The novel NF-κB inhibitor IMD-0354 induces apoptosis in chronic lymphocytic leukemia

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Nuclear factor-κB (NF-κB) is an important regulator of cell survival and has been shown to be constitutively active in chronic lymphocytic leukemia (CLL) cells. Recently, a novel NF-κB inhibitor, IMD-0354 (N(3), 5-bis(trifluoromethyl)-phenyl)-5-chloro-2-hydroxy-benzamide), was shown to specifically inhibit the phosphorylation of IκB by IκB kinases, thus preventing NF-κB release. In this study, we investigated if IMD-0354 can inhibit NF-κB activation and induce apoptosis in CLL cells in vitro. The rate of increase in apoptosis, drug sensitivity and DNA-binding activity of NF-κB were studied using Annexin V stainings, the fluorometric microculture cytotoxicity assay and electrophoretic mobility shift assay, respectively. Finally, the impact of IMD-0354 treatment on the expression of a set of apoptosis-related genes was investigated. The results clearly show that IMD-0354 induced apoptosis (mean 26%, range 8–48%) in CLL cells, independent of immunoglobulin heavy variable (IGHV) gene mutational status, and showed a dose-dependent cytotoxic effect. IMD-0354 treatment also significantly lowered the DNA-binding activity of NF-κB in CLL cells. In addition, we identified differences in expression levels of pro- and antiapoptotic genes following IMD-0354 treatment. In summary, our novel findings show that IMD-0354 can induce apoptosis in CLL cells, and thus merits further investigation as an anticancer agent in vivo.

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Keywords: chronic lymphocytic leukemia; IMD-0354; nuclear factor-κB; apoptosis; drug sensitivity

During the past decade, major advances in the treatment of chronic lymphocytic leukemia (CLL) have been made, including the emergence of promising agents, such as the nucleoside analog fludarabine and the monoclonal antibodies alemtuzumab (anti-CD52) and rituximab (anti-CD20),1,2 which have led to higher response rates and longer periods of remission for CLL patients. However, despite these therapeutic options, the rate of increase in apoptosis, drug sensitivity and DNA-binding activity of NF-κB were studied using Annexin V stainings, the fluorometric microculture cytotoxicity assay and electrophoretic mobility shift assay, respectively. Finally, the impact of IMD-0354 treatment on the expression of a set of apoptosis-related genes was investigated. The results clearly show that IMD-0354 induced apoptosis (mean 26%, range 8–48%) in CLL cells, independent of immunoglobulin heavy variable (IGHV) gene mutational status, and showed a dose-dependent cytotoxic effect. IMD-0354 treatment also significantly lowered the DNA-binding activity of NF-κB in CLL cells. In addition, we identified differences in expression levels of pro- and antiapoptotic genes following IMD-0354 treatment. In summary, our novel findings show that IMD-0354 can induce apoptosis in CLL cells, and thus merits further investigation as an anticancer agent in vivo.

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In this study, we investigated the effect of IMD-0354 in CLL cells in vitro by performing Annexin V stainings and a non-clonogenic cytotoxicity assay. Furthermore, we investigated the effect of IMD-0354 treatment on gene expression of different pro- and antiapoptotic genes. Our data clearly show that IMD-0354 can induce apoptosis of CLL cells directly by inhibiting the NF-κB pathway and should be further investigated as a new potential drug in CLL treatment.

Materials and methods

Patient samples

In total, tumor samples from 20 untreated CLL patients were included in this study. CLL cells displayed the typical immunophenotype and met the recently revised diagnostic criteria.13 Tumor material was obtained from peripheral blood samples. The median age at diagnosis was 64 years (range, 44–85 years), with a male:female ratio of 4 : 1. Eleven cases displayed IGHV unmutated genes and eight cases IGHV mutated genes, whereas in one case the mutation status was unknown. Informed consent was obtained according to the Declaration of Helsinki and ethically approved by the local ethical review committee.
Cell culture, drug treatment and Annexin V stainings

