Sensitization of Tumor Cells toward Chemotherapy: Enhancing the Efficacy of Camptothecin with Imidazolines

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Summary

Activation of nuclear transcription factor κB (NF-κB) by chemotherapeutic agents was found to protect cells from apoptosis. In light of its central role in regulating the cellular resistance to apoptotic agents, inhibition of NF-κB-mediated gene transcription may sensitize tumor cells to chemotherapeutic agents and enhance their efficacy. We describe herein a noncytotoxic imidazoline scaffold that sensitizes leukemia T cells to the chemotherapeutic agent camptothecin.

Introduction

Apoptosis or programmed cell death is a cellular mechanism used to regulate cell number and eliminate damaged or mutated cells [1, 2]. Alterations in apoptotic pathways can disrupt the delicate balance between cell proliferation and cell death, leading to a variety of diseases [3, 4]. In many cancers, apoptosis is abnormally downregulated, either by the mutation of proapoptotic proteins or by the upregulation of antiapoptotic proteins [5]. Aberrant apoptosis provides an intrinsic survival advantage to cancer cells, causing growth and expansion of the tumor as well as resistance to proapoptotic signals such as chemotherapeutic agents [6, 7]. In addition, chemotherapeutic agents may also induce secondary antiapoptotic factors, thereby adding to this intrinsic resistance to chemotherapy [8–11]. The combination of these antiapoptotic mechanisms has resulted in an increased dose intensity of chemotherapeutics, often without the anticipated improved therapeutic results. [12] The search for new chemotherapeutic strategies has therefore shifted to small molecules that can selectively induce apoptosis in cancer cells or retard the cellular chemoresistance [13–15]. Strategies using combinations of inducers of apoptosis and/or inhibitors of antiapoptotic factors and traditional chemotherapeutic drugs may provide an improved alternative to conventional chemotherapy [14, 16–18].

The mammalian nuclear transcription factor, NF-κB, is a multisubunit complex involved in the regulation of gene transcription, including the regulation of apoptosis [19, 20]. Five distinct subunits of NF-κB are found in mammalian cells, which include NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel [20]. These subunits can comprise a variety of homo- or heterodimers, which are used to control the specificity and selectivity of certain DNA control elements [21, 22]. In most unstimulated mammalian cells, NF-κB exists mainly as a homodimer (p50/p50) or heterodimer (p50/p65) in the cytoplasm in the form of an inactive complex with the inhibitory protein I-κB (Figure 1). Many cellular stimuli, including antineoplastic agents [23–31], viruses (e.g., HIV), cytokines, phorbol esters, and oxidative stress, result in the IκB-mediated phosphorylation of I-κB on serines 32 and 36, followed by ubiquitylation and subsequent degradation by the 26 S proteosome [19]. Degradation of I-κB ensures the release of NF-κB. [32] Upon release, NF-κB translocates into the nucleus, where the subunits bind with specific DNA control elements and initiate gene transcription (Figure 1). Prior to DNA binding, additional protein phosphorylation events are required for optimal and specific gene transcription [33–36]. Antiapoptotic genes such as TRAF1, TRAF2, c-IAP1, c-IAP2, XIAP, and IEX-1L are directly regulated by NF-κB and abrogate the apoptotic signals in response to the chemotherapeutic agents [10, 37].

The antitumor agent camptothecin (CPT) is an alkaloid isolated from the extracts of the fruit of Camptotheca acuminata and was identified as a topoisomerase I inhibitor [38, 39]. CPT-11 and several water-soluble analogs including topotecan have successfully passed clinical trials in the United States [40]. Camptothecin exhibits its antitumor activity via the formation of a stable ternary topoisomerase I-DNA cleavable complex [38, 41]. Stabilization of this cleaved DNA complex initiates an apoptotic signaling pathway, ultimately resulting in cell death [38, 42]. Concomitant with the initiation of this apoptotic cell signal, these agents induce antiapoptotic signaling pathways, which have compromised their efficacy in the clinic [26, 43–45]. This cellular resistance has been attributed to the activation of antiapoptotic signaling pathways mediated by several transcription factors, in particular the nuclear transcription factor, NF-κB [11, 28, 46].

