Inhibition of IκB Kinase Subunit 2 in Cutaneous T-Cell Lymphoma Down-Regulates Nuclear Factor-κB Constitutive Activation, Induces Cell Death, and Potentiates the Apoptotic Response to Antineoplastic Chemotherapeutic Agents

Aurore Sors,1 Francette Jean-Louis,1 Elodie Bégué,1 Laurent Parmentier,2 Louis Dubertret,1 Michel Dreano,2 Gilles Courtois,1 Hervé Bachelez,1 and Laurence Michel1

Abstract

Purpose: A key molecular feature of cutaneous T-cell lymphomas (CTCL) is the constitutive activation of the nuclear factor-κB (NF-κB) transcription factor. We investigated in vitro the effects on CTCL survival and chemoresistance of a specific inhibition of IκB kinase subunit 2 (IKK2).

Experimental Design: Selective IKK2 inhibition was carried out by transfection of SeAx and MyLa CTCL lines with an inactive form of IKK2 and by exposing these lines and tumor cells from 10 patients with Sézary syndrome (SS) to AS602868, a new IKK2 inhibitor. The constitutive nuclear translocation of NF-κB was analyzed by electrophoretic mobility shift assay and confocal microscopy. Apoptosis was determined by Annexin V/propidium iodide–positive staining and mitochondrial transmembrane potential alterations as well as poly(ADP-ribose) polymerase cleavage. The expression of Bcl-2 family oncoproteins and survivin was studied by immunoblotting.

Results: Specific IKK2 inhibition resulting from transfection or from incubation with AS602868 allowed a down-regulation of NF-κB transcriptional activity. As shown by electrophoretic mobility shift assay and apoptosis assays, AS602868 down-regulated the nuclear translocation of NF-κB and induced a potent apoptotic response in CTCL lines and in tumor cells from patients with SS while preserving the viability of both peripheral blood lymphocytes from healthy donors and of nonmalignant T cells from SS patients. Moreover, CTCL death induction by conventional antineoplastic agents etoposide and vincristine was potentiated by AS602868. Finally, AS602868–induced apoptosis of CTCL cells was associated with an up-regulation of Bax dimers and a decrease of survivin.

Conclusion: These results indicate that IKK2 inhibition represents a promising strategy for the treatment of advanced stages of CTCL.

One of the main features of epidermotropic cutaneous T-cell lymphomas (CTCL), i.e., mycosis fungoides and its leukemic variant Sézary syndrome (SS), is the striking ability of malignant T cells to resist to apoptotic stimuli such as activation induced cell death and to apoptosis induced by conventional antineoplastic chemotherapy, as emphasized by the usual failure of polychemotherapeutic regimens to provide long-term remission in CTCL patients (1, 2). Recently, molecular studies showed that a constitutive activation of the nuclear factor-κB (NF-κB) signaling pathway is consistently observed in CTCL cell lines and in tumor cells from patients with SS and plays a key role in the resistance of CTCL cells to apoptotic stimuli (3).

NF-κB is a transcription factor consisting in a family of proteins c-rel, p65/RelA, RelB, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), which associate to each other to generate homodimeric or heterodimeric complexes (4). Such transcription factors of the NF-κB family are regulators of cell proliferation and survival and control expression of several genes relevant to the process of oncogenesis (5–8). Under normal conditions, NF-κB is sequestered in an inactive state by the inhibitory (IκB) molecules in the cytoplasm. Several signals, including components of pathogens, such as lipopolysaccharide, proinflammatory cytokines, such as tumor necrosis factor and interleukin-1, mitogens, or oxidative stress, can...
activate NF-κB via phosphorylation of the IκBs, triggering their ubiquitination and degradation by the proteasome 26S. The kinase responsible for phosphorylating IκBs is IκB kinase (IKK), which is composed of two catalytic subunits, IKK1/IKKα and IKK2/IKKβ, and a regulatory subunit called NEMO/IKKγ (9, 10). In addition to the classic NF-κB signaling pathway that requires IKK2 and NEMO, a noncanonical NF-κB cascade has been identified to involve signaling via the NF-κB–inducing kinase and IKK1 homodimers and results in the processing of p100 to mature p52.

According to the shown role of NF-κB in the protection of several types of tumors toward cell death, it has been proposed that an inhibition of this latter pathway could be a relevant strategy for the treatment of cancers characterized by a constitutive activation of the NF-κB pathway, such as multiple myeloma (10–12). Thus, several inhibitors of NF-κB have been developed or are currently under development (13, 14), and promising results have been provided in recent therapeutic trials investigating the proteasome inhibitor bortezomib in patients with refractory multiple myeloma, alone or in combination with dexamethasone (15–17). We recently showed evidence for a constitutive activation of the NF-κB canonical pathway in CTCL and for its key role in the resistance of CTCL cells to apoptosis, including death induced by antineoplastic agents (3). We also showed that proteasome inhibitors, such as bortezomib (Velcade), allowed to reverse NF-κB constitutive activation in CTCL and consequently induced apoptosis of CTCL cells in vitro. Given the significant toxicity associated with bortezomib usage in vivo, which is related to the wide range of molecular targets of this latter class of compounds, the design of new molecules able to down-regulate NF-κB canonical pathway with more specificity is clearly warranted for the treatment of patients with CTCL. Small molecules and viral vectors expressing proteins that inhibit IKK, or other aspects of the NF-κB activation pathway, have been shown to induce apoptosis and to inhibit the proliferation of tumors or tumor-derived cell lines (13). Unfortunately, viral vectors are not currently available for clinical applications. Therefore, more specific and potent inhibitors are needed.

