Research Article

AT7519, a Cyclin-Dependent Kinase Inhibitor, Exerts Its Effects by Transcriptional Inhibition in Leukemia Cell Lines and Patient Samples

Matthew S. Squires¹, Laurence Cooke², Victoria Lock¹, Wenqing Qi², E. Jonathan Lewis¹, Neil T. Thompson¹, John F. Lyons¹, and Daruka Mahadevan²

Abstract

AT7519 is a potent inhibitor of several cyclin-dependent kinases and is currently in early phase clinical development. Recently, cyclin-dependent kinases 7, 8, and 9 have been shown to regulate transcription through phosphorylation of RNA polymerase II. B-cell lymphoproliferative disorders, including chronic lymphocytic leukemia, rely on the expression of transcripts with a short half-life, such as Mcl-1, Bcl-2, and XIAP, for survival. Here, we describe the characterization of AT7519 in leukemia cell lines, and compare and contrast the response in cell lines derived from solid tumors. Finally, we use these mechanistic insights to show activity in peripheral blood mononuclear cells isolated from 16 chronic lymphocytic leukemia patients. AT7519 induced apoptosis at concentrations of 100 to 700 nmol/L and was equally effective regardless of Rai stage or known prognostic markers. Short-term treatments (4-6 hours) resulted in inhibition of phosphorylation of the transcriptional marker RNA polymerase II and downregulation of the antiapoptotic protein Mcl-1, with no effect on either XIAP or Bcl-2 levels. The reduction in Mcl-1 protein level was associated with an increase in cleaved poly(ADP-ribose) polymerase. Together the data suggest AT7519 offers a promising treatment for patients with advanced B-cell leukemia.

Introduction

AT7519 is a novel multitargeted cyclin-dependent kinase (cdk) inhibitor that selectively inhibits cdks 1, 2, 4, 5, and 9, and has no appreciable activity against any other kinases tested except for glycogen synthase kinase-3β. AT7519 was rationally designed with the use of high throughput X-ray crystallography (1), and its activity has been evaluated in preclinical models (2) and a phase I clinical trial in refractory solid tumors (3). Recently, the role of cdks 7, 8, and 9 in the regulation of transcription has been explored (4, 5). Acting through the phosphorylation of the COOH-terminal domain of RNA polymerase II, they promote initiation and elongation of nascent mRNA transcripts (6). Inhibition of the transcriptional cdks has attracted interest because the most sensitive transcripts are those with short half-lives that encode cell cycle regulators, mitotic regulatory kinases, NF-κB-responsive gene transcripts, and apoptosis regulators, such as Mcl-1 and XIAP (5). Diminution of the levels of these transcripts and their encoded proteins may produce anticancer activity or augment apoptotic responses.

AT7519 was shown to be a potent inhibitor of survival in solid tumor cell lines, with a mechanism of action consistent with the compound exerting its activity through the cell cycle. We observed in solid tumor lines that AT7519 is in addition a potent inhibitor of transcription (2). Based on the transcriptional activity of AT7519, we hypothesized that it would promote apoptosis in leukemia cell lines and chronic lymphocytic leukemia (CLL) cells through this mechanism. Human leukemia cell lines are sensitive to AT7519 (2). We showed the inhibition of phosphorylation of RNA polymerase II on incubation with AT7519 and a concomitant rapid reduction in protein levels of the antiapoptotic protein Mcl-1. Reduction in Mcl-1 protein levels in tumors was observed after i.p. administration of AT7519 to HL60 tumor-bearing mice, and optimal efficacy was achieved in this model after once-daily dosing of AT7519 at 15 mg/kg. This is in contrast to the optimal schedule in solid tumor lines, in which twice-daily administration of a lower dose achieved maximal efficacy (2). This is suggestive of AT7519 exerting its effects through a different mechanism in leukemia lines. CLL is characterized by a population of malignant B cells with heterogeneous biological features but representative of a phenotype aberrant in proliferation and apoptosis (7). Treatment of CLL with approved therapies (chlorambucil, fludarabine, alemtuzumab, rituximab, bendamustine, lenalidomide, lumiliximab, flavopiridol, oblimerson)
has not led to significant progression-free and overall survival (8). The cell cycle is a critical regulator of cell proliferation and growth that is tightly regulated by cdks, cyclins (activators), and cdk inhibitors (9, 10). Cdk5 plays a key role in cell cycle progression, and there is evidence that pathways leading to their activation are deregulated in a number of human malignancies, which make them attractive therapeutic targets. Several agents with cdk activity are already in development in this area, with the most advanced being flavipiridol, which has shown robust responses in early phase trials in refractory CLL patient populations (11, 12). Selicilib (CVC202/R-roscovitine; ref. 13) and SNS-032 (BMS 387032; ref. 14) have also shown preclinical activity in CLL and are currently in clinical investigation.