CLL cells were cultured in RPMI 1640 medium with 10% fetal calf serum (Gibco, Paisley, UK) supplemented with the addition of 4 mm glutamine and 1 × penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at a cell density of 1 × 10^6 cells per ml at 37 °C in 5% CO2 atmosphere. CLL cell cultures were maintained for 48 h both with/without the addition of IMD-0354 (Sigma Aldrich, St Louis, MO, USA): 1–10 μM and the survival factor thioredoxin (Trx)14 (1 μg/ml) (Sigma Aldrich). Annexin V staining for detection of phosphatidyl serine exposed on the plasma membrane was carried out using Annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer’s instruction. In brief, cells were washed and incubated for 15 min in the dark with FITC-conjugated Annexin V and propidium iodide provided with the kit and a minimum of 10,000 events were collected on a Beckman Coulter FACSort flow cytometer. Apoptosis was calculated as the percentage of Annexin V-positive cells corrected for the percentage occurring owing to spontaneous apoptosis in control wells.

Fluorometric microculture cytotoxicity assay

The fluorometric microculture cytotoxicity assay is a total cell kill assay, based on the ability of cells with intact cell membranes to convert non-fluorescent diacetate to fluorescent fluorescein.15 Tumor cells from eight CLL samples were cultured in 384-well microtiter plates that contained different concentrations of IMD-0354 ranging from 0.01 to 100 μM, and all samples were run in duplicates. The plates were incubated at 37 °C and 5% CO2 for 72 h and then analyzed using the automated Optimized Robot for Chemical Analysis (Orca, Beckman Coulter, Fullerton, CA, USA). Both the culture medium and drug were aspirated from the plates, followed by two rounds of cell washes in phosphate-buffered saline. Q2 buffer (containing 125 mM NaCl and 25 mM Heps buffer, pH 7.4) was added before the addition of 10 μl/g of fluorescein diacetate (Sigma Aldrich) to each well. The plates were incubated for 50 min and fluorescence was measured in Fluostar Optima (BMG Technologies, Amsterdam, the Netherlands), for the analysis of 35 apoptosis-related genes and four control genes in a single multiplex reaction. MLPA probes were subsequently added and allowed to hybridize for 16 h at 60 °C. Following hybridization, the probes were ligated with ligase 65 and polymerase chain reaction amplified utilizing FAM-labeled M13 primers. All polymerase chain reaction products were analyzed on the Applied Biosystems 9700 DNA sequencer (Applied Biosystems) using the GeneScan software. Peak tables for each sample were exported from GeneScan to Microsoft Excel, in which data normalization to the control gene β-2-microglobulin was carried out. Normalized data sets were grouped and analyzed for significant differences to the Statistica software (ver.7.1, Stat Soft, Tulsa, OK, USA) using the χ2-test (significance P<0.05). A heat map for visualization purposes was generated in the open source multiexperimenter viewer software (http://www.tm4.org/mev), in which data were adjusted to a 0–1 scale for color expression coding.

NF-κB activation by electrophoretic mobility shift assay

CLL cells (1 × 10^6 cells per ml) were treated both with/without 1 μM of IMD-0354 for 24 h. Cytoplasmic and nuclear extracts were prepared using hypotonic buffer (20 mm Heps, pH 7.6, 20% (w/v) glycerol, 20 mm NaCl, 10 mm MgCl2, 0.2 mm EDTA, 1% (w/v) Triton X-100, 1 mm dithiothreitol, 1 mm sodium orthovanadate and 1% aprotinin) and hyperton buffer (20 mm Heps, pH 7.6, 20% (w/v) glycerol, 500 mm NaCl, 1.5 mm MgCl2, 0.2 mm EDTA, 1% (w/v) Triton X-100, 1 mm dithiothreitol, 1 mm sodium orthovanadate and 1% aprotinin), respectively. Briefly, cells were washed twice with phosphate-buffered saline, and lysed in hypotonic buffer, for 10 min on ice. Nuclei were spun down and incubated in hyperton lysis buffer for 20 min on ice. Supernatants were removed and protein concentrations were determined. The NF-κB-specific consensus oligonucleotide (5′-AGTTGAGGGTTTCCCAGGC-3′; Promega, Madison, WI, USA) was 5′ end labeled with [γ-32P]adenosine triphosphate using polynucleotide kinase (Gibco, Gaithersburg, MD, USA). For the binding reactions, equal quantities of nuclear extracts (1–4 μg) were incubated with 10,000 c.p.m. of end-labeled NF-κB oligonucleotide and 1 μg of poly d–dC (GE Health Care, Buckinghamshire, UK) in the binding buffer (20 mm Heps, pH 7.6, 20% (w/v) glycerol, 100 mm NaCl, 10 mm MgCl2, 0.2 mm EDTA, 1% (w/v) Triton X-100, 1 mm dithiothreitol). Free oligonucleotide and oligonucleotide-bound proteins were separated by electrophoresis on a native 6% polyacrylamide gel. Gels were dried under vacuum on Whatman paper and exposed to Kodak BioMax MS film (Sigma Aldrich) for 3–6 h at −80 °C. The absence of binding in the presence of excess unlabeled NF-κB-specific oligonucleotide (competitor oligonucleotide) confirmed NF-κB-binding specificity.