In the present study, we show that a specific inhibition of the IKK2 kinase by the transfection of a dominant-negative version of IKK2 allows to down-regulate NF-κB constitutive activation in CTCL cells. We further show that a pharmacologic inhibitor of the IKK2 kinase (AS602868), which was previously shown to block tumor necrosis factor-α–induced NF-κB activation in Jurkat leukemic cells (18, 19), blocks constitutive nuclear translocation of NF-κB activation in tumor cells from SS patients and in CTCL cell lines, induces an apoptotic response, and potentiates the apoptotic effects of several conventional antineoplastic agents on CTCL cells while preserving the viability of normal T cells.

Materials and Methods

Patients

Patients gave written informed consent after approval by the local Ethical Committee. Essential characteristics of study patients are listed in Table 1. The diagnosis of SS was established according to clinical and morphologic criteria (i.e., a chronic erythroderma, with presence of large size Sézary cells on cytologic examination of a peripheral blood smear, and a dense, bandlike dermal infiltrate with epidermotropism in skin biopsies). A dominant clonal rearrangement of the T-cell receptor (TCR) γ locus was found in both lesional skin and in peripheral blood lymphocytes (PBL) by using PCR multiplex-based analysis at the DNA level, as previously described (20). Patients selected to enter the present study exhibited a Sézary cell ratio exceeding 5% of PBLs (range, 5-51; median, 14.3; Table 1). The quantitative evaluation of peripheral blood tumor cells was assessed by combining CD4/anti-TCRVγ double immunostaining flow cytometric analysis of PBLs and TCRVγ complementary-determining region 3 length analysis by the immunoscope method as previously detailed (21). Human PBLs from normal healthy plasma donors were obtained through the “Etablissement Français du Sang” (Hôpital Saint-Louis, Paris, France).

Table 1. Main characteristics of patients with SS

<table>
<thead>
<tr>
<th>Patient no. (age at diagnosis)</th>
<th>TNM staging*</th>
<th>Previous treatments</th>
<th>PBMC † Lymphocytes † % CD4+/CD3+ TCRVγ † (% of CD4+cells)</th>
<th>% Sézary families</th>
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<tr>
<td>1 75 (74)</td>
<td>T4N3M0 IFNα, ECPC, MTX</td>
<td>12,500 2,880 97.8  NA 50</td>
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<tr>
<td>2 77 (75)</td>
<td>T4N2M0 Cortacyn, MTX, carboxylsine, IFNα</td>
<td>13,400 10,728 99.5  NA 7</td>
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<tr>
<td>3 68 (67)</td>
<td>T4N3M0 Cortacyn, IFNα</td>
<td>15,800 6,320 93.9  Vβ12 (95.2) 9</td>
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<tr>
<td>4 65 (61)</td>
<td>T4N1M0 Carboxylsine, ECPC</td>
<td>5,900 1,400 76.4  Vβ17 (34.4) 7</td>
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</tr>
<tr>
<td>5 63 (58)</td>
<td>T4N1M0 IFNα + PUVA, ECPC</td>
<td>29,490 2,950 97.3  Vβ20 (98) 51</td>
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<tr>
<td>6 64 (59)</td>
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<td>16,000 1,760 94.9  Vβ18 (98.7) 30</td>
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<tr>
<td>7 78 (76)</td>
<td>T4N3M0 MTX, ECPC</td>
<td>16,500 3,140 99  NA 23</td>
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<tr>
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<td>T4N3M0 IFNα, PUVA, MTX</td>
<td>6,700 1,740 82.8  Vβ2 (38.6) 19</td>
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<tr>
<td>9 75 (61)</td>
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</tr>
<tr>
<td>10 79 (69)</td>
<td>T4N3M0 PUVA, IFNα, ECPC, CHOP</td>
<td>11,900 4,044 96.3  Vβ3 (97.5) 5</td>
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Abbreviations: NA, not available; PBMC, peripheral blood mononuclear cells; TNM, tumor-node-metastasis; PUVA, psoralen with UVA radiation therapy; MTX, methotrexate; ECPC, extracorporeal photochemotherapy; CHOP, polychemotherapy regimen containing cyclophosphamide, doxorubicin, vincristine, and prednisone.