In this study we have examined a panel of CLL cells isolated from patients either with low–Rai stage or high–Rai stage disease, and characterized the cytogenetics and ZAP70 status of patient cells. Sensitivity to AT7519 was independent of ZAP70 expression and a variety of cytogenetic abnormalities, including 13q deletion and trisomy 12. Overexpression of ZAP70 has been shown to be associated with shorter progression-free survival (15, 16), and a variety of cytogenetic abnormalities have been identified that have stronger and weaker prognostic significance (17). Aberrations in the p53 pathway, including 17p deletions, whereas trisomy 12 (mdm2 upregulation) and 13q deletion are more common; the latter encodes microRNAs, the targets for which remain unclear (17). Exploiting the transcriptional activity of cdk inhibitors based on the hypothesis that intermittent treatment with these agents will cause rapid reduction in protein levels of antiapoptotic proteins with the highest turnover, triggering apoptosis in the tumor cell but leaving normal cells unaffected, is an area of great interest. The data shown here suggest that AT7519 is a potent inducer of apoptosis in CLL cells independent of the cytogenetic status of the cells due to its transcriptional inhibitory activity and warrants further investigation in a clinical study. Pharmacokinetic data from a phase I study with AT7519 showed that at tolerated doses, exposures consistent with the compound concentrations and incubation times required for induction of apoptosis in CLL cells ex vivo are achieved.

Materials and Methods

Materials

AT7519 is N-(4-piperidinyl)-4-(2,6-dichlorobenzoylamino)-1H-pyrazole-3-carboxamide, synthesized by Astex Therapeutics Ltd. (1), which has a Mr of 382 Da.

Cells. HL60 and HCT116 cells were obtained from European Collection of Cell Cultures, and maintained in RPMI 1640 and DMEM, respectively. The media were supplemented with 10% fetal bovine serum and the cells maintained at 37°C in an atmosphere of 5% CO₂. All reagents were purchased from Invitrogen.

European Collection of Cell Cultures supplies cell lines authenticated by DNA profiling and isozyme analysis. The cultures used in these experiments were passaged for <6 mo after resuscitation of a vial received directly from the cell bank.

Patient samples and preparation. The patients studied had a diagnosis of CLL Rai stage 0/I, II, or III/IV. Patient blood samples (N = 16) were collected after obtaining informed consent on an Institutional Review Board–approved protocol. Whole blood was collected for cells and serum. Mononuclear cells were separated, and either stored frozen at -80°C or maintained in RPMI 1640 with 10% human serum (Gemini Bio-Products) at 37°C in 5% CO₂. Separated serum was stored in 0.5-mL aliquots at -80°C until further studies were done. Mononuclear cells were separated from peripheral blood samples by Ficoll-Hypaque density centrifugation. The CLL cells were then maintained in culture in RPMI 1640 at 37°C for further studies.

Methods

Tritiated uridine assay. mRNA synthesis was measured by monitoring the incorporation of tritiated uridine into mRNA transcripts. HCT116 cells were seeded onto 96-well plates at 2 × 10⁴ cells per well in 72 μL complete medium and allowed to recover overnight. Compounds were diluted in medium and added to non-edge wells to a final concentration of 0.1% DMSO. The plates were incubated at 37°C for 30 min. Tritiated uridine (0.1 μCi) was added to each well and the plates returned to the incubator for a further 3.5 h. The media were removed and the cells fixed by the addition of 5% ice-cold trichloroacetic acid. Plates were washed with trichloroacetic acid and tris(hydroxymethyl)aminomethane (Tris) buffer, and the plates returned to the incubator overnight. AT7519 or vehicle control (0.5% DMSO) was added for 72 h before the addition of 20 μL MTS reagent (Promega) to each well. Plates were incubated for 6 h before reading absorbance at 490 nm. Background data were subtracted and IC₅₀ values calculated with the use of the GraphPad Prism software.

