.

Materials and Methods

Drugs and cell lines

Synthesis of LT-00628, BMN 673, LT-00674 and LT-00878 is described elsewhere (33, 34). Olaparib, rucaparib and veliparib used in this study were either synthesized as previously described (35-37) or obtained from Selleck Chemicals. Drug stock solutions were prepared in DMSO and stored in aliquots at −20°C. Drugs (alone or in combination) were added to cell cultures so that final DMSO concentrations were constant at 1% (v/v). MX-1 was

obtained from National Cancer Institute (Bethesda, MD). All other cell lines were obtained from ATCC and maintained as exponentially growing monolayers according to the supplier's instructions.

PARP enzyme assays

The ability of a test compound to inhibit PARP-1 enzyme activity was assessed using Trevigen's PARP Assay Kit (Trevigen CAT#4676-096-K) following manufacturer's instruction. IC_{50} values were calculated using GraphPad Prism5 software. For PARP inhibitor Ki determination, enzyme assays were carried out in 96-well FlashPlate (Perkin Elmer) with 0.5 unit PARP1 enzyme (Trevigen, Cat#4667-250-EB), 0.25x activated DNA (Trevigen), 0.2 μCi [^{3}H] NAD (Perkin Elmer, Cat#NET443H250UC) and 5 μM cold NAD (Sigma) in a final volume of 50 μL reaction buffer containing 10%glycerol(v/v), 25 mM Hepes, 12.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT and 0.01% NP-40(v/v), pH 7.6. Reactions were initiated by adding NAD to the PARP reaction mixture with or without inhibitors and incubated for 1 min at room temperature. 50 μL of ice-cold 20% TCA was then added to each well to stop the reaction. The plate was sealed and shaken for a further 120 min at RT, followed by centrifugation. Radioactive signal bound to the FlashPlate was determined using TopCount. PARP1 Km was determined using Michaelis–Menten equation from various substrate concentrations (1-100 μM NAD). Compound Ki was calculated from enzyme inhibition curve according to the formula: Ki = IC50/(1+[substrate]/Km). Km for PARP2 enzyme and compound Ki were determined with the same assay protocol except 30 ng PARP2 (BPS Bioscience, Cat#80502), 0.25x activated DNA, 0.2 μCi [^{3}H] NAD and 20 μM cold NAD were used in the reaction for 30min at room temperature.

Biacore binding assay

Recombinant human PARP1 (rhPARP1) catalytic domain (residues 662 – 1011) with N-terminal 6XHis-tag was generated in house at BioMarin Pharmaceutical Inc. and used in binding assay for PARP inhibitor interaction using Biacore T200 (GE Healthcare). rhPARP1 was immobilized on a CM5 sensor chip (GE Healthcare) by amine coupling method. Briefly, one flow cell of a CM5 chip was first activated by a 7-min injection at 10 μL/min of freshly prepared 50 mM NHS: 200 mM EDC (1:1, GE Healthcare) at rate of 10 μL/min. Then rhPARP1 (100 μg/mL, in 10 mM MES pH 6.5) was injected onto the flow cell for 60-sec at 10 μL/min. The remaining active coupling sites were blocked with a 7-min injection of 1M ethanolamine at 10 μL/min. The immobilization buffer contains 10 mM Hepes pH 7.4, 150 mM NaCl, 0.05% Surfectant P20, 5 mM $MgCl_2$, and 0.5 mM TCEP (tris(2-carboxyethyl)phosphine). The immobilization level was ~7600 RU. For binding kinetics measurement, PARP inhibitors at increasing concentrations (12.5, 25, 50, 100, 200 nM) were injected over the chip surface for 60 sec per injection. The exposure was followed by a dissociation phase of 3600 sec in running buffer (immobilization buffer + 1% DMSO) after the last injection. The flow rate was 50 μL/min. After sensorgrams were corrected for signals from a reference flow, kinetics was calculated with Biacore T200 evaluation software ver.1.0 (Biacore, GE Healthcare).