cDNA microarrays on HeLa cells using all annotated human genes have been used to establish the association between characteristic gene expression patterns in response to camptothecin [47, 48]. These studies in HeLa cells provided striking support that administration of camptothecin resulted in upregulation in a large number of genes controlled by NF-κB [47]. NF-κB’s mediated antiapoptotic response induced by DNA-damaging agents may also result in the induction of cellular DNA repair mechanisms [49]. Topoisomerase inhibitors are in this context also considered DNA-damaging
agents, since they exert their cytotoxicity via the stabilization of a ternary DNA-drug-protein cleavable complex [42]. In addition to the induction of antiapoptotic gene transcription, the topoisomerase I inhibitor camptothecin was found to induce the NF-κB-mediated activation of proto-oncogenes such as c-Myc and cyclin D1 and indirect deregulation of the retinoblastoma tumor suppressor protein (Rb protein) [26, 43, 50, 51]. Thus, chemotherapeutic treatment by these agents often fails as a result of an NF-κB-mediated double stimulus, causing chemoresistance and favoring uncontrolled cell growth.

Inhibition of the nuclear translocation of NF-κB blocks the induction of antiapoptotic gene transcription and was found to sensitize tumor cells to chemotherapeutic agents and enhance their antitumor efficacy [17, 29, 50, 52, 53]. Baldwin et al. have demonstrated the control of inducible chemoresistance through inhibition of NF-κB using a mutated form of IκBα, a natural inhibitor of NF-κB (Figure 1) [50]. In another study, Piette et al. showed that the overexpression of IκBα/mutated IκBα regulated the cytotoxicity caused by camptothecin [54]. These pioneering studies illustrated the clinical potential of NF-κB inhibitors in combination chemotherapy. There are numerous natural and synthetic inhibitors of NF-κB reported in the literature [55], which include many antioxidants such as pyrroline-thiocarbamate or PDTC, [56] kinase inhibitors such as hynemialdisine and analogs [57–61], SC-514 [62], inhibitors of IκB degradation such as the proteosome inhibitors lactacystin [63–65] and PS-341 [66], and IKK inhibitors such as the sesquiterpene lactones represented by parthenolide [67, 68]. Even though many of these agents indicate inhibition of the antiapoptotic transcription factor, enhancement of chemotherapeutics has been limited. The most successful example of combination therapy using chemotherapeutic agents with NF-κB inhibitors has been illustrated by the proteosome inhibitor PS-341 (bortezomib), which is currently in phase II clinical trials in the US [53, 69]. PS-341 inhibits the nuclear translocation of NF-κB via the inhibition of the 26 S proteasome-mediated degradation of IκB (Figure 1). Since PS-341 exhibits significant cell cytotoxicity, it may be used as a single agent or in
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Synthesis of Imidazoline 1

The imidazoline scaffold was prepared via a novel silicon-mediated 1,3-dipolar cycloaddition reaction [71, 72]. The low molecular weight scaffold contains a four-point diversity applicable to alkyl, aryl, acyl, and heterocyclic substitutions. After screening a small number of Lewis acid, we found that TMSCl promotes the reaction of oxazolones and imines to afford the imidazolines scaffolds in good yields as single diastereomers (Figure 2).

Compound 1 was isolated as a single diastereomer, and the diastereoselectivity appears to primarily arise from the steric interaction of the bulky silyl group of the azlactone and the phenyl moiety of the imine (Figure 2).

Sensitization of Cancer Cells toward Camptothecin

The imidazolines were evaluated for their ability to enhance the activity of camptothecin (CPT) in cancer cells by evaluating the level of apoptosis induced by camptothecin in the presence and absence of compound 1. Induction of apoptosis is the hallmark of most chemotherapeutic agents, including camptothecin [38, 73]. The level of apoptotic cell death was measured using a caspase-based screen. Caspase activation plays a central role in the execution of apoptosis via the proteolytic cleavage of multiple protein substrates by caspases 3, 6, and 7 [74–76]. The level of induction of apoptosis in cells was quantified using a commercially available Apo-ONE assay, which takes advantage of caspase 3/7 activity. Treatment of the CEM leukemia T cells with compound 1 did not induce significant levels of apoptosis (tested up to 10 μM for 48 hr by Apo-ONE as well as by cell count; data not shown). The imidazoline 1 was subsequently screened for its ability to enhance apoptosis induced by the chemotherapeutic, camptothecin [40].

