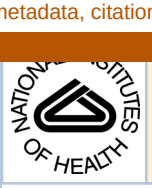

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AT7519, A NOVEL SMALL MOLECULE MULTI-CYCLIN DEPENDENT KINASE INHIBITOR, INDUCES APOPTOSIS IN MULTIPLE MYELOMA VIA GSK-3β ACTIVATION AND RNA POLYMERASE II INHIBITION

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Abstract

Dysregulated cell cycling is a universal hallmark of cancer and is often mediated by abnormal activation of cyclin dependent kinases (CDKs) and their cyclin partners. Overexpression of individual complexes are reported in multiple myeloma (MM), making them attractive therapeutic targets. Here we investigate the preclinical activity of a novel small-molecule multi-CDK inhibitor, AT7519, in MM. We demonstrate the anti-MM activity of AT7519 displaying potent cytotoxicity and apoptosis; associated with *in vivo* tumor growth inhibition and prolonged survival. At the molecular level, AT7519 inhibited RNA polymerase II (RNA pol II) phosphorylation, a CDK9, 7 substrate, associated with decreased RNA synthesis confirmed by [^{3}H] Uridine incorporation. Additionally, AT7519 inhibited glycogen synthase kinase 3 beta (GSK-3β) phosphorylation; conversely pretreatment with a selective GSK-3 inhibitor and shRNA GSK-3β knockdown restored MM survival, suggesting the involvement of GSK-3β in AT7519-induced apoptosis. GSK-3β activation was independent of RNA pol II dephosphorylation confirmed by alpha-amanitin, a specific RNA pol II inihibitor, demonstrating potent inhibition of RNA pol II phosphorylation without corresponding effects on GSK-3β phosphorylation. These results offer new insights into the crucial, yet controversial role of GSK-3β in MM and demonstrate significant anti-MM activity of AT7519, providing the rationale for its clinical evaluation in MM.

Keywords

myeloma; cyclin dependent kinase; GSK-3β; RNA pol II

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Introduction

Cyclin dependent kinases (CDKs) are serine/threonine kinases which control cell cycle progression and transcriptional regulation (Nurse, 2000). They are regulated by the presence of their cyclin partners and specific inhibitors. For example, CDK4/6-cyclin D and CDK2-cyclin E complexes are involved in G1/S transition and S phase progression, whilst CDK2/cyclin A participates in DNA replication during S phase. CDK1 partners with cyclin B1 or A1 and is required for G2/M transition and progression through mitosis (Sherr & Roberts, 2004). Additionally, CDK2, 7, 8 and 9 play a crucial role in transcriptional elongation and initiation by phosphorylating the carboxyl terminal domain (CTD) of the large subunit of RNA polymerase II (RNA pol II) (Oelgeschlager, 2002). CDK9/cyclin T participate in transcriptional elongation by phosphorylating serine 2 and serine 5 of the CTD; CDK7/cyclin H/MAT1 facilitates promoter clearance and transcriptional initiation by phosphorylating serine 5 of the CTD preferentially (Loyer et al., 2005). Besides being involved in cell cycle control, CDK1/cyclin A, CDK2/cyclin E, CDK8/cyclin C complexes participate in transcriptional regulation through phosphorylation of the CTD at serine 2 and serine 5 (Nekhai et al., 2002; Oelgeschlager, 2002). CDK and cyclin complex deregulations are often involved in tumor pathogenesis and growth (Malumbres & Barbacid, 2001). Different human cancers are characterized by cyclin D overexpression, CDK4 and CDK2 hyper-activation, and hyper-expression of anti-apoptotic transcripts (Deshpande et al., 2005; Santamaria & Ortega, 2006). Therefore CDKs represent an interesting therapeutic target, and their pharmacological inhibitors have been proposed for cancer treatment (McInnes, 2008).

Despite advances in therapy during the last decade, multiple myeloma (MM) remains an incurable disease. The use of pharmacological CDK inhibitors may represent an attractive target for MM therapy. The overexpression of D type cyclins have been implicated in MM pathogenesis and progression (Bergsagel & Kuehl, 2005; Hideshima et al., 2004); and the aberrant coactivation of CDK4/cyclin D1 and CDK6/cyclin D2 represents an important factor in myeloma cell proliferation and deregulation of cell cycle control (Ely et al., 2005). Consequently CDK inhibitors, e.g. Flavopiridol, R-Roscovitine and P276-00, have been validated in preclinical studies in MM and PD0332991 and P276-00 are currently in phase I clinical trials in MM (Menu et al., 2008). PD0332991 is a specific CDK4/6 inhibitor, P27600 is a CDK4/CyclinD1 inhibitor. These drugs are able to inhibit CDk4/6 specific phosphorylation of Rb and cell cycle progression through G1 in MM cells. Other CDK inhibitors like Flavopiridol and Roscovitin have shown broader activity against CDK2, CDK7 and CDK9 and affect RNA polymerase II CTD leading to the inhibition of transcription. (Gojo et al., 2002; MacCallum et al., 2005; Raje et al., 2009; Raje et al., 2005)

Although selective CDK inhibitors have shown potent cytotoxic activity in MM cells, the underlying mechanism remains incompletely understood. To date, most studies have focused on inhibitors of CDK1/2 and CDK4/6, even though transcriptional regulation via CDK 7 and 9 may be equally relevant to inducing apoptosis in malignant hematopoietic cells (Shapiro, 2006). Multi-targeted CDK inhibitors may be preferable to specific inhibitors to overcome redundancy within the CDK system thus reducing the potential for acquired resistance (Tetsu & McCormick, 2003). Additionally, CDKs are closely homologous to GSK-3β (Leclerc et al., 2001) and several CDK inhibitors have been shown to inhibit GSK3 (Knockaert et al., 2002) GSK-3β is a serine/threonine protein kinase regulating glycogen synthesis (Embi et al., 1980). Studies have highlighted the role of GSK-3β in different oncogenic pathways, such as PI3K/AKT, Wnt β-catenin and NF-κB signaling cascades (Jope & Johnson, 2004), but its role in MM remains to be elucidated. We studied GSK-3β pathway, known to play a crucial role in several signaling cascades relevant to MM biology (Mitsiades et al., 2002), in the context of CDK inhibition because *in vitro* kinase assays have

shown that CDK inhibitors also inhibits GSK3, yet this effect has not been investigated in the context of MM cells.

