Src kinase inhibitors induce apoptosis and mediate cell cycle arrest in lymphoma cells

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Src kinases are involved in multiple cellular contexts such as proliferation, adhesion, tumor invasiveness, angiogenesis, cell cycle control and apoptosis. We here demonstrate that three newly developed dual selective Src/Abl kinase inhibitors (SrcK-I) (AZM559756, AZD0530 and AZD0424) are able to induce apoptosis and cell cycle arrest in BCR–ABL, c-KIT and platelet-derived growth factor-negative lymphoma cell lines. Treatment of DOHH-2, WSU-NHL, Raji, Karpas-299, HUT78 and Jurkat cells with SrcK-I revealed that the tested substances were effective on these parameters in the cell lines DOHH-2 and WSU-NHL, whereas the other tested cell lines remained unaffected. Phosphorylation of Lyn and in particular Lck were affected most heavily by treatment with the SrcK-I. Extrinsic as well as intrinsic apoptosis pathways were activated and elicited unique expressional patterns of apoptosis-relevant proteins such as downregulation of survivin, Bcl-X_L and c-FLIP. Protein levels of c-abl were downregulated and Akt phosphorylation was decreased by treatment with SrcK-I. Basal expression levels of c-Myc were notably lower in sensitive cell lines as compared with nonsensitive cell lines, possibly providing an explanation for sensitivity versus resistance against these novel substances. This study provides the first basis for establishing novel SrcK-I as weapons in the arsenal against lymphoma cells. ***Anti-Cancer Drugs*** **18:981–995**

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Introduction

Src kinases are nonreceptor intracellular tyrosine kinases implicated in a variety of intracellular signalling pathways including regulation of proliferation, cell motility, adhesion, tumor invasiveness, angiogenesis, immune cell signalling, cell cycle control and apoptosis [1]. In normal cells, Src kinases remain in an inactive state and are only activated transiently during the multiple cellular events for which they are designated [2]. In a large variety of cancer cells, however, protein levels and activation of Src kinases have been observed to be increased [3], and evidence has mounted that aberrant activation of Src kinases is a strong promoting factor for the development of tumors and metastatic phenotypes. Owing to this circumstance but also in particular because of their vital position downstream of imatinib targets such as BCR–ABL, c-Kit and platelet-derived growth factor (PDGF), the development of Src kinase inhibitors (SrcK-I) as substances to circumvent imatinib resistance has led to the emergence of a series of new molecular SrcK-I [4].

Indeed, first results from studies with SrcK-I show that these are able to elicit antiproliferative effects [5], reduce tumor volume [6], induce apoptosis [7], overcome chemoresistance [8], reduce cancer invasiveness [9], and also exert inhibitory effects on imatinib targets such as c-Kit and BCR–ABL [10] offering possibilities to overcome imatinib resistance [11–13].

The development of low-grade non-Hodgkin's lymphoma (NHL) as well as of chronic lymphocytic leukemia (CLL) is thought to be predominated by defects in apoptotic mechanisms rather than increased proliferation [14,15]. This circumstance accounts for the fact that up to now there are no curative treatment possibilities for these diseases as the large majority of antineoplastic substances are targeted at pathways of proliferation rather than malfunctioning apoptosis. In recent studies, we and others [16,17] could demonstrate apoptosis inducing and sensitizing effects of the tyrosine kinase inhibitor imatinib and AMN107 (unpublished observation) in subgroups of CLL cells despite of BCR–ABL, c-Kit and PDGF receptor (PDGFR) negativity in these cells. The availability of new dual inhibitors of Src kinases and Abl may also offer the opportunity to interfere with defective apoptotic pathways present in low-grade NHLs and CLL, which have been shown to include anomalous Src kinases [18]. Furthermore, several studies have shown that

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inhibition of Src kinase activity in lymphoma entities induces growth arrest and cell death [19,20].

In this context, we here analyzed the effect of three new experimental dual selective Src/Abl kinase inhibitors AZM559756, AZD0530 and AZD0424 with respect to their apoptosis-inducing properties in a variety of lymphoma cell lines to elucidate possibilities of employing these substances as new alternative treatment options in these disease entities.

Materials and methods

Cell culture

The cell lines DOHH-2 (human B-cell lymphoma/Epstein–Barr Virus-positive immunoblastic lymphoma), WSU-NHL (human B-cell lymphoma), Jurkat (T-cell leukemia/lymphoma), Karpas-299 (T-cell lineage anaplastic large cell lymphoma) and Raji (human Burkitt lymphoma) were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The cell line HUT78 (T-cell lymphoma) was obtained from the American Type Culture Collection (Manassas, Virginia, USA). All the cells were cultured at 37°C under 5% CO_2 in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Gibco), 2 mmol/l L-glutamine (Gibco) and a 1% penicillin–streptomycin mixture (Gibco). For all experiments cells were seeded at a concentration of 0.5×10^6 cells/ml.

Src kinase inhibitors

The dual selective Src/Abl kinase inhibitors AZM559756, AZD0530 and AZD0424 belong to a group of C-5-substituted (benzodioxolylamino)quinazolines newly developed by Astra Zeneca Pharmaceuticals (Astra Zeneca, Wedel, Germany). For detailed information on structures, chemical properties and pharmacodynamics, refer to Hennequin *et al.* [21]. They were supplied to us by Martin Ruthardt, (Department of Hematology, University Hospital, Frankfurt am Main, Germany), who obtained them directly from Astra Zeneca. For induction of apoptosis the SrcK-I were applied to cell cultures at final concentrations of 1–5 μmol/l.

Assessment of apoptosis

The rate of apoptosis was quantified flow cytometrically with a FACScan flow cytometer (Lysis II; Becton Dickinson, Heidelberg, Germany) after 24 and 48 h using an Annexin V/propidium iodide staining kit according to the manufacturer's instructions (Roche, Mannheim, Germany). Error bars in the graphs represent the standard error of the mean of triplicates calculated using GraphPad Prism Software (San Diego, California, USA). Results are representative for two independent experiments.

Mitochondrial membrane potential (MMP) was measured using the specific fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Alexis Biochemicals, Gruenberg, Germany) employed at a concentration of 5 μg/ml for 20 min. Cells were analyzed with a FACScan flow cytometer (Lysis II). Measurements were carried out after 48 h of incubation with SrcK-I. Data was converted to dot plots using the CellQuest software (Becton Dickinson). Results are representative for two independent experiments.

Cell cycle analysis

DOHH-2, WSU-NHL, Raji and Jurkat cells were incubated with AZD0424 at a final concentration of 5 μmol/l for 48 h. Subsequently cell cycle status was determined flowcytometrically using a CycleTEST PLUS DNA reagent Kit (Becton Dickinson) according to the manufacturer's instructions on a FACSCalibur flow cytometer (Becton Dickinson). Instrument settings were established using DNA QC particles (Becton Dickinson). Data were converted into histograms using the CellQuest software (Becton Dickinson) or Modfit LT (Verity Software House, Topsham, Maine, USA).

Western blot

A total of 15×10^6 cells per sample were pelleted and lysed with sodium dodecyl sulfate (SDS) lysis buffer according to standard protocols. Normalized whole-cell lysates were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) (8–15% gradient gels). Proteins were transferred to a nitrocellulose membrane using a semi-dry electroblotting apparatus (Bio-Rad, Munich, Germany). The membranes were blocked with 5% nonfat dry milk, and incubated with the primary and subsequently secondary horseradish peroxidase-conjugated antibodies. Bands were detected with the enhanced chemiluminescence technique. Blots were stripped using Restore Western Blot Stripping Buffer (Perbio Science, Bonn, Germany) and probed with an antibody against tubulin to confirm equal loading.

Antibodies

Western blot antibodies were obtained as follows: mouse anti-p53 (DO-1), c-myc, α-tubulin, c-Abl; rabbit anti-Bak, Bax, Bcl-$x_{S/L}$, phospho-Akt1 (Ser473), Par-4; goat anti-Bid, ZIP kinase, Rb, Akt1, and the antimouse, antirabbit, antirat and antigoat horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, Heidelberg, Germany).

