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# Bisindolylmaleimides in anti-cancer therapy - more than PKC inhibitors

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## ABSTRACT

Bisindolylmaleimide derivatives were originally described as protein kinase C inhibitors. However, several studies have shown that bisindolylmaleimides target several other signaling molecules. The review presents bisindolylmaleimide-mediated PKC-dependent and PKC-independent biological effects, such as reversal of MDR and modulation of Wnt signaling through GSK-3b and b-catenin. Importantly, the potent proapoptotic properties of bisindolylmaleimides are also described. Bis-IX appears as the most efficient activator of intrinsic apoptotic pathway and additionally, facilitates extrinsic apoptosis. Presented molecular mechanisms indicate that bisindolylmaleimides could be useful agents in anticancer therapy. They repress uncontrolled proliferation and restore the sensitivity to chemotherapy which allows eradication of cancer cells.

**Key words:** bisindolylmaleimide, protein kinase C, Wnt signaling, multidrug resistance, apoptosis, death ligands, anti-cancer therapy

## INTRODUCTION

Cancer is the second leading cause of death in developed countries. Despite of the wide knowledge how cancer develops, the general progress in this field is still insufficient. Nowadays, the emphasis has been put to conduct a variety of basic and clinical studies in order to make use of different compounds, which could be useful in anti-cancer therapy. One of the promising strategies of cancer treatment is immunotherapy. The immunoediting [1], evolutionary developed ability to modify the apoptotic signal transduction, is the major mechanism of cancer cell resistance to apoptogenic stimuli. Immunotherapy is designed to restore the activity of immunological systems against tumor transformed cells by making use of apoptogenic cytokines (i.e. TNF-alpha-related apoptosis inducing ligand, TRAIL or interferons, IFN). The susceptibility of cancer cells to death receptor ligands (TNF-alpha family) could be facilitated by various metabolic inhibitors, which lead to blockade or degradation of antiapoptotic proteins. The review presents the evidences that bisindolylmaleimides could be seriously considered as useful adjuvants in the cancer treatment.

### Bisindolylmaleimide derivatives – well known PKC inhibitors

Bisindolylmaleimides were originally isolated from the culture broths of *Nacardiopsis* sp. by Nakanishi et al. [2]. *Nacardiopsis* sp. K-290 was found to produce several metabolites, among them the staurosporine aglycone, K-252c. K-252c has been shown to display some inhibitory activity against $Ca^{2+}$/calmodulin-activated phosphodiesterase (PDE) and protein kinase C (PKC) [2]. As a consequence, K-252 seriously affected the function of platelets, mast cells and several other cells.

To determine their actual inhibitory potencies and to perform structure-activity relationship studies Toullec et al. [3] carried out the complete synthesis of K-252c and its closely related analogues. The authors used standard method of indolocarbazole system synthesis, reviewed by Bergam et al. [4]. In general, the synthesis followed the pathway: indolyl magnesium bromides were condensed with dibromomaleimide and the resulting indolylmaleimides were subsequently transformed to the hexacyclic derivatives. Described method allowed a stepwise addition of the indolyl residues leading to

asymmetrically substituted compounds. Further, Toullec et al. [3] examined the ability of bisindole derivatives to inhibit PKC and two Ser/Thr protein kinases: the cAMP-dependent protein kinase (PKA) and phosphorylase kinase (PK). More than twenty indolyl derivatives have been tested. However, only one of them, called GF 109203X, containing additional indole residue appeared as a most powerful PKC inhibitor, and at the same time, was much less active against PKA or PK. Moreover, GF 109203X was also inactive against three receptor tyrosine kinases, namely EGF-R, PDGF-R and I-R [3]. Finally, GF 109203 X inhibited myosin light chain kinase (MLCK) in human platelets at higher concentrations than those required to suppress PKC activity in the same cells. The authors concluded that GF 109203X was a potent and selective PKC inhibitor. Importantly, Toullec et al. [3] evaluated the mechanism of GF 109203X-PKC interaction. Nishizuka et al. [5] revealed that PKC has two functional moieties, the catalytic and the regulatory domain. Toullec et al. [3] found that GF 109203 X did not inhibit the binding of [$^3$H]phorbol dibutyrate to PKC suggesting that the inhibition was not due to the interaction of GF 109203X with the diaclyglycerol-binding site of the regulatory domain. Moreover, the inhibitory activity of GF 109203X was not affected when the concentrations of $Ca^{2+}$ or phosphatidylserine were modified in the assay. These results allowed them to conclude that GF 109203X was not inhibiting PKC through the site on the regulatory domain of the kinase. Furthermore, the potency of GF 109203X was not altered by the protein substrate concentration in the assay and this compound, as other analogs of staurosporine, was a competitive inhibitor *versus* ATP. All these results taken together demonstrate that GF 109203X inhibits PKC activity exclusively via the ATP-binding site.