Apoptosis assays. B cells were seeded at 300,000 cells per well in 96-well plates and allowed to recover overnight. AT7519 or vehicle control (0.5% DMSO) was added for 72 h before the addition of 20 μL MTS reagent (Promega) to each well. Plates were incubated for 6 h before reading absorbance at 490 nm. Background data were subtracted and IC₅₀ values calculated with the use of the GraphPad Prism software.

Real-time quantitative PCR for ZAP70 expression. B cells were isolated with the use of the Ficoll gradient...
technique. Fifteen milliliters of blood were layered on top of 12.5 mL of Ficoll. B cells were separated by centrifugation at 500 g for 35 min. The B-cell layer was removed and washed thrice with sterile Dulbecco’s PBS. One hundred nanograms of total RNA were used for reverse transcriptase reactions (20 μL total volume) carried out with the use of SuperScript III Platinum Reverse Transcriptase (Invitrogen). Reactions were incubated at 42°C for 50 min followed by incubation at 37°C with RNase H for 20 min. An Opticon DNA Engine (MJ Research) was used to do real-time fluorescence detection PCR. cDNA (1 μL) was added to 12.5 μL of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 1 μL of gene-specific or β-actin-specific primer pair (see primer design), and 10.5 μL of dH2O (final volume of 25 μL). Amplification (95°C for 15 s, 55°C for 30 s, and 72°C for 30 s) was repeated for 44 cycles. The threshold cycle value (Ct) for each sample indicated the cycle at which a statistically significant increase in fluorescence was first detected. These data were then normalized to β-actin, which served as an unaltered control gene, for each data point and compared with a normal B-cell control to determine relative expression ratios. Each measurement was done in triplicate.

PCR primers were designed with the use of MacVector (Accelrys) to produce amplicons with lengths ranging from 80 to 250 bp to optimize the efficiency of quantitative PCR. β-actin primers from QuantrumRNA β-Actin Internal Standards (Ambion) were used to normalize the quantitative PCR data.

**Cytogenetics.** Standard fluorescence in situ hybridization panel for CLL was done by ARUP Laboratories.

**Immunoblotting.** HL60 or CLL cells were seeded at a concentration of 1 x 10^6 or 4 x 10^6, respectively, onto six-well tissue culture plates and allowed to recover for 16 h. AT7519, at the indicated concentration, or vehicle control (0.1% DMSO) was added for the indicated times. Cells were harvested and lysed in 100 μL of ice-cold Triton lysis buffer (0.1% volume for volume Triton X-100). Lysates were cleared by centrifugation and a sample of the supernatant removed for protein determination. Equivalent amounts of protein lysate had SDS sample buffer added and were boiled for 5 min. Samples were resolved by SDS-PAGE (NuPAGE System, Invitrogen) and blotted onto polyvinylidene difluoride filters. Immunoblotting was done with the specific antibodies described. Mcl-1, Bcl-2, and cleaved poly(ADP-ribose) polymerase antibodies were from Cell Signalling Technology. Anti-total Rb and anti-pRb T^821^ were from Biosource International. RNA polymerase II antibodies were from Cambridge Bioscience. β-actin was from Abcam Plc. Primary antibodies were followed by incubation with either IR dye labeled anti-rabbit or anti-mouse antibodies (LI-COR). Blots were scanned to detect IR fluorescence on the Odyssey Infrared Imaging System (LI-COR).

**Pharmacodynamic studies.** S.c. xenograft tumors were removed from nude mice at the indicated times after administration of a single i.p. dose of 10 mg/kg AT7519. Tumor samples were ground to a fine powder under liquid nitrogen, and protein was extracted by the addition of 1 mL Triton lysis buffer. Western blots were done as outlined above.

**Xenograft studies.** Experiments were done with male BALB/c nu/nu mice according to the UK Animals (Scientific Procedures) Act 1986. Animals were purchased from Harlan UK Ltd. and housed in pathogen-free conditions. Six-to-eight-week-old male BALB/c Hsd:athymic nude-Foxn1^nu^ mice were implanted s.c. with 1 x 10^7 HL60 cells per mouse into the right flank. Eleven to 14 d after implantation, mice were arranged into groups of eight according to tumor volume, with a mean volume of 75 mm^3^ (range, 50-200 mm^3^). Mice were then dosed according to schedule. Tumor volume was measured every 2 to 3 d as above.

