Cyclin-Dependent Kinase Inhibitor AT7519 as a Potential Drug for MYCN-Dependent Neuroblastoma

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Abstract

Purpose: MYCN-dependent neuroblastomas have low cure rates with current multimodal treatment regimens and novel therapeutic drugs are therefore urgently needed. In previous preclinical studies, we have shown that targeted inhibition of cyclin-dependent kinase 2 (CDK2) resulted in specific killing of MYCN-amplified neuroblastoma cells. This study describes the in vivo preclinical evaluation of the CDK inhibitor AT7519.

Experimental Design: Preclinical drug testing was performed using a panel of MYCN-amplified and MYCN single copy neuroblastoma cell lines and different MYCN-dependent mouse models of neuroblastoma.

Results: AT7519 killed MYCN-amplified neuroblastoma cell lines more potently than MYCN single copy cell lines with a median IC50 value of 1.7 compared to 8.1 µmol/L (P = 0.0053) and a significantly stronger induction of apoptosis. Preclinical studies in female NMRI homozygous (nu/nu) mice with neuroblastoma patient-derived MYCN-amplified AMC711T xenografts revealed dose-dependent growth inhibition, which correlated with intratumoral AT7519 levels. CDK2 target inhibition by AT7519 was confirmed by significant reductions in levels of phosphorylated retinoblastoma (p-Rb) and nucleophosmin (p-NPM). AT7519 treatment of Th-MYCN transgenic mice resulted in improved survival and clinically significant tumor regression (average tumor size reduction of 86% at day 7 after treatment initiation). The improved efficacy of AT7519 observed in Th-MYCN mice correlated with higher tumor exposure to the drug.

Conclusions: This study strongly suggests that AT7519 is a promising drug for the treatment of high-risk neuroblastoma patients with MYCN amplification. Clin Cancer Res; 21(22); 5100–9. ©2015 AACR.

Introduction

Neuroblastomas are pediatric tumors that originate from the developing sympathetic nervous system. Current treatment regimens for high-risk tumors comprise a combination of high-dose cytostatics, radiotherapy, surgery, myeloablative therapy with stem cell reinfusion, long-term maintenance therapy with retinoic acid, and immunotherapy using anti-disialoganglioside 2 antibodies. Despite this multimodal treatment strategy, the overall survival of high-risk neuroblastoma patients is still only below 50%. Especially patients with MYCN-dependent tumors have a very poor prognosis. Recent studies have shown that tumors with enhanced MYCN pathway activity not only include MYCN-amplified tumors, but also neuroblastoma tumors with MYCN overexpression, implying that 50% of all high-risk neuroblastoma patients suffer from MYCN-dependent tumors (1).

Synthetic lethality has been widely explored as a mechanism to target oncogenic mutations, the clinical potential of which is exemplified by the use of PARP inhibitors in the treatment of tumors with mutated BRCA tumor suppressor genes (2–4). Synthetic lethality is particularly useful when the activity of a given oncoprotein cannot be directly targeted, as is the case with the myelocytomatosis viral oncogene homolog (MYC) family of oncoproteins (c-MYC, MYCN, and MYCL). Screening efforts have led to the identification of aurora kinase B (AURKB), casein kinase 1 epsilon (CSK1E), checkpoint kinases 1 and 2 (CHEK1, CHEK2), ataxia telangiectasia and Rad3-related (ATR), and several cyclin-dependent kinases (CDKs) as synthetic lethal with aberrant expression of MYC oncoproteins (5–8).

Previously, we described that silencing of CDK2 led to induction of apoptosis in neuroblastoma cells expressing high levels of MYCN (9). These findings were confirmed with the Cdc2/CDK2/...
CDK2 inhibitor roscovitine (seliciclib), but unfavorable pharmacokinetic characteristics (i.e., short elimination half-life and rapid metabolic deactivation; refs. 10, 11) led to failure to sufficiently inhibit CDK2 activity in neuroblastoma xenograft models. Recently, a number of clinical-candidate inhibitors that target CDK2 have been developed, including BMS-387032 and AT7519, with IC₅₀ values of 48 and 47 nmol/L, respectively, for CDK2 (12, 13). In addition to CDK2, AT7519 also displays activity towards other CDKs [i.e., CDK1, CDK4, CDK5, CDK6, and CDK9 (as well as to a lesser extent CDK3 and CDK7 and glycogen synthase kinase 3 beta (GSK-3β)]. AT7519 was identified by Astex using the Pyramid fragment-based drug discovery platform (13), and is the most extensively clinically evaluated inhibitor of CDK2. Currently, phase II clinical trials of AT7519 are ongoing for chronic lymphocytic leukemia, mantle cell lymphoma, and multiple myeloma (http://clinicaltrials.gov), and our previous results suggested potential efficacy for the treatment of high-risk neuroblastoma. In the current study, we explored the potential of AT7519 for inhibiting MYCN-dependent neuroblastoma in vitro and in vivo.

