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The staurosporine analog, Ro-31-8220, induces apoptosis independently of its ability to inhibit protein kinase C

Z Han*,[1], P Pantazis[1], TS Lange[1], JH Wyche[1] and EA Hendrickson[1]

[1] Department of Molecular Biology, Cell Biology and Biochemistry, Box G-J1, Brown University, Providence, RI 02912, USA
* Corresponding author: Z. Han, Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912, USA; Tel: 401-863-9648, Fax: 401-863-2421; E-mail: zhiyong_han@brown.edu

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Abstract

A series of bisindolylmaleimide (Bis) compounds were designed as analogs of the natural compound staurosporine (STS), which is a potent inducer of apoptosis. Many of the Bis analogs appear to be highly selective inhibitors of the protein kinase C (PKC) family, including PKC-α, -β, -γ, -δ, -ε, and -ζ, unlike STS, which is an inhibitor of a broad spectrum of protein kinases. In this report we describe the effects of the Bis analogs, Bis-I, Bis-II, Bis-III and Ro-31-8220 on the survival and proliferation of HL-60 cells, which have been widely used as a model cell system for studying the biological roles of PKC. Treatment of HL-60 cells with Bis-I, Bis-II, Bis-III, or Ro-31-8220 blocked phosphorylation of the PKC target protein Raf-1 with equal potency but did not appear to affect the general phosphorylation of proteins by other kinases. However, the biological effects of the Bis compounds were different: Bis-I and Bis-II had no observable effects on either cell survival or proliferation; Bis-III inhibited cell proliferation but not survival, whereas Ro-31-8220 induced apoptosis. These results indicated that the members of the PKC family which could be inhibited by the Bis analogs were required neither for survival nor proliferation of HL-60 cells. Analyses of cells treated with Ro-31-8220 showed that the apoptotic effect of Ro-31-8220 on HL-60 cells was mediated by a well-characterized transduction process of apoptotic signals: i.e., mitochondrial cytochrome c efflux and the activation of caspase-3 in the cytosol. Moreover, the ability of Ro-31-8220 to induce apoptotic activation was completely inhibited by the over-expression of the apoptotic suppressor gene, Bcl-2, in the cells. Interestingly, proliferation of the Bcl-2-over-expressing cells was still sensitive to the presence of Ro-31-8220, suggesting that the inhibitory effects of Ro-31-8220 on viability and cell proliferation were mediated by different mechanisms. In particular, the apoptotic effect of Ro-31-8220 on cells was not altered by the presence of an excess amount of the other Bis analogs, suggesting that this effect is mediated by a factor(s) other than PKC or by a mechanism which was not saturable by the other Bis analogs. Finally, structure-function analyses of compounds related to Ro-31-8220 revealed that a thioamidine prosthetic group in Ro-311-8220 was largely responsible for its apoptotic activity. *Cell Death and Differentiation* (2000) **7,** 521–530.

Keywords: apoptosis; PKC; analogs; staurosporine

Abbreviations: Bis, bisinodolylmaleimide; PMA, phorbol-12-myristate acetate; STS, staurosporine; CaM kinase, calmodulin-dependent kinase; PKA, protein kinase A; PKG, protein kinase G

Introduction

Apoptosis is an essential mechanism for the development of all mammalian organisms.[1] Recent studies indicate that the fundamental apoptotic machinery, which consists of various effectors and suppressors, is conserved throughout evolution.[2–4] The key apoptotic effectors appear to be a family of aspartate-specific proteases called caspases.[5–7] Caspases exist as inactive proenzymes in healthy cells and are then activated by proteolysis in apoptotic cells. Once activated, caspases cleave a set of cellular substrates,[8] which ultimately result in cell death. The key apoptotic suppressors appear to be members of the Bcl-2 gene family, which regulate apoptosis at the level of cytochrome c efflux from the mitochondria[9] and/or caspase activation.[10] The compound staurosporine (STS), which was originally isolated from *Streptomyces* as a potential anti-fungal agent, has achieved certain notoriety due to its ability to potently induce apoptosis in virtually all mammalian cells. However, the mechanism by which STS causes apoptosis is still poorly understood. One plausible hypothesis has been that STS's effect is mediated through protein kinase C (PKC), given the fact that STS has also been shown to be a potent inhibitor of PKC. This hypothesis was relatively attractive since numerous studies had demonstrated that the inhibition of PKC by a variety of pharmacological agents correlated with apoptotic induction in many cell types.[11–19] In addition, it has been demonstrated that PKC δ is cleaved by caspase-3 in human monocytic leukemia U937 cells during apoptotic induction by etoposide, cisplatinum or Ara-C,[20] suggesting that inhibition of this enzyme is an important aspect of apoptosis. Consistent with these observations, it has also been suggested that the phosphorylation of the apoptotic suppressor, Bcl-2, by mitochondrial PKC α is required for its ability to inhibit apoptosis.[21]

Unfortunately, in order for STS to induce apoptosis it is often used in a concentration range of 1 to 5 μM, which is a concentration at which it acts as a rather non-specific inhibitor of a variety of kinases including, in addition to PKC, protein kinase A (PKA), protein kinase G (PKG),

myosin light-chain kinase, calmodulin-dependent kinase (CaM kinase), and the p60 v-Src protein tyrosine kinase.[22,23] Therefore, even though apoptosis induction in various cell types has been associated with PKC inhibition, a functional connection between PKC inhibition by STS and apoptosis remains unproven.[24,25]

To complicate matters, a positive regulatory role in apoptotic induction for various members of the PKC family (of which there are at least 11 members[26] has been postulated. For example, it was recently reported that (i) PKC δ activity was essential for etoposide-induced apoptosis in salivary gland acinar cells;[27] and (ii) over-expression of PKC α in the prostate epithelial tumor cell line, LNCaP, resulted in apoptosis.[28] Furthermore, it has been demonstrated that PKC α and δ are activated by a caspase-dependent mechanism during apoptosis induction in human promyelocytic leukemia HL-60 cells by 7-hydroxystaurosporine, camptothecin, or etoposide[29] and by UV irradiation in normal human dermal keratinocytes,[30] respectively. Thus, both the activation and inhibition of PKC isoforms have been correlated with apoptosis and the precise role of this important family of protein kinases in cell survival remains controversial.