Rabbit anti-hSurvivin, cIAP-1, cIAP-2 and XIAP were purchased from R&D Systems (R&D Systems, Wiesbaden-Nordenstadt, Germany). Rat anti-FLIP was from Alexis Biochemicals (Alexis Biochemicals, Lausen, Switzerland). Mouse anti-Ras, Src, phospho-(Tyr416)-Src and rabbit anti-Fgr were from Upstate Biotechnology

(Upstate Biotechnology, Billerica, Massachusetts, USA). Rabbit anti-Blk, phospho-(Tyr505)-Lck, phospho-(Tyr507)-Lyn and phospho-c-Abl-(Tyr245) were purchased from Cell Signalling Technologies (Cell Signaling Technologies, Danvers, Massachusetts, USA). Mouse anti-Lck, Fyn, Yes, Hck and Lyn were from BD Transduction Laboratories (BD Transduction Laboratories, Erembodegem, Belgium). Rabbit anti-phospho-($pYpS^{209/211}$)-Hck was from Biosource (Biosource, Camarillo, California, USA). Rabbit anti-PARP was purchased from Roche. Mouse anti-Bcl-2 was from DAKO Hamburg, Germany.

Caspase activity assays and caspase inhibition

For the assessment of caspase activity fluorometric caspase activity assay kits (Biocat, Heidelberg, Germany) were used. Assays were carried out according to the manufacturer's instructions with slight modifications. Free AFC generated in the assays was measured fluorometrically (λ_{ex}: 400 nm; λ_{em}: 505 nm) in a multifunctional reader (Spectrafluor Plus; Tecan, Crailsheim, Germany).

The broad-spectrum caspase inhibitor Z-VAD.fmk and the caspase-3 inhibitor Ac-DEVD.CHO were purchased from Bachem (Weil am Rhein, Germany), the caspase-8 inhibitor Z-IETD.fmk was purchased from Calbiochem (Calbiochem, Darmstadt, Germany). All caspase inhibitors were applied at a concentration of 50 µmol/l as recommended by the manufacturers.

Results

SrcK-I differentially induce apoptosis in lymphoma cells

To assess the apoptosis-inducing properties of the new experimental dual selective Src/Abl kinase inhibitors AZM559756, AZD0530 and AZD0424 on BCR–ABL-negative lymphoma and leukemia cells we selected six well-established cell lines [DOHH-2 (B-cell lineage), WSU-NHL (B-cell lineage), Raji (B-cell lineage), Jurkat (T-cell lineage), Karpas-299 (T-cell lineage) and HUT78 (T-cell lineage)]. The cell lines were incubated with rising concentrations of all three SrcK-I in the range from 1 to 5 µmol/l, and the rate of apoptosis was determined flow cytometrically with Annexin V after 24 and 48 h.

Interestingly, the cell lines could clearly be separated into a group of sensitive and resistant cell lines to these SrcK-I (Fig. 1a–d). The cell line DOHH-2 was the most sensitive cell line followed by WSU-NHL cells, which showed an increase in apoptosis at higher concentrations of SrcK-I. AZD0424 and AZD0530 emerged to be the more effective of the three tested inhibitors, inducing the highest levels of apoptosis with the lowest concentrations, whereas AZM559756 was the least effective. The remaining four cell lines Jurkat, Raji, Karpas-299 and HUT78 showed no sensitivity towards the applied SrcK-I. For further evidence of apoptosis-inducing capabilities of the tested SrcK-I Fig. 1e and f show the results of assessment of the disruption of MMP in DOHH-2 cells after 48 h incubation and demonstrate that in analogy to apoptosis measurement with Annexin V, the SrcK-I were able to induce rising rates of MMP disruption, i.e. apoptosis, in DOHH-2 cells.

Treatment with SrcK-I leads to cell cycle arrest in sensitive cell lines

As Src kinases have, among other fields, been implicated in the regulation of cell cycle [22,23] we were interested in the effect of SrcK-I on the cell cycle progression in our lymphoma cell lines. Experiments were carried out with AZD0424. DOHH-2, WSU-NHL, Raji and Jurkat cells were incubated with rising concentrations of AZD0424 in the range from 1 to 5 µmol/l for 48 h and subsequently their cell cycle status was assessed flow cytometrically. Treatment with AZD0424 led to increased G_0/G_1 cell cycle arrest in DOHH-2 and WSU-NHL cells, whereas the cell cycle progression of the nonsensitive cell lines Raji and Jurkat remained unaffected by AZD0424 (Fig. 2a and b).

Lymphoma cell lines display deregulated expression profiles of Src kinases

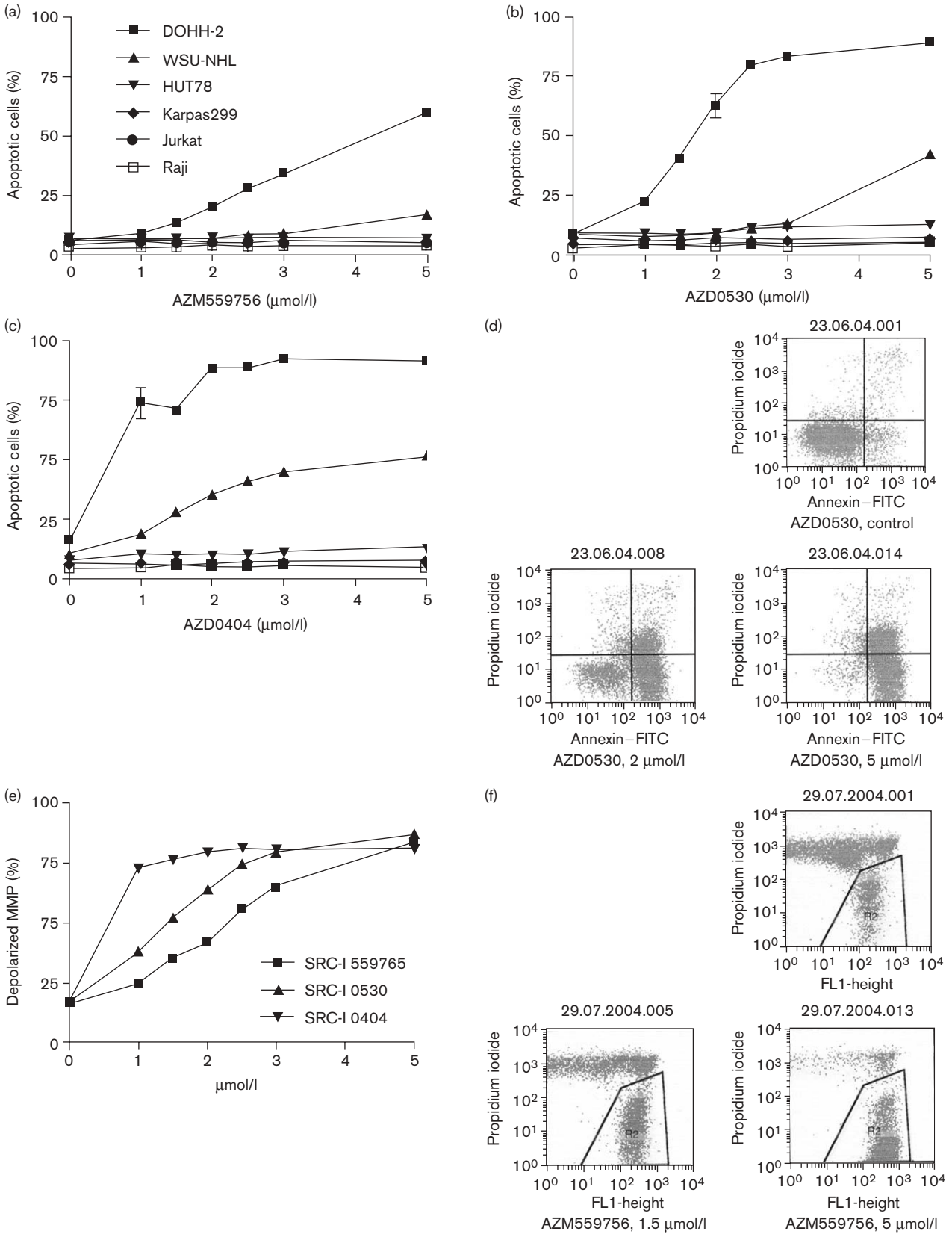
To further elucidate the molecular mechanisms underlying sensitivity and resistance towards SrcK-I, we analyzed the basal expression levels of Src, Hck, Lck, Lyn, Yes, Fyn, Fgr and Blk.