The biological functionality of GF 109203X Toullec et al. [3] tested in two cellular models: in human platelets and in Swiss 3T3 fibroblasts. For example in Swiss 3T3 cells the involvement of PKC in the induction of DNA synthesis has been determined by its ability to stimulate 80K phosphorylation and by the abrogation of the induction after down-regulation of PKC. These and other authors [6, 7] demonstrated that GF 109203X abrogated [3H]thymidine incorporation into DNA when cells were treated with agents reported to activate PKC. In contrast, GF 109203X did not affect the reinitiation of DNA synthesis elicited by growth factors or agents which activated signal transduction pathways other than PKC, i.e. cAMP-dependent and protein tyrosine kinases. To summarize, Toullec et al. [3] proved that GF 109203X is a potent and specific inhibitor of PKC *in vitro* and in intact cells. The authors estimated that in the future, GF 109203X will represent an important experimental tool to determine the PKC dependence of particular signal transduction mechanisms in living cells. At the same time, such an inhibitor should enable the role played by PKC in different pathophysiological processes to be explored. In fact, nowadays, ten bisindolylmaleimide derivatives, also GF 109203X, known as bisindolylmaleimide I (Bis-I) are routinely used as metabolic inhibitors of PKC (from Bis-I to Bis-XI). For example, Bis-II was effective PKC inhibitor in C6 glioma cell line. The inactivation of PKC abolished phorbol 12-myristate 13-acetate PMA-induced increase in sodium-dependent transporters activity related to EAAC1 (neuronal glutamate transporter) expression [8]. On the other hand, in HL-60 cell line Bis-I and Bis-III were able to completely block PKC-dependent Raf-1 activation, the known downstream target of PKC [9]. Numerous data indicate that bisindolylmaleimides influence PKC [10–13]. However, most recent reports showed that some of the bisindolylmaleimide derivatives are able to modulate other, PKC-independent signaling pathways.

## Bisindolylmaleimides regulate c-Jun N-terminal kinase (JNK)/glycogen synthase kinase-3 (GSK-3) pathway

Beltman et al. [14] investigated the selectivity of bisindolylmaleimides for PKC isozymes in Rat-1 fibroblasts. It is known, that PKC is a multifunctional protein kinase family whose members exhibit distinct properties including sensitivity to calcium and the phorbol ester family of tumor promoters. Various PKC kinases have been implicated in diverse cellular processes including inflammation, mitogenesis and differentiation. It was therefore of considerable interest to identify, whether the observed effects are the consequences of PKC family or their individual isozymes action. Beltman et al. [14] used five different bisindolylmaleimides, among them Bis-I (GF 109203X) and Bis-IX (Ro-31-8220), to evaluate targeted PKC isoforms. They found that Bis-IX was selective inhibitor of calcium-, diterpine-, and phorbol ester-activable PKC isozymes $\alpha$ and $\varepsilon$, while Bis-I was an equally effective inhibitor of basal PKC activity composed of $\alpha$, $\varepsilon$ and $\xi$ activity of activated PKC. Based on several analyses, Beltman et al. [14] concluded, that Bis-I is less selective for particular PKC isozymes than Bis-IX, although the molecular reasons for these observations were not explained. More interestingly, the authors noticed that Bis-IX has also unique and novel properties. Firstly, Bis-IX inhibited growth factor-stimulated expression of MAPK phosphatase-1 (MKP-1) and c-Fos. Activation of PKC by PMA is sufficient to induce both *mkp-1* and *c-fos* expression indicating that PMA-sensitive isoforms can at least impinge on the signaling pathways regulating the expression of these two genes. Inhibition of PKC or the depletion of PKC by chronic PMA treatment inhibited PMA-stimulated *mkp-1* and *c-fos* expression. However, detailed analysis revealed that LPA- or EGF-stimulated *mkp-1* and *c-fos* expression is mainly inhibited by Bis-IX but not by Bis-I. In the case of *mkp-1*, this inhibition of expression resulted in the potentiation of the sustained phase of LPA- and EGF-stimulated ERK1 activity [14]. In contrast, Bis-I did not potentiate LPA- and EGF-stimulated ERK activity, suggesting that it is not a PKC-mediated effect but rather specific effect of Bis-IX.

