Rocaglamide Derivatives Are Potent Inhibitors of NF-κB Activation in T-cells*

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Crude extracts from different Aaglia species are used as anti-inflammatory remedies in the traditional medicine of several countries from Southeast Asia. Because NF-κB transcription factors represent key regulators of genes involved in immune and inflammatory responses, we supposed that the anti-inflammatory effects of Aaglia extracts are mediated by the inhibition of NF-κB activity. Purified compounds of Aaglia species, namely 1H-cyclopenta[b]benzofuran lignans of the rocaglamide type as well as one aglain congenere were tested for their activity. We show that a group of rocaglamides represent highly potent and specific inhibitors of tumor necrosis factor-α (TNFα) and phorbol 12-myristate 13-acetate (PMA)-induced NF-κB-dependent reporter gene activity in Jurkat T cells with IC50 values in the nanomolar range. Some derivatives are less effective, and others are completely inactive. Rocaglamides are able to suppress the PMA-induced expression of NF-κB target genes and sensitize leukemic T cells to apoptosis induced by TNFa, cisplatin, and γ-irradiation. The suppression of NF-κB activation correlated with the inhibition of induced IκBα degradation and IκBα kinase activation. The level of interference was determined and found to be localized upstream of the IκBα kinase complex but downstream of the TNF receptor-associated protein 2. Our data suggest that rocaglamide derivatives could serve as lead structures in the development of anti-inflammatory and tumoricidal drugs.

In recent years Aaglia species have attracted considerable interest due to their unique 1H-cyclopenta[b]benzofuran lignans, which have been isolated from more than ten Aaglia species so far and are exclusively confined to members of this genus (1–4). Rocaglamide derivatives are potent natural insecticides that are active against their activity to azadirachtin from the Neem tree Azadirachta indica (1–4). Moreover, these compounds were found to have cytostatic activity in human cancer cell lines (5–7). These cytostatic effects were comparable to the established anticancer drugs such as vinblastine sulfate and actinomycin D (8, 9). Furthermore, rocaglamides have been shown to block protein biosynthesis and to induce growth arrest in the G1/M phase in certain tumor cells lines (8). However, the one or more underlying molecular mechanisms of these rocaglamide mediated effects have not been identified.

The members of the Rel/NF-κB transcription factor family bind to DNA as homo- and/or heterodimers. They are critically involved in the regulation of genes mediating inflammatory responses and cellular processes such as cell survival, apoptosis, development, differentiation, cell growth, and neoplastic transformation (reviewed in Ref. 10). In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm due to the interaction with proteins of the IκB family. Stimulation of cells, e.g., by pro-inflammatory agents results in the rapid activation of the IκB kinase (IKK) complex. This complex consists of two kinases IKKα and IKKβ as well as a regulatory component called NEMO/IKKγ (10, 11). Its activation results in the de novo phosphorylation of conserved serine residues in the N-terminal domain of the IκB proteins marking them for ubiquitination and subsequent degradation by the proteasome. This allows nuclear translocation of NF-κB and binding to cognate DNA motifs in the promoter region of target genes, which subsequently initiates transcription of these genes and finally starts a genetic program responsible for e.g. inflammatory responses (12).

Numerous efforts have been initiated to develop or to identify specific low molecular weight compounds to inhibit this pathway (13, 14). Substances that inhibit the proteasome as well as radical scavengers have been shown to block NF-κB activation. These inhibitors have been valuable for many studies of NF-κB functions in cell culture systems. However, because these compounds also affect multiple other cellular reactions, they are not useful as therapeutic agents in vivo (13). Substances that either block the action of the IκB kinase complex or interfere with its activation are thought to be more specific.

* This work was supported in part by Grants DFG Wi 789/2-3 and SFB 497/B1 from the Deutsche Forschungsgemeinschaft (to T. W. and B. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ Supported by the Bundesministerium für Bildung and Forschung/ Bayer AG and Fond der Chemischen Industrie.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 47, Issue of November 22, pp. 44791–44800, 2002

This paper is available on line at http://www.jbc.org

Published, JBC Papers in Press, September 16, 2002, DOI 10.1074/jbc.M208003200

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and should have fewer side effects. Recently, a novel reversible inhibitor of the proteasome, PS-341, a dipeptidyl boronic acid analogue, has been described to be a potent inhibitor of NF-κB activation (15, 16). This compound has excellent bioavailability and stability and has been shown to yield effective anti-cancer responses both in vitro and in vivo (17, 18). Phase II trials in several hematological malignancies and solid tumor types are now in progress (19).