Enhancement of CPT-induced apoptosis in CEM cells was first investigated at concentrations of ≤ 10 nM CPT, at which CPT has been reported to cause DNA aberrations but no significant levels of apoptosis in leukemia cells [77]. Figure 3 illustrates the effect of the imidazoline on CEM cells when incubated with CPT (10 nM) over a 48 hr time period. Treatment of the cells with compound 1 (10 nM) had no apparent cytotoxicity; however, further investigations revealed that this agent drastically enhanced the level of apoptosis induced by chemotherapeutics. Even though micromolar concentrations of compound 1 did not induce any apoptosis when used as a single agent, it was found to enhance the anticancer efficacy of the anticancer agent camptothecin approximately 75-fold at 100 nanomolar concentration. Investigation into its potential mode of action revealed that the imidazoline blocks the nuclear translocation of NF-κB via the inhibition of phosphorylated I-κB degradation. The inhibition of NF-κB by compound 1 may provide a potential rationale for its enhancement of CPT-induced apoptosis. These findings illustrate that this class of antiapoptotic inhibitors might provide an improved therapeutic strategy in the treatment of cancer.

Results and Discussion

Synthesis of Imidazoline 1

Figure 2. Synthesis of Imidazoline 1 and Proposed Rationale for Diastereoselectivity of Compound 1

(A) Synthesis of imidazoline 1. a: benzoyl chloride, NaOH (aq), Et2O, overnight; b: EDCI, CH2Cl2, 2 hr, room temperature, 68% overall; c: TMSCl, benzylamine, benzaldehyde, CH2Cl2, reflux 4 hr, room temperature, overnight, 65%.

(B) Proposed rationale for diastereoselectivity of compound 1.

We describe herein a noncytotoxic agent that drastically enhances the activity of camptothecin in leukemia T cells. This class of NF-κB inhibitors is structurally different from any reported NF-κB inhibitor and unique with respect to its insignificant toxicity and remarkable ability to enhance the anticancer efficacy of the chemotherapeutic agent camptothecin. The agent was prepared via our recently reported diastereoselective multicomponent one-pot synthesis of imidazolines (Figure 2) [71, 72]. A large library of imidazoline scaffolds was prepared via this silicon-mediated cycloaddition reaction and evaluated for biological activity. Upon screening of these agents, we found that the imidazoline 1 exhibited no apparent cytotoxicity; however, further investigations revealed that this agent drastically enhanced the level of apoptosis induced by chemotherapeutics. Even though micromolar concentrations of compound 1 did not induce any apoptosis when used as a single agent, it was found to enhance the anticancer efficacy of the anticancer agent camptothecin approximately 75-fold at 100 nanomolar concentration. Investigation into its potential mode of action revealed that the imidazoline blocks the nuclear translocation of NF-κB via the inhibition of phosphorylated I-κB degradation. The inhibition of NF-κB by compound 1 may provide a potential rationale for its enhancement of CPT-induced apoptosis. These findings illustrate that this class of antiapoptotic inhibitors might provide an improved therapeutic strategy in the treatment of cancer.

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the cells to an initial exposure of the drug, followed by removal of drug and supplementation with fresh media. Cells were preincubated with 1, 10, and 100 nM concentrations of the imidazoline followed by exposure to a titration range of CPT concentrations. Various time points were obtained and the data were analyzed. The data are summarized in Figure 4 and illustrate the effect on the CC₉₅ for camptothecin on cells pretreated with imidazoline (for the complete data set, see Supplemental Figure S1).

The CC₉₅ of CPT was decreased 75.08-fold when the cells were pretreated with 0.1 μM imidazoline for 1 hr. This fold enhancement was reduced to 29.1-fold enhancement of efficacy when the amount of imidazoline was decreased to 1 nM. As anticipated, the continuous exposure resulted in an overall higher cell cytotoxicity due to a longer exposure time. When the cells were washed with 1× PBS after 1 hr of camptothecin treatment, the fold enhancement dropped to 20.9-fold of CPT-induced cell death (CC₉₅). Similar potentiation was seen with pretreatment of ALLN followed by camptothecin treatment (see Supplemental Figure S2). Post-treatment of CPT-exposed cells with the imidazoline after 1, 2, or 4 hr resulted in no enhancement of apoptosis over a 48 hr time period, suggesting the possible inhibition of an antiapoptotic event induced by CPT (data not shown).