Materials and Methods

Chemicals

AT7519 (>98%) was kindly provided by Astex. Etoposide, vincristine, cisplatin, topotecan, doxorubicin, irinotecan, and temozolomide were purchased from Sigma Aldrich. For in vitro studies, AT7519 was formulated in saline in final concentrations of 1.5 and 1.875 mg/mL.

Cell culture

Classical human neuroblastoma cell lines and neuroblastoma tumor-initiating cell (TIC) lines were cultured as previously described (14, 15). Cell culture protocols are described in detail in the Supplementary Materials and Methods.

IC₅₀ and LC₅₀

Cells were seeded in triplicate in 96-well plates using the most optimal confluency for each cell line (Supplementary Table S1). Cells were incubated overnight and treated with 0.64 nmol/L to 10 μmol/L AT7519 using 5-fold dilution steps. Control samples were treated with 0.1% DMSO. Cell viability was established prior to and at 72 hours after treatment using the MTT colorimetric assay (16). Half maximal effective concentration (IC₅₀) and half lethal concentration (LC₅₀) values were derived from dose-response curves. IC₅₀ values at 72 hours were calculated by determining the AT7519 concentrations needed to achieve a 50% reduction in cell viability observed for DMSO-treated cells at 72 hours (set at 100%). LC₅₀ values at 72 hours were calculated by determining the AT7519 concentrations needed to achieve a 50% reduction in the cell viability at 0 hour.

Western blotting

The following antibodies were used: mouse anti-human Rb (clone G3-243) monoclonal antibody (1:1,000, BD Biosciences); rabbit anti-human p-Rb (Thr187) polyclonal antibody (1:1,000, BioSource International); mouse anti-human MYCN (clone B8.4B) monoclonal antibody (1:5,000, BD Biosciences); rabbit anti-human PARP polyclonal antibody (1:2,000, Cell Signaling Technology); rabbit anti-human NPM polyclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human p-NPM (Thr199) polyclonal antibody (1:500, Cell Signaling Technology); rabbit anti-human β-actin (clone 13E5) monoclonal antibody (1:1,000, Cell Signaling Technology); mouse anti-human β-actin (clone AC-15) monoclonal antibody (1:20,000, Abcam); mouse anti-human α-tubulin (clone DM1A) monoclonal antibody (1:1,000, Cell Signaling Technology) and IRDye 800CW goat anti-rabbit and goat anti-mouse secondary antibodies (1:5,000, Li-COR). See Supplementary Materials and Methods for a detailed protocol.

Immunohistochemistry

The following antibodies were used: rabbit anti-human NPM polyclonal antibody (1:1,000, Cell Signaling Technology); rabbit anti-human p-NPM (Thr199) polyclonal antibody (1:400, Cell Signaling Technology); rabbit anti-human Ki-67 (clone SP6) monoclonal antibody (1:1,000, Thermo Scientific), rabbit anti-human cleaved caspase-3 (Asp175) polyclonal antibody (1:100, Cell Signaling Technology), and BrightVision horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (1:5,000, Li-COR). See Supplementary Materials and Methods for a detailed protocol.

FACS analysis

Cells were treated with 0.1% DMSO (control), 150 nmol/L AT7519 (100 nmol/L for IM3R2) or AT7519 concentrations equal to the IC₅₀ for each individual cell line (see Supplementary Table S1 for the IC₅₀ values). After 72-hour treatment, floating and adherent cells were harvested for FACS analysis to determine the cell-cycle distribution and the apoptotic sub-G₁ fraction. See Supplementary Materials and Methods for a detailed protocol.