Here, we have explored the pharmacology of a multi-targeted CDK inhibitor that potently inhibits CDK1, 2, 4, 5, 6 and 9 *in vitro* (Squires et al., 2009; Wyatt et al., 2008). AT7519 exhibited potent anti-myeloma activity both *in vitro* and *in vivo*. Cell death occurred through the dephosphorylation of the CTD of RNA pol II, consistent with inhibition of transcription. Additionally, we observed that AT7519 induced rapid dephosphorylation of GSK-3β at serine 9 resulting in apoptosis *in vitro* and antitumor activity *in vivo* resulting in prolonged survival. The results of this study provide the rationale for future clinical trials of this agent in patients with MM.

Results

AT7519 induces dose dependent cytotoxicity in MM cells and partially overcomes the proliferative effects of BMSCs and cytokines

The effect of AT7519 (Fig. 1A, table 1), was determined in MM cell lines sensitive (MM. 1S, RPMI, U266) and resistant (LR-5, Dox40, MM.1R) to conventional therapy, as well as patient derived MM cells by MTT assays. Cells were cultured in the presence of increasing doses of AT7519 (0–4 μM) for 24, 48 and 72 h. AT7519 resulted in dose-dependent cytotoxicity with IC_{50}s ranging from 0.5 to 2 μM at 48 hours, with the most sensitive cell lines MM.1S (0.5 μM) and U266 (0.5 μM) and the most resistant MM1R (> 2 μM) and in patient derived MM cells (Fig. 1B). Exposure of MM cells to AT7519 for 72 hours did not show additional cytotoxicity, suggesting maximum effect at 48 hours (data not shown). Importantly, AT7519 did not induce cytotoxicity in PBMNC from five healthy volunteers (Fig. 1C). Given that BM microenvironment confers growth and survival in MM cells (Hideshima et al., 2004), we next evaluated the effect of AT7519 on MM cells cultured in the presence of BMSCs. AT7519 resulted in a partial inhibition of DNA synthesis of MM cells adherent to BMSCs at 48 h in a dose-dependent manner. Both IL-6 and IGF-1 are known to inhibit apoptosis (Chauhan et al., 1997) and stimulate growth (Hallek et al., 1998) of MM cells. AT7519 partially inhibited the growth conferred by IL6 and IGF-1 at 48 h (Fig. 1D). Therefore, AT7519 overcomes the proliferative advantage conferred by cytokines and the protective effect of BMSC.

AT7519 induces cell cycle arrest and apoptosis of MM cells in a time- and dose- dependent manner

MM cell cytotoxicity due to AT7519 was characterized by cell-cycle analysis on MM.1S cells cultured with media alone and AT7519 (0.5 μM) for 6, 12 and 24 h. AT7519 treated MM.1S cells showed an increase of cells in G0/G1 and G2/M phase as early as 6 hours. AT7519 increased the proportion of cells in sub-G1 phase starting from 12 h indicating that the compound induced cell death (Fig. 2A). To confirm AT7519 induced apoptosis, PI and Annexin V staining demonstrated apoptosis starting from 12 h onwards with maximal effect at 48 h (Fig. 2B). This time frame was consistent with observed caspase -9,-3 and -8 cleavage (Fig. 2C).

AT7519 inhibits phosphorylation of RNA polymerase II CTD and partially inhibits RNA synthesis in MM.1S cells

MM.1S cells were cultured for 1/2, 1, 2, 4 and 6 h with media alone and AT7519 (0.5 μM). The effect of AT7519 on the expression of CDKs and cyclins was determined (Fig. 3A). Although levels of the relevant CDKs and cyclins were unaffected by AT7519 treatment at early time points, cyclin D1, cyclin A and cyclin B1 were downregulated by AT7519 treatment within 2 hours. We investigated the phosphorylation state of substrates specific to

individual CDKs (e.g. p-Rb serine807/811, p-Rb serine780, phospho-PP1alpha Threonine320) and observed that dephosphorylation of these proteins was noted 6 h after exposure to AT7519 (Supplementary data, Fig S2). Since AT7519 inhibits CDKs responsible for transcriptional regulation, we next investigated its effect on phosphorylation status of RNA pol II CTD at both the serine 2 and serine 5 sites. AT7519 induced rapid dephosphorylation at both sites within 1 hour, without significant variations in total protein expression (Fig. 3B). AT7519 induced dephosphorylation of RNA pol II CTD at serine 2 and serine 5 in dex-resistant MM.1R and melphalan-resistant LR5 MM cells after 3 hours of treatment in a dose dependent manner (data not shown). AT7519 induced dephosphorylation of RNA pol II CTD at serine 2 and serine 5 suggests that cytotoxicity correlates with the inhibition of transcription. Based on the hypothesis that transcriptional repression affects proteins with rapid turnover, we investigated the effect of AT7519 on Mcl-1 and XIAP. AT7519 treated cells showed decreased expression levels of Mcl-1 and XIAP within 4 h (Fig. 3C) as is consistent with other CDK inhibitors in the context of MM (MacCallum et al., 2005; Raje et al., 2005). Total RNA synthesis by [^{3}H] uridine incorporation was measured after exposure to AT7519. After 48 hours, RNA synthesis levels in AT7519 treated MM.1S cells was approximately 50% of control values, confirming that the mechanism of action of AT7519 induced cytotoxicity of MM cells was via inhibition of transcription (Fig. 3D). Because the effect was only in part due to transcriptional repression, our results also suggest that other mechanisms contribute to AT7519 induced apoptosis in MM.