Interestingly, the expression of Src kinases emerged to be very heterogenous in the tested cell lines and not in accordance with published expressional patterns detected in leukocytes [24]. In addition to Fyn and Lck which are reported to be expressed in normal T-lymphocytes [24], Jurkat, Karpass-299 and HUT78 cells heterogeneously expressed Src, Hck, Lyn, Yes and Fgr. Similarly, cell lines of B-cell origin in addition to Fyn, Fgr, Lyn, Blk and Yes also aberrantly expressed Src, Lck and Hck (Fig. 3). Nevertheless, the heterogenous expression of the tested Src kinases was not able to discriminate between sensitive and nonsensitive cell lines, i.e. there were no certain types of Src kinases which were only expressed in the sensitive cell lines while not being expressed in the resistant ones.

Treatment with SrcK-I leads to dephosphorylation of Lck-Tyr505 and Lyn-Tyr507 in sensitive and resistant cell lines

To examine the effects of SrcK-I on the expression of apoptosis-relevant proteins and Src kinase activation, we conducted experiments with DOHH-2, WSU-NHL, Jurkat and Raji cells. The cell lines were treated with the IC_{50} (inhibiting concentration leading to 50% apoptosis) of the SrcK-I AZD0530 and AZD0424 for 48 h. For each cell line, the protein expression in

Fig. 1



untreated samples was compared with the expression in treated samples. To avoid repetitions, only the Western blots of samples treated with AZD0424 are displayed here. Samples treated with AZD0530 displayed comparable effects to AZD0424 (data not shown).

Analysis of the phosphorylation of Src-Tyr416, Hck-Tyr209/Ser211, Lck-Tyr505 and Lyn-Tyr507 showed that Lyn and in particular Lck were the kinases influenced most heavily upon treatment with the SrcK-I AZD0424 and AZD0530 (Fig. 4), whereas Src and Hck did not display changes of phosphorylation. Interestingly, the phosphorylation of Lck-Tyr505 as assessed by a specific antibody was strongly reduced in all cell lines treated with the SrcK-I independently of their sensitivity properties (Fig. 4). A similar observation was applied for Lyn-Tyr507, which was moderately downregulated in all treated cells that expressed Lyn at detectable levels (DOHH-2, WSU-NHL and Raji) (Fig. 4). Furthermore, apart from the downregulation of phospho-Lck-Tyr505 the enzyme itself was downregulated upon treatment with the SrcK-I (Fig. 4).

The analysis of phosphorylated Src-Tyr416 and Hck-Tyr209/Ser211 (Fig. 4) showed no substantial changes of expression of these antigens and no expressional changes of the enzymes themselves. During the assessment of the enzyme expression levels of the kinases Yes, Fyn, Fgr and Blk it stood out that expression levels of Fyn were reduced in sensitive cell lines treated with the SrcK-I as compared with untreated cells, an event which could not be observed in nonsensitive cell lines. No fundamental change in protein expression of the kinases Yes, Fgr or Blk could be ascertained during treatment with the SrcK-I (Fig. 4).

SrcK-I induced apoptosis is caspase dependent and induces poly(ADP-ribose) polymerase cleavage

After analysis of Src kinase expression and activation, the next question was how key proteins of apoptosis cascades are influenced by the new SrcK-I.

Western blot analysis of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) demonstrated that PARP was only cleaved in the sensitive cell lines DOHH-2 and WSU-NHL treated with AZD0424 whereas it remained unaffected in Jurkat and Raji cells (Fig. 5a).

To assess the pattern of caspase activation induced by SrcK-I, we employed fluorometric caspase activity assays. The cell lines DOHH-2, WSU-NHL, Jurkat and Raji were treated with the IC_{50} of AZD0424 and incubated for 60 h. At the time points 0, 12, 24, 36, 48 and 60 h cells were extracted, lysed and kept for subsequent caspase-activity analysis of caspase-8, caspase-9, caspase-3, caspase-7 and caspase-6. The sensitive cell lines featured activation of caspases under treatment with AZD0424, whereas the caspase activity in the resistant cell lines remained on the level of untreated control cells (Fig. 5b). The activity of the initiator caspase-8 and caspase-9 chronologically peaked before the executioner caspase-3, caspase-7 and caspase-6. As compared with WSU-NHL, the caspase activity in DOHH-2 cells progressed in an enhanced manner.

To further confirm caspase dependency of SrcK-I-induced apoptosis, we conducted an experiment employing the broad-spectrum caspase inhibitor Z-VAD.fmk and the specific caspase-3 and caspase-8 inhibitors Ac-DEVD. CHO and Z-IETD.fmk. DOHH-2 cells were incubated with 4 μmol/l AZM559756, 2.5 μmol/l AZD0530 and 1.5 μmol/l AZD0424, and supplemented with the respective caspase inhibitors for 48 h. Subsequently, the rate of apoptosis was determined. Figure 5c confirms that apoptosis induced by all three SrcK-I is caspase-dependent as evidenced by the near-to-complete abrogation of apoptosis induction by treated samples coincubated with the broad-spectrum caspase inhibitor Z-VAD.fmk. The observation that the specific caspase-8 and caspase-3 inhibitors only reduce apoptosis rates partially furthermore reflects the circumstance that both intrinsic and extrinsic apoptosis pathways are activated by the SrcK-I.