Cell Death Determined by Trypan Blue Exclusion
Parallel to the Apo-ONE assay, cells were counted by the trypan blue exclusion method to determine the number of viable cells. Cells were preincubated with 0.1 μM, 0.01 μM, and 1 nM imidazoline, then various concentrations of camptothecin were added, and the cells were incubated for 0, 24, and 48 hr. Well-mixed aliquots of these cell cultures were subsequently mixed with equal volumes of 0.04% trypan blue dye in 1× PBS and counted on a hemacytometer under the microscope. The results clearly confirmed the enhancement of camptothecin activity in the presence of imidazoline, as seen in the Apo-ONE assays (see Supplemental Figure S3).

Combination treatment of compound 1 with varying concentrations of CPT (10–0.1 μM) caused a strong induction of apoptosis, resulting in a dose-dependent response.

Pathway of Apoptotic Modulation
In order to determine the potential molecular pathway through which the imidazoline modulates apoptosis, we evaluated its ability to inhibit CPT induction of NF-κB activation. Inhibition of the NF-κB signaling cascade may occur at several different steps in its activation pathway (Figure 1). In order to determine where the imidazoline intersected the NF-κB activation pathway, we examined the effect of imidazoline 1 on CPT-induced NF-κB activation in each of the steps shown in Figure 1.

NF-κB Activation by Camptothecin
Activation of NF-κB by chemotherapeutic agents such as camptothecin has been well documented by several groups in different cell lines, including CEM leukemia T cells [26, 43, 44]. The stabilization of a topoisomerase I-DNA cleavable complex by camptothecin activates cell
survival signaling pathways mediated by the nuclear transcription factor NF-κB [26, 43, 44, 49]. The activation of NF-κB by camptothecin was confirmed in CEM cells using an EMSA assay [43]. The cellular nuclei were isolated and evaluated for NF-κB activation and (0.16 pmol/λ) + nuclear extract (1.0 nM CPT); lane 11, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.1 nM CPT). All incubations with CPT were performed for 2 hr. The positive control with TNF-α was incubated for 15 min.

Figure 4. EMSA Assay for NF-κB Activation by Camptothecin

Lane 1, NF-κB consensus oligonucleotide (0.16 pmol/λ); lane 2, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α); lane 3, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α + p65 antibody); lane 4, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT); lane 5, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1 nM CPT); lane 6, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT) + p65 antibody; lane 7, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1 μM CPT); lane 8, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.1 μM CPT); lane 9, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.01 μM CPT); lane 10, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1 nM CPT); lane 11, NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.1 nM CPT). All incubations with CPT were performed for 2 hr. The positive control with TNF-α was incubated for 15 min.

Activation of NF-κB is transient and depends on exposure time. This was confirmed by treating CEM cells at log phase with 10 μM camptothecin or DMSO at 30 min and 1, 2, 4, 6, 14, and 24 hr (see Supplemental Figure S4). Maximal NF-κB activation was at 2 hr and decreased after 4 hr, confirming a post-induction self-repression loop during the early hours of incubation with camptothecin consistent with earlier reports [43].