In each case a statistically significant slowing of increase in xenograft volume or regression of tumor volume over time compared with a matched control group was used to characterize efficacy. A complete regression was defined as a decrease in tumor volume to an undetectable size, taken as measurements of <3 mm in any dimension. Tolerance was estimated by monitoring body weight loss, clinical signs, and survival over the course of the study.

**Results**

**AT7519 inhibits transcription in human tumor cell lines**

AT7519 is a potent inhibitor of a number of cyclins, including cyclin 9, which is associated with the regulation of transcriptional activity (1, 2). The ability of AT7519 to inhibit phosphorylation of RNA polymerase II on serines 2 and 5 of the COOH-terminal domain repeats was shown in the HL60 human leukemia cell line (Fig. 1A). Complete inhibition of phosphorylation was observed at concentrations >400 nmol/L. A downstream consequence of this transcriptional inhibition was the reduction in protein levels of short–half-life transcripts, such as Mcl-1. Mcl-1 protein levels were rapidly decreased after exposure of HL60 cells to AT7519 at concentrations >400 nmol/L for 4 h (Fig. 1B). The inhibitory effect on transcription was confirmed, showing that AT7519 inhibited incorporation of tritiated uridine into the mRNA fraction (Fig. 1C). In HCT116 cells tritiated uridine incorporation was inhibited with a mean IC^50^ of 54 nmol/L, confirming that AT7519 inhibits transcription and can affect cell survival through this mechanism. These observations were confirmed in a s.c. xenograft model with HL60 cells. A single dose of AT7519 at 10 mg/kg i.p. was sufficient to inhibit markers of cyclin 2 inhibition (pNPM, pRb) for between 8 and 16 h; however, protein levels of Mcl-1, a surrogate for the transcriptional activity, were reduced by 1 h after dosing and remained depressed at 24 h after a single dose (Fig. 2A). This observation was consistent with the proposal that the transcriptional activity of AT7519 dominates in leukemia cell lines and that the optimum dose schedule for efficacy in the HL60 xenograft was a larger dose given daily rather than the twice-daily schedule. The fact that cell cycle pharmacodynamic
markers (pNPM and pRb) are only transiently inhibited after dosing (Fig. 2B) and that previous studies in solid tumor cell lines have shown that the optimum dosing schedule in solid tumor lines is twice-daily administration suggests a requirement to inhibit cell cycle markers more completely over the dosing period in these cell types (2).

We hypothesize that AT7519 exerts its effects primarily through cell cycle inhibition in most solid tumor cell lines, but through transcriptional inhibition in cell lines and tumors that are dependent on short-lived mRNA transcripts (e.g., Mcl-1) for survival. Inhibition of cell cycle targets are nevertheless observed in this cell population and may make a contribution to the apoptotic activity observed.

**AT7519 is cytotoxic to CLL patient-derived cells**

CLL cells were isolated from 16 patients with low–Rai stage and high–Rai stage disease (18). They were pathologically well characterized and were either pretreated or treatment naïve (Table 1). Eight patients were described as having Rai stage 0 to II and eight with Rai stage III/IV. The samples were further characterized for ZAP70 expression, which was expressed as fold expression over normal B-cell samples taken from healthy volunteers, and fluorescence in situ hybridization analysis to identify cytogenetic abnormalities commonly associated with CLL prognosis was done.

After 72-h exposure to AT7519, the viability of CLL samples was assessed by MTS assay. Effective concentration 50% (EC50) values of between 100 and 700 nmol/L were obtained across patient samples tested with a mean EC50 of 264 nmol/L (Table 1). AT7519 seems to be active against CLL cells regardless of Rai stage, number of prior therapies, and prognostic factors, such as ZAP70 expression and cytogenetic abnormalities. To investigate further the kinetics of this cytotoxic activity, a number of cell samples were exposed to AT7519 for different times up to 72 h (Fig. 3). For each of the four patients tested, the EC50 decreased as time of exposure to AT7519 was increased, reaching a minimum at around 24 h. However, significant cytotoxicity was observed even at 4 h after exposure to AT7519, with EC50 values <1 μmol/L in three of four patients tested, suggesting that short exposure to concentrations ~1 μmol/L is sufficient to trigger significant cell death in these CLL samples.