Beltman et al. [14] hypothesized that Bis-IX acts by inhibiting PKC-related kinases (PRK-1 or PKN), however,

they did not verify this assumption. Finally, also Bis-IX, but not Bis-I stimulated c-Jun N-terminal kinase (JNK1) and induced c-Jun expression. First, PMA, a potent PKC activator, was not able to stimulate JNK1 activity. Secondly, neither initial down-regulation of PKCα, -β and -ε, nor the inhibition of PKCα, -β and -γ by Bis-I had any effect on the ability of Bis-IX to activate JNK1 or to stimulate c-Jun expression. All these results indicate that the presence of PKC protein and PKC catalytic activity are unrelated to the ability of Bis-IX to stimulate stress signaling pathway [14]. Based on Beltman et al. [14], Standaert et al. [15] investigated Bis-IX-induced c-Jun activation in context of insulin action in rat adipocytes and L6 myotubes. Standaert et al. [15] previously noticed in studies on insulin action that Bis-IX provoked increase in glycogen synthase (GS) activity in rat adipocytes. They initially postulated that stimulation of GS might be due to an inhibition of basal activity of PKC, which directly phosphorylates and thereby inhibits GS [16,17]. PKB, in turn, appears to be largely regulated through insulin-induced increase in phosphatidylinositol-3-kinase (PI3-K) activity [18], presumably via its lipid products [19]. However, from the performed studies it was evident that despite its inhibitory influence of Bis-IX on ERK1/2, the activation of GS by Bis-IX cannot be explained by changes in the activity of either ERK1/2 or PKB evidenced by PKB electrophoretic mobility or immunoprecipitation analyses. Moreover, because other PKC inhibitors did not affect glycogen synthesis, it seems more convincing that the activating effect of Bis-IX on GS cannot be explained solely by Bis-IX impact on basal PKC activity. Finally, unlike insulin, activating effect of Bis-IX on GS was independent of PI3-K. Thus, the JNK pathway appears as the most likely candidate to explain the stimulatory effect of Bis-IX on GS activation. This belief was supported by previous report concerning the ability of anisomycin, another activator of JNK [14] that induces GS in skeletal muscle [20]. In rat adipocytes anisomycin was also able to activate GS, albeit much less efficiently than Bis-IX [15]. In spite of significant role of JNK activity in GS stimulation, Standaert et al. [15] suggested that JNK activation was unlikely to serve as a major mechanism for stimulatory effects of insulin on glycogen metabolism. Indeed, although insulin provoked increase in JNK activity in both rat adipocytes and L6 myotubes, these insulin-induced increases were not additive to increase in JNK activity that were provoked by Bis-IX. Thus, it was concluded that insulin and Bis-IX-induced GS activation is mediated by two distinct signaling pathways. The molecular mechanisms Bis-IX-induced GS activation was explained further by Hers et al. [21]. Based on the Woodgett [22] and Shepherd et al. [23] that glycogen synthase kinase-3 (GSK-3) phosphorylates and inhibits glycogen synthase, Hers et al. [21] investigated the role of GSK-3 in bisindolylmaleimides action in rat adipocytes. They found that Bis-I and Bis-IX strongly inhibited GSK-3 activity when added directly to cell lysates and in GSK-3β immunoprecipitates. Bis-IX was the more potent inhibitor of GSK-3 activity, with an approximately 100 times lower $IC_{50}$ value than Bis-I. The authors supposed that Bis-I and Bis-IX compete reversibly with ATP for binding to the nucleotide-binding site of GSK-3, as proposed for PKC [3]. The loss of the inhibitory effect of the agents during immunoprecipitation of GSK-3β from cells previously exposed to them was compatible with this hypothesis. The fact, that Bis-I had a little effect on insulin-induced inhibition of GSK-3, under conditions where it would be expected to potently inhibit PKC, suggested that PKC was not implicated in GSK-3 regulation. With regard to Bis-IX it was concluded that glycogen synthase stimulation is the result of GSK-3 inactivation in a PI3-K-independent manner. Either effect of insulin or Bis-IX on GSK-3 inhibition, which were not additive, were explained by the ability of insulin to activate protein phosphatase-1 [24], and hence the dephosphorylation of glycogen synthase by a mechanism independent of GSK-3.