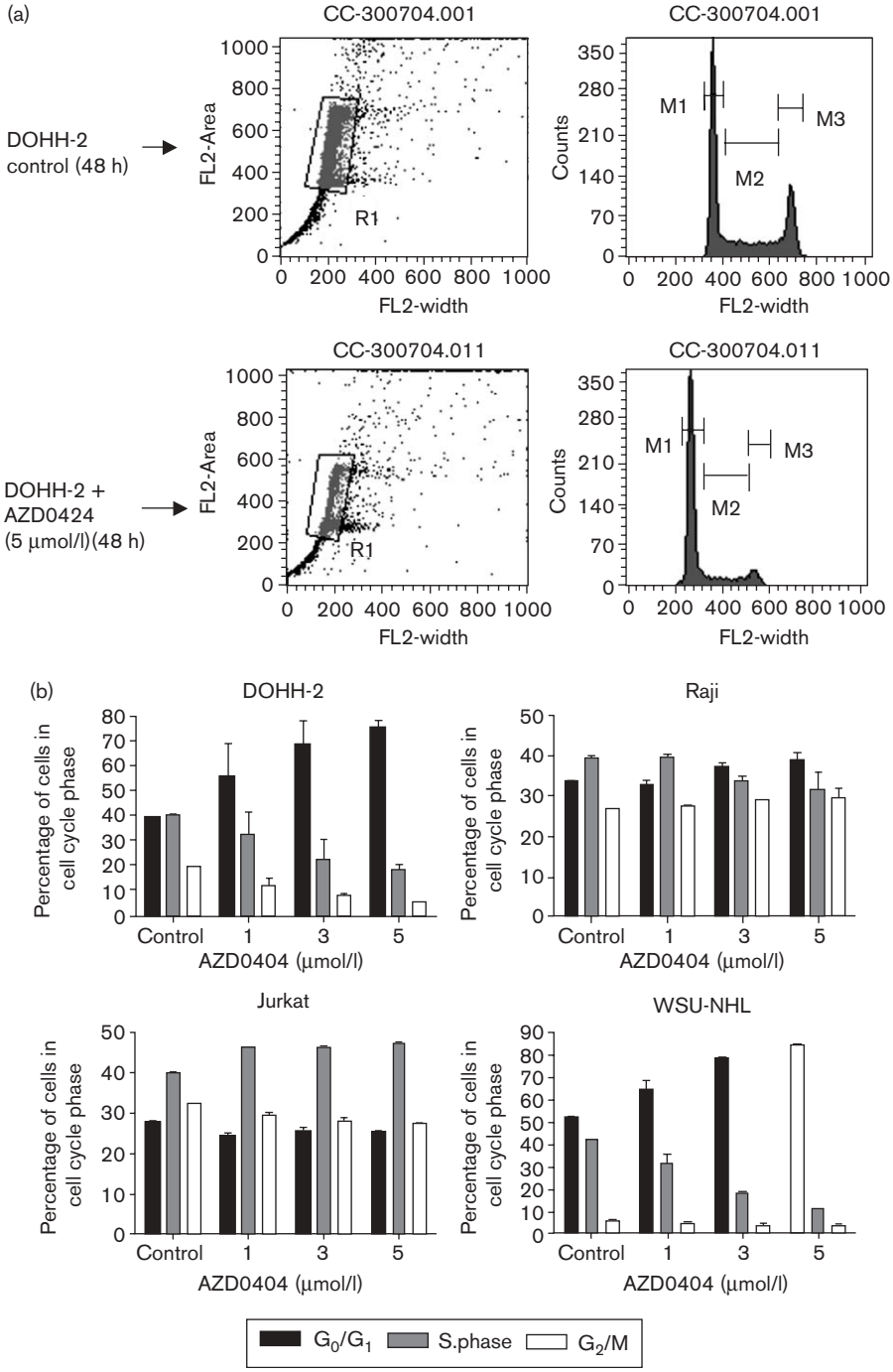
Treatment of lymphatic cells with SrcK-I affects expression of Bcl-x_L and truncation of Bid

To further analyze possible differential mechanisms of apoptosis induced by SrcK-I, we assessed the expression levels of crucial Bcl-2 family members.

In the above-described experimental setup composed of treatment of the cell lines with AZD0424 and AZD0530, we analyzed the expression levels of Bcl-2, Bcl-x_L, Bak, Bid and Bad. Figure 6a shows that the Bcl-2 family members Bcl-2, Bak, Bax and Bad remained unaffected by treatment with SrcK-I in all cell lines, whereas the

(a–c) Rates of apoptosis induced in lymphoma cell lines by rising concentrations of the dual selective Src/Abl kinase inhibitors AZM559756, AZD0530 and AZD0424 after 48 h of incubation as determined flow cytometrically with Annexin V/propidium iodide staining. (d) Example dot plots of flow cytometric measurement of apoptosis with Annexin V/propidium iodide staining in DOHH-2 cells treated with AZD0530. (e) Percentages of cells (DOHH-2) treated with rising concentrations of AZM559756 (SRC-I 559756), AZD0530 (SRC-I 0530) or AZD0424 (SRC-I 0424) for 48 h which displayed depolarized mitochondrial membrane potential (MMP) as determined flow cytometrically with JC-1 staining. (f) Example dot plots demonstrating the flow cytometric determination of MMP depolarization induced in DOHH-2 cells by treatment with AZM559756.

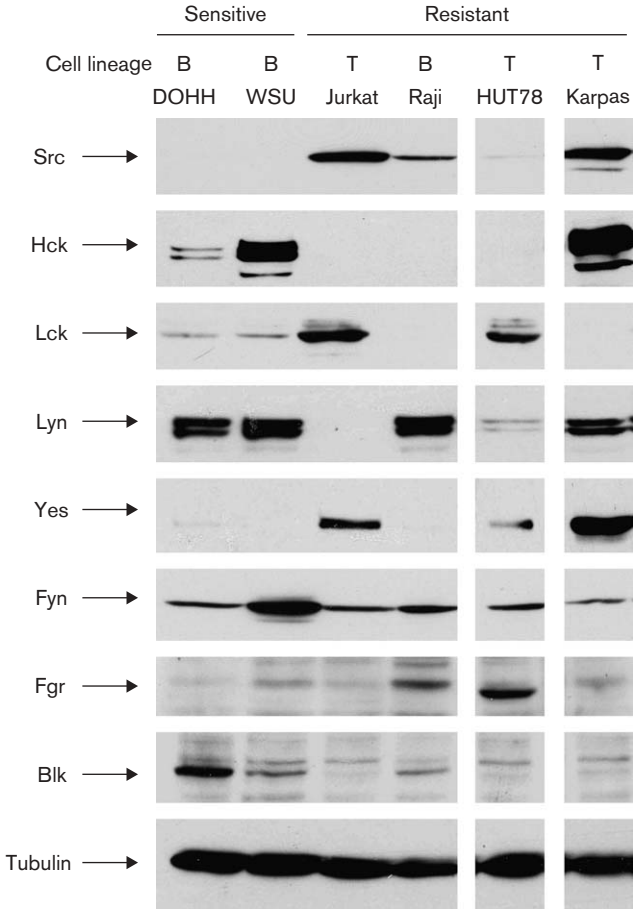
Fig. 2



(a) Example histograms of flow cytometric assessment of cell cycle progression. M1 = cells in G_0/G_1 phase, M2 = cells in S phase, M3 = cells in G_2/M phase. (b) Summary of results gained in the cell cycle analysis of DOHH-2, WSU-NHL, Raji and Jurkat cells treated with rising concentrations of AZD0424 for 48 h. DOHH-2 and WSU-NHL cells were sensitive towards treatment with AZD0424, and displayed increasing cell cycle arrest with rising concentrations of AZD0424, whereas Raji and Jurkat cells remained unaffected.

antiapoptotic member Bcl-x_L was downregulated upon treatment with AZD0424 (and AZD0530, data not shown) in all cell lines albeit in a more pronounced manner in DOHH-2 and WSU-NHL cells. Expressional differences could also be observed for the proapoptotic Bcl-2 family member Bid (Fig. 6a). Western blot signals for Bid were decreased in samples of DOHH-2 and WSU-NHL, but also slightly in Jurkat and Raji cells treated with SrcK-I. Even though Bid is a proapoptotic member, the decrease in expression should be interpreted as a

Fig. 3



Western blots displaying the basal expression levels of the Src kinases Src, Hck, Lck, Lyn, Yes, Fyn, Fgr and Blk in untreated DOHH-2, WSU-NHL, Jurkat, Raji, HUT78 and Karpass-299 cells. Equal loading of lanes was controlled with tubulin blots of stripped membranes.

proapoptotic event owing to the circumstance that upon induction of apoptosis, Bid is truncated, i.e. activated, by caspase-8. Therefore, decrease of its inactive full-length form as detected by the employed antibody can be understood as a transition into its active truncated form that is not detected by this antibody anymore, possibly owing to a rapid proteosomal degradation [25].

Inhibitor of apoptosis proteins survivin and cIAP-1 and the FLICE inhibitory protein c-FLIP are downregulated by treatment with SrcK-I in sensitive cell lines

Another important family of apoptosis regulators is the inhibitor of apoptosis proteins (IAPs), which have the main function to directly and indirectly inhibit caspases [26]. The most potent inhibitor is the XIAP followed by others such as cIAP-1/2 or survivin. Interestingly, the analysis of these proteins showed that expression of XIAP and cIAP-2 was not influenced at all by treatment with SrcK-I (Fig. 6b). In contrast, survivin the smallest IAP protein was profoundly downregulated in sensitive cell lines treated with the SrcK-I and cIAP-1 could be observed to be downregulated in treated DOHH-2 cells (Fig. 6b).