In vivo efficacy in neuroblastoma xenograft mouse models

Female NMRI nu/nu mice (6–15 weeks old; 20–30 g) were obtained from Harlan and experiments were performed with permission from and according to the standards of the Dutch animal ethics committee (DEC 102389 and 102690). NMRI nu/nu mice were subcutaneously injected with 1 × 10⁶ cells/flank of AMC711T or KCNR. The size of the tumors was recorded twice weekly and when tumors reached a size of approximately 1,000 mm³, tumor pieces were serially xenotransplanted in recipient mice. Parts of the xenotransplanted tumors were analyzed by FACS as described above.
formalin-fixed and paraffin-embedded sections were routinely checked by hematoxylin–eosin staining. For the in vivo efficacy studies, recipient mice with AMC711T or KCNR neuroblastoma xenografts with a mean volume of 268 mm³ were subsequently treated intraperitoneally with 5, 10, or 15 mg/kg/d AT7519 or vehicle (saline) in a 5 days on, 2 days off schedule for up to 3 weeks consecutively. Tumor sizes were measured by an external caliper. Blood samples were collected by cheek puncture (5 mg/kg AT7519 or saline). Blood and tumor samples were collected at 1 and 4 hours after AT7519 administration and processed and analyzed as described above.

In vivo efficacy in Th-MYCN transgenic mice

All experimental protocols were monitored and approved by The Institute of Cancer Research Animal Welfare and Ethical Review Body, in compliance with guidelines specified by the UK Home Office Animals (Scientific Procedures) Act 1986 and the United Kingdom National Cancer Research Institute guidelines for the welfare of animals in cancer research (17) and ARRIVE guidelines (18). In Th-MYCN mice (129S1/SvJ-Tg(Th-MYCN) 41Wav/NiJ), expression of a human MYCN transgene is directed by a rat tyrosine hydroxylase (Th) promoter to neural crest cells during early development (19). Th-MYCN mice were genotyped to detect the presence of human MYCN transgene. After weaning, animals were palpated for intra-abdominal tumors twice weekly. Mice (40–80 days old) with palpable tumors (mean size of 960 mm³) were randomized and treated intraperitoneally with 15 mg/kg/d AT7519 or vehicle (saline) in a 5 days on, 2 days off schedule for up to 3 weeks consecutively. Tumor volume was determined by MRI on a 7T horizontal bore microimaging system (Bruker Instruments) using a 3 cm birdcage coil. Anatomical T2-weighted coronal images were acquired from 20 contiguous 1 mm thick slices through the mouse abdomen, from which tumor volumes were determined using segmentation from regions of interest drawn on each tumor-containing slice. Mice were allowed access to food and water ad libitum.

In vivo pharmacokinetics

Mice with AMC711T neuroblastoma xenografts (∼268 mm³) were treated with a single intraperitoneal injection of 5, 10, or 15 mg/kg AT7519 or saline. Blood samples were obtained by centrifugation twice at 1,150 × g for 15 minutes. Tumor samples were homogenized in saline in a final concentration of 0.1 g/mL using the Precellys 24-Dual. Samples were stored at −80°C until pretreatment and analysis by LC/MS-MS as described for plasma by Dolman and colleagues (20). Pharmacokinetic parameters were calculated by fitting to a first-order two-compartment model with MultiFit pharmacokinetic software (Dr. Hans Proost, University of Groningen, the Netherlands).

Results

Neuroblastoma cells with MYCN amplification are sensitized to AT7519 treatment

The efficacy of AT7519 was evaluated in a panel of 20 MYCN-amplified and 9 non–MYCN-amplified cell lines using the MTT cell viability assay (Supplementary Fig. S1A; Supplementary Table S1). MYCN-amplified neuroblastoma cell lines were found to be significantly more sensitive to AT7519 compared with non–MYCN-amplified cell lines, as was demonstrated by the median IC₅₀ values of 386 and 1227 nmol/L (P = 0.0074), respectively (Fig. 1). Differences in sensitivity became more obvious when taking the induction of cell death into account by means of the LC₅₀ values (Fig. 1). Less overlap was observed between the LC₅₀ values obtained in MYCN-amplified and non–MYCN-amplified neuroblastoma cell lines, with median LC₅₀ values of 1.7 and 8.1 μmol/L (P = 0.0053), respectively. The sensitivity of neuroblastoma cell lines to AT7519 did not correlate with MYCN mRNA (Supplementary Fig. S1B) or MYCN protein levels (Supplementary Fig. S1C), indicating that the presence of MYCN amplification is the best predictive biomarker for AT7519 efficacy.