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Intracellular PAR formation assay

Cellular PAR synthesis assay assesses the ability of a test compound to inhibit polymerization of PAR. LoVo human colorectal tumor cells grown in 96-well microtiter plates overnight were pre-treated with increasing concentrations of PARP inhibitors for 30 min before H_2O_2 was added at a final concentration of 50 mM. After a 5-min treatment at room temperature, cells were fixed for 10 minutes with pre-chilled methanol/acetone(7:3) at −20 °C. Fixed cells were incubated with anti-PAR monoclonal antibody (Trevigen) for 60 min, followed by incubation with FITC coupled goat anti-mouse IgG (diluted 1:100) and 1 μg/mL DAPI for 60 min. FITC signal was normalized with DAPI signal, and EC_{50} values were calculated using GraphPad Prism.

siRNA screen

CAL51 cells plated in 96-well plates were transfected 24 h later with siRNA (final concentration 100 nM), using Oligofectamine (Invitro- gen, USA) as per manufacturer's instructions. At 48h after transfection, three replica plates were treated with 0.01% (v/v) dimethylsulphoxide (DMSO) vehicle in media and three replica plates with each PARP1/2 inhibitor in media. Cell viability was assessed after five days of PARP1/2 inhibitor exposure using CellTiter-Glo Luminescent Cell Viability Assay (Promega, USA) as per manufacturer's instructions. Data from each screen was analyzed as described previously(18).

Colony formation survival assays

Colony formation assays were performed as described previously (6). In brief, cells were seeded into 6-well plates at a concentration of 500-2000 cells/well. After 24 hours, media was replaced with fresh media containing PARP1/2/ inhibitor. This procedure was repeated twice weekly for 14 days, at which point colonies were fixed with trichloroacetic acid (TCA) and stained with sulforhodamine B. Colonies were counted and Surviving Fractions calculated by normalizing colony counts to colony numbers in vehicle treated wells. Survival curves were plotted using a four-parameter logistic regression curve fit.

Single agent cytotoxicity and chemo-sensitization assays

In single-agent assays, cells are seeded in a density that allows linear growth for 10-12 days in 96-well plates (typically 500-3000 cells/well). Cells were treated in their recommended growth media containing increasing concentrations of PARP inhibitors for 10-12 consecutive days (media changed with fresh compounds every 5 days). In chemo-sensitization assays, PARP inhibitors at various concentrations were either combined with 200 μM Temozolomide (TMZ) to treat LoVo cells or with SN-38 (0-10 nM), an active metabolite of Irinotecan to treat MX-1 cells for 5 days. After treatments, cell survival was determined by CellTiter Glo assay (Promega), expressed as relative to mock treatment control (0.1-0.5% DMSO), and IC_{50} or GI_{50} values were calculated using GraphPad Prism5 software.

Confocal microscopy

Cells were seeded on coverslips placed in 6-well plates and after 24 hours treated with several concentrations of olaparib or BMN 673. 24 hours after treatment the cells were fixed

in 10% formalin (3.7% PFA) for 1 hour. Cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes, treated with 50 μL DNase I (Roche) (diluted 1/10 in PBS) for 1 hour at 37°C and then blocked with IFF (PBS + 1% BSA and 2% FBS followed by filter sterilization) for 1 hour. The coverslips were then incubated with rabbit anti-γH2Ax primary (Millipore) and mouse anti-RAD51 primary (Epitomics Lot YI031608C) (both 1:1000 in 50μL IFF) overnight at 4°C. The next day cells were incubated with anti-mouse Alexafluor 546 secondary and anti-rabbit Alexafluor 488 secondary (both 1:1000 in 50μL IFF) for one hour. Cells were then washed in PBS containing DAPI 1:10.000 for 10 minutes and attached on glass plates using Vectashield and nail polish. A minimum of four pictures were made of each coverslip using the Leica confocal microscope, and cells were subsequently counted. At least 100 cells were assessed per coverslip, being positive for γH2Ax if they had more than 5 foci per nucleus. The percentage of positive cells was plotted.