Additionally the CD95 receptor-associated FLICE inhibitory protein (c-FLIP$_L$) was downregulated by SrcK-I treatment in the sensitive cell lines (Fig. 6b).

Nuclear proteins Daxx, Par-4 and ZIP kinase are not influenced by SrcK-I treatment

The death-associated protein (Daxx) and prostate apoptosis response gene-4 (*Par-4*) are proapoptotic proteins, which we identified to be potent enhancers of apoptosis induced by various antineoplastic substances [27–30], and may represent key mediators of synergistic drug efficacy in CLL and NHL treatment [31]. Therefore, we were interested in the effects of the SrcK-I on the expression of these proteins. Furthermore, as an interaction of these two factors in collaboration with a third factor, ZIP kinase, within promyelocytic leukemia (PML) nuclear bodies has recently been demonstrated [32], we also analyzed expression levels of ZIP kinase. In contrast to apoptosis induced by cytotoxic drugs in which Par-4 and Daxx are profoundly downregulated, the changes in expression of these factors evoked by AZD0424 treatment were only marginal if detectable at all (Fig. 7). Only DOHH-2 cells treated with AZD0424 showed a slight tendency of reduced Daxx and Par-4 expression in treated samples. Moreover, ZIP kinase was not influenced by Src kinase inhibition.

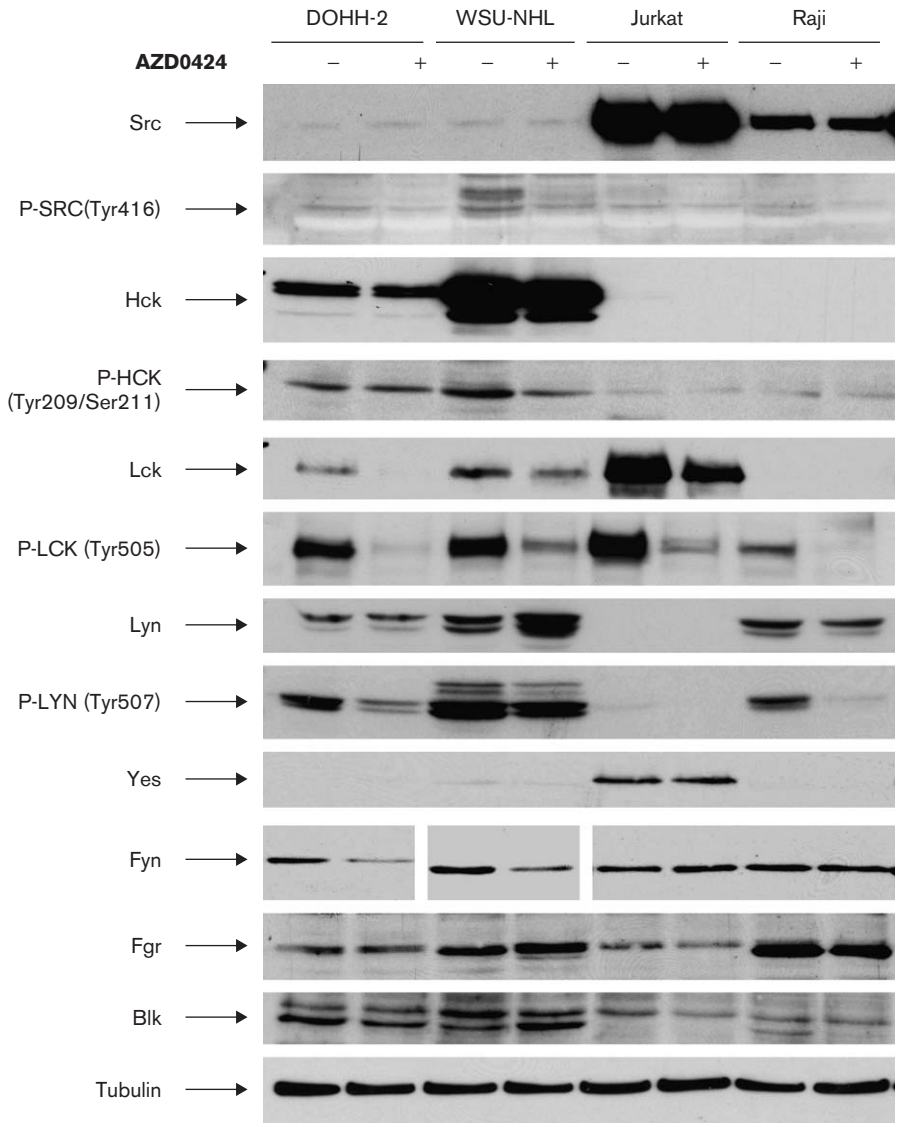
p53 expression remains unaffected, Rb is down-regulated upon Src kinase inhibition

Owing to the induction of cell cycle arrest by the tested SrcK-I in sensitive cell lines, we also assessed the expression of the tumor suppressors and cell cycle regulators p53 and retinoblastoma protein (Rb) [33]. The basal expression of p53 in the different cell lines was heterogeneous and remained uninfluenced by treatment with SrcK-I (Fig. 7). In contrast, Rb was observed to be downregulated in DOHH-2, WSU-NHL and Jurkat cells treated with AZD0424.

Treatment with SrcK-I leads to inhibition and down-regulation of c-Abl and reduced Akt phosphorylation

As several substances primarily designated as SrcK-I were subsequently also shown to exert inhibitory effects on Abl and Akt phosphorylation [4,10,34], we also analyzed the expression and phosphorylation of Abl and Akt in the tested lymphoma cell lines. Interestingly, treatment with the SrcK-I AZD0424 and AZD0530 caused a substantial downregulation of the c-Abl kinase in the sensitive cell lines, but not in the resistant ones (Fig. 7). Concomitantly a tendency of decreased Tyr-245 phosphorylation, i.e. a decreased activation of Abl, could be observed in all cell lines (Fig. 7).

Fig. 4



Westerns blot analysis of Src kinase expression of Src, Hck, Lck, Lyn, Yes, Fyn, Fgr, Blk and phosphorylated Src kinases P-SRC(Tyr416), P-HCK (Tyr209/Ser211) P-LCK (Tyr505) and P-LYN (Tyr507) in DOHH-2, WSU-NHL, Jurkat and Raji cells treated with the IC_{50} concentrations of AZD0424 for 48 h (+) and untreated control cells (–). Equal loading of lanes was controlled with tubulin blots of stripped membranes.

Similarly, Akt1 Ser473 phosphorylation was clearly decreased in the sensitive cell lines treated with AZD0530 or AZD0424, whereas Akt1 protein levels remained stable in all cell lines under all conditions (Fig. 7).