Next, we treated MYCN-amplified and non–MYCN-amplified cell lines with increasing doses of AT7519 to study effects on retinoblastoma protein (Rb) phosphorylation (p-Rb) and PARP cleavage. Rb is a direct target of CDK2, activated by phosphorylation on threonine 821 (Thr⁸²¹). AT7519 treatment led to a

![Figure 1](image_url)

MYCN-amplified neuroblastoma cells are more sensitive to AT7519 than non–MYCN-amplified neuroblastoma cells. IC₅₀ (left) and LC₅₀ values (right) of AT7519 were determined for 20 MYCN-amplified and 9 non–MYCN-amplified neuroblastoma cell lines. The lower values observed for MYCN-amplified neuroblastoma cells showed that these cells are more sensitive to AT7519. Statistical analysis was performed using a one-tailed unpaired Student t-test, with P < 0.05 (indicated as *) as the minimum level of significance. Horizontal lines indicate median values.
dose-dependent diminution of p-Rb levels, independent of MYCN status (Fig. 2A). However, p-Rb inhibition in MYCN-amplified neuroblastoma cell lines resulted in more potent PARP cleavage induction than observed for non–MYCN-amplified cell lines. Treatment of MYCN-amplified cell lines with IC25, IC50, or IC75 concentrations of AT7519 resulted in average increases in PARP cleavage of 1.1%, 7.6%, and 14.1%, respectively, versus 0.4%, 2.1%, and 1.9% for non–MYCN-amplified cell lines (Fig. 2A and Supplementary Fig. S2). In some neuroblastoma cell lines, total Rb and/or MYCN protein levels were inhibited at higher concentrations of AT7519 treatment. This may relate to additional inhibitory activities of the compound.

The apoptotic effects of AT7519 were further studied by flow cytometry after treatment with AT7519 concentrations equal to 150 nmol/L or the IC50 for each individual cell line (Supplementary Fig. S3). For 7 of 9 MYCN-amplified neuroblastoma cell lines treated with 150 nmol/L AT7519 larger increases in sub-G1 fraction were observed than for non–MYCN-amplified cell lines, with average increases in sub-G1 fraction of 12.4% and 0.6%, respectively (Fig. 2B and Supplementary Table S2). As expected, treatment with the IC50 dose of AT7519 resulted in a greater sub-G1 population in all MYCN-amplified cell lines for which the IC50 was >150 nmol/L. In contrast, no significant increases in sub-G1 fraction were observed for 2 of 4 non–MYCN-amplified cell lines (i.e., SKNSH and SY5Y), despite of treatment with micromolar concentrations of AT7519. Instead, high AT7519 doses caused G1 arrest of SKNSH and SY5Y. Together, these findings indicate that AT7519 treatment leads...
AT7519 inhibits the growth of MYCN-amplified neuroblastoma xenografts

The efficacy of AT7519 was subsequently studied in vivo in mice with MYCN-amplified AMC711T neuroblastoma xenografts. AMC711T cells are short-term cultured primary neuroblastoma cells and xenografts of these cells have shown to properly reflect high-risk neuroblastoma (14). Mice were treated daily with intraperitoneal injections of 5, 10, or 15 mg/kg AT7519 in a 5 days on, 2 days off schedule for 3 weeks consecutively. AT7519 inhibited the growth of AMC711T neuroblastoma xenografts in a dose-dependent manner, with even the lowest dose of 5 mg/kg providing a statistically significant reduction in tumor growth (Fig. 3A and Supplementary Fig. S5A). Treatment with either 10 or 15 mg/kg AT7519 almost completely blocked tumor growth, resulting in a significantly improved anticancer effect compared with 5 mg/kg AT7519 (Fig. 3A). More rapid tumor growth was observed after terminating treatment with AT7519 (Supplementary Fig. S5B). We also tested AT7519 (15 mg/kg) in MYCN-amplified KCNR neuroblastoma xenografts and found a 50% reduction in tumor growth compared with saline control at day 17 after treatment initiation (Supplementary Fig. S5C).