In recent years, a class of bisindolylmaleimide (Bis) analogs, modeled on the polyaromatic aglycon portion of the STS molecule, were synthesized and found to be highly selective inhibitors of multiple PKC isoenzymes, including at least PKC-α, -β, -γ, -δ, -ε, and -ζ.[31–35] Impressively, when given orally to experimental rats, one analog, Ro-32-0432, prevented T-cell-driven induction of host *versus* graft responses and secondary paw swelling in adjuvant-induced arthritis.[36] These findings suggested that the Bis compounds might be useful as drugs to treat inflammatory and autoimmune diseases which are linked to apoptosis. In addition, the high selectivity of the Bis analogs of STS towards PKC as opposed to other protein kinases also suggested that these analogs might be useful tools for probing the various biological roles of PKC including apoptosis regulation.

To investigate whether the selective inhibition of PKC in cancer cells by the Bis analogs of STS results in inhibition of cell proliferation, induction of cell differentiation, or apoptosis we tested the effect of these compounds on HL-60 human promyelocytic leukemia cells, which have been used as a model cell system for studying PKC. In this report, we demonstrate that the Bis analogs Bis-I, Bis-II, Bis-III, and Ro-31-8220 had different effects on proliferation and survival of HL-60 cells, although they inhibited PKC activity equally well. Specifically, Bis-I, Bis-II, and Bis-III had cytostatic effects whereas Ro-31-8220 potently induced apoptosis. Additional results strongly suggested that the apoptotic activity of Ro-31-8220 was due to the presence of a unique prosthetic group in this compound and that its apoptotic effect on cells was not altered by the presence of other Bis analogs. These results demonstrate that apoptotic induction in HL-60 cells proceeds in a PKC-independent fashion and they shed light on the mechanism by which STS induces cell death.

Results

The effects of bisindolylmaleimide analogs on protein phosphorylation and cell proliferation

The STS bisindolylmaleimide analogs Bis-I, Bis-II, Bis-III, and Ro-31-8220 (Figure 1) are equipotent and highly selective inhibitors of PKC in *in vitro* kinase assays.[32] In contrast, Bis-V (Figure 1) does not inhibit PKC nor several other protein kinases.[33] Therefore, we tested these compounds for their effects on the phosphorylation of proteins in, and the proliferation of, HL-60 cells *in vivo*. Treatment of HL-60 cells with 100 nM phorbol-12-myristate acetate (PMA) caused the hyperphosphorylation of c-Raf-1 a known PKC family target[37] (Figure 2A, compare lanes '$-$' and '+'). However, pretreatment of the cells with 10 μM Bis-I, Bis-II, Bis-III, or Ro-31-8220, but not Bis-V, completely blocked this phosphorylation (Figure 2A). In contrast, none of these compounds showed significant inhibitory effect on the general phosphorylation of proteins in HL-60 cells stimulated by serum (Figure 2B). Thus, these results confirmed the *in vitro* data obtained by Davis *et al*.[32,33] that Bis-I, Bis-II, Bis-III, and Ro-31-8220 were relatively selective and potent inhibitors of PKC.

Induction of apoptosis by Ro-31-8220

Next, the biological effects of these compounds on HL-60 cells were investigated. Treatment of HL-60 cells with 10 μM Ro-31-8220, but not the other Bis compounds, for 24 h induced apoptosis. DAPI staining of the cells at the end of the treatment showed that Ro-31-8220 induced formation of densely stained granular nuclear bodies, which are a classic hallmark of apoptosis,[38] in almost all the cells (Figure 3). In contrast, the percentage of apoptotic cells caused by treatment with the other compounds remained at the basal level, approximately 1–5%. Biochemical analyses showed that Ro-31-8220, but not other compounds, also induced (i) the cleavage of Numa (Figure 4A), a nuclear target of caspases during apoptosis;[39] and (ii) the internucleosomal degradation of chromatin DNA into nucleosomal fragments (Figure 4B), another hallmark of apoptosis.[40] In addition, treatment of HL-60 cells with Ro-31-8220 rapidly induced the efflux of mitochondrial cytochrome c into the cytosol, which was followed by the activation of caspase-3 but not caspase-2 (Figure 5A). In contrast, Bis-I, Bis-II, Bis-III, and Bis-V failed to induce these biochemical changes (Figure 5B). Therefore, the apoptotic effect of Ro-31-8220 on HL-60 cells appeared to be mediated by a well-characterized transduction process of apoptotic signals, i.e., mitochondrial cytochrome c efflux, and activation of caspase-3 in the cell cytosol. Prolonged treatment of HL-60 cells with 10 μM of Bis-I, Bis-II, Bis-III, or Bis-V for up to 4 days failed to induce apoptosis. Interestingly, Bis-I, Bis-II, and Bis-V did not have observable effects on cell proliferation, whereas Bis-III profoundly inhibited proliferation in cell growth assays (Figure 6). Taken together, these results indicated that although Bis-I, Bis-II, Bis-III, and Ro-31-8220 were equally potent PKC inhibitors (Figure 2A);[32,33] the effects of these compounds on cell survival and growth were very different. More importantly, these experiments clearly demonstrated that the inhibition of PKC was a separable, distinct and unrelated event from apoptotic induction and cell proliferation.