Induction of NF-κB activation can proceed via a wide range of signaling pathways [78, 79]. Thus, inhibition of NF-κB activation can proceed via the mediation of many different pathways [55]. Modulators of these pathways may therefore act as general activation inhibitors, whereas others may inhibit specific induction pathways [55]. In order to investigate whether the imidazoline 1 inhibits the specific pathway of camptothecin-induced NF-κB activation, we examined the inhibition of camptothecin-induced NF-κB-DNA binding in the presence of compound 1. CEM cells were treated with various concentrations of imidazoline 1 30 min prior to treatment by camptothecin (1 μM). Pyrrolidine dithiocarboxylic acid (PDTc) is a nonselective NF-κB inhibitor and was used as a positive control. After a 2 hr incubation period, the nuclei were isolated and treated with the labeled κ-B consensus sequence. As illustrated in Figure 5, the addition of imidazoline 1 inhibited camptothecin-induced NF-κB-DNA binding in a dose-dependent manner. Control lanes included DNA only (lane 1), TNF-α-activated NF-κB (lane 2), TNF-α-
activated NF-κB treated with a p65 antibody, which provided a supershift (lane 3), and the unactivated control (lane 4). Treatment of the cells with camptothecin resulted in the activation of NF-κB, as indicated by the strong band of the NF-κB-DNA complex (lane 5). Inhibition of DNA binding in the presence of the non-selective NF-κB inhibitor PDTC resulted in reduced binding as anticipated (lane 7). A similar reduction in camptothecin-induced NF-κB-DNA binding resulted upon treatment of imidazoline 1 (concentrations ranging from 10 μM to 10 nM), as illustrated in lanes 8–12. Comparison of lane 5 (activated by 1 μM CPT) with lane 8 (activated by 1 μM CPT + 1 μM compound 1) clearly indicates a significant decrease in NF-κB-DNA complex formation.

**Inhibition of Camptothecin-Induced Nuclear Translocation of NF-κB by Imidazoline 1**

The previously described EMSA studies indicate that imidazoline 1 inhibits the binding of NF-κB to its consensus sequence. This may be due to a direct inhibition of DNA binding or to a decrease in the amount of NF-κB in the nucleus. We therefore investigated the ability of compound 1 to inhibit the nuclear translocation of NF-κB using a p65 sandwich ELISA assay (Figure 6). Activation of NF-κB was carried out for a period of 0.5 hr for TNF-α and 2 hr for camptothecin, after which the nuclear extracts were isolated. TNF-α activation of NF-κB was used as positive control, and the level of translocated p65 was normalized to 100%. The negative control included the level of nuclear p65 without activation [57, 58]. Treatment of the cells with TNF-α or CPT (1.0 μM) resulted in a significant increase in nuclear NF-κB, as illustrated in Figure 6 (first and third columns, respectively). Pretreatment of the cells with the non-selective NF-κB inhibitor PDTC (5 μM) indicated a significant inhibition of nuclear translocation after CPT activation (fourth column). Pretreatment of the cells with imidazoline 1 30 min prior to activation also resulted in a significant inhibition of translocation (fifth column). Various concentrations of the imidazoline (1–100 nM) resulted in inhibition of translocation (Figure 6). Similar results were obtained with HeLa cells activated with TNF-α (Figure 6) as well as Jurkat cells activated with PMA/PHA (data not shown). These findings indicate that imidazoline 1 inhibits the nuclear translocation of NF-κB at the nanomolar level.

**Inhibition of Kinase Activity**

We have demonstrated that imidazoline 1 inhibits the translocation of NF-κB across the nuclear membrane in response to stimulation by camptothecin. The translocation of NF-κB requires the phosphorylation and subsequent degradation of its inhibitory protein IκB by the 26 S proteosome, resulting in the liberation of NF-κB [81]. Thus, the effect of the imidazoline on IκB phosphorylation and degradation was investigated. We first evaluated the imidazoline for its effect on the phosphorylation of the inhibitory protein IκB by its endogenous kinases IKKα and IKKβ (data not shown) [26, 79, 80]. No inhibition of kinase activity was observed (tested at 10 μM). Alternatively, inhibition of NF-κB translocation may be the result of the upstream inhibition of kinase activity in a variety of kinase pathways.

We therefore evaluated imidazoline 1 for its inhibition of the several kinases either related to the NF-κB pathway or other apoptotic signaling pathways. No inhibition of any kinase activity was detected (tested up to 10 μM concentrations of the imidazoline). Based on these studies, it is unlikely that imidazoline 1 inhibits nuclear translocation via the inhibition of kinase activity.