In vivo efficacy of AT7519 correlates with tumor drug exposure

To further evaluate the tumor-specific effects of AT7519, intratumoral and plasma drug concentrations were correlated with the dose-dependent tumor growth inhibitory effects in AMC711T xenografts. AT7519 concentrations in plasma and tumor were measured up to 24 hours after intraperitoneal administration of a single dose of 5, 10, or 15 mg/kg AT7519. Plasma clearance and tumor accumulation curves were fitted for each individual dose.

Figure 3.
Loss of Rb and NPM phosphorylation are biomarkers for the inhibitory effects of AT7519 on the growth of MYCN-amplified AMC711T neuroblastoma xenografts in mice. A, inhibitory effects of AT7519 on the growth of AMC711T neuroblastoma xenografts in mice. Relative tumor volume was calculated as the volume at the indicated day after start of treatment divided by the volume prior to treatment initiation. Data represent the mean relative tumor volume \( \pm \) SEM (Group sizes: \( n = 10 \) (saline), \( n = 8 \) (5 mg/kg), \( n = 8 \) (10 mg/kg), and \( n = 11 \) (15 mg/kg)). Statistical differences between AT7519-treated and saline-treated groups are indicated on the first day after treatment initiation at which a statistically different effect was observed (*). Statistical differences between the different doses of AT7519 are indicated as *. B, plasma and intratumoral drug levels after administration of a single intraperitoneal injection of 15 mg/kg AT7519 to mice bearing MYCN-amplified AMC711T neuroblastoma xenografts. Plasma AT7519 concentrations established at 1 minute after administration have been averaged for curve fitting. Each symbol represents a single data point (tumor, solid red circles; plasma, solid black diamonds) and continuous lines represent the fitted curves. Outliers were calculated using the Dixon Q test at a 95% confidence level. One tumor sample (i.e., 214.3 ng/g AT7519 at 0.5 hours after administration) has been considered outlier based on corresponding plasma levels. Outliers are indicated by unfilled red circles for tumor samples and unfilled black diamonds for plasma samples. C, in vivo correlation between drug exposure (AUC_{0-24}) and AT7519 tumor dose (tumor, red circles; plasma, black diamonds). D and E, in vivo inhibitory effects of AT7519 on the tumor phosphorylation states of the CDK2 targets Rb (D) and NPM (E). Phosphorylation states have been expressed as the ratio between phosphorylated and nonphosphorylated protein levels. \( \beta \)-Actin was used as household protein. Data represent mean values \( \pm \) SEM (\( n = 4 \) per group).
AT7519 on tumoral p-Rb and p-NPM levels were evaluated 1 hour after administration of the compound regardless of dose. AT7519 rapidly entered the circulation from the peritoneal cavity, as demonstrated by the short time needed to reach maximum plasma levels ($T_{\text{max}} < 7$ minutes). Plasma AUC$_{0-\infty}$ values correlated with AT7519 doses (Fig. 3C), such that the biologic availability after intraperitoneal administration of AT7519 was equivalent for all three doses evaluated. When comparing the maximum AT7519 plasma levels ($C_{\text{max}}$), no increase in $C_{\text{max}}$ was observed after administration of 15 mg/kg AT7519. Also, a slightly longer plasma terminal half-life was observed with 15 mg/kg AT7519 compared with the lower doses (Supplementary Table S3). These observations indicate a slight change in pharmacokinetic behavior, potentially due to partial precipitation of AT7519 in the peritoneal cavity. Gradual redissolution of AT7519 might eventually lead to the same biologic availability as observed for the lower doses.

AT7519 rapidly entered the tumors (Fig. 3B and Supplementary Fig. SSD; Supplementary Table S3), with maximum intratumoral drug concentrations reached within 25 to 40 minutes after AT7519 administration. Maximum intratumoral AT7519 concentrations linearly increased with increasing dose. However, as shown by the tumor AUC$_{0-\infty}$ values, administration of 15 mg/kg AT7519 did not result in a linear increase in tumor exposure to the inhibitor (Fig. 3C and Supplementary Table S3). This might explain why treatment of AMC711T neuroblastoma xenografts with 15 mg/kg AT7519 did not result in significantly improved efficacy as compared with 10 mg/kg AT7519. Independent of dose, AT7519 was eliminated from the tumor with a terminal half-life of approximately 1.8 hours. The results obtained with AMC711T neuroblastoma xenografts were reproduced with KCNR xenografts (15 mg/kg), with plasma and intratumoral AT7519 levels detected at 1 and 4 hours after administration of a single intraperitoneal injection (Supplementary Fig. SSE). Taken together, these observations show that the efficacy of AT7519 correlates to the overall tumor exposure to AT7519.