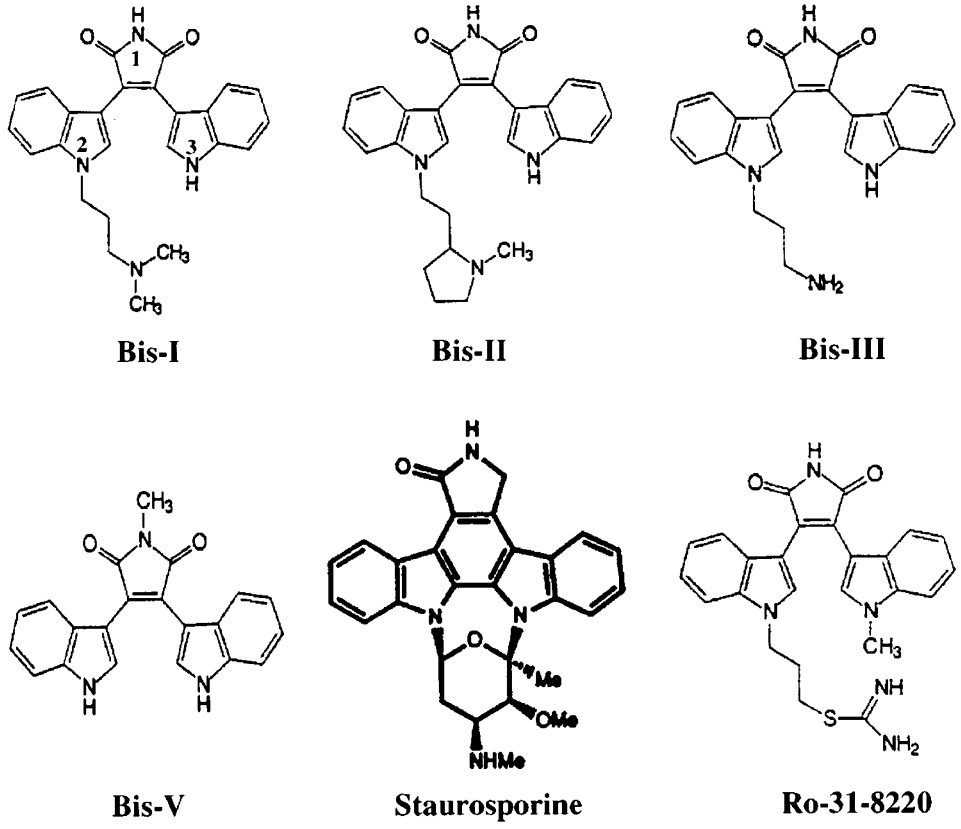

Figure 1 Structures of the Bis analogs. For description of the synthesis of the analogs, see references by Davis *et al*.[32,33] For convenience, the three nitrogen atoms in the compounds are denoted as 1, 2, and 3. For IC50 or K_i values of the Bis compounds, see reports by Davis *et al*.[32,33] and Wilkinson *et al*.[35]

The structural similarities amongst the Bis analogs suggested that they might interact with the same cellular target protein(s) regardless of their ability to induce apoptosis. If this were true, then the non-apoptotic Bis analogs might compete with the apoptotic Ro-31-8220 compound for binding to the same target protein(s) and, therefore, partially or completely, inhibit the ability of Ro-31-8220 to induce apoptosis through this protein(s). To experimentally test this hypothesis, HL-60 cells were pretreated with 20 μM of Bis-I, Bis-II, Bis-III, Bis-IV, or Bis-V for 60 min and then 10 μM of Ro-31-8220 was added to the cell cultures and incubation continued for 20 h. The ability of Ro-31-8220 to induce apoptosis was not affected by the presence of any of the Bis analogs (data not shown), suggesting that it interacts with a unique cellular target(s).

Structural changes in Ro-31-8220 alters its apoptotic-inducing capability

The alteration of the N-1 prosthetic group in Bis V (Figure 1) correlated with the lack of its inhibitory effect on PKC.[32,33] In addition, the presence of widely disparate prosthetic groups at the N-2 nitrogen seemed not to affect the ability of these compounds to inhibit PKC (Figure 1). Therefore, it seemed likely that the PKC inhibitory activity of the Bis analogs was largely associated with the basic polyaromatic aglycon portion of their structures and not with their respective constituent prosthetic groups. However, it was possible that the prosthetic group of Ro-31-8220 was responsible for its apoptosis-inducing activity. If this were true, then substitution of this prosthetic group with a different moiety, such as the one in Ro-31-7549 or Ro-31-8425 (Figure 7), should alter its apoptosis-inducing activity. Indeed, Ro-31-7549 and Ro-31-8425 were significantly less active than Ro-31-8220 in inducing apoptosis (Figure 7). Therefore, the presence of the thioamidine prosthetic group at the N-2 position of Ro-31-8220 was responsible for its potent apoptotic activity. It should also be noted that Bis-III is a N-3 de-methylated form of Ro-31-7549 (Figure 7). Thus, in contrast to Ro-31-7549 which showed some apoptotic activity, the complete lack of apoptotic activity of Bis-III indicated that the N-3 methyl group was also important for the apoptotic activity of Ro-31-7549.

Overexpression of Bcl-2 blocks induction of apoptosis, but not inhibition of cell proliferation, by Ro-31-8220

Overexpression of the apoptosis suppressor, Bcl-2 in different cell types blocks the chemical induction of apoptosis, but not necrosis.[10,41] Therefore, the specific apoptosis-inducing activity of a chemical can be best demonstrated by the resistance of cells overexpressing an apoptosis suppressor to the apoptotic effect of the chemical. To experimentally test if the transduction pathway for the apoptotic signals of Ro-31-8220 was inhibitable by Bcl-2, transgenic Bcl-2:HL-60 cells