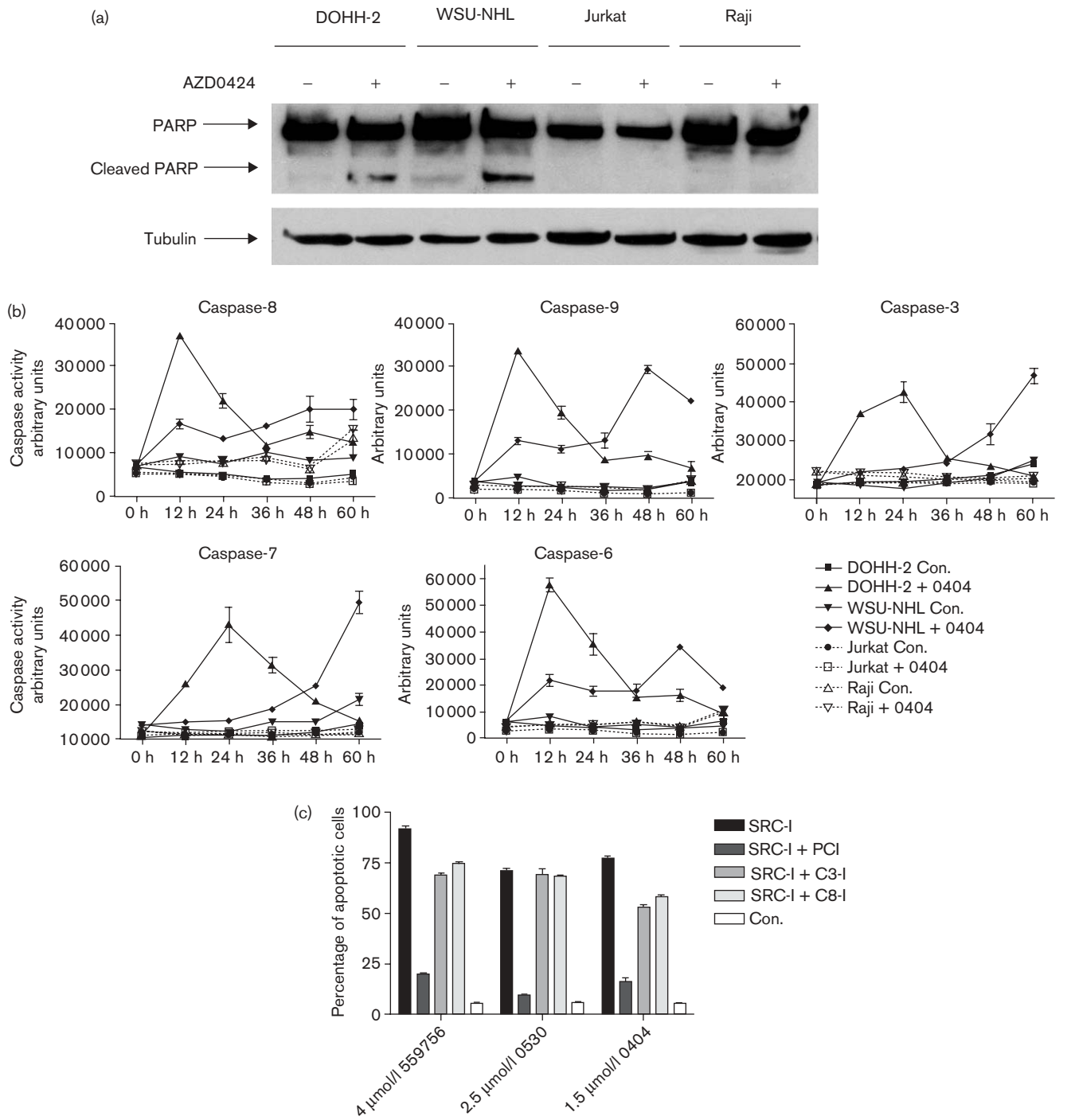
Sensitivity towards SrcK-I is associated with low c-myc expression levels

To further elucidate differences discriminating sensitive from nonsensitive cell lines we analyzed the expression of the oncogenes Ras and c-myc [35,36] as to possibly detect expressional patterns suggesting a predisposition for sensitivity towards apoptosis. Expression levels of Ras were identical in all samples and uninfluenced by treatment with SrcK-I (Fig. 7). The assessment of c-myc expression revealed that c-myc displayed low protein levels in the sensitive cell lines and high expression in nonsensitive ones (Fig. 7). Furthermore, expression levels remained unchanged upon treatment with SrcK-I as compared with the untreated control cells.

Discussion

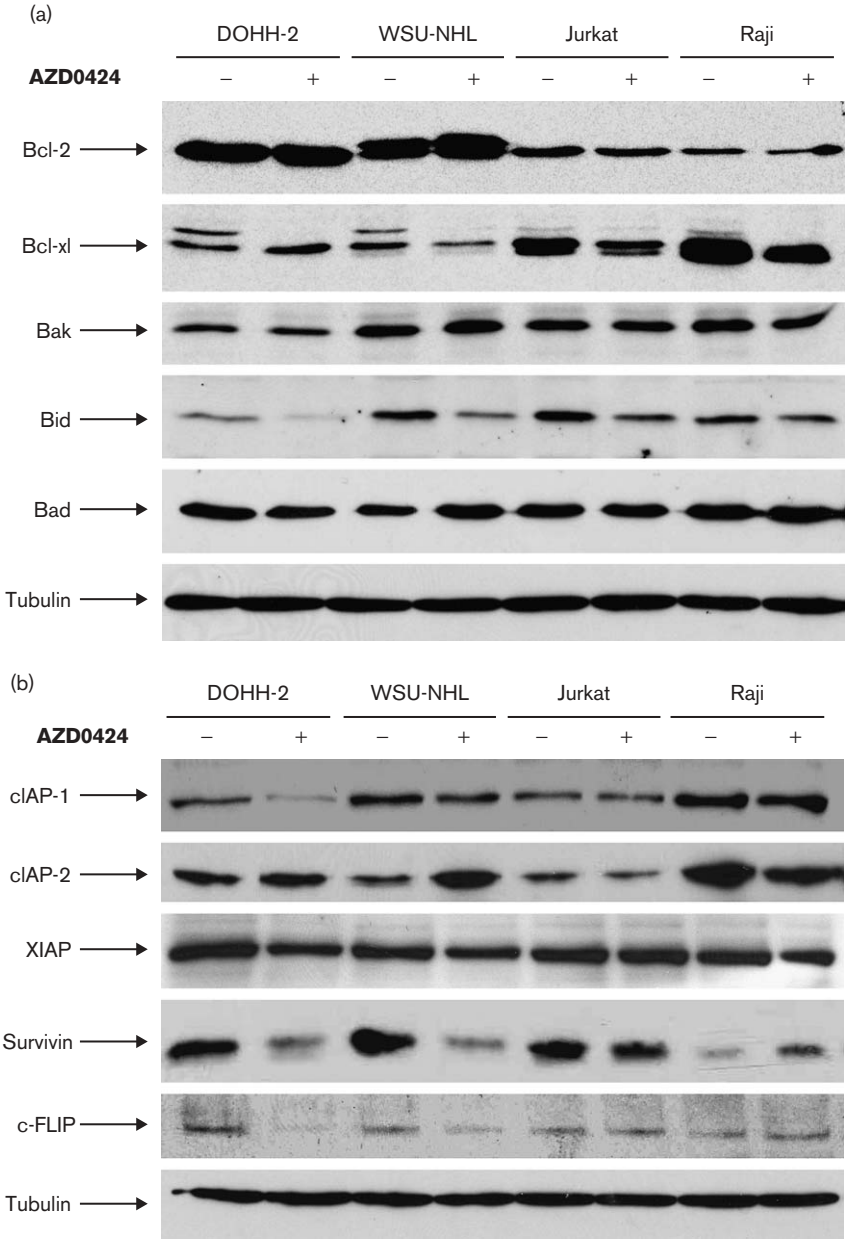
Recently, we [37] and others [17] could demonstrate susceptibility of certain subgroups of lymphoma cells towards treatment with imatinib and its successor substance AMN107 (unpublished observation) despite

Fig. 5



(a) Western blot analysis of PARP cleavage in DOHH-2, WSU-NHL, Jurkat and Raji cells treated with the IC_{50} concentrations of AZD0424 (+) and untreated control cells (–) for 48 h. Equal loading of lanes was controlled with tubulin blots of stripped membranes. (b) Caspase activities of caspase-8, caspase-9, caspase-3, caspase-6 and caspase-7 determined in DOHH-2, WSU-NHL, Jurkat and Raji cells treated with the IC_{50} concentrations of AZD0424 (0424) for 60 h and concomitant untreated controls (Con.) at the time points 0, 12, 24, 36, 48 and 60 h of incubation. Caspase activity was assessed using specific fluorometric caspase activity assays performed in microtiter plates and measured on a corresponding multifunctional reader. (c) Rates of apoptosis induced in DOHH-2 cells after 48 h of treatment with 4 µmol/l AZM559756, 2.5 µmol/l AZD0530 or 1.5 µmol/l AZD0424 (SRC-I) and coincubated with the broad-spectrum caspase inhibitor Z-VAD.fmk (+PCI), the specific caspase-3 inhibitor Ac-DEVD.CHO (+C3-I) or the specific caspase-8 inhibitor Z-IETD.fmk (+C8-I), respectively. PARP, poly(ADP-ribose) polymerase.