Xenograft experiments

Female athymic nu/nu mice (8-10 week old) were used for all *in vivo* xenograft studies. Mice were quarantined for at least 1 week before experimental manipulation. Exponentially growing cells (LNcap, MDA-MB-468) or *in vivo* passaged tumor fragments (MX-1) were implanted subcutaneously at the right flank of nude mice. When tumors reached an average volume of ~150 mm^3, mice were randomized into various treatment groups (6-8 mice/group) in each study. Mice were visually observed daily and tumors were measured twice weekly by calliper to determine tumor volume using the formula [length/2] × [$width^2$]. Group median tumor volume (mm^3) was graphed over time to monitor tumor growth. In single agent studies, olaparib (100mg/kg), BMN 673 (various doses as indicated), or vehicle (10% DMAc, 6% Solutol and 84% PBS) was administered by oral gavage (p.o.), once daily or BMN 673 (0.165 mg/kg) twice daily for 28 consecutive days. Mice were continuously monitored for 10 more days after last day of dosing. In cisplatin combination study, BMN 673, olaparib, or vehicle was administered p.o. once daily for 8 days starting on day 1. Cisplatin at a dosage of 6 mg/kg or its vehicle (saline) was administered intra-peritoneally (i.p) as a single injection on day 3, 30 minutes after PARP inhibitor was administered. Combination with carboplatin was conducted in a similar way in MX-1 model in which BMN 673 was administered p.o. once daily for either 8 days or 5 days and carboplatin was injected i.p. at single dose of 35 mg/kg, 30 min after BMN 673 on day 3.

PAR assay *in vivo*

MX-1 tumor xenografts were prepared as described in methods. When tumors reached an average volume of ~150 mm^3, olaparib (100 mg/kg), BMN 673 (1 mg/kg) or vehicle was administered in a single p.o. dosing. Tumors were harvested at 2, 8 and 24 hours after drug dosing, snap frozen in liquid N_2. Tumor tissue was then homogenized in PBS on ice and extracted with lysis buffer (25mM Tris pH 8.0, 150mM NaCl, 5mM EDTA, 2mM EGTA, 25mM NaF, 2mM Na3VO4, 1mM Pefabloc, 1% Triton X-100, and protease inhibitor cocktail) containing 1% SDS. Levels of PAR in the tumor lysates were determined by ELISA using PARP *in vivo* PD Assay II kit (Trevigen).

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Statement of Translational Relevance

PARP1/2 inhibitors are a class of anticancer drugs that target tumor-specific defects in DNA repair. Here, we describe a novel PARP1/2 inhibitor, BMN 673, which shares features such as tumor selectivity with existing inhibitors but has strikingly increased anti-tumor potency and markedly improved pharmacokinetic attributes. The anticancer activity and selectivity of existing PARP1/2 inhibitors has been proven in early proof-of-concept clinical trials where patient benefit has been seen with limited toxicity. However, research to define patient selection, scheduling and whether these agents should be used in combination with other anticancer drugs is still on-going. BMN 673 is already being assessed in clinical trials and represents an exciting new PARP1/2 inhibitor at a time when optimal clinical use of these agents is being established.