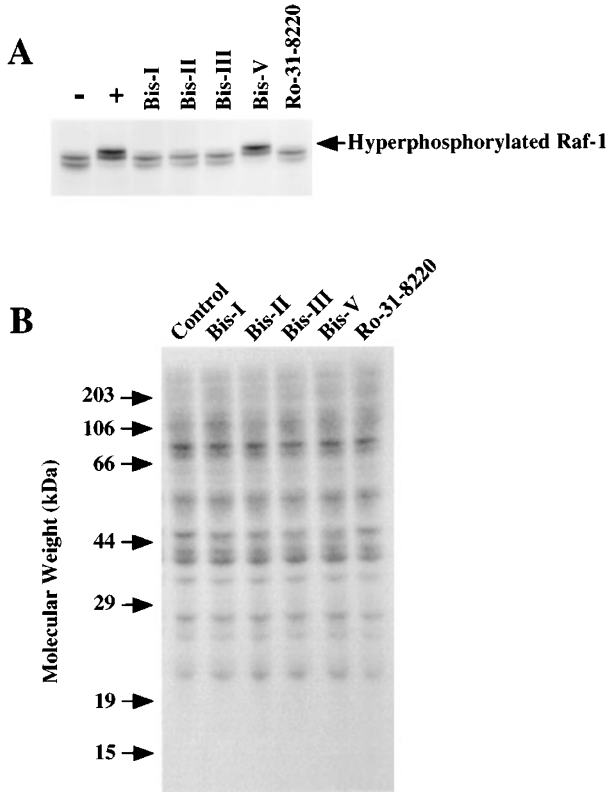

Figure 2 Effects of the Bis compounds on protein phosphorylation. (A) Bis compounds inhibit the phosphorylation of Raf-1 by PKC. HL-60 cells were left untreated or treated with 10 μM of the indicated compound for 1 h. Then, the cells were left unstimulated (−) or stimulated with 100 nM PMA (+) for 30 min. The status of Raf phosphorylation was then determined by Western blot analysis. (B) Lack of inhibitory effect of Bis compounds on the general phosphorylation of cellular proteins. HL-60 cells arrested in serum-free medium were metabolically radiolabeled with ^{32}P-phosphoric acid (see Materials and Methods). The cells were then either left untreated (Control) or treated with 10 μM of the indicated compound for 1 h. The cells were then stimulated with 10% fetal bovine serum for 10 min. Incorporation of [^{32}P] into proteins was subsequently analyzed by electrophoresis and autoradiography

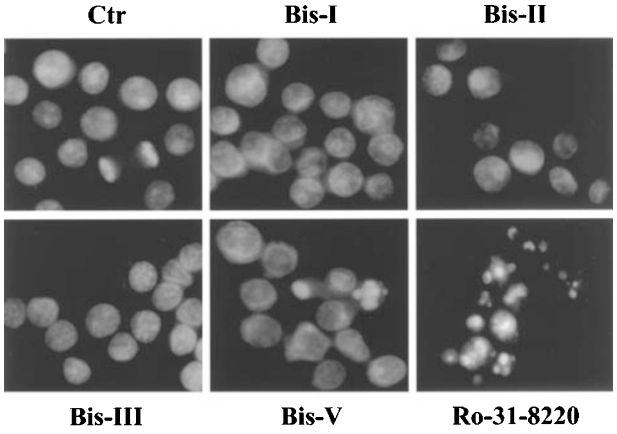

Figure 3 Induction of nuclear apoptotic bodies by Ro-31-8220. HL-60 cells were left untreated (Control) or treated with 10 μM of the indicated compound for 24 h. The cells were then fixed, stained with DAPI, and photographed under a fluorescent microscope

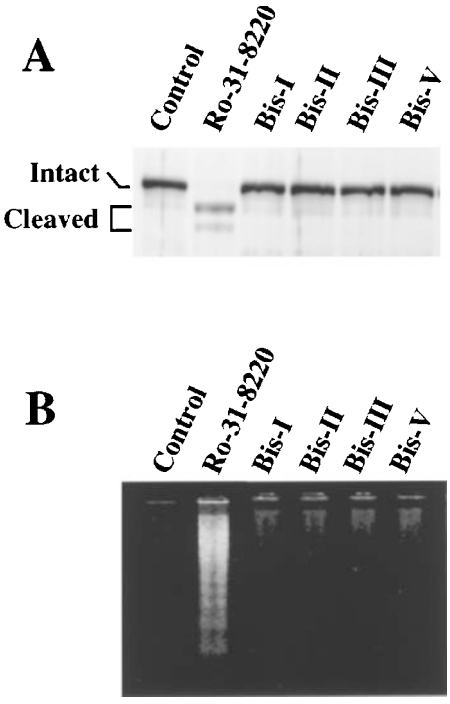

Figure 4 Induction of apoptosis by Ro-31-8220. HL-60 cells were left untreated (Control) or treated for 24 h with 10 μM of the indicated compound. At the end of the treatment, whole cell extract and DNA samples were prepared for (A) Western blot (WB) analysis of Numa cleavage, and (B) agarose gel electrophoresis analysis of DNA fragmentation, respectively

that overexpress Bcl-2[42,43] were treated with 10 μM of Ro-31-8220 for up to 24, 48 and 72 h and then examined for visible features of apoptosis. Microscopic examination of DAPI-stained cells showed that only a small fraction ($<$10%) of the Bcl-2:HL-60 cells treated with 10 μM of Ro-31-8220 for up to 72 h exhibited typical nuclear apoptotic bodies (Figure 8A). In addition, biochemical analysis showed that treatment of Bcl-2:HL-60 cells with Ro-31-8220 did not result in detectable mitochondrial cytochrome c efflux into the cytosol, activation of caspase-3, degradation of Numa, nor degradation of DNA (Figure 8C,D). These results indicated that over-expression of Bcl-2 in HL-60 cells essentially blocked the apoptotic effect of Ro-31-8220. In striking contrast, however, the proliferation of Bcl-2:HL-60 cells remained very sensitive to the presence of Ro-31-8220 (Figure 8B) and appeared to arrest predominately at the G2/M phase of the cell cycle (data not shown). Therefore, over-expression of Bcl-2 blocked the apoptotic effect of Ro-31-8220, whereas it failed to prevent its inhibitory effect on cell proliferation.

Discussion

In this study, we have attempted to dissect out the role that PKC enzymes inhibitable by the Bis family compounds play in cellular growth and survival. Experimentally, we took advantage of the STS analogs Bis-I, Bis-II, Bis-III and Ro-31-8220, which are synthetic and equally potent PKC inhibitors, but which had different effects on the proliferation and survival of HL-60 cells. More specifically, treatment of the