**AT7519 induces apoptosis in CLL patient-derived cells**

The mechanism of this cytotoxicity was investigated by scoring nuclear morphology after staining with acridine orange. Apoptotic cells were scored as a percentage of the total cell population after exposure to AT7519 (Fig. 4). Figure 4A shows the percentage of apoptotic cells after 4-h or 6-h exposure to a range of concentrations of AT7519 for patient samples 9 and 13, respectively. Significant numbers of apoptotic cells were observed after 6-h exposure to AT7519 in each case, with a smaller
effect observed at 4 h. This general trend for significant induction of apoptosis after 6-h exposure at 1 \( \mu \)mol/L AT7519 was observed across several patient samples (Fig. 4B). These data suggest that short-term exposure to AT7519 is sufficient to commit CLL cells to apoptosis.

**AT7519 inhibits RNA polymerase II and reduces antiapoptotic protein levels**

CLL cells from patients 11 and 13 were treated with AT7519 for 6 h, and phosphorylation of RNA polymerase II, cell cycle markers, and levels of apoptosis-related proteins were assessed by western blotting (Fig. 5A and B). RNA polymerase II phosphorylation on the C terminal domain (CTD) repeats was inhibited at concentrations \( \geq 300 \) nmol/L. At the same concentration, levels of Mcl-1 were reduced, consistent with AT7519 inhibiting transcription of Mcl-1 mRNA. A concomitant increase in the cleaved form of poly(ADP-ribose) polymerase was also observed, indicative of the induction of apoptosis. In patient 11 we observed induction of cleaved poly(ADP-ribose) polymerase after only a modest reduction of Mcl-1 protein levels. This suggests that these cells were either particularly sensitive to loss of Mcl-1 or that the reduction of other prosurvival proteins contributes to this effect in certain patient samples. Levels of the anti-apoptotic protein Bcl-2 remained unchanged as did phosphorylation of the cdk 2 substrate Rb. Lack of inhibition of Rb probably reflects the fact that CLL cells isolated from the peripheral blood are not proliferating. As such the phospho-Rb signal reflects a low basal level of phosphorylation in quiescent cells that is not turned over. The time course of these inhibitory events was investigated in patient sample 13 (Fig. 5C). Cells...
were exposed to 500 nmol/L AT7519 for varying lengths of time and prepared for western blotting. In this case we observed rapid inhibition of phosphorylation of pRNA polymerase II that was complete between 4-h and 6-h exposure. Mcl-1 protein levels declined by 4 h, and significant amounts of cleaved poly(ADP-ribose) polymerase were observed by 6 h, peaking at 24-h exposure. These observations are consistent with AT7519 exerting its effect primarily through inhibition of transcription in CLL cells.

Discussion

AT7519 is a potent inhibitor of multiple cdks, including those implicated in the regulation of transcription through phosphorylation of the CTD repeats of RNA polymerase II. We show here that inhibition of RNA polymerase II phosphorylation occurs in human tumor cell lines in a manner consistent with the inhibition of global transcription as measured by tritiated uridine incorporation. In leukemia cell models, this activity leads to a reduction in the protein levels of short-half-life transcripts, including antiapoptotic proteins, such as Mcl-1, which occurred rapidly and was followed by an increase in tumor cell apoptosis. These effects were observed in vitro at AT7519 concentrations of 400 nmol/L and in vivo after a dose of 10 mg/kg i.p. The optimal dosing schedule in vivo also suggested an antitranscriptional effect in that greater efficacy was achieved after a single daily dose of AT7519 in leukemia lines. This contrasts with the optimal dosing schedule in solid tumor lines, in which maximal efficacy was achieved by dividing the dose over a twice-daily schedule to increase coverage of the cell cycle (2). The solid tumor lines tested as xenografts responded to AT7519 in a manner consistent with cell cycle

Table 1. Patient sample characteristics

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<th>Patient</th>
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<th>No. of prior treatments</th>
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<th>Relative ZAP70 expression</th>
<th>Cytogenetic abnormalities</th>
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NOTE: Patient sample set characteristics and response to AT7519. Sixteen CLL samples were isolated from a diverse patient sample characterized by a variety of Rai stages and prognostic markers, including ZAP70 expression and additional cytogenetic abnormalities. The isolated cells were exposed to AT7519 for 72 h and the cytotoxic EC50 calculated by MTS assay.