In the past, several natural occurring compounds like sesquiterpene lactones, curcumin, tetrandrine, and triptolide have been shown to inhibit NF-κB activation (20–27). However, their broad side effects and the relatively high doses required for NF-κB inhibition make them less feasible as therapeutic drugs (13). The rationale for studying a series of rocaglamide derivatives for their influence on NF-κB activity was based on the observation that leaves and flowers of Aglaia dupreana and Aglaia odorata are used in the traditional medicine of several countries from Southeast Asia (e.g. Vietnam) for the treatment of asthma and inflammatory skin diseases. Here we show that certain rocaglamide derivatives are efficient inhibitors of NF-κB activation and NF-κB target gene expression, which could explain the anti-inflammatory function of these herbal remedies. Interestingly, they show a high degree of cell type specificity being much more active in T lymphocytes than in other cell types. Furthermore, inhibition of NF-κB by rocaglamides is responsible for sensitization of resistant leukemic T cells toward cancer therapy induced apoptosis. The mechanism of NF-κB inhibition by rocaglamides is predominantly localized upstream of the IκB kinase complex.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—CEM-S (acute T cell leukemia) cells die rapidly in response to γ-irradiation, treatment with chemotherapeutic agents, or direct triggering of death receptors. Subclones of CEM-S have been selected from the parental cells by periodical triggering of CD95 with αAPO-1 (50 ng/ml to 10 μg/ml) for at least 1 year (28). These subclones (CEM-R) do not die in response to αAPO-1 and exhibit a strongly delayed percentage of apoptosis following γ-irradiation or treatment with chemotherapeutic agents. These cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Hamburg, Germany), 100 units/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 2 mM L-glutamine (all from Invitrogen, Karlsruhe, Germany). Jurkat T and Jurkat T cells deficient in IKKα and IKKβ target gene expression, and the endogenous IKK complex were immunoprecipitated from 1 mg of extract using IκK2-specific antibodies (Santa Cruz Biotechnology, sc-7607). The precipitated IKK complex was incubated in kinase assay mixture containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 25 mM β-glycerophosphate, 10 mM MgCl2, 1 mM dithiothreitol, 10 μg of γ−32 ATP and 600 ng of GST-IκBα substrate. Coupled in vitro kinase assays were performed in kinase assay mixture supplemented with 1 μM unlabeled ATP. After 20 min (30 °C) the reaction was terminated by boiling with SDS sample buffer, and the proteins were separated on 10% polyacrylamide gels. Finally, the gel was either dried or the proteins were transferred to a polyvinylidene difluoride membrane, and radioactive bands were visualized by phosphorimaging or autoradiography. Kinase activities were quantified by Phosphoimager (Amersham Biosciences) analysis. The membranes were used for immunoblot analysis as described elsewhere (37) and were labeled with antibodies specific for IκKα (Santa Cruz Biotechnology, sc-7183) to determine total amounts of immunoprecipitated IκKα. Western immuno blot analysis for monitoring Bcl-2 degradation and RelA expression were performed as described earlier (37) using IgE (Cell Signaling, #9242)- and RelA (Santa Cruz Biotechnology, sc-372)-specific antibodies.

RNase Protection Assay Analysis—Jurkat cells (10 × 10⁶) were treated with PMA (50 ng/ml) and dox (200 ng/ml) as indicated. Total RNAs were extracted with the RNA INSTAPURE kit (Euregentech, Burgundy, California). The presence of the indicated transcripts was detected using RPA analysis using the Multi-Probe template sets (Pharmingen, Hamburg, Germany) IAP-2h (Bcl family members), IAP-3c (death receptor-related proteins), and hCK-3 (ligands). Probe synthesis, hybridization, and RNase treatment were performed with the RiboQuant Multi-Probe RNase Protection Assay system (Pharmingen). After RNase treatment, protected transcripts were resolved by electrophoresis on 5% urea-polyacrylamide-bis-acrylamide gels and visualized on a Phosphorimager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Measurement of Apoptosis—Early apoptotic changes were identified by staining of cells with fluorescein thiocyanate-conjugated annexin V and propidium iodide (Becton Dickinson, Heidelberg, Germany) and analyzed by flow cytometry (FACScan, Becton Dickinson) as described earlier (38).