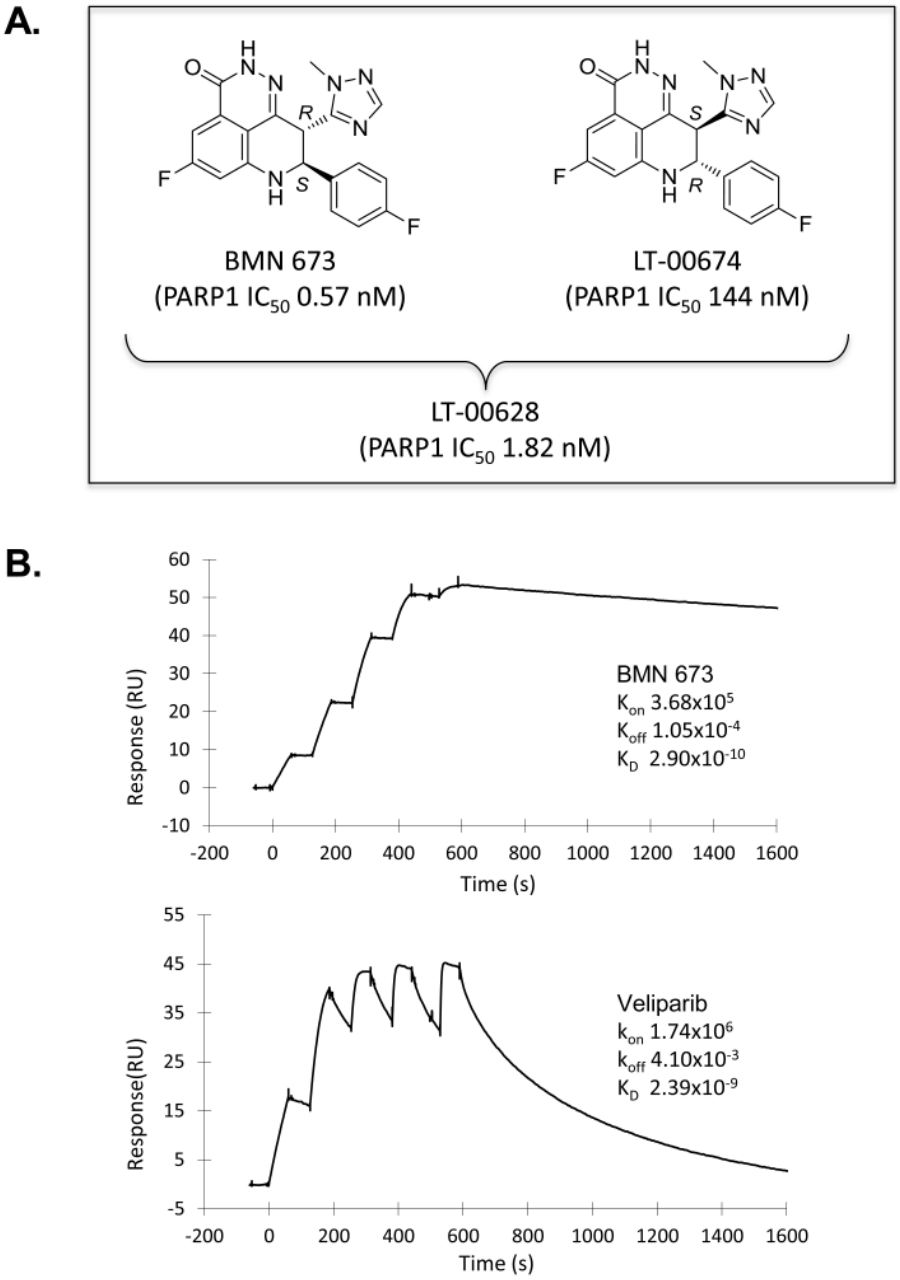

Figure 1. BMN 673 is a potent PARP inhibitor

A) Structure and PARP1 IC_{50} of BMN 673, its corresponding isomer LT-00674, and the racemic mixture LT-00628. **B)** Biacore T200 sensorgrams of BMN 673 and Veliparib binding to immobilized recombinant human PARP1. Top: BMN 673 binding sensorgram, bottom: veliparib. Each compound was injected at increasing concentrations (12.5, 25, 50, 100, 200 nM, 60 sec per injection) over a chip surface that was pre-coated with recombinant PARP1. The on-rates, off-rates and dissociation constants (K_D) were determined as described in Materials and Methods.