**Effect of Imidazoline 1 on IκBα Phosphorylation**

Liberation of the NF-κB subunits from the NF-κB/IκB complex is required for efficient translocation and is dependent on the degradation of the inhibitory protein IκB. The CPT-induced phosphorylation and subsequent degradation of IκB was evaluated in the presence and absence of the imidazoline 1 using a pS32 ELISA on the cellular cytoplasmic extracts. A time course experiment using 2 μM CPT indicated that 60 min was the optimum time period for IκB phosphorylation (see Supplemental Figures S5 and S6). Cytoplasmic extracts from CPT-treated cells with or without pretreatment with imidazoline were analyzed for phosphorylation at ser-32 and ser-36 (Figure 7). ALLN (N-acetyl-Leu-Leu-norleucinal), a proteasome inhibitor [82, 83], and parthenolide, an IKK inhibitor [67, 68], were used as controls to mark the two steps involved in IκB degradation leading to NF-κB translocation and gene transcription. Parallel to the pS32 ELISA, the total IκB levels were measured to accurately determine the percent phosphorylation. As anticipated, the proteasome inhibitor ALLN shows accumulation of phosphorylated IκB, whereas the IKK kinase inhibitor indicated a decrease in phosphorylation. Upon pretreatment of the cells with imidazoline 1, accumulation of phosphorylated IκB was observed. The values were normalized to the total amount of IκB, resulting in an IC50 value of 0.19 μM.

Similar experiments were performed using TNF-α to activate the leukemia cells. Both TNF-α and CPT are known to activate NF-κB by IκB degradation following phosphorylation at ser-32 and ser-36 in CEM cells, although with varying kinetics [54]. Consistent with these studies, we found that the imidazolines inhibited the NF-κB signaling pathway in TNF-α-activated cells more effectively than with CPT, illustrated by an IC50 value of 2.1 nM (see Supplemental Figure S6). These studies clearly demonstrate that imidazoline 1 inhibits nuclear translocation of NF-κB via the inhibition of IκB degradation of TNF-α as well as CPT-activated CEM cells. This suggests that the imidazoline may target the 26 S proteosome or the ubiquitination of IκB. Studies toward the identification of the potential molecular target and its binding interactions are actively ongoing in our lab.

**Inhibition of CPT-Induced NF-κB Transcription by Imidazoline 1**

To demonstrate the ability of imidazoline 1 to affect NF-κB-mediated gene transcription, we evaluated the inhibition of NF-κB-mediated luciferase production using a luciferase reporter assay. HeLa cells were transfected with 6×κB-driven reporter gene pNF-κB-Luc (Strategene) and were activated with TNF-α (10 ng/μl) or camptothecin (3 μM) in the presence and absence of
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Figure 6. Levels of Nuclear Translocation Measured by p65 ELISA Assay of Compound 1 in CEM and HeLa Cells with Camptothecin and TNF-α.

All values have been normalized to the positive control to show percentage of translocation, and the data are reported as an average of two independent experiments.

imidazoline 1, parthenolide, ALLN, or DMSO control, and luciferase production was assayed using the Dual-Glo reporter assay. A constitutively activated plasmid pFC-MEK was used as a positive control for transfection. pCIS-CK was used as the negative control for the κB-driven reporter gene. pRL-TK was used as a coreporter. The imidazoline inhibited the NF-κB-induced transcription by 52% and an EC50 value of 0.95 μM when cells were activated by TNF-α. The imidazoline inhibited the CPT induced NF-κB transcription by up to 35%, providing an EC50 value of 9.8 μM (Supplemental Figure S9).

Significance

Antitumor drugs exert their antitumor activity via the initiation of apoptotic signaling pathways, ultimately resulting in cell death. Concomitant with the initiation of this apoptotic cell signal, many chemotherapeutic agents, including camptothecin, induce antiapoptotic signaling pathways, which have compromised the efficacy of these antitumor agents in the clinic. Inhibition of the antiapoptotic transcription factor, NF-κB, retards this cellular resistance. We discovered a class of nontoxic agents that drastically enhance the efficacy of these antitumor agents in the clinic.
leukemia cells at nanomolar concentrations to camptothecin and drastically enhance the level of camptothecin-induced apoptosis. Combination therapy of CEM cells with camptothecin and the imidazoline 1 provided ~75-fold enhancement of efficacy after 48 hr. The ability of the imidazoline to enhance the apoptotic events initiated by camptothecin may proceed via the inhibition of the NF-κB signaling pathway. Inhibition of the NF-κB pathway was found to occur via the inhibition of degradation of NF-κB inhibitory protein IκBα. These findings are consistent with the lack of enhancement of CPT-induced apoptosis in the post-treatment of cells with compound 1. These studies indicate that combination therapy of classical anti-cancer agents with small molecule NF-κB inhibitors has the potential to target chemoresistance and provides a potential new strategy to treat chemoresistant tumors. Work toward a detailed molecular mechanism and the clinical potential of these agents is currently under investigation in our laboratory.