Fig. 6



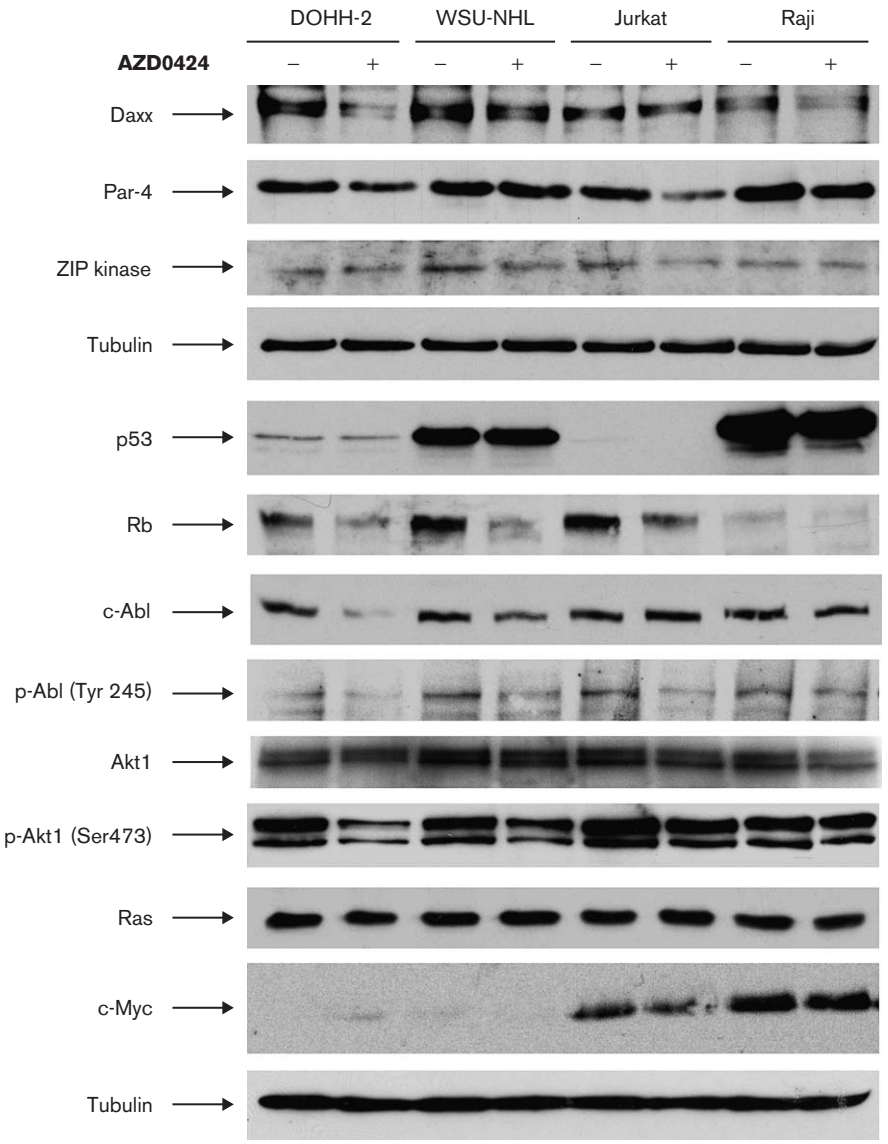
(a) Western blot analysis of Bcl-2 family proteins Bcl-2, Bcl-x_L, Bak, Bid and Bad in DOHH-2, WSU-NHL, Jurkat and Raji cells treated with the IC_{50} concentrations of AZD0424 (+) and untreated control cells (–) for 48 h. (b) Western blot analysis of inhibitor of apoptosis proteins cIAP-1, cIAP-2, XIAP and survivin and FLICE inhibitory protein c-FLIP in DOHH-2, WSU-NHL, Jurkat and Raji cells treated with the IC_{50} concentrations of AZD0424 (+) and untreated control cells (–) for 48 h. Equal loading of lanes was controlled with tubulin blots of stripped membranes.

of the fact that these cells lack the classic imatinib/AMN107 targets BCR–ABL, c-Kit and PDGFR. Following these observations, we wanted to know whether lymphoma cells might also be sensitive to new dual selective inhibitors of BCR–ABL and Src kinases.

The tested substances AZM559756, AZD0530 and AZD0424 belong to a group of newly developed C-5-substituted (benzodioxolylamino)quinazolines, which have been shown to be potent inhibitors of c-Src and Abl kinases, and are described in detail in the recent publication by Hennequin *et al.* [21].

As the deregulation of apoptosis is considered to be one of the main prerequisites for the emergence of CLL or low-grade NHL [15], we hypothesized that the tested SrcK-I may possibly act by influencing key pathways of apoptosis in lymphoma cell lines.

Fig. 7



(a) Western blot analysis Daxx, Par-4, and ZIP kinase, p53, Rb, Abl and degree of Abl-Tyr245 phosporylation, Akt1 expression and Akt1-Ser473 phosphorylation, Ras and c-Myc in DOHH-2, WSU-NHL, Jurkat and Raji cells treated with the IC_{50} concentrations of AZD0424 (+) and untreated control cells (–) for 48 h. Equal loading of lanes was controlled with tubulin blots of stripped membranes.

Our experiments showed that two of the tested substances indeed induced apoptosis in two of the tested cell lines when concentrated in the range of 1–5 µmol/l. To date, little information exists concerning the concentrations of these inhibitors required to exert biological effects. In-vitro cellular activity tests carried out with AZD0530 on mouse NIH 3T3 cells transfected with constitutively active human c-Src showed inhibitory effects on enzyme activity and proliferation at far lower concentrations of below 10 and 100 nmol/l, respectively [21]. Antiproliferative effects on less artificial systems such as human K562 cells or A549 nonsmall cell lung cancer cells, however, were exerted with an IC_{50} of 220 nmol/l or even 14 ± 1.5 µmol/l, respectively [21], indicating that the concentration range of 1–5 µmol/l lies within the upper field of in-vitro concentrations assessed by the developers. Importantly, the observation that the tested cell lines could clearly be divided into a group of sensitive and nonsensitive cell types in this range of concentrations served as an argument to use this system to elucidate the molecular alterations elicited by SrcK-I on apoptotic pathways.

After ascertaining a clear impact on apoptosis and cell cycle on two of the tested cell lines by the SrcK-I, the subsequent analysis of Src kinase expression in all cell

lines revealed an apparent heterogeneous and irregular expression of these enzymes, which was not consistent with published expressional patterns of Src kinases in leukocytes [24]. This strengthened once more the notion of aberrant Src kinase expression and development of malignancy. The expression patterns could not, however, be used to discriminate between the sensitive and resistant cell lines, ruling out that there are specific kinases responsible for the sensitivity towards the tested SrcK-I only present in sensitive cells and absent in nonsensitive ones.