RESULTS

Dose-dependent Inhibition of NF-κB by Rocaglamide Derivatives—Sixteen naturally occurring 1H-cyclopent[a]benzo[furan lignans of the rocaglamide type as well as one naturally occurring aglaim congenere were tested for their ability to inhibit NF-κB function. The different substances were isolated from various Aglaia species as described earlier (3). All compounds were prepared to at least 98% purity as assayed by high performance liquid chromatography. These compounds (named 1–16) differed mainly in their amide side chains and/or in the substitution patterns of aromatic rings A and B (Fig. 1).

Jurkat T cells bearing an integrated NF-κB-dependent luciferase reporter (3xIkB.luc) served as a test system for NF-κB activity. Jurkat T cells were pre-treated with the different rocaglamide compounds at a concentration of 100 nM. One hour

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later the cells were stimulated with TNF, and at 6 h post-stimulation luciferase activities were determined. TNF stimulation resulted in a potent activation of NF-κB-dependent transcription (>30-fold). Interestingly we found that pretreatment of the Jurkat T cells with several of the different rocaglamide compounds led to inhibition of TNF-induced NF-κB-dependent luciferase activity (Fig. 2A and data not shown). The most efficient inhibitors were N,N-didesmethyl-N-4-hydroxybutyl-rocaglamide (compound 8), didesmethyl-rocaglamide (synonym with RocB, compound 1), and compounds 3 and 4. These substances show an inhibition activity ranging from 70% up to complete inhibition. Compounds 2, 5–7, 9–12, 14, and 15 are less powerful inhibitors that repress TNF-induced NF-κB-dependent luciferase activity to levels less than 50% (Fig. 2A and data not shown). The aglain derivative (compound 17), 8β-methoxyrocaglaol (compound 13), and compound 16 were completely inactive even at concentrations up to 2 μM (Fig. 2A and data not shown).

We next investigated the dose dependence of the inhibition and determined the IC₅₀ values for several rocaglamides following TNF treatment. All investigated active compounds showed a dose-dependent inhibition of TNF-induced NF-κB-driven gene expression. An example of the dose-dependent inhibition of the transcription factor NF-κB by RocB is shown in Fig. 2B. A complete inhibition of the transcription factor NF-κB-dependent gene expression in Jurkat T cells was observed, when the T-cells were treated with RocB at a concentration of 200 nM. The IC₅₀ value was determined to be 58 nM, and several other compounds showed IC₅₀ values in the range of 200 nM (Table I).

A wide variety of stimuli has been shown to induce NF-κB activity. We therefore asked whether rocaglamides would specifically inhibit TNFα-induced NF-κB or whether they might also interfere with PMA-induced NF-κB activation. RocB (compound 1) was able to inhibit PMA stimulation in the same concentration range as shown for TNFα stimulation. Other rocaglamide congeners were somewhat less efficient in their inhibitory action for PMA-induced NF-κB activity. IC₅₀ values of five rocaglamide derivatives were determined for their inhibition of PMA stimulation (Table I). The aglain derivative and 8β-methoxyrocaglaol were again completely inactive up to the final concentration tested. Our results demonstrate that, depending on their chemical structure, rocaglamide derivatives represent potent inhibitors of the NF-κB pathway.

We next asked whether rocaglamide derivatives specifically interfere with the NF-κB pathway or whether they affect the activity of other transcription factors, too. For this purpose we analyzed the influence of rocaglamides on AP-1 activity. AP-1 is a dimeric transcription factor, which regulates gene expression in response to a great variety of stimuli, including TNF and PMA (39). Jurkat T cells transiently transfected with an AP-1-dependent reporter gene (5xTRE-tk-luc) showed a strong basal activity already (in comparison to tk-luc). This activity was only marginally induced by TNF or PMA. We investigated the influence of rocaglamides on this basal AP-1 activity in Jurkat T cells. We found that AP-1 activity was not altered in the presence of active rocaglamide derivatives 1–5 and the previously inactive aglain derivative (compound 17). Interestingly, compound 8 strongly reduced reporter gene activity (Fig. 2C). Similar results were obtained when we investigated the PMA/ionomycin-induced activity of the Oct-coactivator BOB.1/OBF.1 (40, 41). PMA/ionomycin-induced Oct-dependent activity was not inhibited by rocaglamides (data not shown), but again compound 8 showed a dramatic reduction of Oct-dependent activity. From this and several other experiments we concluded that the effects of compound 8 were unspecific. This rocaglamide derivative also showed strong cytotoxic effects (data not shown) and was therefore excluded from further experiments. In conclusion, rocaglamide derivatives are specific inhibitors of the NF-κB system and do not block the activity of the AP-1 and Oct transcription factors.