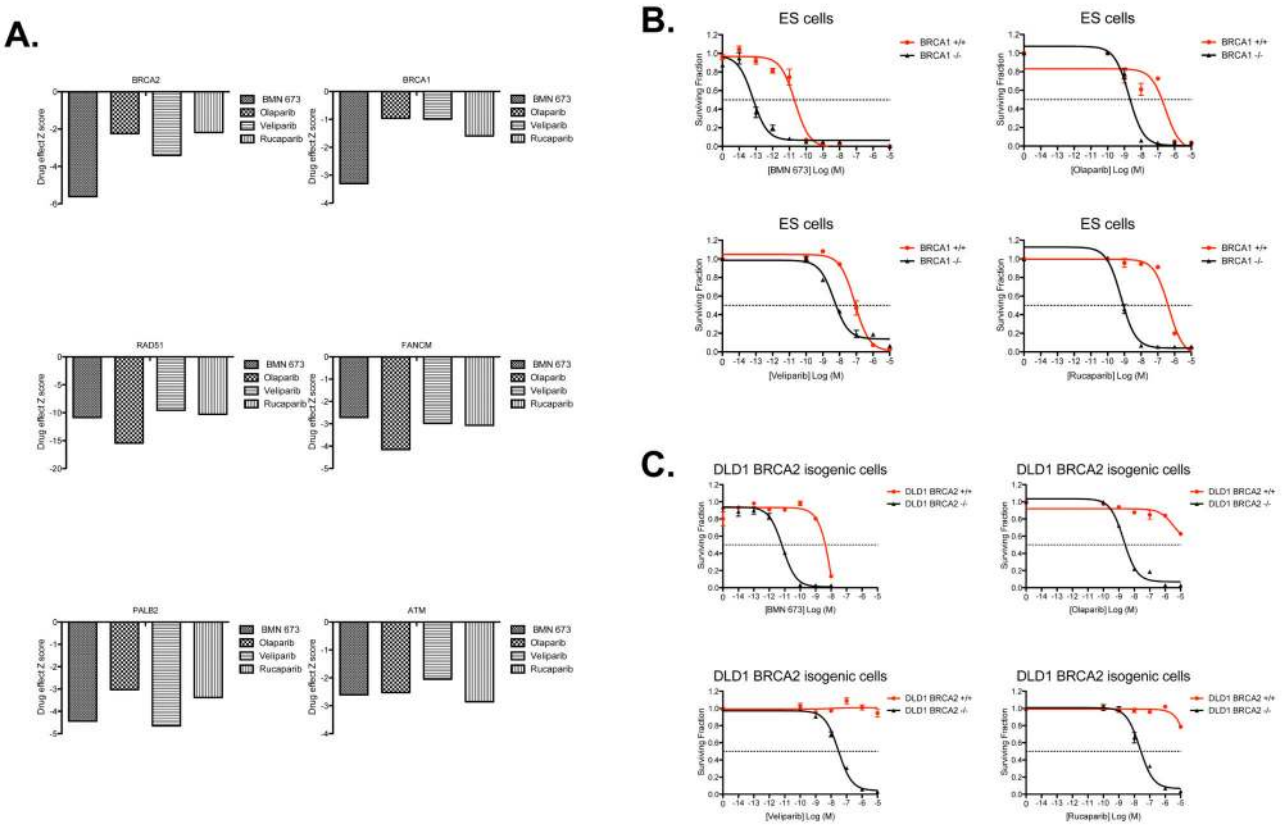

Figure 2.

A) siRNA targeting HR genes sensitize to PARP1/2 inhibitors. CAL51 cells were transfected with a library of siRNAs and treated with BMN 673, olaparib, veliparib or rucaparib. Each drug was used at its respective SF_{80} concentration. The effect of each siRNA on drug sensitization was quantified by the calculation of a Drug Effect (DE) Z score. Out of 960 genes, those involved in HR-mediated DNA repair were a prominent feature of the sensitivity profile of all PARP1/2 inhibitors. DE Z scores for *BRCA1 BRCA2, ATM, FANCM, RAD51* and *PALB2* are shown here. **(B and C) BMN 673 is selectively toxic to BRCA1 or BRCA2 deficient cells.** Dose response curves from clonogenic survival assays in a variety of isogenic models are shown. The response of BRCA1 deficient and proficient ES cells (generated by gene targeting) are shown as are the responses in human DLD1 tumor cell lines with *BRCA2* gene targeted alleles. Cells were exposed to different PARP1/2 inhibitors as shown for 10-14 days, after which surviving colonies were counted and Surviving Fractions calculated by normalizing surviving colony numbers to colony numbers in control (vehicle-treated) cells.