Loss of Rb and NPM phosphorylation are biomarkers for AT7519 efficacy in MYCN-driven tumors

Because Rb and nucleophosmin (NPM) are direct targets for phosphorylation by CDK2, we investigated the use of p-Rb and p-NPM as potential biomarkers of AT7519 efficacy. Mice bearing AMC711T neuroblastoma xenografts were treated with 5, 10, or 15 mg/kg/d AT7519 for 5 consecutive days. Inhibitory effects of AT7519 on tumoral p-Rb and p-NPM levels were evaluated 1 hour after administration of the last dose. AT7519 significantly reduced tumor levels of p-Rb and p-NPM, despite interanimal variation (Fig. 3D and E and Supplementary Fig. S6A). Furthermore, treatment with either 10 or 15 mg/kg/d AT7519 resulted in decreased levels of p-Rb and p-NPM compared with 5 mg/kg/d AT7519. Inhibitory effects on phosphorylated NPM levels were also studied in situ (Fig. 3F and Supplementary Fig. S6B). Again, significant inhibition of p-NPM was observed, even after treatment with 5 mg/kg/d AT7519. No effects of AT7519 on total NPM levels were observed (Supplementary Fig. S6A and S6B). Hematoxylin–eosin staining of the tumor tissues did not indicate clear phenotypic changes between vehicle and AT7519-treated mice (Supplementary Fig. S6C).

AT7519 causes tumor volume reduction in Th-MYCN transgenic mice

Given that Th-MYCN transgenic mice develop tumors in an immunocompetent background, we reasoned that the preclinical evaluation of AT7519 in this mouse model would be complementary to our xenograft data. Consistent with both neuroblastoma xenograft models tested, Th-MYCN transgenic mice were treated with 15 mg/kg/d AT7519 in a 5 days on, 2 days off schedule for 3 weeks consecutively. Magnetic resonance imaging at day 7 after treatment initiation with AT7519 showed significant reductions in tumor volume (Fig. 4A and Supplementary Fig. STA). Tumor volume reduction was observed for all treated mice, with 6 of 7 partial responses (PR; i.e., 50%–95% tumor volume reduction) and 1 of 7 very good partial response (VGPR; i.e., 95%–99.99% tumor volume reduction; Fig. 4B). The average reduction in tumor volume was approximately 86% at day 7 after treatment initiation with AT7519. In contrast, all saline-treated mice showed progressive disease (PD), with an average increase in tumor volume of approximately 218% (Fig. 4B and Supplementary Fig. STA). Three weeks treatment with AT7519 significantly increased the overall survival of Th-MYCN transgenic mice (Fig. 4C). However, consistent with tumor regrowth observed for our neuroblastoma xenograft studies, Th-MYCN mice relapsed while on AT7519 treatment (Supplementary Fig. S7B and S7C).

Unexpectedly, intratumoral concentrations of AT7519 at 1 hour after a single intraperitoneal injection of 15 mg/kg were almost three times higher than the predicted levels based on the xenograft models, while average plasma levels were approximately 40% lower (Fig. 4D). This corresponds with an approximately 5-fold higher tumor/plasma ratio for the Th-MYCN transgenic mouse model. No differences in observed and predicted plasma and intratumoral AT7519 levels were obtained at 4 hours after administration of AT7519. Thus the tumor exposure to AT7519 is higher for the Th-MYCN transgenic mouse model than both neuroblastoma xenograft models tested in this study. This might explain the improved efficacy of AT7519 observed for Th-MYCN transgenic mice.

Pharmacodynamic analysis showed that AT7519 strongly induced cell death in Th-MYCN tumors. Treatment of these mice with 15 mg/kg/d AT7519 resulted in increased cleavage of caspase-3 (Fig. 5A and Supplementary Fig. S8A and S8B) and the occurrence of nuclear condensation and fragmentation and necrosis (Fig. 5B). Effects on apoptosis after AT7519 treatment of Th-MYCN transgenic mice were consistent with the observed tumor regression.