* According to the tumor-node-metastasis classification.
† Expressed as peripheral blood absolute cell counts per cubic millimeter.
‡ The TCRVγ segment expressed by tumor cells was identified by TCRVγ/C4 double immunostaining flow cytometric analysis of PBLs from SS patients, although PBLs from patients 1, 2, and 7 did not immunoreact with any anti-TCRVγ monoclonal antibody. Nevertheless, the monoclonal nature of the CD4+ expansion was confirmed in all cases by PCR analysis of TCRVγ-γ rearranged DNA segments, by Vγ family-specific RT-PCR, and by complementary-determining region 3 length analysis of expanded TCRVγ families.
Reagents

AS602868 was kindly provided by Merck Serono International (Geneva, Switzerland) and dissolved at 13.19 mmol/L final concentration in cycloextrin (excipient). AS602868 is an anilinopyrimidine derivative and ATP competitor selected for its inhibitory effect in vitro on IKK2 EE, a constitutively active version of IKK2. The proteasome inhibitor bortezomib (Velcade, formerly PS-341) was kindly supplied on IKK2 EE, a constitutively active version of IKK2. The proteasome inhibitor bortezomib (Velcade, formerly PS-341) was kindly supplied by Millenium Pharmaceuticals, Inc. under Materials Cooperative Research and Development Agreements, dissolved at 0.825 mol/L final concentration in DMSO, and stored at -20°C. Doxorubicin (Sigma) was prepared as a stock solution at 17.2 mmol/L in H2O and stored at -20°C

Cell lines and PBLs from CTCL patients and healthy donors

The HuT-78 cell line (SS) and the Jurkat cells (human acute T-cell leukemia, clone E6.1) were purchased from European Collection of Animal Cell Cultures. SeAx (SS) and MyLa 2059 (mycosis fungoides) cell lines were kindly provided by Dr. Keld Kaltoft (University of Aarhus, Aarhus, Denmark). Cell lines were grown at 37°C with 5% CO2 at 1 x 10^5 cells/mL in RPMI 1640 (Life Technologies) supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1% HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; RPMI 1640 complete medium). PBLs were obtained by venipuncture and separated from heparinized whole blood by Ficoll-Hypaque gradient centrifugation followed by elimination of monocytes by adherence in RPMI 1640 complete medium at 37°C for 2 h.

Cell proliferation assay

Cells exposed to AS602868 (1-30 μmol/L), with or without etoposide (1 or 10 μmol/L) or doxorubicin (0.1 or 0.5 μmol/L), or exposed to bortezomib (20 nmol/L) were cultured in 96-well microplates (1 x 10^4/100 μL/well) at 37°C for 24, 48, and 72 h and pulsed with 1 μCi/well [3H]thymidine during the last 16 h of culture. Incorporated tritium was quantified using a MicroBeta1450 counter (Wallac).

Analysis of apoptosis, mitochondrial membrane potential, and cell cycle

PBLs from SS patients or CTCL cell lines incubated in the presence of AS602868, associated or not with etoposide, vincristine, or bortezomib,
for 6 to 72 h were analyzed for apoptosis according to the following protocols.

**Annexin staining.** Detection of phosphatidylserine on apoptotic cells was done by using Annexin V/propidium iodide (PI) detection kit (Beckman Coulter Immunotech) according to the manufacturer’s instructions. Briefly, 5 x 10⁵ cells were incubated in the dark at 4°C with Annexin V-FITC and 2.5 μg/mL PI in phosphate buffer for 10 min before 18,000 nongated events were collected by flow cytometry (FACSCalibur, BD Biosciences) and analyzed with the ProCellQuest software provided by the manufacturer.

**Mitochondrial membrane potential.** The 3,3'-diethylthiacarbocyanine (DiOC₆) fluorescence probe (Molecular Probes) was used to quantify mitochondrial transmembrane potential ΔΨm as previously described (3). In brief, 5 x 10⁵ cells were incubated in RPMI 1640 complete medium with 0.1 μmol/L DiOC₆ for 30 min at 37°C, washed, and resuspended in complete medium with 10 μg/mL PI before immediate analysis by flow cytometry. The percentage of cells exhibiting a low level of DiOC₆ uptake, which reflects loss of ΔΨm, was determined.

**Cell cycle analysis.** Cells (5 x 10⁵) were washed twice with ice-cold PBS, fixed in cold 70% ethanol for 1 h at 4°C, washed twice in cold PBS, treated with 200 units/mL DNase-free RNase A (Sigma) for 30 min at 37°C, and stained with 25 μg/mL PI. Distribution of cell cycle phases was determined using PI fluorescence emission (15,000 events) on FACS-Calibur and analyzed with ModFit software (BD Biosciences).

**Immunoblot analysis**

Cells (10 x 10⁵) were lysed for 1 h at 4°C in buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1% NP40, 1 mmol/L NaF, 1 mmol/L NaVO₃, 0.25% sodium deoxycholate, 1 μmol/L phenylmethylsulfonyl fluoride, 19 μg/mL aprotinin, 10 μmol/L pepstatin, 10 μmol/L leupeptin). After centrifugation (14,000 rpm, 15 min, 4°C), whole-cell protein extracts (50 μg) were loaded onto a 10% polyacrylamide gel containing SDS, subjected to electrophoresis, and dried, and exposed to autoradiographic films for 24 h before being developed. Binding reactions were done by adding 10⁵ cpm of [³²P]-labeled oligonucleotide for 20 min at 4°C. Samples were analyzed by electrophoresis in a 4% polyacrylamide gel. Gel was fixed, vacuum dried, and exposed to autoradiographic films for 24 h before being scanned using ScanQuant. The specificity of binding was examined by competition with the unlabeled oligonucleotide (50×). Normalization of constitutive nuclear translocation of NF-κB was obtained by comparison with a nonspecific binding band as well as by using OCT-1 probe obtained from Promega.