Inhibition of NF-κB Activity by Rocaglamides Is Cell Type-specific—We next tested the inhibition properties of rocaglamides in other cell lines to address the question whether the inhibition of the NF-κB-dependent gene expression might be due to a cell-type-specific mechanism. First we analyzed the

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**Fig. 1. Chemical structures of the 1H-cyclopentatetrahydrobenzofuran derivatives (rocaglamide congeners).** The list below shows the substituents (R₁, R₂, R₃, and R₄) indicated in the chemical structures.

| Rocaglamide | R₁ | R₂ | R₃ | R₄ |
|------------|----|----|----|----|
| [1]        | OH | OH | CONH₂ | H |
| [2]        | OH | OH | CONH₂ | OH |
| [3]        | OH | OH | CON(CH₃) | H |
| [4]        | OH | OH | CON(CH₃) | H |
| [5]        | OH | OH | CON(CH₃) | OH |
| [6]        | OH | OH | CON(CH₃) | OCH₃ |
| [7]        | OH | OCOCH₃ | CON(CH₃) | OH |
| [8]        | OH | OH | CON(HCH₃)OH | OH |
| [9]        | OH | OH | COOCH₃ | H |
| [10]       | OH | OH | COOCH₃ | OH |
| [11]       | OH | OH | H | H |
| [12]       | OH | OH | H | O-Benz |
| [13]       | OCH₃ | OH | H | H |
| [14]       | OH | OH | COOCH₃ | . |
| [15]       | OH | OH | COOCH₃ | H |
| [16]       | OH | OH | CON(CH₃) | OCH₃ |

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**Table I.**
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A301 (human) and EL-4 (mouse) T-cell lines, which were transiently transfected with an NF-κB-dependent reporter gene construct. Similar to the results obtained with Jurkat T cells, the TNF (Fig. 3A) or PMA (Fig. 3B) stimulated activation of the NF-κB-driven reporter gene could be inhibited by RocB in EL-4 cells and in A301 cells (data not shown). The concentrations of RocB were in the same range as those needed for the inhibition of the NF-κB activation in Jurkat T cells. Again, the inactive compounds (the aglain derivative and 8β-methoxyrocaglaol) showed no inhibitory activity. Thus, we conclude that rocaglamides inhibit NF-κB induction in mouse and human T cell lines.

Surprisingly, a remarkable resistance of several other cell lines toward inhibition of NF-κB by rocaglamide congeners was observed. In PC-12 cells bearing an integrated NF-κB-dependent reporter gene and transiently transfected human HeLa cells, rocaglamide derivatives were not able to block NF-κB activation. Even RocB concentrations up to 800 nM failed to inhibit NF-κB activation by PMA (Fig. 4A) or TNF in PC-12 cells (data not shown). In HeLa cells several rocaglamides used at a concentration of 200 nM showed no inhibitory effect on TNF and PMA-induced NF-κB activity (Fig. 4, B and C). In contrast, when PC-12 and HeLa cells were treated with highly active rocaglamide derivatives, an increase of the NF-κB-dependent gene expression was found. The aglain derivative and 8β-methoxyrocaglaol showed no signs of inhibition or induction on the NF-κB system in these cells. Similar results were obtained in NIH3T3 fibroblasts and A549 human lung epithelial cells bearing an integrated NF-κB-dependent reporter gene (data not shown). We therefore conclude that rocaglamides do not block NF-κB in these non-T cells.

All the cell lines that exhibited no rocaglamide-dependent inhibition of NF-κB were of non-lymphoid origin. We therefore asked whether B lymphocytes might respond to rocaglamides. For this purpose we used the variant plasmacytoma cell line S107, which lacks constitutive NF-κB activity typical for mature B cells but is still highly responsive to TNF (42). NF-κB-dependent gene expression was investigated using S107 cells with an integrated reporter gene. Although the required rocaglamide concentrations for inhibiting TNF-induced NF-κB activity in S107 cells were ~5 to 7 times higher than in Jurkat T cells, inhibition could be observed (Fig. 4D). Again, 8β-methoxyrocaglaol showed no inhibitory activity. From these results we conclude that NF-κB activation in lymphocytes, but not in several other cell types, is controlled by a rocaglamide-sensitive pathway.