Results

IMD-0354 induces apoptosis in CLL cells in vitro

To determine whether IMD-0354 is capable of inducing apoptosis in CLL cells in vitro, cells from 20 CLL patients were incubated in the presence and absence of IMD-0354. The survival factor Trx was added in cells from 10 of these patients (five IGHV mutated and five IGHV unmutated samples). The percentage of early apoptotic and viable cells was evaluated by Annexin V/PI flow cytometry after 48 h of incubation. The
Annexin V-positive percentage of cells was calculated as an increase over spontaneous apoptosis in untreated controls at that time point. Overall, the majority of CLL samples investigated showed an increase in the percentage of apoptotic cells following treatment with IMD-0354 only (mean 26%, range 8–48%), whereas treatment with IMD-0354 + Trx gave a somewhat lower mean percentage of apoptosis (mean 20%, range 10–30%) (Figures 1a and b). On the contrary, CLL cells treated with Trx alone (n = 10) did not show any apoptosis. Out of total 20 CLL samples with IMD-0354 treatment, the percentage of average mean increase in apoptosis for all unmatured samples was 30% and for all mutated samples 25%.

CLL cells in duplicate (from two patients) were also incubated with increasing amounts of IMD-0354, with concentrations ranging from 1 to 10 μM. A dose-dependent increase in the apoptotic response of CLL cells was observed after 24 h of IMD-0354 incubation (Figure 1c).

**Dose-dependent inhibition of CLL cell survival in vitro**

To measure the cytotoxicity of IMD-0354 and calculate the percentage of surviving CLL cells, we performed fluorometric microculture cytotoxicity assay. Tumor cells from eight CLL samples were cultured in 384-well microtiter plates with different concentrations of IMD-0354. The percentage of cell survival was calculated as outlined in the Materials and methods section. As shown in Figure 2, all CLL patient samples showed a dose-dependent decrease in the percentage of survival index, with increasing concentrations of IMD-0354.

**Inhibition of NF-κB activation in CLL cells**

To determine the DNA-binding activity of NF-κB in both IMD-0354-treated and -untreated CLL cells, for four CLL samples, we performed electrophoretic mobility shift assay, using a small consensus NF-κB binding site. As shown in Figure 3, the DNA-binding activity of NF-κB is almost lost in the IMD-0354-treated CLL cells (lanes 6–9), as compared with the untreated cells, which displayed a high DNA-binding activity (lanes 2–5). The cold competition experiment, which was performed using 100-fold excess of unlabeled oligonucleotide, showed a complete loss of DNA-binding activity (lane 10). These results indicate that the activity of NF-κB is significantly lowered following 24 h of IMD-0354 treatment in all four CLL samples.

**Induction of apoptotic-related gene expression in CLL cells**

The above results indicated that IMD-0354 significantly induced apoptosis by targeting NF-κB-induced genes and hence decreased the cell survival in CLL patient samples in vitro. To test for changes in the expression of apoptotic genes in both IMD-0354-treated and -untreated CLL cells, the expression changes of 35 different apoptosis-related genes was investigated by applying the RT-MLPA kit in five CLL samples. Cells were first treated with tumor necrosis factor-α for 24 h, with subsequent addition of IMD-0354 for another 24 h and thereafter total RNA was extracted. Significant differences (P < 0.05) in gene expression were only observed for three genes (BIRC2, BIRC3 and BBC3) (Figure 4). To confirm gene expression levels observed with MLPA, we selected four genes (BCL2, BCL2A1, BAX and MCL2) and verified the results using quantitative real-time-polymerase chain reaction (data not shown).
Discussion