**Reverse transcription-PCR**

Total cellular RNAs were extracted with the RNA-Plus kit based on the manufacturer's instructions (Quantum Applgene), and 1 μg of total RNA was converted to cDNA by using a RT-for-PCR kit (Invitrogen) according to the manufacturer's instructions.

**Real-time quantitative PCR**

The resulting first-strand cDNA (1 μL) was assayed by real-time PCR using SYBR Green PCR Core Reagents (ABI PRISM 7700 Fast Real-Time PCR System, Applied Biosystems) according to the manufacturer's instructions. The sets of primers were designed with the primer design software Primer Express version 3.0 (Applied Biosystems). The sequence of the forward primer for IκBα mRNA was 5'-TGTTGTCTTGGCGTGGCCTGAT-3' and that of the reverse primer was 5'-AGGTCCACCTGGAGGTAAG-3'. For survivin mRNA, the forward primer was 5'-GCAAGGAAAA-CCAAACATAGAAGA-3' and the reverse primer was 5'-ATGGCAACGCC-GCAGTT-3'. For Bax mRNA, the forward primer was 5'-GGATGTTGTC-CCCTTACTGTTTG-3' and the reverse primer was 5'-CAGTTCCGCACCTTGTGTTG-3'. The forward primer for β2microglobulin mRNA was 5'-TGTTGCTTGCATGGTAGTATCTC-3' and the reverse primer was 5'-TCTTCTGCTCCCCCCACTCTAAGT-3'. For GAPDH mRNA, the forward primer was 5'-GGTTCTCCTGGACAGCTCA-3' and the reverse primer was 5'-ACCTTCCCCATTGTTGCTGTA-3'. The conditions for the one-step reverse transcription-PCR (RT-PCR) were as follows: 2 min at 50°C followed by 10 min at 95°C, then 40 cycles of amplification at 95°C for 15 s and 60 s at 60°C, and finally 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C. Each assay was run in triplicate. All samples were normalized to either β2microglobulin or GAPDH. Quantification of the target gene expression was done using the comparative cycle threshold (Ct) method according to the manufacturer's instructions (Applied Biosystems Primer Express version 3.0).

**Fig. 2.** AS602688 treatment down-regulates IκBα phosphorylation. AS602688 treatment inhibits the constitutive phosphorylation of IκBα subunit without altering IκBα expression. MyLa cells (top) and tumor cells from SS patient 9 (bottom) were incubated with 3,10, and 30 μmol/L of AS602688 for 10 min and subjected to Western blot analysis. Whole-cell extracts (50 μg) were fractionated on 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane. Western blot analysis was done with phosphorylated-specific anti-IκBα antibody. The same membrane was then reblotted with anti-IκBα antibody and then with GAPDH antibody as an internal control for loading.
An average Ct was calculated for the duplicate reactions and normalized to H2 microglobulin (ΔΔCt = Ct sample - Ct H2 microglobulin). The ΔΔCs were then compared between different treatments and time points (ΔΔCt) with normalization to the untreated samples. Finally, a fold change was calculated from the ΔΔCt (fold change = 2ΔΔCt).

Gene transfer by nucleofection

CTCL cell lines (2 × 10^5/100 μL electroporation buffer) were transiently transfected using the Human T Cell Nucleofector kit according to the manufacturer (Amnax Biosystems). Four hundred nanograms of a β-dependent reporter plasmid (Promega) were cotransfected with 0.4 to 2.4 μg of empty vector (pcDNA3) or plasmid vector expressing a mutant form of IKK2 (IKK2 SS/AA), kindly provided by Dr. F. Mercurio (Cellgen Corporation, Summit, New Jersey), and characterized by the substitution of two serine residues with alanines, leading to inhibition of IKK2 autophosphorylation required for IKK activity (22). Transfection efficiency was determined by electroporating 2 μg of vector containing green fluorescent protein gene driven by the cytomegalovirus promoter.

Two hundred nanograms of plasmid containing the β-galactosidase gene (EF1LacZ) were used to normalize luciferase activity. After electroporation, cells were immediately cultured in RPMI 1640 complete medium at 37°C for 18 to 48 h. Cells (1 × 10^6) were harvested by centrifugation, washed with PBS, and lysed with reporter lysis buffer for luciferase assay detection, which was done in duplicate (Luciferase Assay System, Promega). Measurement of β-galactosidase activity (β-gal Reporter Gene Assay, Roche) was done according to the manufacturer. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector. Determination of green fluorescent protein expression in transfected cells by flow cytometry analysis indicated that transfection efficiency ranged between 25% and 30%.