RocB Suppresses PMA-induced Expression of NF-κB-dependent Target Genes—All of the NF-κB inhibitory effects of rocaglamides described here were observed in reporter gene assays.
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Rocaglamides were then prepared and analyzed by RNase protection assay analysis. These mutant Jurkat cells are unable to activate NF-κB.

Therefore, we next investigated whether rocaglamides also influence the activation of endogenous NF-κB target genes. Jurkat T cells were pretreated with RocB, a highly active rocaglamide derivative and subsequently exposed to PMA. Total RNAs were then prepared and analyzed by RNase protection assay (RPA) analysis for the expression of known NF-κB target genes. To prove the NF-κB dependence of the respective genes, Jurkat cells deficient in IKKγ (43), the essential component of the NF-κB signaling pathway, were used in parallel for RNase protection assay analysis. These mutant Jurkat cells are unable to activate NF-κB in response to various NF-κB inducers, including PMA and TNF, but show normal activation of NF-AT and AP-1 (44). We found a strong up-regulation of LT-β (Fig. 5A), Bfl1/A1 (Fig. 5B), and TRAIL (Fig. 5C), mRNA in Jurkat cells upon PMA treatment. At the same time PMA-induced expression of these genes was completely absent in IKKγ-deficient cells. This indicates a critical involvement of NF-κB transcription factors in the activation of these genes as described elsewhere (45–49). In the presence of RocB the PMA-stimulated expression of LT-β, Bfl1/A1, and TRAIL was completely abolished in Jurkat T cells indicating that the induced transcription of NF-κB target genes is specifically inhibited by rocaglamides. The transcription of other genes for example L32, glyceraldehyde-3-phosphate dehydrogenase, BcL-XL, BID, Bak, Bax, and caspase-8 (Fig. 5, A–C), which are not regulated by NF-κB, are unaffected in the presence of PMA and/or RocB. However, independently of the effects on NF-κB target genes rocaglamides enhanced the PMA-induced expression of Mcl-1, a protein primarily under transcriptional regulation of CREB and E2F (50, 51).

**RocB Interferes with DNA-binding Activity of NF-κB**—We next asked where the one or more molecular targets of rocaglamide action are localized in the NF-κB signaling pathways induced by PMA and TNF. We first investigated the effects of RocB on NF-κB DNA-binding activity. For this purpose we performed EMSA experiments using extracts from Jurkat T cells, which were pretreated with RocB and subsequently stimulated with TNFα and PMA. In the absence of RocB maximal NF-κB DNA-binding activity was detected after 10 min of TNF treatment, remained at this level until 1 h, and decreased after 2 h (Fig. 6A). PMA stimulation resulted in maximal NF-κB DNA-binding activity after 30 min, and this activity decayed from 2 to 8 h. RocB pretreatment resulted in a virtually complete block of the NF-κB induction for both TNF and PMA stimulation (Fig. 6A). Also a second wave of NF-κB activation, which was seen after 4 and 8 h of TNF treatment, was inhibited by RocB. At the same time no significant alterations in the DNA-binding activity of the Sp1 transcription factor were detected in the presence of RocB, suggesting that RocB specifically interferes with induced bind-
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Rocaglamides Inhibit NF-κB Activation “Upstream” of the IKK Complex—An essential prerequisite for induced IκBα degradation is the de novo phosphorylation of conserved serine residues on the N-terminal domain of IκBα catalyzed by the IκB kinase complex. The IKK1 and IKK2 kinases present in the complex are both capable of phosphorylating IκBα in response to extracellular signals. We therefore asked whether the IκB kinase complex is a target of rocaglalide action. To address this question, immune complex kinase assays were performed. Consistent with the induced degradation of IκBα, stimulation of Jurkat T cells with TNF and PMA resulted in an activation of the IKK complex monitored via phosphorylation of the GST-IκBα substrate (Fig. 7A). However, in the presence of RocB the IκBα-induced phosphorylation of the substrate was completely blocked, and in the case of TNF induction a significant reduction was observed. Immunoblot analysis revealed equal protein levels of precipitated IKK1. These data argue for a rocaglalide inhibition mechanism upstream of the IKK complex or at the level of the IKK complex.