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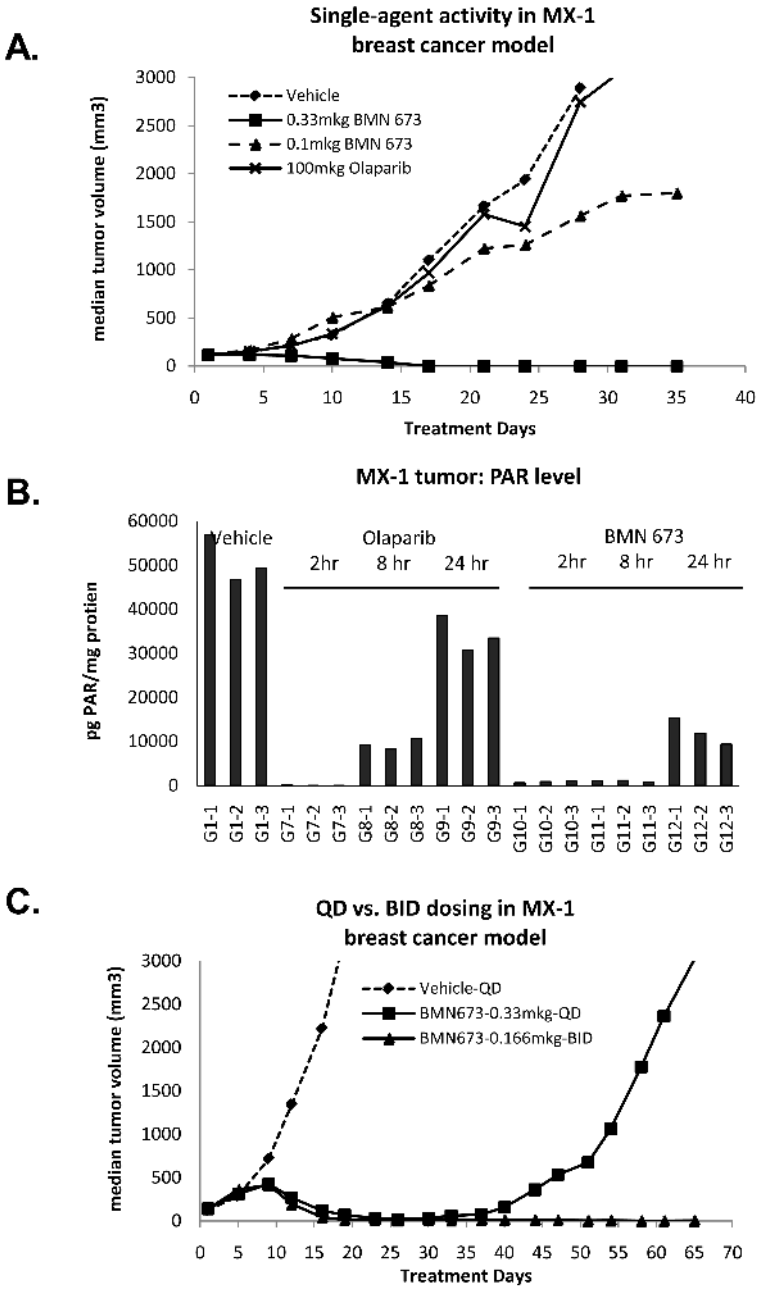

Figure 3. BMN 673 exhibits anti-tumor activity against a BRCA mutant tumor model in mice

A) MX-1 human mammary xenografts were inoculated subcutaneously in female athymic nu/nu mice. When tumors reached an average volume of ~150 mm^3 (range 100-196 mm^3), mice were randomized into various treatment groups, and were treated orally, once-daily for 28 consecutive days, with BMN 673 (0.33 or 0.1 mg/kg/day), olaparib (100 mg/kg/day) or empty vehicle. Median tumor volume was plotted against days of treatment (first day of treatment is defined at Day1). **B)** Inhibition of PARP activity by a single oral dose of BMN 673 (1 mg/kg) was demonstrated *ex vivo* by measuring MX-1 tumor PAR levels at 2, 8 hours

and the inhibition was partially recovered 24 hours after dosing. Intratumoral PARP inhibition was also observed with olaparib at 100 mg/kg oral administration, but the effect was much shorter. Each bar represents an individual tumor from an individual animal. **C)** BMN 673 is more effective in mouse xenograft models with 0.165mg/kg/dose twice-a-day dosing than 0.33 mg/kg/dose once-a-day dosing. In the MX-1 model, not only did all 6 mice treated with 0.165mg/kg/dose 2X/day regimen reached complete response, but also none of the mice had tumor re-growth until the end of the study, eight weeks after BMN 673 dosing stopped. Median tumor volume was plotted against days of treatment (first day of treatment is defined at Day1).