Confocal microscopy

Cells were cytospined on slides (5 × 10^4 per slide) during 2 min at 700 rpm, fixed in acetone/methanol (1:1) for 10 min, blocked in a solution of 30% bovine serum albumin in PBS for 20 min, and
permeabilized using 0.2% Tween 20-PBS for 30 min. Slides were incubated with rabbit polyclonal anti-p65 or goat polyclonal anti-p50 (both at 4 μg/mL; Santa Cruz Biotechnology) for 1 h, washed twice with PBS, and incubated with the secondary antibody labeled with FITC or Texas red (1:50) for 1 h. Mounting was done using Vectashield medium (Vector Laboratories) including 4′,6-diamidino-2-phenylindole fluorescence for nuclei staining. Images were acquired on a Zeiss LSM510 META laser scanning confocal microscope equipped with a Zeiss Plan Apochromat 63×1.4 numerical aperture oil immersion objective using the LSM510 software (version 3.2).

Statistical analysis

For analysis of apoptosis and mitochondrial Δψm, values represent the means ± SD for at least three separate experiments done in triplicate, unless otherwise noted. The significance of differences between experimental variables was determined by ANOVA followed by the Student’s t test, and P < 0.05 was considered as significant.

Results

Constitutive NF-κB activation in CTCL cell lines is IKK2 dependent. To assess the contribution of the catalytic subunit of IKK, IKK2, to the constitutive activation of NF-κB in CTCL, we investigated the effect of its specific inhibition. For this, we first transfected CTCL cell lines MyLa and SeAx with a catalytically inactive form of IKK2 (IKK2 SS/AA) and monitored NF-κB activity using reporter plasmids expressing firefly luciferase under either a NF-κB–dependent or a NF-κB–independent minimal conalbumin promoter.

In both untreated CTCL cell lines, the NF-κB–dependent reporter plasmid was reproducibly much more active than the control reporter plasmid, with a 20 ± 4.4–fold and 26.1 ± 3.8–fold difference in SeAx and MyLa cells, respectively. In contrast, only a 1.7 ± 0.5–fold difference was observed in control Jurkat cell line (Fig. 1A). Importantly, transfection of both SeAx and MyLa cells with IKK2 SS/AA resulted in a decrease of NF-κB–dependent luciferase activity, which reached ~80% following transfection of SeAx with 2 μg of IKK2 SS/AA plasmid (Fig. 1B). Similar results were obtained with HuT-78 cell line (data not shown).

We then tested whether a newly developed pharmacologic inhibitor of IKK2, anilinopyrimidine AS602868 (18), would also reduce NF-κB activity in CTCL cells using the same assay. As shown in Fig. 1C, a treatment with AS602868 potently decreased NF-κB–dependent luciferase activity in both transfected SeAx and MyLa cells (Fig. 1C). A reduction of 60% of luciferase activity was observed at 3 μmol/L and reached >90% at 30 μmol/L for both cell lines.

To confirm that AS602868 was indeed able to inhibit NF-κB transcriptional activity in CTCL cells and to affect expression of endogenous genes, which are under NF-κB control, we analyzed its effect on the expression of IkBα, one of the major targets of NF-κB. On incubation with increasing concentrations of AS602868 during 4 to 48 h, IkBα expression decreased substantially in both CTCL cell lines and tumor cells from SS patients, as shown by quantitative PCR analysis (Fig. 1D).

Finally, we addressed whether the phosphorylation status of IkBα in CTCL cell lines or tumor cells derived from SS patients, which is an indirect readout of IKK catalytic activity, would be altered by AS602868 treatment. As shown in Fig. 2, AS602868...
A

Annexin-V staining
Propidium Iodide

<table>
<thead>
<tr>
<th></th>
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<th>10</th>
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<tr>
<td>Etoposide</td>
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B

Mitochondrial membrane depolarization (ΔΨm)

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<td>17.7%</td>
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C

AS602868 - induced apoptosis

- SS PBLs
- HD PBLs

Mean ± SEM (n=10)

Mean ± SD (n=6)
induced a strong decrease of IκB phosphorylation as early as after 10 min of incubation without inducing any detectable change in the total amount of IκB at this time. This first set of data shows that IKK2 catalytic activity is required to maintain a high constitutive NF-κB activity in CTCL cells.

The IKK2 inhibitor AS602868 down-regulates the constitutive activation of the NF-κB canonical pathway in CTCL cell lines and in peripheral blood tumor cells from patients with SS. We then analyzed the effects of AS602868 on NF-κB DNA-binding activity in CTCL by using electrophoretic mobility shift assays. AS602868, from 3 to 30 μmol/L, inhibited the binding capacity of p50 and p65/RelA subunits of NF-κB in SeAx and MyLa cell line and in peripheral blood tumor cells from patients with SS (Fig. 3A; data not shown). In all electrophoretic mobility shift assay experiments, normalization of constitutive nuclear translocation of NF-κB was obtained by using OCT-1 probe. This observation supports the view that the canonical pathway of NF-κB activation is sensitive to IKK2 inhibition by AS602868 in CTCL. Accordingly, an additional IKK2 inhibitor, IMD-0354 (Inhibitor V, Calbiochem) inhibited NF-κB DNA-binding activity in CTCL cells (Supplementary Fig. S1).

Results from electrophoretic mobility shift assay were confirmed by analyzing p65 and p50 intracellular localization using confocal microscopy. We also observed a decrease of p65 and p50 nuclear staining following treatment of CTCL cell lines and of PBLs from SS patients with AS602868 (Fig. 3B and C).