AT7519-induced cytotoxicity is associated with GSK-3β activation independent of transcriptional inhibition

Since the amino acid sequence of GSK-3β has high homology to CDKs (Leclerc et al., 2001), and many CDK inhibitors have shown activity (at least in vitro kinase assays) against GSK-3 (Knockaert et al., 2002), we investigated if GSK-3 was involved in AT7519 induced MM apoptosis. The effect of AT7519 on the phosphorylation status of GSK-3β at serine 9 was studied. We observed that AT7519 induces GSK-3β activation, as demonstrated by decreasing levels of phosphorylated GSK-3β within 2 hours of treatment, without significant impact on total protein expression level (Fig. 4A left panel). In order to confirm the induction of GSK-3β activity by AT7519, we tested its effect on the expression level of phospho-glycogen synthase, a downstream substrate of GSK-3. The upregulation of phosphorylation of glycogen synthase occurred with similar kinetics to the downregulation of phosphorylation of GSK-3β induced by treatment with AT7519 at 0.5 μM (Fig. 4A right panel). Similar results were observed in MM.1R and LR5 MM cells after 3 hours of AT7519 treatment (data not shown). Importantly, decreased phosphorylation of GSK-3β was not associated with the inhibition of its upstream pathway; in contrast AT7519 upregulated p-AKT and p-p70SK6 within 30 minutes. Since Akt plays a crucial role in MM cell survival (Tu et al., 2000), AT7519 induced Akt phosphorylation may be due to a compensatory feedback loop. No effect was noted on p44/42MAPK. Because AT7519 induced the activation GSK-3β, we also investigated its downstream targets c-Myc and cyclin D1, and demonstrated their inhibition (Fig. 4B).These results suggest that GSK-3β activation may contribute to MM apoptosis induced by AT7519. In contrast, the addition of AR-A014418, a chemical small molecule ATP pocket-site binding inhibitor of GSK-3 (Bhat et al., 2003), triggered an increase phosphorylation of GSK-3β at serine 9 and a decrease in phosphorylation of glycogen synthase in a dose dependent manner after 24 hours of treatment (Fig. 5A). To further characterize the role played by GSK-3β, MM.1S cells were treated with increasing doses of AR-A014418 (0.25–1 μM) for 30 minutes prior to AT7519 treatment. The cytotoxicity induced by AT7519 was partially abrogated by pretreatment with AR-A014418 (Fig. 5B). To further confirm the role of GSK-3β in AT7519-induced apoptosis we used specific GSK-3β shRNA sequences to knock down GSK-3β expression in

MM.1S cells. GSK-3β was differentially inhibited by the various shRNAs. We selected three different shRNAs to perform our experiment and the scrambled shRNA as control (Fig. 5C). As shown in figure Fig 5 D, MM.1S cells with knocked-down GSK-3β, were more resistant to AT7519-induced cytotoxicity in 48 h culture with respect to control shRNA-transfected cells. These findings support the hypothesis that AT7519-induced apoptosis in MM cells is, at least in part, a result of increasing GSK-3β activity. Since AT7519-induced apoptosis correlates with inhibition of RNA pol II, we investigated if the decreased phosphorylation of GSK-3β at serine 9 was due to transcriptional inhibition. MM. 1S cells were incubated for 24 hours with increasing doses of alpha-amanitin, a cyclic peptide which binds the large subunit of RNA pol II with high affinity and inhibits the initiation of transcription and its subsequent elongation. Although dephosphorylation of RNA pol II at serine 2 and serine 5 and downregulation of RNA pol II was induced by 10 μM of alpha-amanitin, no effect on the dephosphorylation of GSK-3β at serine 9 was noted (Fig. 6A). We next evaluated the effect of alpha-amanitin on the viability of MM.1S cells using the MTT assay in order to ensure that the effect on RNA pol II observed by western blotting was not associated with cytotoxicity. Alpha-amanitin induced 20 % cytotoxicity after 24 hours of treatment (Fig 6 B). Thus the observed effect of alpha-amanitin on expression of phosphorylated GSK-3β suggests that the activation of GSK-3β by AT7519 occurs independently from inhibition of transcription.

AT7519 inhibits human MM cell growth *in vivo*

We examined the *in vivo* efficacy of AT7519 using a human MM xenograft mouse model. As shown in Fig 7A, tumor growth in AT7519-treated mice was inhibited compared to controls ($p < 0.05$). Immunohistochemistry confirmed increased caspase 3 activation (Fig 7B) in AT7519 treated tumor samples. Using Kaplan-Meier and log-rank analysis, the median overall survival (OS) of animals treated with either 15 mg/kg once a day for five days for 2 weeks or 15 mg/kg once a day three days per week was significantly prolonged (40 and 39 days versus 27.50 days respectively; $p < 0.05$) (Fig. 7C). In contrast, treatment with AT7519 did not affect the body weight of the animals (Fig. 7D).