Many studies have shown that constitutive activation of NF-κB is a hallmark for most types of human cancer including leukemia and lymphoma.16,17 Given the considerable contribution of NF-κB to proliferation in many cancers, NF-κB could be one of the important molecular targets of therapy and inhibition of NF-κB may also have a therapeutic potential for the control of tumor progression. In this study, we applied a novel drug, IMD-0354, to inhibit NF-κB activation in CLL cells in vitro. IMD-0354 has been shown to inhibit proliferation of neoplastic cells and results in suppressed progression in human breast cancer and mast cells.12,18 It has also been shown in mice models that IMD-0354 treatment can significantly reduce colitis-associated tumorogenesis.19 However, to our knowledge, the effect of IMD-0354 in CLL has previously not been investigated.

The phenotype of CLL cells is characterized by defects in both programmed cell death and control of proliferation.20 When applying IMD-0354, a clear increase in apoptosis was observed in the majority of CLL samples investigated (Figure 1a). As it is well known that CLL cells will rapidly undergo spontaneous apoptosis when cultured in vitro,21 we also added a survival factor (Trx)14 when performing the IMD-0354 experiments, to prevent or reduce apoptosis in CLL cells. Despite this, a similar increase in the level of apoptosis was observed for treatments, including IMD-0354 alone or IMD-0354 along with Trx, which indicates that IMD-0354 indeed can induce apoptosis in CLL cells in vitro (Figure 1). Importantly, IMD-0354 also induced apoptosis at similar levels in both IGHV mutated and unmutated subgroups of CLL cases, indicating that both these prognostic subgroups respond similarly to the effect of IMD-0354. In addition, we measured the cytotoxic effect of IMD-0354 on the CLL cells using fluorometric microculture cytotoxicity assay, and all cases showed an increase in cytotoxicity in a dose-dependent manner (Figure 2).

IMD-0354 is thought to induce apoptosis by inhibiting the degradation of IκB and thereby protecting it against phosphorylation, which in turn inhibits NF-κB activation. To show the direct inhibition of NF-κB activity, we performed electrophoretic mobility shift assay to determine and compare the
DNA-binding activity of NF-kB before and after the IMD-0354 treatment in CLL cell nuclear extracts. Significant reduction in DNA-binding activity of NF-kB was observed after IMD-0354 treatment (Figure 3), hence indicating that IMD-0354 induces apoptosis in CLL cells by direct targeting of NF-kB.

Reduction of NF-kB may result in significant changes in expression patterns of apoptotic-related genes, which may include both anti- and proapoptotic BCL2 family members. The BCL2 proteins are well-characterized regulators of apoptosis and can both promote survival (antiapoptotic) and induce cell death (proapoptotic), and hence play an important role in oncogenesis.22,23 We explored possible changes in gene expression of several apoptosis-related genes before and after the IMD-0354 treatment in CLL cells using the MLPA technique. After the IMD-0354 treatment, significant reduction of gene expression was observed for the antiapoptotic BIRC3 and BIRC2 genes. These two genes are IAP family members, which are known to be highly expressed in cancer cells compared with normal cells.24 BIRC3, also known as PUMA, was the only proapoptotic gene that showed significant increase in the expression after IMD-0354 treatment. PUMA is a proapoptotic member of the BCL2 family, which mediates p53-dependent and -independent apoptosis.25 Hence, these results suggest that IMD-0354 induces apoptosis in CLL cells by decreasing and increasing anti- and proapoptotic genes, respectively.

Taken together, we conclude that IMD-0354 specifically targets the NF-kB pathway, and significantly induces apoptosis in CLL cells in vitro. This effect was retained when adding the survival factor Trx and the drug was equally effective in subgroups defined by the IGKV mutational status. We encourage further investigations of this drug as it may represent a novel potential drug in CLL treatment.

Conflict of interest

At the time of the study Anna Åleskog worked as a hematologist at the Department of Hematology, Uppsala University Hospital, but is currently employed as medical affairs manager at MSD, Sweden. Gerard Tobin worked at the time of the study at Rudbeck Laboratory, Uppsala University, Sweden, but is currently employed by Hofmann La Roche, Switzerland.

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