The AS602868 IKK2 inhibitor reduces proliferation of CTCL cells, induces their apoptosis, and potentiates the effect of conventional antineoplastic agents in vitro. We further studied the effect of an IKK2 inhibition by AS602868 on the proliferative and cycling capacities of MyLa and SeAx CTCL lines. Treatment of SeAx cells or MyLa cell lines with AS602868 induced a dose-dependent inhibition of their proliferative capacity (Fig. 4A; data not shown). This inhibitory effect was already dramatic at a 10 μmol/L concentration of AS602868, and proliferation was almost completely abolished at 30 μmol/L (Fig. 4A). In contrast, Jurkat cell proliferation was much less affected by AS602868, the highest concentration of AS602868 only leading to a 50% inhibition. We also investigated the effects on CTCL proliferation of a combination of AS602868 with conventional antineoplastic agents etoposide, vincristine, and doxorubicin. As shown in Fig. 4A, AS602868 potentiated the effect of etoposide, allowing a 90% inhibition of SeAx proliferation in the presence of 10 μmol/L etoposide. Similarly, AS602868 potentiated vincristine and doxorubicin (data not shown). In contrast, no additive effects could be evidenced on Jurkat cells, and only antiproliferative effects of etoposide, vincristine, and doxorubicin were observed (Fig. 4A). The thymidine incorporation results were further confirmed by flow cytometry analysis of SeAx DNA content changes. These experiments showed an increase of the sub-G₁ population, together with a decrease in G₂-M, after treatment with AS602868 (Fig. 4B).

To investigate the effects of IKK2 inhibition on CTCL cell survival/apoptosis, cells were incubated for 24 to 72 h in the presence or absence of AS602868, and apoptosis was measured by Annexin V binding and PI staining. AS602868 induced a dose- and time-dependent apoptosis of CTCL cell lines and PBLs from SS patients, as shown for one representative patient with a very high proportion of peripheral blood tumor cells (Fig. 5A). The combination of 10 μmol/L AS602868 with etoposide induced a 2-fold increase in the dead cell population, which, for half of it, showed a necrotic postapoptotic status, in comparison with both drugs alone. The combination of 10 μmol/L AS602868 with 12.5 μmol/L vincristine leads to a ~2-fold increase in the apoptotic cell ratio and less necrotic postapoptotic cells, in comparison with vincristine alone (Fig. 5A). The rate of AS602868-induced apoptosis was also analyzed by investigating the disruption of Δψm, an essential step in mitochondrial apoptosis, by flow cytometry analysis of DiOC₆ fluorescent probe staining. Both CTCL cell lines and SS tumor cells exhibited a dose- and time-dependent disruption of Δψm.

Fig. 5 Continued. D, AS602868-induced apoptosis is predominant in CTCL cells compared with nonmalignant CD4⁺ T lymphocytes from SS patients. Peripheral blood cells from three patients (cases 3, 4, and 10) were treated with AS602868 (3-10 μmol/L) in combination or not with 10 μmol/L etoposide or with 20 nmol/L bortezomib for 48 h. The percentages of TCRV⁺/CD4⁺/Annexin V− cells and TCRV⁺/CD4⁺/Annexin V⁺ cells in each patient were determined in duplicate and normalized using respective control untreated cells, which were arbitrarily set as 1. Results obtained with nonmalignant PBLs (TCRV⁺/CD4⁺) and peripheral blood tumor cells from the same SS patient (TCRV⁺/CD4⁺) are represented by white and gray columns, respectively. Columns, mean (n = 3); bars, SD. *, P < 0.05, significant apoptosis in tumor peripheral cells when compared with apoptosis in nontumor peripheral lymphocytes from the same patient. E, AS602868 induces cleavage of poly(ADP-ribose) polymerase (PARP) in CTCL cells. SeAx cells were treated with increasing doses of AS602868 (3-10 μmol/L) in combination or not with 10 μmol/L etoposide or with 20 nmol/L bortezomib for 24 h. Cells were analyzed by Western blotting for expression of poly(ADP-ribose) polymerase as an intact (116 kDa) or cleaved (85 kDa) protein. GAPDH signal was used as an internal control for loading. Data represent one of two independent experiments giving similar results.
Δψm (Fig. 5B; data not shown). Again, combination of AS602868 with etoposide and vincristine allowed a potentiation of proapoptotic effects.

We comparatively analyzed AS602868-induced apoptosis in peripheral blood tumor cells from SS patients (n = 10) and in PBLs from 6 healthy donors. As shown in Fig. 5C, a 2.5-fold increase of Annexin V+ cells was observed in SS patients in response to 30 μmol/L AS602868 during 48 h, whereas viability of PBLs from six healthy donors was much less altered, showing only a 1.5-fold increase of apoptosis. Of interest, the proportion of apoptotic cells following incubation with AS602868 was notably lower in nonmalignant PBLs versus peripheral blood tumor cells from the same SS patient, as shown by TCRVh+/CD4/Annexin V triple staining analysis of PBLs from three patients (Fig. 5D). The malignant nature of TCRVh+ PBLs in these patients was confirmed by showing the monoclonal pattern of the CD4+ expansion through TCRVh complementary-determining region 3 length-based analysis and by showing evidence for expression of T-plastin mRNA (23) in purified-positive (CD4/TCRVh) PBLs by RT-PCR analysis (data not shown).