Hers et al. [21] focused also on the second substrate of activated JNK, c-Jun. c-Jun is a part of activating protein-1 complex (AP-1), which could be phosphorylated by JNK in two regulatory sites Ser-63 and Ser-73. Phosphorylation of these sites transactivates c-Jun, and may also explain the increased *c-jun* expression induced by bisindolylmaleimide IX [14]. Stimulation of AP-1 activity in response to Bis-IX was, therefore, the result of increased c-Jun synthesis and its phosphorylation by increased JNK activity (25). On the other hand, GSK-3 phosphorylates c-Jun on three sites in a region proximal to the DNA-binding domain resulting in decreased c-Jun DNA binding and transcriptional activity [26]. Indeed, transfection experiments had shown that AP-1 activity is inhibited by co-expression of GSK-3 [27]. Inhibition of GSK-3 activity by Bis-IX might therefore abolish this negative restraint, thereby increasing c-Jun/AP-1 activity [21]. The ability of bisindolylmaleimide IX to inhibit GSK-3 activity prompted Zhang et al. [28,29] to synthesize new bisindolylmaleimide derivatives, which selectively targeted GSK-3. Especially, novel bisindolylmaleimide pyridinophanes seems to be possible potent and specific GSK-3 inhibitors.

## Bisindolylmaleimides modulate Wnt signaling pathway

Wnt signaling is initiated by binding of the Wnt proteins to their receptors called frizzled (FZ) [30]. Two major signaling pathways have been described for Wnts. The canonical Wnt pathway involves inactivation of GSK-3β/APC complex. Inactivation of GSK-3β results in decreased phosphorylation and thus increased stability of β-catenin. Elevated level of β-catenin allows it to enter the nucleus and interact with Lef-1/Tcf transcription factor [30]. Wnt activates dishevelled (Dsh) protein which can also signal through Rho GTPase and JNK instead of β-catenin and Lef-1/Tcf [31]. A second major mechanism of Wnt signaling involves a G protein-mediated activation of a phosphoinositide pathway (PI3-K-dependent) resulting in increased activities of $Ca^{2+}$-activated enzymes, such as PKC [32,33]. Wnt pathways are one of the major mechanisms of embryonic development and may be

functionally redundant. However, the high activity of Wnt proteins is also characteristic for cancer cells. It is well known, for example, in the absence of APC, hyperactive canonical pathway components (β-catenin-mediated), govern the colon cancer tumorigenesis [34]. Loss of APC was found to induce the inappropriate stabilization and accumulation of β-catenin [35]. Consequently, β-catenin/Tcf complex is constitutively active and lead to uncontrolled cell proliferation. Thus, the compounds which are able to destabilize β-catenin could be promising tool in anticancer research. There is only one paper concerning the ability of bisindolylmaleimide to directly regulate Wnt canonical pathway. Cho et al. [36] found that Bis-I treatment increased β-catenin cytoplasmic level in 3T3-L1 adipocytes. However, the β-catenin mRNA level did not change in response to different concentrations of Bis-I. The authors concluded that Bis-I activated the Wnt/β-catenin signaling pathway via the up-regulation of β-catenin protein stability. Further, it was found that 3T3-L1 adipocytes treated with Bis-I showed a consistent and robust, concentration-dependent increase in Tcf transcription factor activity.