Experimental Procedures

Chemicals and Antibodies
Camptothecin and TNF-α were purchased from Sigma. ALLN was purchased from Calbiochem. Parthenolide and monoclonal antibody for supershifts for p65 were purchased from Santa Cruz Biotechnology. HeLa cells were a kind gift from Prof. John LaPres, Dept. of Biochemistry and Molecular Biology, Michigan State University.

Apoptosis Assay with Apo-ONE
CEM cells (CCRF-CEM; Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 media (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (614 ng/ml), streptomycin (10 μg/ml), and HEPES buffer (pH 7.2) at 37°C, 5% CO₂, DMSO was used as the vector for all drugs and was added in the control experiments. Cell cultures were treated with 0.1 μM, 0.01 μM, or 1 nM of the imidazoline 1 and allowed to incubate at 37°C, 5% CO₂ atmosphere for 30 min. Camptothecin was added at concentrations 50 μM, 1 μM, 0.3 μM, 0.1 μM, 0.03 μM, 0.01 μM, 3 nM, 1 nM, or 0.3 nM and further incubated. An aliquot was transferred to a 96-well plate at various time points, 0, 6, 12, 24, and 48 hr, and mixed with an equal volume of Apo-ONE homogenous caspase 3/7 assay (Promega Corporation) reagent. The contents of the plate were gently mixed and allowed to incubate for 1 hr. The fluorescence of each well was then measured on a Molecular Imager FX Pro at 532 nm. All reported data are the average of two independent experiments unless otherwise indicated. The data were analyzed using GraphPad prism. Data were normalized between maximum and minimum cell death relative to the respective control sets, and CC₅₀ values were calculated using the equation for the sigmoidal curve for variable slope.

EMSA Assay for NF-κB-DNA Binding
EMSA assay for NF-κB-DNA binding: CEM cells (CCRF-CEM; Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 media (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (614 ng/ml), streptomycin (10 μg/ml), and HEPES buffer (pH 7.2) at 37°C, 5% CO₂. Cells (1.6 x 10⁶ cells/ml) were subsequently treated with various concentrations of compound 1 for 30 min at 37°C and 5% CO₂ followed by TNF-α (0.4 pg) or camptothecin (Sigma-Aldrich) (1.0 μM) stimulation for an additional 30 min or 2 hr, respectively. The cells were harvested by centrifugation, washed in ice-cold PBS, and the nuclear extracts were prepared as previously described [52]. The protein concentration of the extract was determined according to the Bradford method (1976) with BioRad reagents. Nuclear extracts (20 μg total protein) were incubated for 20 min at room temperature with a double-stranded Cy3-labeled NF-κB consensus oligonucleotide, 5'-AGTTGGAGGGGACTTTC CCAGGC-3' (0.16 pmol). The binding mixture (25 μl) contained 10 mM HEPES-NaOH (pH 7.9), 4 mM tris-HCl (pH 7.9), 6.0 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.3 mg/ml bovine serum albumin, and 1 μg poly (dl,dC). The mixture was loaded on a 4% polyacrylamide gel prepared in 1 x Tris borate/EDTA buffer and electrophoresed at 200 V for 20 min. After electrophoresis, the gel was analyzed using a phosphorimager (Biorad FX plus) for detection of NF-κB-DNA binding.