During the apoptotic process through the mitochondrial pathway, mitochondrial disruption leads to activation of the effector caspase-3 and the cleavage of poly(ADP-ribose) polymerase. We confirmed by Western blot that AS602868-induced apoptosis of CTCL cell lines and tumor cells from two SS patients was associated with decrease in 32 kDa procaspase-3 amount and with proteolytic degradation of the

![Fig. 6. Effects of AS602868 on Bcl-2 family oncoprotein and survivin expression.](image)

**A**. AS602868-induced apoptosis in CTCL is associated with an up-regulation of Bax dimers. MyLa cells, peripheral blood tumor lymphocytes from one patient with SS (case 9), and PBLs from one healthy donor were treated with or without increasing concentrations of AS602868 (3-30 μmol/L) in combination or not with 10 μmol/L etoposide for 48 h as indicated. Whole-cell extracts were prepared, and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against the Bcl-2 family members Bcl-2, Bcl-XL, and Bax. The membrane was stripped and reprobed for expression of GAPDH to control for loading. The numbers indicate the relative modifications of expression, in comparison with control untreated cells, which are arbitrarily set as 1 under normalization with GAPDH. Bottom, the percentages of Annexin V+ cells obtained in each cell type just before whole-cell extracts were done were analyzed by Annexin V/PI staining using flow cytometry. B, up-regulation of survivin transcription in CTCL. Total RNAs were isolated from 10 millions of MyLa, SeAx, and Jurkat cells, freshly isolated tumor PBLs from 10 patients with SS (SS PBLs), or PBLs from 8 healthy donors (HD PBLs). Gene expression of survivin was assessed by real-time RT-PCR. β2microglobulin was used as the internal control. Columns, mean survivin expression (arbitrary unit) from two independent experiments done in duplicate; bars, SD. C, AS602868 negatively regulates survivin gene transcription in SeAx cell line and in tumor cells from SS patients. SeAx cells and peripheral blood tumor lymphocytes from three patients with SS (cases 2, 7, and 9) were treated with or without increasing concentrations of AS602868 (3-30 μmol/L) for 48 h. Total RNAs were extracted from 1 x 10⁷ cells and cDNAs were prepared for assessing survivin expression by real-time RT-PCR. β2microglobulin was used as the internal control. Columns, mean results analyzing survivin expression from two independent experiments in SeAx cells (white column) or tumor lymphocytes (gray columns), normalized using control untreated cells, which were arbitrarily set as 100; bars, SD. *, P < 0.05; significant inhibition by AS602868 compared with control untreated cells.
caspase-3 substrate poly(ADP-ribose) polymerase into a fragment of 85 kDa (Fig. 5E).

**AS602868-induced apoptosis of CTCL cells is associated with an up-regulation of Bax dimers and with a repression of survivin expression.** To decipher the mechanisms underlying CTCL apoptosis resulting from targeted inhibition of IKK2, we assessed changes in the expression of Bcl-2 family oncoproteins following treatment of CTCL cells with AS602868. Thus, immunoblot analysis of Bcl-2, Bcl-X<sub>L</sub>, and pro-apoptotic protein Bax was done in SeAx and MyLa cell lines and in PBLs from patients with SS, in comparison with PBLs from healthy donors. Results showed that treatment of CTCL cells with AS602868 induced an increase in expression of the 46 kDa dimeric form of Bax in both CTCL cell lines and PBLs from patients with SS, whereas no dimers were detected in PBLs from healthy donors (Fig. 6A). In contrast, the amount of the 23 kDa monomer remained unchanged whatever the cells. This dimer accumulation was further amplified by cotreating the cells with etoposide (Fig. 6A). In contrast, no significant difference in Bcl-2 or Bcl-X<sub>L</sub> protein levels was detected. Similar results were observed with tumor cell extracts from three other patients with SS.

We also analyzed the expression levels of prosurvival factors that are regulated by NF-κB at the transcription level. For this, a real-time quantitative RT-PCR analysis of cIAP1, cIAP2, XIAP, and survivin genes was carried out. No significant changes in transcription of cIAP1, cIAP2, and XIAP genes could be detected after IKK2 inhibition (data not shown). In contrast, expression of the survivin gene in CTCL cells was 22- and 118-fold higher than in control Jurkat cells or healthy PBLs, respectively, as shown in Fig. 6B, and this higher survivin expression in CTCL cells was confirmed by Western blot analysis (data not shown). Importantly, AS602868 treatment for 4 to 48 h down-regulated expression of the survivin gene in CTCL cell lines and tumor cells from three patients with SS (Fig. 6C).