Discussion

The critical role played by cyclin D and CDK4/6 deregulation in MM pathogenesis led us to study the pharmacology of CDK inhibitors in models of the disease. One such inhibitor is AT7519, which inhibits CDKs 1, 2, 4, 5, 6 and 9 with lower potency against CDK3 and 7 in *in vitro* kinase assays. Our results demonstrate that AT7519 induces apoptosis not only by a mechanism similar to other CDK inhibitors tested in MM (e.g., Seliciclib), i.e., via the dephosphorylation of the CTD of the large subunit of RNA pol II, but also, unlike other CDK inhibitors, through the rapid dephosphorylation and subsequent activation of GSK-3β at serine 9 which was in contrast to *in vitro* kinase assay data.

This study investigated the hypothesis that, because AT7519 inhibits not only the CDKs involved in cell cycle control but also CDKs involved in transcriptional regulation, its mechanism of action in MM may be a consequence of transcriptional repression. Although CDK7 and CDK9 are the primary transcriptional activating kinases that phosphorylate CTD, both CDK2 and CDK1 also phosphorylate RNA pol II CTD at serine 2 and serine 5 *in vitro* (Cai et al., 2006). Moreover, CDK inhibition with flavopiridol and seliciclib is also associated with inhibition of phosphorylation of RNA pol II CTD, resulting in a decrease in transcription. The present study demonstrates that AT7519 decreased dephosphorylation of RNA pol II CTD at both serine 2 and serine 5 leading to transcriptional repression. Because the most sensitive targets of transcription inhibitors are mRNAs coding for proteins with short half lives (Chen et al., 2005; MacCallum et al., 2005), we evaluated the expression level of antiapoptotic proteins with rapid turnover, such as Mcl-1 and XIAP. As expected,

AT7519 decreased the level of Mcl-1 and XIAP. Mcl-1 is a Bcl-2 family antiapoptotic protein essential for MM cell survival (Zhang et al., 2002). Inhibition of Mcl-1 by antisense oligonucleotides induces apoptosis in MM cells (Derenne et al., 2002). XIAP overexpression renders myeloma cells resistant to apoptosis induced by chemotherapeutic agents, and its high-level expression has been associated with a poor prognosis (Nakagawa et al., 2006). The ability of AT7519 to reduce levels of both Mcl-1 and XIAP demonstrated here suggests that it may have promise in the treatment of MM.

Our data demonstrated that the inhibition of RNA synthesis, measured by [3H] Uridine incorporation, was only partial suggesting that other mechanisms are implicated in AT7519 induced MM cytotoxicity. The fact that CDKs are closely homologous to GSK-3β (Cohen & Frame, 2001; Leclerc et al., 2001; Wyatt et al., 2008), led us to investigate the role of this kinase in the biological effects of AT7519. Because of their structural similarity, many CDK inhibitors are inhibitors of GSK-3β in isolated biochemical assays (Knockaert et al., 2002). Given its inhibitory role in the pathogenesis of cancers, GSK-3β had not until recently been considered as a therapeutic target. More recently, several lines of evidence have challenged this view. Whilst GSK-3β promotes oncogenesis and supports cell proliferation in mixed lineage leukemia (MLL), a similar effect has not been seen in other leukemia cell lines (Wang et al., 2008). Inhibition of GSK-3 induces apoptosis in colon (Shakoori et al., 2005) prostate cancer cells (Vene et al., 2008) as well as in chronic lymphocytic leukemia B cells (Ougolkov et al., 2007); and suppresses cell growth in MM (G-Amlak et al., 2002). AKT inhibitors induce apoptosis in MM cell lines by decreasing phosphorylation of AKT and GSK-3β at serine 9 (Hideshima et al., 2007; Neri et al., 2008), suggesting that it may play a dual role based on cell and cancer type. The role of GSK-3 in MM cell biology has yet to be fully defined. Surprisingly, we observed a rapid dephosphorylation of GSK-3β at serine 9 (its active form). Because GSK-3β is an important kinase involved in several signaling pathways (Grimes & Jope, 2001), its activity is regulated by several mechanisms and at multiple levels. GSK-3β is constitutively active in MM cells; AKT and other kinases inhibit GSK-3 by phosphorylating the regulatory residues at serine 21 (alpha isoform) or serine 9 (beta isoform). The substrates of GSK-3β include many signaling proteins and transcription factors that regulate growth and survival e.g., cyclin D, cyclin E, c-Myc, NF-KB, beta catenin, p53 (Cohen & Frame, 2001). Among these substrates, c-Myc, and cyclin D1 were all downregulated whereas p53 was upregulated (data not shown) by AT7519 treatment. No effect was noted on beta catenin (data not shown). In contrast, the upstream pathways of GSK-3 (AKT and p70S6K) were upregulated, suggesting that the activation of GSK-3β was independent of these upstream pathways, and that GSK-3β was a direct target of AT7519. To further understand the role of the activation of GSK-3β in AT7519 induced cytotoxicity, we used a specific inhibitor of GSK-3β, AR-A04414. This inhibitor increased GSK-3β phosphorylation in a dose-dependent manner, associated with a dephosphorylation of glycogen synthase (a primary GSK-3 substrate). Importantly, the inhibition of GSK-3β using AR-A04414 at low doses prior to treatment with AT7519 and GSK-3β knock down using shRNA resulted in partial rescue of cell death. Our findings therefore suggest that the activation of GSK-3β plays a role in the inhibition of MM cell survival. This was interesting given that the in vitro kinase assay demonstrated inhibition of GSK-3β.

Since AT7519 inhibits transcription, we investigated if dephosphorylation of GSK-3β was a consequence of transcriptional repression by using a specific and selective inhibitor of RNA pol II (alpha-amanitin) (Wieland & Faulstich, 1991). Treatment with alpha-amanitin did not correlate with GSK-3β dephosphorylation, suggesting that dephosphorylation of GSK-3β occurs independently from the RNA pol II inhibition induced by AT7519.