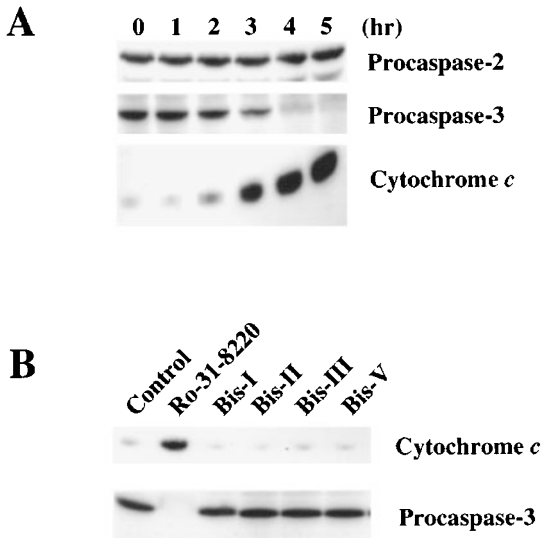

Figure 5 Rapid induction of mitochondrial cytochrome c efflux and caspase-3 activation by Ro-31-8220. (A) HL-60 cells were treated with 10 μM of Ro-31-8220 for 0, 1, 2, 3, 4 and 5 h. At the end of each treatment, S-100 extract was prepared from the cells, and aliquots were subjected to SDS–PAGE and Western blot analysis to determine the status of caspase activation and the accumulation of cytochrome c in the cytosol. The reduction in the level of procaspase-3 indicates the activation of caspase-3 and the accumulation of cytochrome c in the S-100 indicates mitochondrial cytochrome c efflux. (B) Selective induction of mitochondrial cytochrome c efflux and caspase-3 activation by Ro-31-8220. HL-60 cells were left untreated (Control) or were treated with 10 μM of the indicated compound for 5 h. S-100 extract was then prepared from the cells and analyzed for the status of procaspase-3 and cytochrome c

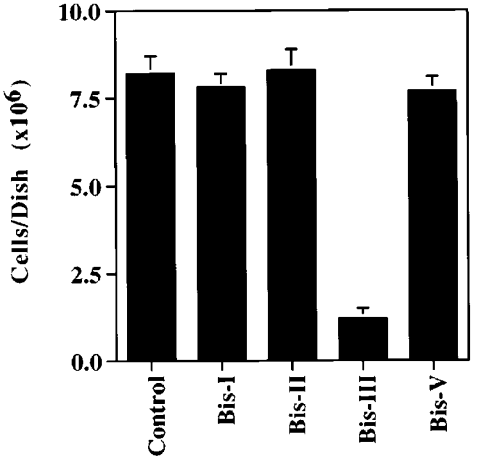

Figure 6 Effects of the Bis compounds on cell proliferation. HL-60 cells were left untreated (Control) or treated with 10 μM of the indicated compound for 4 days. Cell numbers in triplicate cultures were determined at the end of the treatment. The data presented here represent the result from a typical experiment. The assay was repeated twice

cells with Bis-I and Bis-II at concentrations that completely blocked PKC activity had no effect on either cell proliferation or survival. These results strongly suggested that members of the PKC family which are regulated by Bis compounds are not absolutely required for either of these cellular functions and, by extension, implied that these PKC isoforms are not the critical targets of STS. The STS analog, Ro-31-8220, however, induced apoptosis of HL-60 cells as well as the parental compound. Additional studies suggested that the apoptotic activity of Ro-31-8220 was due to the presence of a unique prosthetic group which enabled the compound to activate apoptosis via a mechanism which was PKC-independent, but suppressible by Bcl-2.

Induction of apoptosis by STS

All mammalian cells appear capable of carrying out apoptosis when provoked by the appropriate environmental cues.[2,4] Productive responses to apoptotic signals, however, appear to be quite cell-type specific. For example, low levels of ionizing radiation, to which most fibroblastic cells are refractory, potently induces apoptosis in lymphoid precursor cells.[44,45] Similarly, the withdrawal of growth factors drives most cells into quiescence, but causes hematopoietic cell lineages to undergo rapid apoptosis.[46] STS, is relatively unique in its ability to rapidly and completely drive virtually all mammalian cell lines into apoptosis.[23,47,48] Since STS is a nonselective inhibitor of a diverse array of protein kinases,[22,23] it is likely that the inhibition of one or more of these kinases is directly responsible for STS's apoptotic activity. In this study, we demonstrated that Bis-I and Bis-II had no effects on either proliferation or survival of HL-60 cells (Figures 2, 3, and 4). Others have demonstrated that Bis compounds including Bis-I are potent inhibitors of the PKC isozymes, PKC-α, -β, -γ, -δ, -ε, and -ζ, which are the same PKC isozymes inhibitable by STS.[31,34] These results, taken together, clearly demonstrate that the PKC inhibitory activity of STS can be separated from its ability to induce apoptosis and thus imply that PKC is not the relevant downstream kinase for STS-induced apoptosis. In addition, our experiments rule out the oncogenic kinase, Raf-1, as the downstream kinase. Thus, complete inhibition of Raf-1 phosphorylation – and therefore its activation – by the STS analogs, Bis-I, Bis-II and Bis-III did not induce HL-60 cells to undergo apoptosis (Figures 2, 3 and 4). Therefore, while the experiments described here cannot delineate which kinase(s) is targeted by STS, our results unequivocally rule out multiple PKC isozymes and Raf-1 as being that kinase. In a broader sense, the identification of the kinase targeted by STS begs the larger question of what are the relevant biological targets related to apoptosis of that kinase. The ultimate identification of the protein(s) affected by STS treatment is important since that protein(s) would be an extremely attractive target for chemotherapeutic intervention in malignant cells. The use of Ro-31-8220 or its derivatives may permit the identification of the target(s).

The roles of PKC in proliferation and survival of HL-60 cells

It has long been established that the activation of PKC in HL-60, as well as in other monocytic leukemia cell lines, leads to the differentiation of these cells into monocytes/macrophages.[49] This PKC-mediated cell differentiation process is inhibitable by PKC inhibitors. Indeed, we observed that all of the Bis compounds that were capable of inhibiting PKC blocked differentiation of HL-60 cells into monocytes following induction by the potent PKC activator, PMA (data not shown). However, the issue of whether or not PKC also played roles in