The examination of 3T3-L1 preadipocytes differentiation by Oil Red O staining revealed the inhibition of this phenomenon. Additionally, Cho et al. [36] showed that Bis-I administration reduced adipocytes lipid binding protein aP2, which is downstream of C/EBPα and PPARγ transcription factors, known hallmarks of adipocytes differentiation. The same group reported previously, that PKC phosphorylates Ser33/37 of β-catenin, resulting in the promotion of β-catenin degradation [37]. In addition, the small interference RNA (siRNA)-mediated knock-down of PKCα inhibited Ser33/37 phosphorylation of β-catenin and induced the accumulation of this cytoskeleton protein in cells. The authors hypothesized that the observed Bis-I effect is mediated by PKC inhibition, however, they did not perform any analysis to visualize the PKC phosphorylation status in the presence of Bis-I. As described in previous section it is possible that Bis-I-induced inhibition of adipocytes differentiation was not only the result of PKC but also GSK-3β inactivation. Hence, it is believed that PKC family plays a major role in non-canonical Wnt signaling pathway. Koyanagai et al. [38] reported that non-canonical pathway activation determines the circulating progenitor cells (CPCs) to differentiate into cardiomyogenic cells. The use of bisindolylmaleimides: Bis-I or Bis-III resulted in decreased PKC phosphorylation and significantly reduced differentiation of CPCs. Controls confirmed that the inhibition of cardiac differentiation by the use PKC inhibitor did not result from reduced cell survival. In contrast, inhibition of JNK did not affect cell differentiation, whereas JNK appeared to be of importance. Bis-I-mediated inhibition of non-canonical Wnt pathway was also reported by Chou et al. [39]. They found that Bis-I was able to reverse the inhibition by Wnt1 or by Wnt3a of NGF-induced neurite outgrowth and the inhibition by Wnt1 of NGF-induced SCG10, late response gene expression. However, Bis-I did not reverse the activation by Wnt1 of Lef-1/Tcf, demonstrating that Bis-I did not affect canonical Wnt pathway.

The authors concluded that Bis-I acted by affecting either a pathway downstream from Lef-1/Tcf in the canonical pathway or a Wnt signaling pathway other than the canonical pathway. Additional studies with pertussis toxin and anti-activated JNK antibody showed, that neither the Wnt/$Ca^{2+}$ nor Dsh/JNK pathway were involved in the inhibition by Wnt1 of NGF-induced neurite outgrowth. Interestingly, none of other tested PKC inhibitors, among them Bis-IX (Ro 31-8220) and staurosporine, reversed the effect of Wnt1. Two possibilities were accounted for these results. First, the compounds selectively inhibit different PKC isozymes. Although, Bis-IX is structurally related to Bis-I, it does not possess a different specificity for PKC kinases [40]. The second possibility is that Bis-I affected Wnt signaling pathway affecting other protein than PKC. Chou et al. [39] recalled the results published by Hers et al. [21] and possible modulation of GSK-3β by Bis-I. However, the exposure of PC12 cells to myo-inositol, which also inhibits GSK-3β activity and activates down-stream portion of the canonical Wnt pathway [41] did not block NGF-induced SCG10 expression even though Wnt1 was not expressed. The new results published by Cho et al. [36], on the involvement of PKC in canonical-pathway of Wnt signaling, suggest that the Bis-I action in PC12 cells is similar to above-described Bis-I action in adipocytes. Regardless of cellular target of bisindolylmaleimides, in particular Bis-I, it is evident that Bis-I is able to modulate Wnt pathway and reduces cell differentiation [36, 38] and uncontrolled proliferation [36]. It is possible that similar effects might be observed in cancer cells, however, up today, no studies concerning the Wnt pathway and bisindolylmaleimides action in cancer cells were done in detail.

## Bisindolylmaleimides reverse multidrug resistance (MDR) in tumor cells

First report concerning the modulation of MDR proteins by bisindolylmaleimide derivatives were published by Gekeler et al. [42]. Human leukemia cell line HL60 and its non-P-gp MDR subline were incubated with GF 109203X (Bis-I) and afterwards, the accumulation of rhodamine 123 within the cells was analyzed by flow cytometry. In the absence of Bis-I, the enhanced efflux of the fluorescent dye rhodamine 123 was observed from MDR overexpressing HL60 subline in comparison to parental HL60 cells. However, when Bis-I (10 μM) was added, a strong inhibition of the dye efflux was seen. It demonstrated that GF 109203X affected transport function was most likely associated with MRP/P-gp protein (the main representative of MDR proteins family). Importantly, 10 μM Bis-I concentration did not reveal any effect on cellular proliferation or vitality of the cells, what was confirmed by flow cytometry and MTT assay. Furthermore, the cDNA-PCR gene expression analysis showed that the GF 109203X did not significantly alter the expression of the *mrp/P-gp* gene at the mRNA level [42]. The authors supposed that, either a PKC dependent function could be disturbed by GF 109203X, i.e. phosphorylation of MRP/P-gp, or/and the compound interacts