In conclusion, we have demonstrated that AT7519, a novel small molecule multi-CDK inhibitor, has potent anti MM activity both *in vitro* and *in vivo*. In addition, although the

inhibition of transcription is an important mechanism common to many CDK inhibitors, molecular studies of AT7519 revealed that GSK-3β plays a crucial role in AT7519-mediated antimyeloma effect. These results thus provide the rationale for future clinical trials of AT7519 in MM patients, as well as provide insights into the potential role of GSK-3β as a therapeutic target in cancer treatment.

Materials and Methods

Cell lines and reagents

Dexamethasone (Dex) sensitive (MM.1S) and Dex resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). RPMI8226 and U266 human MM cells were obtained from American Type Culture Collection (Rockville, MD). Melphalan-resistant (LR5) RPMI8266 human MM and doxorubicin-resistant RPMI-Dox40 (Dox40) cell lines were provided by Dr William Dalton (H Lee Moffitt Cancer Center, Tampa, FL). OPM1 cells were provided by Dr P. Leif Bergsagel (Mayo Clinic, Tucson, AZ). All MM cell lines were cultured as previously described (Raje et al., 2009). Fresh peripheral blood mononuclear cells (PBMNC) were obtained from four healthy volunteers. BM aspirates from MM patients were obtained following approval from the institutional review board. After mononuclear cells were separated, MM cells were purified by positive selection using CD138 (Syndecan-1) Micro Beads and the Auto Macs magnetic cell sorter (Miltenyi Biotec Inc., Auburn, CA). Bone marrow stromal cells (BMSCs) were generated as previously described (Raje et al., 2009). BMSCs were incubated in 96-well culture plates (10 000 BMSCs/well) for 24 h, after washing off the medium, MM cell lines were added to the wells (2×10^4cells/well) and incubated with media or with increasing doses of AT7519 for the specified time at 37°C.

AT7519 is N-(4-piperidinyl)-4-(2,6-dichlorobenzoylamino)1H-pyrazole-3-carboxamide. AT7519 was obtained from Astex therapeutics Ltd, Cambridge, UK (Fig. 1A). It was dissolved first in dimethyl sulfoxide (DMSO; Sigma Chemical) at a concentration of 10mM, and then in culture medium (0.25–4 μM) immediately before use. Alpha-amanitin was obtained from Axxora LLC (San Diego, CA). GSK-3β inhibitor was obtained from Calbiochem (La Jolla, CA).

Cell viability and proliferation assays

AT7519's effects on viability of MM cell lines, primary MM cells, and PBMNCs was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrasodium bromide (MTT; Chemicon International) dye absorbance as previously described (Raje et al., 2009). DNA synthesis was measured by tritiated thymidine uptake (^{3}H-TdR; Perkin Elmer). MM cells ($2–3\times10^4$cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) with media and different concentrations of AT7519 and/or recombinant IL-6 (10 ng/mL) or IGF-1 (50 ng/mL) for 24 or 48 h at 37°C and ^{3}H-TdR incorporation was measured as previously described (Raje et al., 2009).

Detection of RNA synthesis

RNA synthesis was evaluated by measuring [^{3}H] uridine (Perkin Elmer) incorporation. MM.1S cells (3×10^4cells/well) were incubated in 96-well culture plates in the presence of media or AT7519 (0.5 μM) for 4, 6, 24 and 48h. Cells were incubated with [^{3}H] uridine (1 μCi (37 KBq)/well) for 3.5 h at 37°C, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). ^{3}H uptake analyses were performed in triplicate.

Cell cycle analysis and detection of apoptosis

MM cells (1×10^6) were cultured for 48h in media alone or with varying concentrations of AT7519. Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), fixed with 70% ethanol for 20 minutes, and pretreated with10 μg/mL RNase (Sigma) for 20 minutes as previously described (Raje et al., 2009). Apoptosis analysis was also confirmed by using Annexin V/PI staining after MM cells were cultured in media or 0.5 μM of AT7519 at 37°C for 6, 12, 24 hours as previously described (Raje et al., 2009). Annexin V^+PI^- apoptotic cells were enumerated by using the Epics flow cytometer. The percentage of cells undergoing apoptosis was defined as the sum of early apoptosis (AnnexinV-positive cells) and late apoptosis (Annexin V-positive and PI-positive cells).

Western blotting

MM cells were cultured with AT7519 0.5 μM, harvested, washed, and lysed using lysis buffer as previously described (Raje et al., 2009). The protein concentration of lysate was measured, mixed with gel electrophoresis loading buffer, boiled for 5 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane. The membranes were blocked in TBS plus 5% non fat milk powder and 0.1% TWEEN20 for 1 hour before incubating with the following antibodies overnight at 4°C: anti phospho -RNA pol-II serine 2 and serine 5, RNA pol II (Astex therapeutics, Cambridge, UK), phospho-GSK-3β (serine 9), GSK-3β, phospho-Akt (serine 473), Akt, phospho-p44/42-MAPK, p44/42 MAPK, phospho-p70SK6, p70SK6, CDK4, CDK9, XIAP, Mcl-1, caspase 3, caspase 9 and caspase 8 (Cell Signaling Technology); anti-cyclin D1, c-Myc (BD Biosciences); anti-CDK1, CDK2, CDK5, CDK6, cyclin B1, cyclin A, Mcl-1, (Santa Cruz Biotechnology). Antigen-antibody complexes were detected using secondary antibodies conjugated to HRP and visualized using enhanced chemiluminescence (GE Healthcare). Blots were stripped and reprobed with anti-α-tubulin, GAPDH or α-actin (Santa Cruz Biotechnology) antibodies to ensure equal protein loading. Quantitation of band intensity was performed using Image J software.