To distinguish between a direct inhibition of IKK1 and IKK2 enzyme activity by RocB or effects on upstream factors of the IKK complex, we performed in vitro kinase assays with purified recombinant IKK proteins (57). In a coupled kinase assay with the GST-IκBα substrate we observed no repression of IKK1 and IKK2 kinase activities in the presence of 100, 200, or 1000 nM RocB. Both IKK proteins showed some level of autophosphorylation, which also was not affected by the addition of RocB (Fig. 7B). Even very high RocB concentrations of up to 330 μM did not result in any alteration of IKK1 and IKK2 kinase activity (data not shown). These observations suggest that rocaglamides have no direct influence on IKK1 and IKK2 enzyme activity but rather act on factors regulating or activating the kinase activity.

To investigate whether upstream signaling events in the
signal transduction cascade initiated by TNFα and PMA are likely to be targeted by rocaglamides, we performed transient transfections with known components of the TNF signal transduction cascade, namely TRAF2, NIK, MEKK1, and IKK2. Upon overexpression, these proteins induce NF-κB activity measured via activation of a κB-dependent luciferase reporter (Fig. 8). We could observe that, in the presence of RocB the NF-κB activity was even seen in experiments, where RelA was overexpressed (data not shown). This suggests that rocaglamides predominantly affect a activation step upstream of the IKK complex, the IKK2 kinase (a constitutive active version, IKK2-EE) or the wild type version (data not shown), was inhibited about 2-fold. Interestingly, such a 2-fold reduction of activity was also seen in experiments, where RelA was overexpressed (data not shown). This suggests that rocaglamides do not interfere with IKK1 and IKK2 kinase activity.

Rocaglamides promote apoptosis in therapy-resistant Leukemic T Cells—The role of NF-κB in apoptosis has been well established over the past years. In most cell types, NF-κB has an anti-apoptotic function by up-regulation the expression of protective factors such as cIAP1 and 2, Bfl-1, Bcl-XL, and c-FLIP (58–64). Some tumor cell lines show increased resistance to the induction of apoptosis, which contributes to the tumorigenic potential of these cells (65, 66). Interestingly, suppression of NF-κB activity increases the apoptotic response to chemotherapy (67, 68). In two variants of the CEM T cell leukemia line, which were either sensitive (CEM-S) or resistant (CEM-R) toward therapy-induced apoptosis, we could recently show that inhibition of NF-κB activity by proteasome inhibitors and dominant-negative IκBα augments induction of apoptosis and abrogates apoptosis resistance (69). We therefore asked whether rocaglamide derivatives could affect apoptosis...
induction in these cell lines. CEM-S cells and the derived resistant cell line CEM-R were γ-irradiated or treated with cisplatin and TNFα. 48 h later apoptosis was determined by annexin-V staining and fluorescence-activated cell sorting analysis. Strongly enhanced apoptosis was detected in γ-irradiated CEM-S cells and to a lower extent after treatment with cisplatin and TNFα. In contrast, apoptosis due to these insults was not significantly altered in CEM-R cells (Fig. 9). However, cotreatment with RocB increased apoptosis in CEM-R cells, whereas the high percentage of apoptosis in CEM-S cells was not further enhanced. RocB also enhanced basal apoptosis in sensitive and resistant CEM cells. The inactive aglain congener failed to induce apoptosis at any concentration (data not shown). Thus, whereas RocB does not influence cancer therapy-induced apoptosis in sensitive cells, the therapeutic effect in resistant tumor cells is increased by RocB. These data suggest RocB as being a potentially helpful therapeutic agent that may overcome the apoptosis defect of therapy-resistant cancer cells by blocking the anti-apoptotic effect of NF-κB.