Inhibition of Translocation with p65-ELISA Assay
The quantity of p65/p50 heterodimer that had translocated into the nucleus was measured using a NF-κB p65 sandwich ELISA assay (Imgenex Corp.). CEM cells were grown to 1.6 x 10⁶ cells/ml and were subsequently treated with various concentrations of the compound 1 for 30 min at 37°C and 5% CO₂ followed by TNF-α (0.4 pg) or camptothecin (1.0 μM) stimulation for an additional 30 min or 2 hr, respectively. The cells were harvested and nuclear extracts were prepared as previously described by Dignam and coworkers [84]. The NF-κB p65 sandwich ELISA assay was used to monitor and quantify p65 translocation into the nucleus according to the manufacturer’s protocol. Results were read on Biorad’s Microplate Benchmark reader at 405 nM.
Inhibition of IκBα Degradation in CEM Cells

CEM cells (CCRF-CEM; Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 media (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (614 ng/ml), streptomycin (10 μg/ml), and HEPES buffer (pH 7.2) at 37°C, 5% CO₂. Cells (1.6 × 10⁵ cells/ml) were subsequently treated with various concentrations of compound 1, ALLN (50 μM), parthenolide (50 μM), or vehicle (DMSO) for 1 hr at 37°C and 5% CO₂ followed by TNF-α (0.4 μg/ml) or camptothecin (Sigma-Aldrich) (2.0 μM) stimulation for an additional 4 min or 1 hr, respectively. The cells were harvested by centrifugation, washed in ice-cold PBS, and the cytoplasmic extracts were prepared as described by the manufacturer's protocol. The protein concentration of the extracts was determined by centrifugation, washed in ice-cold PBS, and the cytoplasmic extracts were prepared as described by the manufacturer's protocol.

The protein concentration of the extracts was determined according to the method of Bradford (1976) with BioRad's Bradford reagent. Extracts were diluted and subjected to IκBα (total) ELISA (Biosource International, CA) to measure and quantify the effect of imidazoline on the phosphorylation status of IκBα. Results were read on Biorad's Microplate Benchmark reader at 450 nM.

Inhibition of Kinase Activity

The in vitro kinase assay was conducted as follows: compound 1 was tested in vitro against the kinases AMPK, Axl, BK, CDK2/cyclinE, CDK5/p35, c-RAF, Fes, Fyn, IGF-1R, IR, JNK1/2, JNK3, Lck, Lyn (human), MAPK1, MAPK2 (human), MEK1, MKK4, MKK6, MKK7b, MSK1, p70S6K, PDK1, PAK2, PKA (human), PKβ1, PKβ2, PKCy, PDK1, PKCδ, PKCθ, PKCμ, SGK, Yes, Syk, TrkB, and ZAP-70 by Upstate, UK using a kinase profiler assay according to the manufacturer's protocol. Briefly, in a final volume of 25 μl, the kinase was incubated with the desired buffer and the required polypeptide substrate in the presence of 10 mM magnesium acetate and γ32P-ATP (10 μM). After incubation for 40 min at room temperature, the reaction was stopped by the addition of 3% H3PO4 (5 μl). Ten microliters of the reaction was then spotted on a P30 filtermat, washed three times in 75 mM H3PO4, and finally in methanol. Samples were then dried and signals were counted on a scintillation counter.

Inhibition of NF-κB-Mediated Transcription in HeLa Cells

Transient transfections were performed using lipofectamine 2000. Briefly, 0.8 μg of plasmid DNA was combined with 1.5 μl lipofectamine 2000 (Invitrogen). The mixture was incubated at room temperature for 20 min and mixed with the cells without serum. Cells were transfected for 5 hr at 37°C in 5% CO₂. Cells were allowed to grow in complete medium for 15 hr in 5% CO₂. Various treatments with various concentrations of imidazoline 1, parthenolide (50 μM), or ALLN (50 μM) were performed for 1 hr followed by a treatment with TNF-α (10 ng/ml) or camptothecin (3 μM) for 5 or 10 hr, respectively. Cells were washed with 1× PBS. Washed cells were assayed for luciferase production using the manufacturer's protocol for both luciferase as well as renilla luciferase (coreporter) using the Dual-Glo reporter assay (Promega). The results were read on a Veritas microplate luminometer (Turner Biosystems, CA) as relative light units. Values from the luciferase activity were normalized to those with renilla luciferase.

Supplemental Data

Supplemental Data including ten figures are available at http://www.chembiol.com/cgi/content/full/11/12/1689/DC1/.

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