Transfection and Lentivirus infection

To determine the role of GSK-3β in AT7519-induced apoptosis, we used shRNA sequences to knock down GSK-3β in MM.1S cell line using a lentivirus transfection system. The shRNA was kindly provided by RNAi Screening Facility of Dana Farber Cancer Institute. The sequence for of the GSK-3β shRNA construct was as follows: clone no.1: 5'-CCACTGATTATACCTCTAGTA-3'; clone no.2: 5'-CCCAAACTACACAGAATTTAA-3'; clone no 3: 5'-GCAGGACAAGAGATTTAAGAA-3'; clone no 4: 5'-GCTGAGCTGTTACTAGGACAA-3'; clone no 5: 5'-GACACTAAAGTGATTGGAAAT-3'. pLKO.1 plasmid (Supplementary data, Fig S1) with GSK-3β shRNA or pLKO.1 control plasmid were cotransfected with pVSV-G and delta 8.9 plasmids into 293T cells with FuGENE 6 transfection reagent (Roche, IN, USA). At 48 and 72 hours post transfection, supernatant containing pseudoviral particles were collected; aliquots with 8 μg/ml polybrene were added to MM.1S cells (1×10^6 per ml) as previously described (Raje et al., 2009). Two days after infection, cells were analyzed for GSK-3β and GAPDH expression by western blotting. In order to obtain GSK-3β null MM cell line, cells were selected in puromycin (1.5 μg/ml, Invitrogen). The transfection efficiency was 40% after puromycin selection.

MM xenograft mouse model

To evaluate the in vivo anti-MM activity of AT7519, male SCID mice were inoculated subcutaneously with 5×10^6 MM.1S cells in 100 μl serum-free RPMI 1640 medium. When tumors were measurable, mice were treated intraperitoneally (IP) with vehicle or AT7519

dissolved in saline 0.9%. The first group of 10 mice was treated with 15 mg/kg once a day for five days for 2 weeks, and the second group was treated with 15 mg/kg once a day three times a week for four consecutive weeks. The control group received the carrier alone at the same schedule. Tumor size was measured every alternate day in 2 dimensions using calipers, and tumor volume was calculated with the formula: $V= 0.5\ a \times b^2$ (a= long diameter of the tumor, b= short diameter of the tumor). Animals were sacrificed when the tumor reached 2 cm^3 or when the tumor was ulcerated. Survival and tumor growth were evaluated from the first day of treatment until death. All animal studies were approved by the Dana-Farber Animal Care and Use Committee.

Statistical analysis

All *in vitro* experiments were performed in triplicate and repeated at least 3 times; a representative experiment was selected for figures. Statistical significances of differences for both *in vitro* and *in vivo* experiments were determined using Student *t* test, with minimal level of significance $P < 0.05$. Overall survival was measured using the Kaplan-Meier method, and the results are presented as the median overall survival, with 95% confidence intervals. All statistical analyses were determined using GraphPad Prism software (GraphPad Software, Inc. San Diego, CA,)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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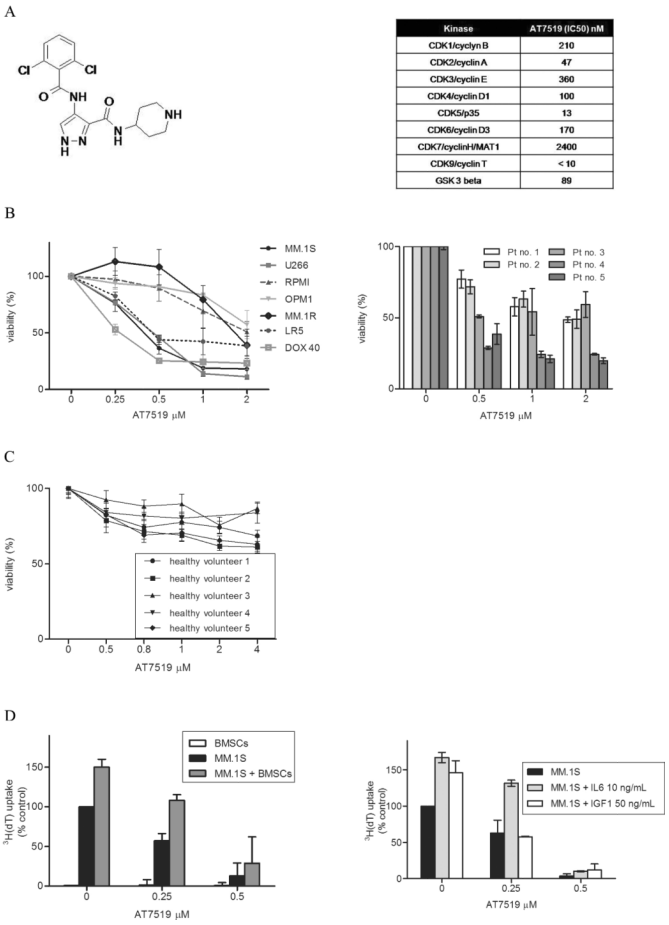

FIG 1. AT7519 treatment decreases viability of MM cells in a dose dependent manner and overcomes proliferative advantage conferred by cytokines and the protective effect of BMSC

(A) Chemical structure of AT7519 (left panel). *In vitro* kinase inhibition (right panel). (B) MM cell lines (MM.1S, U266, OPM1, RPMI, LR5, DOX 40, MM.1R) and primary CD138+ MM cells from five different patients were cultured in the presence of increasing doses of AT7519 for 48 hours.. The effect of AT7519 was determined by MTT assay. IC_{50} ranged from 0.5 to 2 μM. (C) AT7519 does not affect viability of peripheral blood mononuclear cells (PBMNCs) from healthy volunteers. (D) MM.1S cells were cultured with BMSCs, IL-6 (10 ng/ml), IGF-1 (50 ng/ml). AT7519 induced inhibition of DNA synthesis at 48 hours in dose dependent manner. The results represent an average of triplicate experiments ± SD.