**DISCUSSION**

In this study we analyzed the effect of plant-derived natural products on NF-κB activity. We show that rocaglamides are potent inhibitors of NF-κB activity. They block inducible NF-κB DNA-binding activity and IκBα degradation as well as expression of NF-κB target genes in T lymphocytes. We characterized the NF-κB inhibition properties of 16 rocaglamide derivatives and one naturally occurring aglain congener. We could define a group (compounds 1, 3, and 4) of rocaglamide derivatives, which show a dose-dependent inhibition of PMA- and TNF-induced NF-κB activation in Jurkat T cells and which mediate an almost complete inhibition at a final concentration of 200 nM. The results correspond well with earlier experiments, in which the insecticidal properties of rocaglamides (2) and their anti-proliferative activities were determined (8, 9). RocB (didesmethylrocaglamide) was found to be the most active NF-κB-specific compound with IC_{50} values of 58 nM for TNF-induced NF-κB activity and 44 nM for PMA-induced NF-κB activity, respectively. RocB, compounds 3 and 4 have a polar aminoacyl side chain at carbon C-2 and an unsubstituted C-3' at the phenyl ring system B in common. A replacement of the polar amide group at carbon C-2 either by a polar COOCH₃...
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Another family of plant-derived NF-κB inhibitors. They are active in concentrations of 50 nM, which turned out to be helpful tools to identify such pathway and cell type-specific signaling intermediates.

Interestingly, the RocB also showed the highest insecticidal activity against larvae of the pest insect Spodoptera littoralis and the highest antiproliferative activity on Mono Mac 6 cells in vitro (3, 8). The molecular mechanism responsible for insecticidal activity of rocaglamides has not been resolved yet. Interestingly, the NF-κB system is conserved in insects not only at the level of the Rel/NF-κB transcription factors (Dorsal, Dif, and Relish in Drosophila) but also with respect to the signaling components involved in regulation of the function of these factors (70). This cascade is involved in regulation of embryonic development and “innate immune responses” in insects. Therefore, it seems possible that inhibition of this pathway by rocaglamide derivatives contributes to its insecticidal effects. The mechanism of the rocaglamide-induced block of cell cycle progression observed in some cell lines may also be mediated by NF-κB inhibition. It was shown that expression of cyclin D1, a key factor in the regulation of the cell cycle, is regulated by NF-κB (71–74).

When the active concentrations of rocaglamide derivatives 1, 3, and 4 are compared with other NF-κB inhibitors, it becomes clear that these rocaglamides are highly active compounds (13). They are active in concentrations of 50–100 nM, whereas most other inhibitors work in the micromolar to millimolar range. For example, aspirin and sulfasalazine inhibit NF-κB activity at concentrations of 20–50 μM, proteasome inhibitors, and inhibitors of the anti-oxidant class act at 20–100 μM, curcumin at 40–60 μM and herbizymyc A at 200–2000 nM. Recently, another family of plant-derived NF-κB inhibitors, namely sesquiterpene lactones, were described previously (20, 21). These compounds were shown to block NF-κB activation induced by PMA and TNF at a concentration of 5–20 μM, which is still 5- to 20-fold higher than the active concentration of rocaglamide derivatives. So far only glitoxin, a metabolite produced by Aspergillus fumigatus and other pathogenic fungi, was shown to inhibit NF-κB in nanomolar concentrations (50–1000 nM (75)). Glucocorticoids such as dexamethasone are also potent inhibitors of NF-κB activity, which act in a range of 100 nM (76, 77). From this comparison we suggest that rocaglamide derivatives act in a concentration range that is of future therapeutic relevance. In addition, the observed reduction of the inhibitory capacity found in several compounds due to variations in the chemical structure may help to develop structural modifications that allow the generation of derivatives with optimized features.

An interesting outcome of our study was the obvious cell type specificity of NF-κB inhibition. The reason for this specificity is not clear, yet, but two different hypotheses could account for this result. One possibility would be that TNF and PMA initiated signal transduction cascades differ between T cells and other cell types. Although the signaling components from the cell surface to the IKK complex have been studied extensively, not all intermediates have been unequivocally identified. Indeed, more than 20 protein kinases are implicated in the activation and the regulation of the IKK complex. These kinases, including NIK, NAK, MEKK1, -2, and -3, Akt1, HPK-1, Raf, several members of the protein kinase C family, TPL-2/Cot, MLK3, BTK, and PAK1 may form signaling cascades that differ in their composition depending on the NF-κB inducer (reviewed in Refs. 11 and 14). It is therefore conceivable that different cell types may utilize different components/isoforms for the activation and/or regulation of the IKK complex and the component used in T cells is susceptible to rocaglamide inhibition. It is not clear whether there is a common factor in TNF- and PMA-induced signaling pathway, which is affected by rocaglamide action. In that respect, rocaglamide derivatives may turn out to be helpful tools to identify such pathway and cell type-specific signaling intermediates.