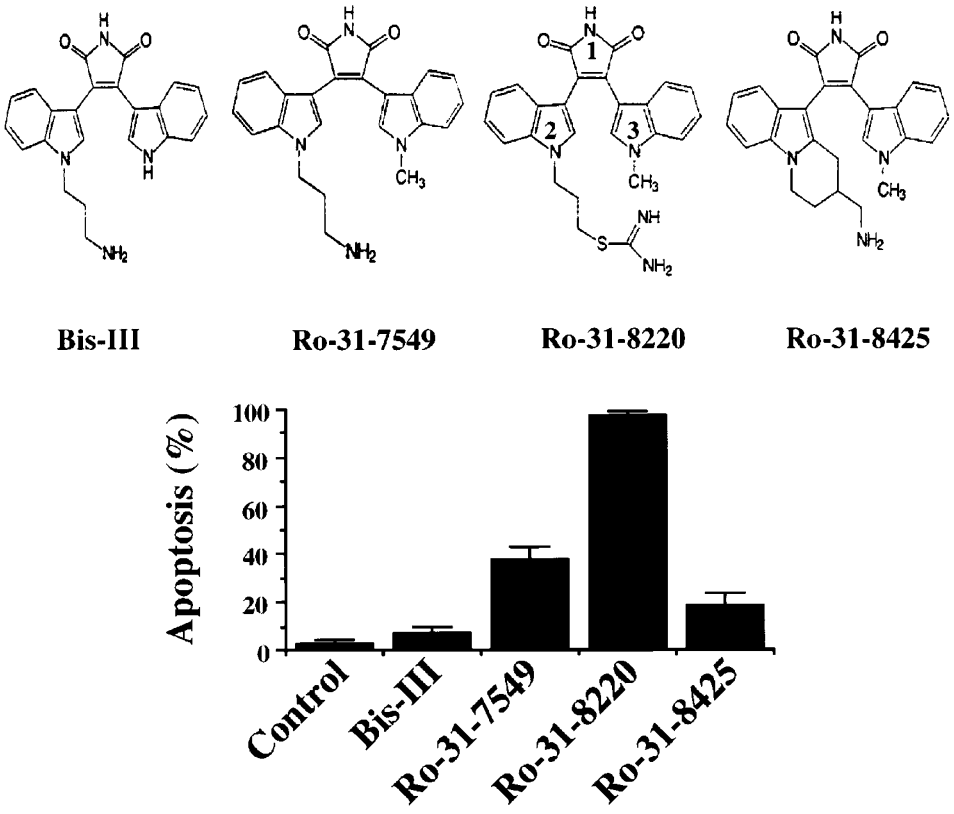

Figure 7 Structure-function analyses of Ro-31-8220 related compounds. For convenience, the three nitrogen atoms in the compounds are denoted as 1, 2 and 3. HL-60 cells were left untreated or treated with 10 μM of each compound for 24 h. The cells were then fixed, stained with DAPI, and the percentage of cells with apoptotic nuclear bodies was quantified. The average of two experiments is presented here

the proliferation and survival of HL-60 and other monocytic leukemia cells was much less clear, though numerous studies had suggested that this was likely to be the case.[19] Using the STS analogs, Bis-I, Bis-II, Bis-III, and Ro-31-8220, however, we were able to demonstrate that PKC is required neither for proliferation nor for cell survival in the HL-60 cell system. Thus, although Bis-I, Bis-II, Bis-III, and Ro-31-8220 were equipotent in their ability to inhibit PKC activity in HL-60 cells (Figure 2), Bis-I and Bis-II did not effect proliferation and survival of HL-60 cells while Bis-III and Ro-31-8220 inhibited the proliferation (Figures 4 and 6) and only Ro-31-8220 induced apoptosis of HL-60 cells (Figures 3 and 4). Bis-V, which does not inhibit PKC,[32,33] lacked any effect on the proliferation and survival of HL-60 cells (Figures 3, 4 and 6) and did not block PMA-induced monocytic differentiation (data not shown). Thus, these experiments strongly suggest that while PKC is required for cellular differentiation it is not essential for either proliferation or survival. Similar to our study, Wang *et al*. using a different STS analog, 7-hydroxystaurosporine (UCN-01), demonstrated that the inhibition of PKC by UCN-01 was not correlated with UCN-01's ability to induce apoptosis.[50] These authors suggested instead that the ability of UCN-01 to activate the histone H1 kinase activities of cdk-1 and cdk-2 might be casually related to the induction of apoptosis by UCN-01.[50] Therefore, it is also possible that the simultaneous inhibition of PKC and the activation of other target(s) such as cdk-1 or cdk-2 underlie the mechanism by which Ro-31-8220 induces apoptosis. However, this hypothesis remains to be investigated.

Induction of apoptosis by Ro-31-8220

Activation of the Jun N-terminal kinase (JNK) has been correlated with apoptosis induction by a diverse group of chemicals.[51–56] Further, it was demonstrated that treatment of Rat-1 fibroblast cells with Ro-31-8220 quickly induced JNK activation.[57] However, treatment of rat mesangial cells for a long period with Ro-31-8220 alone did not induce JNK activation, although this could be attenuated by tumor necrosis factor α (TNF).[54] Thus, although the treatment of the rat mesangial cells with Ro-31-8220 or TNF alone had no significant effect on the cell viability, co-treatment of the cells with Ro-31-8220 and TNF induced apoptosis in the majority of the cells within a period of 4–6 h.[54] It was then suggested that sustained JNK activation in the cells in the presence of Ro-31-8220 was required for apoptosis induction by TNF-α.[54]. In contrast to these studies, however, we detected no apparent changes in JNK activity in HL-60 cells during the apoptosis induction process by Ro-31-8220 as confirmed by JNK activity assays and Western blot analyses for the identification of activated (or phosphorylated) JNK (data not shown). We did detect a significant increase in JNK activity only in cells that had progressed to the end of the apoptosis process, i.e., after caspase-3 activation, degradation of caspase-3 target