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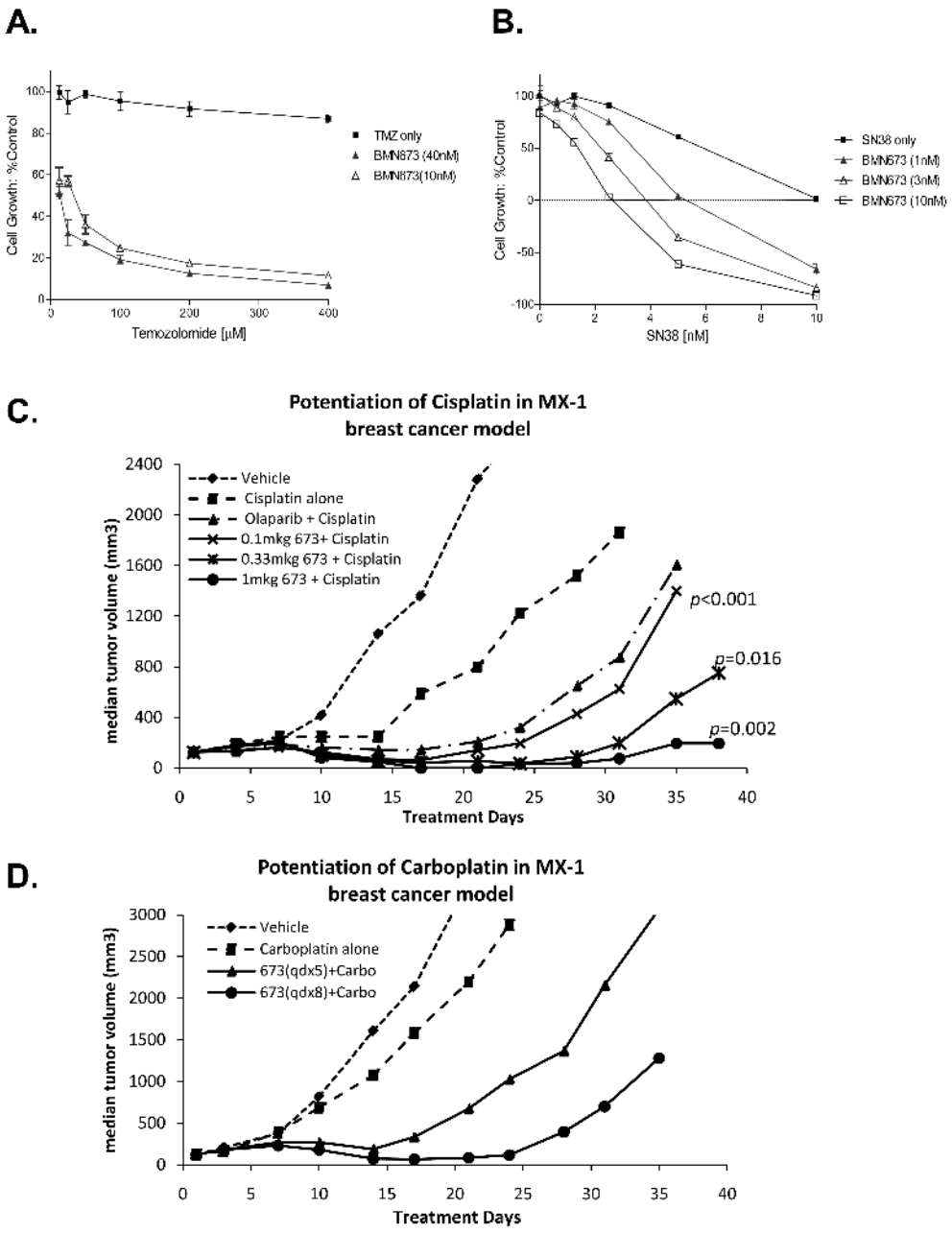