A second explanation for the cell type specificity could be that rocaglamide derivatives are only active after an obligatory metabolizing step. Only certain cell types might possess the machinery for rocaglamide uptake and/or for the enzymatic conversion of rocaglamides into its active derivate. In support of this explanation comes from insecticidal (2) and cell proliferation assays with RocB (8, 9). In these assays rocaglamide-dependent effects were only observed at late time points (48–96 h), and this finding was explained with a potential metabolic step. Also in Jurkat T cells conversion of rocaglamides seemed to be a rate- or time-limiting step. In EMSA experiments it turned out that preincubation with RocB for at least 2 h before stimulation with TNF or PMA was necessary for complete inhibition at early induction time points.

Our data virtually rule out the possibility that the IsB kinases themselves are the target for inhibition by rocaglamides. First of all we show that active IKK2 is only slightly affected by rocaglamides in transient transfection assays. More important, we have analyzed IKK activity in vitro using purified recombinant IKK1 and IKK2. In both cases rocaglamides had no effect on the IsB kinase activity measured in vitro. Furthermore, constitutive NF-κB activity in mature B cells was not affected by RocB (data not shown) also suggesting that RocB does not interfere directly with NF-κB activity but, rather, blocks a step in the signaling cascades leading to the activation of NF-κB.

The NF-κB transcription factor family functions broadly in the host control of immunoregulatory gene expression, inflammation, and apoptosis. Our results demonstrate that the expression of NF-κB-dependent target genes involved in these processes such as Bfl-1/A1, lymphotoxin-β (LT-β), and TRAIL can be efficiently blocked by rocaglamides. Bfl-1/A1 is an anti-apoptotic protein of the bcl-2 family, whose preferential expression in hematopoietic, leukemic, and endothelial cells is controlled by inflammatory stimuli. Bfl-1/A1 is a direct transcriptional target of NF-κB that is able to block TNF-induced apoptosis and mediates resistance to chemotherapy-induced apoptosis (47, 78). Interestingly, in the presence of RocB, TNF and chemotherapy-induced apoptosis is strongly enhanced in otherwise TNF-resistant leukemic T cells (CER). In this context it is possible that up-regulation of Bfl-1/A1 in response to TNF and other NF-κB inducers like γ-irradiation or cisplatin confers resistance to these inducers, which is overcome by rocaglamide action. However, it remains open whether the different stimuli induce expression of Bfl-1/A1 in CEM cells and/or whether there are differences in the expression level in CEM-S and CEM-R cells.

Lymphotoxin-β (LT-β) is a tumor necrosis factor-related membrane-bound cytokine that forms a heterotrimeric complex with LT-α on the surface of lymphoid cells. LT-β is produced by activated lymphocytes and functions as a mediator of inflammatory processes. The PMA-induced up-regulation of LT-β mRNA in Jurkat T cells is consistent with previous findings.
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(45) demonstrating that PMA-inducible promoter activity depends on the integrity of a conserved NF-κB binding site. The inhibition of PMA-induced expression of LT-β by RocB offers a possible explanation for the use of rocaglamide-containing plant extracts as anti-inflammatory remedies in traditional medicine, although the effects on other pro-inflammatory cytokines require further detailed investigation. TNF-related apoptosis-induced ligand (TRAIL) is involved in apoptosis in a broad range of tumor cells with apparently no cytotoxic effects on most non-transformed cells. It was recently shown that the regulation of TRAIL expression depends critically on NF-κB. Therefore, a specific role of rocaglamides in cancer therapy is suggested. However, a more detailed characterization of rocaglamides' effects on TRAIL expression requires further detailed investigation. Rocaglamides also induce apoptosis in a broad range of tumor cells, including leukemia cells, although the effects on other pro-inflammatory cytokines remain to be investigated. A possible explanation for the use of rocaglamide-containing plant extracts as anti-inflammatory remedies in traditional medicine is suggested. However, a more detailed characterization of rocaglamides' effects on TRAIL expression requires further investigation.
Rocaglamide Derivatives Are Potent Inhibitors of NF-κB Activation in T-cells
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J. Biol. Chem. 2002, 277:44791-44800.
doi: 10.1074/jbc.M208003200 originally published online September 16, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208003200

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