Supplementary we attempted to gain insight into the effects of SrcK-I on the activity of the Src kinases. Activation of Src kinases can putatively be gauged by analyzing phosphorylation at specific phosphorylation sites of the kinases [38]. This approach was somewhat hampered owing to the circumstance that the number of commercially available antibodies against phosphorylated Src-kinases was limited. Nevertheless, the phosphorylation of Src-Tyr416, Hck-Tyr209/Ser211, Lck-Tyr505 and Lyn-Tyr507 could be assessed, and led to the interesting result that Lyn-Tyr507 and especially Lck-Tyr505 were dephosphorylated upon treatment with SrcK-I. These observations were somewhat intricate because according to primary data the phosphorylation sites Lck-Tyr505 and Lyn-Tyr507 are regarded as negative regulation elements of these kinases, i.e. according to a traditional model, hyperphosphorylation at these sites is supposed to inhibit the kinase activity [39–42], whereas phosphorylation at tyrosine residues Lck-Tyr394 and Lyn-Tyr397 should cause their activation. Following this interpretation would mean that the Src kinases Lck and Lyn are activated by treatment with SrcK-I, which seems very unlikely. Hence, our data are more consistent with subsequent studies about Lck and Lyn regulation, which demonstrate that the matter of activation of these kinases cannot be understood in such a simplistic model. Several approaches addressing this problem have shown that despite of Lck-Tyr505 hyperphosphorylation, the kinase activity of this enzyme was constitutively elevated [43–45] and evidence is mounting that a decrease of Lck kinase activity is associated with dephosphorylation of both regulatory sites [46]. We therefore hypothesize that the observed dephosphorylation of Lck-Tyr505 and Lyn-Tyr507 in our experiments marks an inhibition of these kinases. This interpretation also gains support by the unexpected observation of a concomitant downregulation of the Lck enzyme itself under the influence of the SrcK-I. It would be hard to conceive elevated total kinase activity by an enzyme of which the expression is downregulated. It is of note that also Fyn levels were downregulated upon SrcK-I treatment. Summarizing these results leads to the conclusion that in these tested cell lines the Src kinases Lyn, Fyn and in particular Lck are manipulated by the employed inhibitors, and may therefore represent the candidate Src kinase responsible for mediating the apoptotic effects.

In the further course of this study, we concentrated on the apoptotic pathways induced by the treatment with AZD0530 or AZD0424 in the two sensitive cell lines DOHH-2 and WSU-NHL in comparison to the two nonsensitive cell lines Raji and Jurkat.

It could be observed that apoptosis induced by these SrcK-I was mediated by the activation of both the extrinsic and intrinsic apoptosis signalling as evidenced by the activation of caspase-8 as well as caspase-9, which represent the main initiator caspases of these two branches, respectively. Furthermore, activation of the mitochondrial apoptosis pathway was demonstrated by the disruption of MMP in DOHH-2 cells. Activation of the initiator caspases was consequently followed by an enforced activation of executioner caspase-3, caspase-7 and caspase-6, activation of Bid, and cleavage of PARP in the sensitive cell lines.

An interesting finding was that these processes were supported by the downregulation of antiapoptotic factors such as Bcl-x_L, c-$FLIP_L$ and in particular the IAP survivin which was downregulated by SrcK-I treatment in sensitive cell lines, whereas it remained unchanged or even slightly upregulated in the nonsensitive Jurkat and Raji cells. Survivin is a potent inhibitor of apoptosis and vital regulator of cell cycle [47–49], and has been shown to be upregulated in cancer, especially hematological malignancies. The observation that it is so strongly affected by the Src inhibitors and possible inversely influenced in sensitive versus nonsensitive cells may represent an important link to the mechanisms by which these substances take influence on cell cycle regulation.

Moreover, it is noteworthy because these proteins are specifically influenced by SrcK-I-induced apoptosis, whereas they remain uninfluenced in apoptosis induced by cytotoxic drugs in the same cell lines [31]. In experiments analyzing cytotoxic drug-induced apoptosis in these cells, however, other members of the IAP family such as cIAP-1 and XIAP were dramatically downregulated [31,50], whereas expression of survivin and Bcl-x_L remained unaffected. These observations suggest that the two apoptosis inducers – cytotoxic drugs versus SrcK-I – activate fundamentally different apoptosis pathways. Another result corroborating this notion is the finding that the proteins comprising an apoptosis-mediating complex in PML nuclear bodies consisting of Daxx, Par-4 and ZIP kinase [32] which feature strong downregulation of Par-4 and Daxx in apoptosis induced by cytotoxic drugs, histone deacetylase inhibitors or in particular imatinib [28,30,31,37] remain uninfluenced by treatment with the SrcK-I. This fundamental difference in mode of action could be the rationale for incorporating these substances in future therapeutic regimens employed against lymphoma as the combination of substances with different mechanisms of apoptosis induction

may have a chance of achieving synergistic pharmacological effects. This could be assessed in in-vitro chemosensitivity tests of cell cultures and primary cells as previously carried out [31,51,52].

As the SrcK-I not only induced apoptosis but also cell cycle arrest we analyzed the expression of p53 and retinoblastoma protein Rb as central regulators of cell cycle progression [33]. p53, which is upregulated in cytotoxic drug-induced apoptosis owing to the inflicted DNA damage, remains unaffected by treatment with SrcK-I. In contrast, Rb is downregulated upon SrcK-I treatment in both sensitive and nonsensitive cell lines. The downregulation of a classical tumor suppressor protein appears counterintuitive in this situation. It, however, is known that besides regulating cell cycle, Rb may also block apoptosis [53,54] and that in certain cases downregulation of Rb is associated with differentiation and apoptosis [55], and Rb is degraded by a caspase-3-like activity during apoptosis [56]. Therefore, downregulation of Rb in this context may be associated with progression of apoptosis and is not an entirely devious observation.

As it was recently demonstrated that imatinib is able to induce apoptosis in CLL lymphocytes by the putative mechanism of c-Abl inhibition [17,37], and AZD0530 and AZD0424 are regarded as a dual inhibitors of Abl and Src kinases [4], we also analyzed c-Abl expression and Abl phosphorylation. A substantial downregulation of the c-Abl protein was detected in sensitive cell lines treated with SrcK-I. Such a downregulation can again be seen in coherence with reduced activity, which is further supported by reduced levels of Abl-Tyr245 phosphorylation, indicating that inhibition or expressional regulation of this tyrosine kinase may represent an important mechanism for the induction of apoptosis by SrcK-I in lymphoid cells. Similarly, the detected inhibition of Akt1 as evidenced by reduced Akt1 (Ser473) phosphorylation [34] in sensitive cell lines can be hypothesized to be a mechanism contributing to the induction of apoptosis. Akt is a pivotal regulator and oncogene implicated in apoptosis and proliferation [57], and the Akt signalling pathway is regarded as one of the most critical pathways in regulating cell survival [58], therefore its inhibition is in consistency with apoptosis induction and may also represent one of the key mechanisms by which the tested SrcK-I elicit their apoptotic effects in the sensitive cell lines.

In the light of these results, it was also interesting to ascertain that the 'resistant' cell lines displayed a notedly higher basal c-myc expression than the two sensitive cell lines. The proto-oncogene c-myc is a potent transcription factor implicated in the regulation of the cell cycle and proliferation, and it is one of the most commonly upregulated factors in cancer [36,59,60]. One of the key biological functions of c-myc is the ability to promote cell cycle progression [61,62]. In this context, it was recently demonstrated that c-myc is able to antagonize cell growth arrest provoked by Akt inhibition in lymphoid cells [63], results corroborated by other studies carried out in lymphoid cells [58,64] showing that c-myc and Akt cooperate for tumorigenesis and survival. Furthermore, Myc overexpression has been demonstrated to be a resistance factor against antitumor therapy in numerous cases [65–67]. Therefore, the elevated Myc expression in nonsensitive cell lines may represent a lead in the search for their resistance against the SrcK-I.

In conclusion, we here provide first evidence that the new experimental dual selective Src/Abl kinase inhibitors AZM559756, AZD0530 and AZD0424 are able to induce apoptosis and cell cycle arrest in lymphoma cell lines, which are BCR–ABL, c-Kit and PDGFR-negative. We demonstrate that apoptosis induction by these agents is associated with the activation of a unique pattern of apoptotic pathways which considerably differ from pathways activated by other apoptosis inducers such as cytotoxic drugs which have been extensively studied in the same cell lines. Besides manipulating Src kinase signalling, the tested SrcK-I disrupt the c-Abl and Akt pathways. As a possible explanation for responsiveness towards these substances we have demonstrated differential expression of c-myc in sensitive versus resistant cell lines. Taken together, this study provides the first basis for possibly establishing novel Src kinase inhibitors as treatment in lymphoma.

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