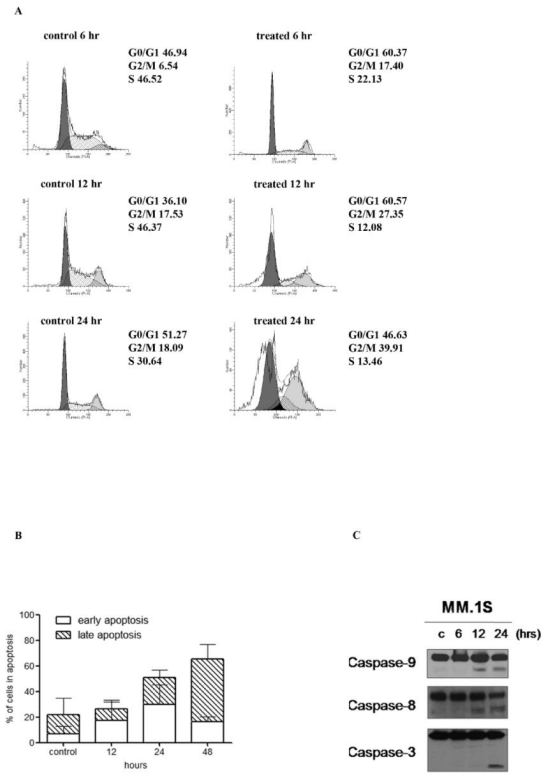

FIG 2. AT7519 treatment induces apoptosis of MM cells in a time-dependent manner

(A) Cell cycle analysis by PI staining was performed on MM.1S. MM cells cultured with media alone or AT7519 (0.5 μM) for the indicated time points. AT7519 resulted in an increase G0/G1 phase and G2/M phase starting at 6 h. (B) Apoptosis was evaluated by Annexin/PI staining. The percentage of cells undergoing apoptosis was defined as the sum of early apoptosis (AnnexinV-positive cells) and late apoptosis (Annexin V-positive and PI-positive cells). Apoptosis induction was observed starting from 12 hours. (C) The apoptosis induced by AT7519 was further confirmed by western blotting. Whole cell lysates (30 μg/lane) were subjected to Western blotting using the specified antibodies.

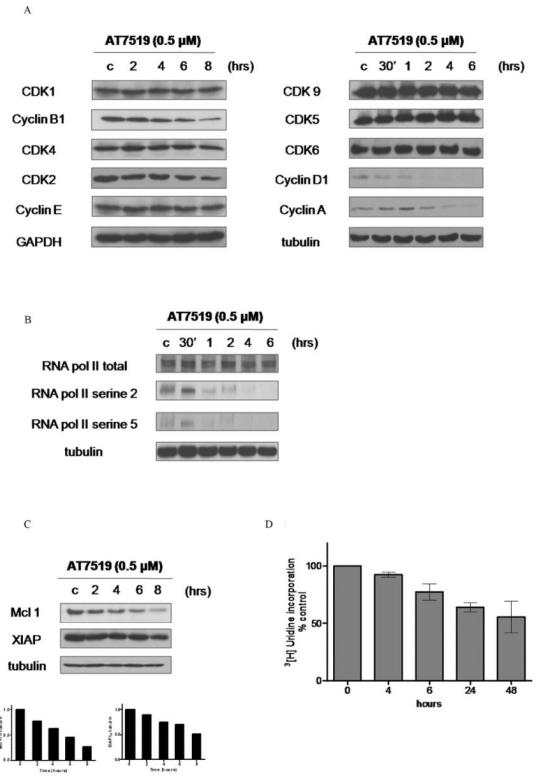

FIG 3. AT7519 does not affect the expression of relevant cyclins and CDKs at early points but induces dephosphorylation of RNA pol II CTD

(A) MM.1S cells were incubated with AT7519 0.5 μM. At indicated time points, cells were harvested and whole lysates were subjected to Western blotting using the antibodies indicated. (B) MM.1S cells were incubated with AT7519 0.5 μM. At indicated time points cells were harvested and whole lysate were subjected to Western blotting using anti-RNA pol II, phospho RNA pol II serine 2, phospho RNA pol II serine 5, α-tubulin, α-actin antibodies. (C) MM.1S cells were incubated with AT7519 0.5 μM. At indicated time points cells were harvested and whole lysate were subjected to Western blotting using anti-Mcl-1, anti-XIAP and anti-α tubulin antibodies. Densitometry is demonstrated for each panel. (D) AT7519 inhibited RNA synthesis in MM.1S cells. MM.1S cells were incubated with media alone and AT7519 0.5 μM for 4, 6, 24 and 48 hours; then 3.5 hours prior of harvesting, [^{3}H] Uridine was added to the cell culture and RNA synthesis measured. The results represent an average of triplicate experiments ± SD.

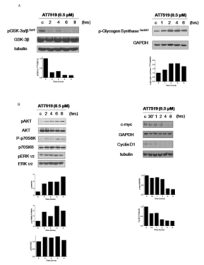

FIG 4. AT7519 induced cytotoxicity was associated with dephosphorylation of GSK-3β at serine 9

(A) MM.1S cells were incubated with media alone and AT7519 0.5 μM. At indicated time points cells were collected and whole lysates were subjected to Western blotting using anti-phospho GSK-3β serine 9, GSK-3β, glycogen synthase serine 641, α-tubulin, GAPDH antibodies. (B) MM.1S cells were incubated with AT7519 0.5 μM. At indicated time points, cells were collected and whole lysates were subjected to Western blotting using the antibodies indicated. Densitometry is demonstrated for each panel.