Figure 4. BMN 673 potentiates the effects of DNA-damaging cytotoxic agents

A) LoVo cells were treated with BMN 673 and temozolomide either alone or in combination for 5 days. Surviving fraction was determined using CellTiterGlo assay. BMN 673 significantly increased the cytotoxicity of temozolomide in LoVo cells. **B)** BMN 673 sensitized MX-1 cells to SN-38 cytotoxicity in a dose dependent fashion. **C)** Nude mice carrying MX-1 tumor xenografts were treated with oral administration of BMN 673, olaparib, or vehicle once-a-day from day 1 to 8. Cisplatin was dosed intraperitoneally at 6 mg/kg on the third day of PARP inhibitor treatment. While 0.1, 0.33 and 1 mg/kg of BMN

673 showed dose-dependent sensitization of the cisplatin, 100 mg/kg olaparib showed a sensitizing effect that is equivalent to the lowest dose of BMN 673. **D)** Once-a-day dosing of BMN 673 for 8 days potentiated carboplatin more than 5 daily dosing, which in turn generated tumor growth inhibition more than carboplatin alone. BMN 673 was dosed orally, once-a-day for either 5 or 8 days starting on Day 1, at a dosage level of 0.33 mg/kg. Carboplatin at a dosage of 35 mg/kg or its vehicle (saline) for the control group was administered intraperitoneally on Day 1, half an hour after BMN 673 administration. Median tumor volume was plotted against days of treatment (first day of treatment is defined at Day 1).

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Table 1

Summary of BMN 673 *in vitro* activities

Activities of BMN 673, LT-00628, and three clinical PARP1/2 inhibitors veliparib, rucaparib and olaparib, were compared in four *in vitro* assays. 1) Concentration for 50% inhibition in PARP1 enzyme assay (IC_{50}); 2) Concentration for 50% inhibition in Cellular PAR synthesis assay in LoVo cells (EC_{50}); 3) Concentration for 50% Capan-1 cell survival reduction in single-agent cytotoxicity assay (IC_{50}), and 4) Concentration that, when combined with 200 μM of temozolomide, resulted in 50% growth inhibition of LoVo cells in temozolomide potentiation assay (GI50). Assay conditions are described in the Materials and Methods. Values are average data from three to four independent experiments.

	PARP1 Enzyme Inhibition IC_{50} (nM)	Cellular PAR Synthesis EC_{50} (nM)	Capan-1 Cytotoxicity IC_{50} (nM)	Temozolomide Potentiation GI_{50} (nM)
Veliparib	4.73	5.9	>10,000	6203
Rucaparib	1.98	4.7	609	144
Olaparib	1.94	3.6	259	237
LT-00628	1.82	4.5	8	5
BMN 673	0.57	2.5	5	3

Table 2

Selective killing of tumor cells with BRCA1, BRCA2 or PTEN mutations

Cultured human tumor cell lines were treated with veliparib, rucaparib, olaparib or BMN 673, and tumor-killing effects were assessed either by colony formation assays (SUM149) or by 2-D cytotoxicity assays (all other cell lines). Survival curves were plotted and the IC_{50} was calculated as the concentration required to kill 50% of the cells. Known deficiency of HR DNA repair genes is indicated in parenthesis. Cells with *BRCA1/BRCA2* mutations or PTEN deficiency were more sensitive to all PARP inhibitors than cells lacking these mutations. (ND = not determined).

	SF_{50} (μM)								
	MX-1 (BRCA1 deficient)	**SUM149 (BRCA1 deficient)**	**Capan-1 (BRCA2 deficient)**	**MB-468 (PTEN deficient)**	**LNCap (PTEN deficient)**	**PC-3 (PTEN deficient)**	**SW620**	**MDA-MB-231**	**MRC-5 (Normal)**
Veliparib	ND	0.818	>10	ND	ND	ND	ND	ND	>10
Rucaparib	0.0053	0.0079	0.609	0.220	0.737	0.293	ND	5.53	8.53
Olaparib	0.0232	0.0198	0.259	0.368	0.589	0.787	ND	6.41	5.83
BMN 673	0.0003	8.57E-6	0.005	0.006	0.003	0.004	0.13	1.85	0.31