Figure 3. AT7519 induces apoptosis in CLL cells after short-term exposure. The cytotoxic EC50 was determined by MTS assay for four CLL cell samples isolated from the indicated patients. Cell samples were exposed to AT7519 for the indicated time, and EC50 values were determined from six replicate dose-response curves generated from each individual.
inhibition and are generally thought to be less reliant on, and therefore less sensitive to, the loss of short-lived antiapoptotic proteins. CLL is dependent on short-lived antiapoptotic proteins, including Mcl-1 (19). CLL cells that accumulate in the peripheral blood are generally quiescent, and as such the cell cycle activity of AT7519 in an ex vivo setting was unlikely to be significant. On the basis of these observations, we hypothesized that AT7519 would be an effective inhibitor of CLL cell survival.

CLL can be stratified by Rai staging and by prognostic markers, such as the expression of ZAP70, in which high levels of ZAP70 are associated with more aggressive disease or a variety of cytogenetic abnormalities that predict for disease progression, response to therapy, or time to initial therapy. Patients were chosen to exemplify the heterogeneous nature of CLL. The cytotoxic effect of AT7519 on these samples was characterized ex vivo in terms of both the concentration response and time of exposure. These data show that 4- to 6-h exposure of AT7519 is sufficient to commit CLL cells to apoptosis at concentrations <1 µmol/L. The characterized variables of Rai stage, ZAP70 expression, and cytogenetic abnormality made no significant difference to sensitivity to AT7519, with the compound having similar activity across all samples tested, including high-risk disease that was or would be predicted to respond poorly to conventional therapy. The cytotoxicity observed was consistent with inhibition of RNA polymerase II phosphorylation, as judged by a reduction in Mcl-1 protein levels but not in the levels of Bcl-2 protein. All of these changes occurred rapidly after <4-h compound exposure and at concentrations <400 nmol/L. Similar studies have examined the activity of other pan-cdk inhibitors in CLL. The furthest advanced in development is flavopiridol, currently in phase III clinical development. Flavopiridol has similar effects on transcription and Mcl-1 protein turnover in CLL ex vivo samples and has shown encouraging responses in clinical studies (13). It is a highly protein-bound compound in human plasma with a relatively low volume of distribution and high clearance, and as such is dosed with a short loading infusion followed by an extended maintenance phase. The reported dose-limiting toxicity is tumor lysis syndrome elicited by the rapid apoptosis of CLL blasts in patients with high white cell counts. SNS-032, another pan-cdk inhibitor, is also...

Figure 4. AT7519 induces apoptosis in CLL cell samples in a time-dependent and dose-dependent fashion. CLL cell samples from patients 9 and 13 were exposed to the indicated concentrations of AT7519 for either 4 or 6 h (A). After exposure to the compound, the percentage of apoptotic cells was estimated by nuclear morphology scoring. Scoring was undertaken in triplicate or more response curves in each case. Additional CLL cell samples were exposed to AT7519 for 6 h only to estimate the range of response across the subject group (B).
undergoing clinical investigation in CLL with the use of a similar two-stage schedule to flavopiridol. CYC-202 has also shown activity in CLL samples, although with a much lower potency than AT7519 or the two compounds described above (14).

During the dose-escalation phase of phase I clinical studies, AT7519 had a favorable pharmacokinetic profile that achieved plasma levels, at tolerated doses, which we would predict from our studies described here to be effective at targeting CLL blasts (3). These doses maintain pharmacologically active levels of up to 500 nmol/L for >12 h. AT7519 has a low protein binding of 57% in human plasma and a high volume of distribution at 1.4 L/kg in mice. This dosing regimen would therefore be expected to be efficacious in a CLL population dosed on an intermittent schedule. AT7519 would be expected to be efficacious in a broad CLL patient population, including those refractory to purine analogue-based therapy, and therefore a single-agent phase II trial is planned.

Disclosure of Potential Conflicts of Interest

D. Mahadevan: research funding from and consultant and advisory board member for Astex Therapeutics Ltd. M.S. Squires, V. Lock, N.T. Thompson, E.J. Lewis, and, J.F. Lyons: employees of and have shares and share options in Astex Therapeutics Ltd. The other authors declared no potential conflicts of interest.

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