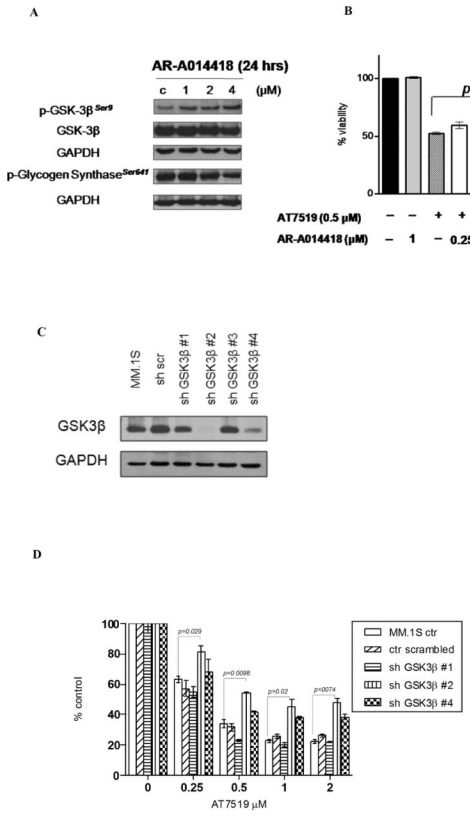

FIG 5. Inhibition of GSK-3β attenuates AT7519-induced apoptosis

(A) MM.1S cells were incubated with DMSO and AR-A014418 at indicated doses for 24 hours. Whole lysates were subjected to western blotting using anti-phospho GSK-3β serine 9, GSK-3β, glycogen synthase serine 641, GAPDH antibodies. These results are representative of at least three independent experiments. (B) MM.1S cells were cultured for 30 minutes with media alone and indicated doses of AR-A014418, and then incubated for 48 hours with AT7519 0.5 μM. The viability was determined by MTT assay. The results represent an average of triplicate experiments ± SD. (C) Silencing GSK-3β with shRNA lentiviral constructs in MM.1S was evaluated by western blotting. GSK-3β knock down was evident in clone no. 2 and observable in clone no.4. (D) MM.1S cells and transfected MM. 1S were treated with increasing doses if AT7519 for 48 h. MM.1S cells with knocked-down GSK-3β, were more resistant to AT7519-induced cytotoxicity with respect to control shRNA-transfected cells. The effect of AT7519 was determined by MTT assay.

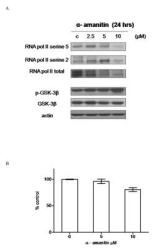

FIG 6. AT7519 dephosphorylates GSK-3β independent of inhibition of transcription

(A) MM.1S cells were incubated with media alone and with increasing doses of α-amanitin as indicated. At 24 hours, cells were collected and whole lysate were subjected to Western blotting using the specified antibodies. (B) The effect of alpha-amanitin on MM.1S cells viability was determined by MTT assays after 24 hours of treatment. The results represent mean (± SD) of triplicate experiment.

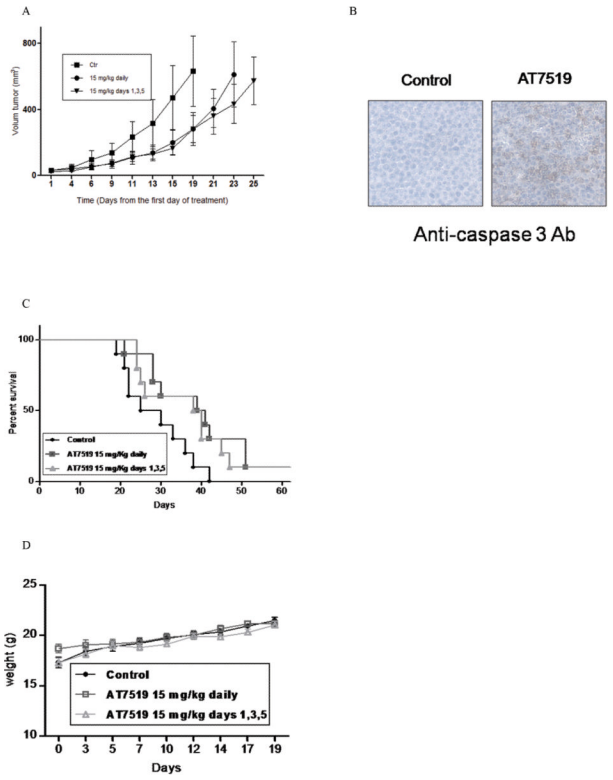

FIG 7. AT7519 inhibits MM cell growth *in vivo*

(A) Average ±SEM of tumor volume (mm^3) from groups of mice (n=10/group) versus time (days) from first day of treatment after the development of measurable tumor. (B) Immunohistochemestry confirmed caspase 3 activation. (C) Survival was evaluated from the first day of treatment until death. Using Kaplan Meier and log-rank analysis, statistically significant prolongation in median OS was observed in treated mice compared with the control group ($p = 0.0324$ and $p= 0.0411$, respectively). The median overall survival (OS) in the group treated with 15 mg/kg once a day for five days for 2 weeks was 40 days versus 27.50 days in the control cohort. The median OS in the group treated with 15 mg/kg once a day three days week for four consecutive weeks was 39 days versus 27.50 days in the control group. (D) Average ±SEM of weight (gr) from groups of mice (n=10/group) versus time (days) from first day of treatment. AT7519 did not affect the body weight.