**Discussion**

A constitutive activation of the NF-κB pathway is a characteristic feature shared by several human malignancies, such as multiple myeloma, melanoma, and a subset of B-cell nodal NHL called activated B-cell diffuse large cell lymphoma (24–26). We recently showed that the NF-κB canonical pathway is constitutively activated in CTCL lines and in peripheral blood tumor cells from patients with SS and that this abnormal signaling pathway predominantly contributes to the resistance of CTCL cells to apoptosis (3). However, the molecular mechanism underlying this activation remains unelucidated thus far. The data presented herein show that the IKK2 kinase provides a key contribution to the constitutive activation of the NF-κB canonical pathway in CTCL cells, at least in the context of SS and of CTCL lines, and promotes the survival of these malignancies. These results also show that molecular mechanisms responsible for NF-κB constitutive activation take place at the level of or upstream of the IKK complex. In support of this hypothesis, we have recently shown that inhibiting NEMO, the regulatory subunit of IKK, also down-regulates NF-κB activity in CTCL. Taking these new insights into account, it would be of interest to investigate the respective contributions of upstream IKK2-activating elements, such as CARD11, MALT1, and BCL10, to the NF-κB status and the resistance to apoptosis in CTCL cells, as recently described in the survival of activated B-cell NHL cells (27–29). The fact that CARD11 is also positively regulating NF-κB activity following stimulation via the TCR in T lymphocytes emphasizes the need for studies addressing the contribution of this latter signaling factor to the IKK activity in CTCL. However, it remains unclear whether up-regulation of IKK2 is a common feature shared by CTCL subtypes other than SS, such as mycosis fungoides. Indeed, evidences for NF-κB activation are scarce in the context of mycosis fungoides, beside data obtained with MyLa cell line, which are not necessarily relevant to the in vivo situation, and preliminary immunohistochemical studies that need confirmation at the molecular genetic level (30).

Another insight from the present study is the demonstration that an inhibition of IKK2 negatively alters the proliferative capacities of CTCL lines and promotes CTCL death. The pharmacologic inhibitor AS602868 has initially shown antitumor activity _in vitro_ against acute myelogenous leukemia blasts, inhibiting proliferation and promoting apoptosis of leukemic cells, through IKK2 inhibition (19), although in this latter model, it was also found to interfere with FLT3 kinase activation, thus appearing to target two different kinases that play a crucial role in the pathogenesis of acute myelogenous leukemia (31). Nevertheless, our results provide the first evidence that a pharmacologic inhibition at the IKK2 level is efficiently inducing apoptosis of a human lymphoid malignancy. One of the main interests of the targeted inhibition of IKK2 by AS602868 is its specificity for tumor cells because T lymphocytes from healthy donors and nonmalignant peripheral blood T cells from SS patients were relatively more preserved by the apoptosis-inducing effect of this latter compound, making this strategy appealing in an _in vivo_ therapeutic perspective in patients with CTCL, which would ideally aim at sparing as much as possible the pool and the diversity of nonmalignant T lymphocytes. The relative specificity of AS602868-induced cell death for tumor cells differs from the broader effects of bortezomib, which strongly alters the viability of nonmalignant T cells (3). Although it is still unclear whether IKK2 inhibition strategy should be used _in vivo_ as single-agent therapy or in combination with classic antineoplastic chemotherapy, one might expect that AS602868 specificity would be responsible for less toxicity compared with proteasome inhibitors. Furthermore, given results from pro apoptotic and antiapoptotic protein expression studies, it is likely that the specificity of AS602868 antitumor effect relies at least in part on the ability to specifically induce in CTCL cells, and not in normal T cells, an up-regulation of the dimeric form of Bax proapoptotic oncoprotein in CTCL cells without any significant change in the quantity of the monomeric form. This latter feature is according to previous studies showing that oligomerization of Bax was required but not sufficient to lead to cytochrome c release and further apoptosis of neuroblastoma cells following exposure to Taxol (32, 33). This latter set of effects of AS602868 on Bax dimerization emphasizes further the discrepancy between mechanisms underlying apoptosis induced by AS602868 and those associated with proteasome inhibitor-induced cell death, as there is no evidence for a change.
in Bax dimerization in the presence of the latter of these drugs (3). On the other hand, it has been previously shown that a high level of forced Bcl-2 expression prevented Bax oligomerization and insertion into the mitochondrial membrane (32). The present data show that proapoptotic effects of Bax complexes occurred in CTCL despite the high level of expression of Bcl-2 protein, a consistent finding in CTCL cells (34). It is also noteworthy that the transcriptional activity of the gene coding for survivin, a protein that has been shown to contribute to the protection of several malignancies against apoptosis (35–38), has been found up-regulated in CTCL cells compared with normal T cells and that inhibition of the NF-κB canonical pathway induces a striking down-regulation of this latter gene (39, 40). These latter results are in keeping with the NF-κB-regulated feature of the survivin gene and with recent studies reporting its down-regulation following IKK inhibition in myeloid tumor cells (41, 42). It remains to be determined whether the time interval required for cell death after exposure to AS602686 is related to the long half-life of the survivin protein or to mandatory syntheses of proteins that negatively regulate the prosurvival function of this latter compound, two hypotheses that are not mutually exclusive.

In conclusion, it seems that major alterations responsible for a constitutive activation of the NF-κB canonical pathway are a major feature in different types of malignancies. Although these features do not rule out a putative contribution of the alternative pathway, which relies on another kinase called NF-κB–inducing kinase, the present data identify IKK2 as an appealing therapeutic target in patients with advanced stages of CTCL.

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References

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Aurore Sors, Francette Jean-Louis, Elodie Bégué, et al.


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