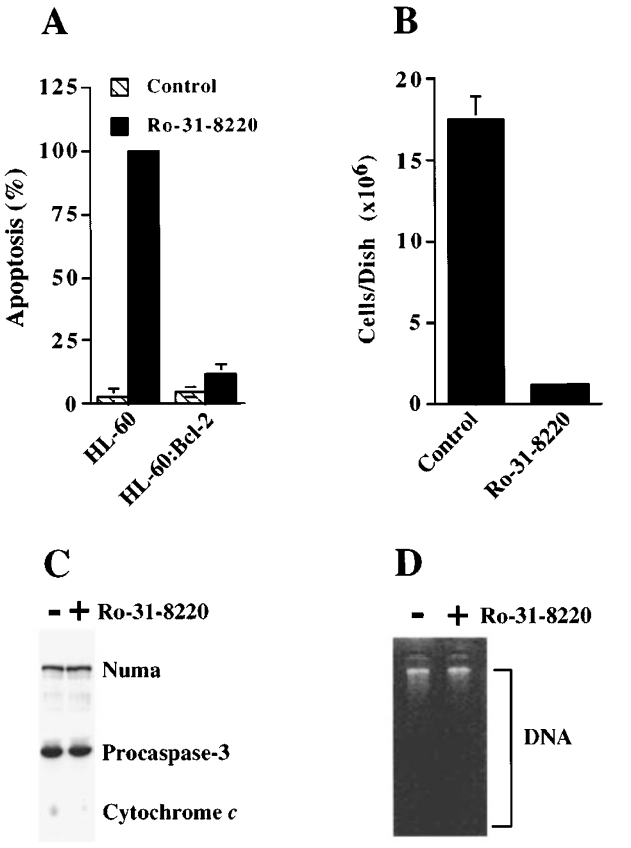

Figure 8 Overexpression of Bcl-2 in HL-60 cells blocks the apoptotic, but not the anti-proliferative, effect of Ro-31-8220. (A) Bcl-2 inhibits apoptosis induction by Ro-31-8220. DAPI staining analysis of apoptosis in HL-60 and Bcl-2:HL-60 cells cultured in medium in the absence or presence of 10 μM Ro-31-8220 for 72 h. (B) Ro-31-8220 inhibits proliferation of Bcl-2:HL-60 cells. HL-60 cells were cultured in the absence or presence of 10 μM Ro-31-8220 for 6 days. Cell numbers were then determined in triplicate. This figure represents the result from a typical experiment. (C) HL-60 cells were left untreated (−) or treated (+) with 10 μM Ro-31-8220 for 24 h. Whole cell or S-100 extracts were then prepared from the cells and used for the analysis of Numa cleavage, procaspase-3 activation and mitochondrial cytochrome c efflux. (D) Lack of DNA degradation in Ro-31-8220-treated Bcl-2:HL-60 cells. Bcl-2:HL-60 cells were left untreated (−) or were treated (+) for 24 h with 10 μM of Ro-31-8220. DNA samples were then prepared from the cells and analyzed by agarose gel electrophoresis

proteins, and DNA degradation (data not shown). Therefore, it is unlikely that JNK activation is required for the transduction of the apoptotic signal caused by Ro-31-8220 in HL-60 cells.

Another cellular factor that has been proposed to mediate, directly or indirectly, the apoptotic effect of Ro-31-8220 on glioblastoma cells is p53.[58] It was demonstrated that the treatment of two glioblastoma cell lines with Ro-31-8220 caused these cell lines to undergo apoptosis, which was preceded by the nuclear accumulation of wild-type p53, a protein that has been shown to be required for apoptosis induction in certain cell types.[41] However, since HL-60 cells are deficient in p53 expression due to large deletions in the gene,[59] it is clear that the Ro-31-8220-induced apoptosis in HL-60 cells occurred by a p53-independent mechanism.

Lastly, it should be noted that Ro-31-8220 and other Bis analogs have been reported to block apoptosis induction in cerebellar granule cells following deprivation of depolarizing concentrations of KCl.[25] In addition, it has been demonstrated that Ro-31-8220 not only lacked an apoptotic effect on immature rat thymocytes, but blocked the apoptotic effect of thapsigargin.[24] In contrast, our results show that Ro-31-8220 is a very potent inducer of apoptosis in HL-60 cells. These studies suggest that the apoptotic effect of Ro-31-8220 may be cell-type specific and suggest that caution should be used in extrapolating these data directly to other cell lines and/or tissues.

Ro-31-8220 is a model compound that can be used for the development of chemicals that modulate apoptosis and for the identification of cellular factors that regulate apoptosis

Given the extensive structural similarities of the Bis analogs, we expected that some of these compounds would compete with Ro-31-8220 for binding to the same cellular apoptosis regulator(s) and thus partially or completely block the apoptotic effect of Ro-31-8220. However, pre-exposure of HL-60 cells to excess amounts of Bis-I, Bis-II, Bis-III, or Bis-V had no detectable effect on the apoptotic response of the cells to Ro-31-8220. These results suggested that the apoptotic effect of Ro-31-8220 was mediated via a specific cellular factor(s) involved in apoptosis regulation, which did not interact with or was not regulated by the other Bis analogs. It can be envisioned that binding of Ro-31-8220 to this factor alters its activity, either positively or negatively, which then rapidly activates the apoptosis process. Therefore, identification of this putative Ro-31-8220 binding factor should provide information for the development of novel, specific inducers of apoptosis.

Our results showed that the N-2 thioamidine prosthetic group is largely responsible for the potent apoptotic activity of Ro-31-8220, and substitution of this group with an amine group such as the one in Ro-31-7549 reduced its apoptotic activity (Figure 7). However, it is also interesting to note that both Ro-31-8220 and Ro-31-7549 contain an N-3 methyl prosthetic group, which is absent on the other non-apoptotic Bis analogs (Figures 1 and 7). For example, Bis-III is an N-3 de-methylated version of Ro-31-7549 (Figure 7), and Bis-III completely lacked apoptotic activity (Figures 3 and 7). Therefore, as far as the apoptotic activity of Ro-31-7549 is concerned, the presence of the N-3 methyl group is required. By extension, we predict that the presence of the N-3 methyl group will also be required for the apoptotic activity of Ro-31-8220, and in its absence the apoptotic activity of Ro-31-8220 would probably be reduced. Therefore, the potent apoptotic activity of Ro-31-8220 is likely due to the presence of both the N-2 thioamidine and N-3 methyl groups.

The chemical pathways for the synthesis of Bis analogs including Ro-31-8220 are known,[32,33] and it is therefore possible to produce combinatorial libraries of Ro-31-8220 analogs with various modifications at the N-2 and/or N-3 positions in the maleimide ring. Further, the use of cell systems such as HL-60 and Bcl-2:HL-60 should make it possible to rapidly screen these combinatorial libraries. We envision that compounds which induce apoptosis of HL-60, but not Bcl-2:HL-60 cells can be quickly identified. Such compounds may have potential chemotherapeutic value.