Discussion

The work presented here describes the preclinical evaluation of the novel fragment–derived small-molecule CDK inhibitor AT7519 in neuroblastoma treatment. In agreement with our previous results, the sensitivity of neuroblastoma cells to AT7519 partly depends on the MYCN amplification status. The degree to which AT7519 specifically killed cells with MYCN amplification versus non–MYCN-amplified cells, however, was less pronounced than the synthetic lethal interaction between knockdown of CDK2 and MYCN amplification (9). The underlying reason for this is likely that while AT7519 was originally described as a unique CDK2 inhibitor that binds within the active site cleft of CDK2 overlapping with the ATP-binding pocket (13), it also effectively targets additional kinases, including CDK1, CDK4, CDK5, CDK6,
AT7519 causes tumor volume reduction in Th-MYCN transgenic mice and improves overall survival. A, representative anatomical T2-weighted coronal MRI images for one of the AT7519-treated mice, showing the tumor prior to treatment (day 0; left) and at day 7 after treatment initiation (right). B, waterfall plot of the in vivo effects of AT7519 on tumor volume in Th-MYCN transgenic mice. The waterfall plot displays the % change in tumor volume at day 7 after treatment initiation for each individual mouse. Blue and white block bars represent the saline-treated mice. The red bar and red and white block bars represent the mice treated with 15 mg/kg AT7519. PD = Progressive disease, PR = partial response (i.e., 50%–95% tumor volume reduction) and VGPR = very good partial response (i.e., 95%–99.9% tumor volume reduction). Statistical differences between the tumor size before and after treatment were calculated using a one-tailed paired Student t test and are indicated as *. Statistical differences between the AT7519-treated and saline-treated group were calculated using a one-tailed unpaired Student t test and are indicated as #. C, Kaplan-Meier curve to show the long-term survival after 3 weeks treatment with saline (n = 8; blue line) or AT7519 (n = 6; red line). Statistical analysis was performed using the log-rank (Mantel-Cox) test, with P < 0.05 as the minimum level of significance. D, comparison between plasma (left) and intratumoral (right) drug levels obtained in Th-MYCN transgenic mice and the AMC711T neuroblastoma xenograft model after administration of a single intraperitoneal injection of 15 mg/kg AT7519. Grey and white line print areas in both graphs show again the fitted curves for the AMC711T neuroblastoma xenograft model. For the Th-MYCN transgenic mice, drug levels were established at 1 and 4 hours after administration of AT7519 (n = 3 per group) and individual data points (●) as well as average drug levels (—) are shown in the graphs.
immunocompetent Th-MYC transgenic mice express MYCN in the developing neural crest, leading to the spontaneous development of neuroblastoma tumors (24, 25). Treatment with AT7519 resulted in a more potent anticanter activity in Th-MYC transgenic mice compared with the anticanter effects obtained in the AMC711T and KCNR xenograft models. Our data suggest that this may be caused by an increased tumor exposure to AT7519 in Th-MYC transgenic mice, which might be the result of a higher degree of tumor vascularisation. There are indications that MYCN promotes tumor angiogenesis (26) and consistent with results obtained clinically in human promyelocytic leukemia xenografts, where treatment with AT7519 also resulted in inhibition of tumor-associated p-Rb and p-NPM (31). In the Th-MYC model, we saw that treatment with 15 mg/kg AT7519 caused an increase in cleaved caspase-3.

Additional studies are needed to validate if this apoptotic marker can be used as non-target-specific biomarker of efficacy of AT7519.

The clinical use of AT7519 has been associated with dose-limiting toxicities, including QTc prolongation (22). However, follow-up studies showed that treating patients at days 1, 4, 8, and 11 once every 3 weeks instead of at days 1 to 5 appears to limit the risk of QTc prolongation (32). Currently running clinical trials with AT7519 are therefore performed using the new intermittent treatment schedule (https://clinicaltrials.gov).

Taken together, the results presented in this study strongly suggest that AT7519 is a clinical candidate drug for the treatment of MYCN-amplified, high-risk neuroblastoma. The ability of AT7519 to rapidly decrease tumor volume in Th-MYC mice indicates its possible use in the induction phase of treatment. Finally, the potential utility of AT7519 is increased by the identification of target-specific efficacy biomarkers.
Disclosure of Potential Conflicts of Interest

H.N. Caron is an employee of and holds ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.

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