Materials and Methods

Materials

Fetal bovine serum (FBS), sodium fluoride, sodium orthovanadate, EDTA, phenylmethylsulfonyl fluoride (PMSF), aprotinin, antipain, and leupeptin, were purchased from Sigma Chemical Co. (St Louis, MO, USA). Mouse monoclonal caspase-3 and c-Raf-1 antibodies were purchased from Transduction Laboratories, Inc. (Lexington, KY, USA). Mouse monoclonal Numa antibodies were purchased from Oncogene Research Products, Inc. (Cambridge, MA, USA). ECL Western blot analysis reagents were from Amersham Life Science, Inc. (Arlington Heights, IL, USA). The compounds Ro-31-8220, Ro-31-7549, Ro-31-8425, Ro-32-0432, and bisindolemaleimide-I, -II, -III, -IV and -V (Bis-I, Bis-II, Bis-III, Bis-IV and Bis-V, respectively) were purchased from Calbiochem-Novabiochem, Inc. (Boston, MA, USA), dissolved in dimethyl sulfoxide at a 10 mM concentration, and stored at -20°C. Under this storage condition, the biological activities of these compounds are very stable. HCl-free [^{32}P] phosphoric acid was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA, USA).

Cells stocks

HL-60 and HL-60 cells expressing a bcl-2 cDNA transgene (Bcl-2:HL-60 cells) have been described.[42,43] The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and 50 U/ml streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Cell proliferation

Cells ($0.5-1\times10^6$) were seeded in 5 ml of medium in 60-mm dishes in the presence or absence of various concentrations of the compounds tested. The total cell number per dish was then determined in triplicate every 24 h.

Determination of apoptosis by DAPI staining, analysis of DNA, and protein degradation

Cells were washed in PBS once and fixed in a 2% paraformaldehyde-0.1% Triton X-100 PBS solution for 30 min at 4°C. The cells were then washed in PBS once and stained with 100 ng/ml DAPI in PBS for 10 min at room temperature. An aliquot of the stained cells was then observed under a fluorescent microscope. Apoptotic cells were identified as those with densely stained granular nuclear bodies.[60] For the analysis of DNA degradation, fragmented DNA was isolated from cells and analyzed by agarose electrophoresis.[61,62] For the analysis of protein degradation, whole cell extract was prepared, and aliquots were subjected to electrophoresis and Western blot analyses for caspase-catalyzed cleavage of proteins.[60,63]

Isolation of cytosolic S-100 extract and detection of mitochondrial cytochrome c efflux, caspase-3 activation

HL-60 cells were either left untreated or were treated with various compounds. The cells were then washed with PBS and cytosolic S-100 extracts were prepared.[60] The protein concentration in the S-100 extract was determined and then adjusted to 10 μg protein/mL plus 50 mM KCl, 5% glycerol and then stored in small aliquots at -80°C. To assay for the presence of cytochrome c and the status of caspases, S-100 aliquots of 10 μL were subjected to SDS–PAGE and Western blot analyses.[60]

Detection of PKC-induced phosphorylation of c-Raf-1

Preliminary investigation showed that the pretreatment of HL-60 cells with Bis-I, Bis-II, Bis-III, or Ro-31-8220, but not Bis-V, caused a dose-dependent inhibition of PKC-catalyzed phosphorylation of Raf-1 when the cells were stimulated by phorbol-12-myristate acetate (PMA), a potent and specific PKC activator. Maximal inhibition of PMA-induced Raf-1 phosphorylation by the Bis analogs occurred between 7.5–10 μM (data not shown). Therefore, for all subsequent studies, we utilized the Bis analogs at a concentration of 10 μM. HL-60 cells were either left untreated or treated with 10 μM of each of the Bis analogs for 60 min and then the cells were stimulated with 100 nM PMA for 30 min. The cells were washed three times in PBS, pelleted by centrifugation, and then resuspended in three volumes of buffer B (10 mM HEPES, pH 7.5, 2 mM EDTA, 50 mM β-glycerophosphate, 5 mM sodium orthovanadate, 10 nM okadaic acid, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.3% Triton X-100 and 10 mg/ml each of antipain, aprotinin, and leupeptin), and incubated on ice for 5 min. The cells were broken by passing them five times through a 26-gauge needle fitted to a syringe, and the lysate was centrifuged at $12\,000\times g$ for 10 min at 4°C. The supernatant was further centrifuged at $100\,000\times g$ for 30 min at 4°C. The protein concentration of the clarified supernatant was determined, adjusted to 4 μg/μL and then small aliquots were stored at -80°C. An aliquot of 40 μg of S-100 extract from each sample was subjected to Western blot analyses to determine the phosphorylation status of c-Raf-1.

General phosphorylation of proteins in HL-60 cells

HL-60 cells were cultured in serum-free medium at a density of 3.5×10^6 cells/ml for 48 h with a medium change at the 24 h interval. [^{32}P]-Phosphoric acid was added to the culture medium (71 μCi/ml of cell culture), and the cells were incubated for another 3.5 h to allow the cells to accumulate an adequate pool of [^{32}P]ATP. The cells were then washed three times with serum-free medium and approximately 1.5×10^7 cells were cultured in 9 ml of serum-free medium containing either no drug or 10 μM Bis-I, Bis-II, Bis-III, Bis-IV, or Ro-31-8220. The cells were then incubated for 60 min after which time each culture received 1 mL of fetal bovine serum and then the incubation was continued for an additional 10 min. The cells were then pelleted by centrifugation, washed in serum-free medium once, and pelleted by centrifugation again. The cell pellet was lysed in 100 μL of lysis buffer (10 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 10 mM sodium orthovanadate, 100 nM okadaic acid, 1 mM PMSF, 1 mM DTT, 0.5% Triton X-100 and 50 μg/mL each of antipain, aprotinin, and leupeptin). The lysate was incubated on ice for 5 min, and centrifuged at $12\,500\times g$ for 10 min. The supernatant was combined with 30 μL of 4×-SDS-loading buffer (40% glycerol, 4% SDS, 20% β-mercaptoethanol, and 0.001% brilliant blue) and heated at 100°C for 5 min. Aliquots of 20 μL were then subjected to electrophoresis in 10% polyacrylamide gels and autoradiographic analyses.

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