directly with the drug transporter. Because of the rather high concentration of GF 109203X necessary to modulate the resistance of non-P-gp MDR cells, the assumption of a direct interaction with the transporter appeared to be reasonable. It was postulated that Bis-I affected ATP-binding site(s) of MRP/P-gp, similarly to PKC inhibition.

A year later the same group published results from similar studies performed on MDR-1/P-gp-transfected cells [43]. The efficacy of GF 109203X in modulation of MDR was examined using P-gp-overexpressing several cell lines, such as MDR-1/P-gp HeLa cells, and was compared with the activities of dexniguldipine-HCI (DNIG) and dexverapamil-HC1 (DVER), both of which essentially act via binding to P-gp. As PKCα has been suggested to play a major role in P-gp-mediated MDR [44], cell lines exhibiting different expression levels of this PKC isozyme were chosen for study. At up to 1 μM final concentrations of the PKC inhibitor GF 109203X, at which many PKC isozymes should be blocked substantially, no cytotoxic or MDR-reversing effects were seen, as monitored by 72 h MTT or a 90 min rhodamine 123 accumulation assays. Moreover, depletion of PKCα by phorbol ester in MDR-1/P-gp HeLa transfectants had no influence on rhodamine 123 accumulation after 24 and 48 hours. Thus, similarly to previous results from HL-60 cells, Gekeler et al. [43] concluded, that GF 109203X influenced MDR mostly via direct binding to P-gp, but did not confirm the concept of a major contribution of PKC to P-gp-associated MDR. The role of bisindolylmaleimide derivatives in multidrug resistance of cancer cells was investigated also by Budworth et al. [45]. In contrast to previously described observations, they found that neither GF 109203X (Bis-I) nor Ro 31-8820 (Bis-IX) decreased efflux of the P-gp probe rhodamine 123 from MCF-7/Adr cells [45]. In MCF-7/Adr cells, apparently the most potent P-gp inhibitor was staurosporine. It is possible that in MCF-7 breast cancer cells, P-gp activity depends on isozyme of PKC family, which is not inhibited by tested bisindolylmaleimide derivatives, but could be blocked by staurosporine. The possibility that bisindolylmaleimides-mediated MDR modulation is cell-type specific is supported by results published by Merritt et al. [46].

It was shown that Ro 32-2241 completely blocked the efflux of [$^3$H]-daunomycin from P-gp-expressing KB-8-5 and KB-8-5-11carcinoma cells, resulting in increased daunomycin accumulation. Moreover, Ro 32-2241 was more potent than Verapamil and showed potency similar to that of cyclosporine A, both well known P-gp antagonists, in reversing resistance in these cells. Since Ro 32-2241 inhibits PKC in an ATP-competitive manner and binding of ATP to P-gp is required for drug efflux, the possibility that Ro 32-2241 might interact directly with P-gp was considered. Evidence was obtained suggesting that Ro 32-2241 is a substrate for P-gp. [$^{14}$C]-Ro 32-2241 efflux from the KB-8-5 cells appeared to not differ from the very small baseline leak seen from drug-sensitive KB-3-1 cells, whereas there was a higher level of efflux from the KB-8-5-11 cells. Ro 32-2241 clearly inhibited binding of [$^3$H]-azidopine to P-gp. Thus, the effects of Ro 32-2241, like the effects of other PKC inhibitors, may be a result of direct interaction with P-gp, inhibition of PKC, or some combination of both mechanisms. Regardless of the model of Ro 32-2241 action it appeared highly effective at inhibiting drug efflux from MDR cells *in vitro*. According to Merritt et al. [46] Ro 32-2241 completely restored the sensitivity of KB-8-5 and KB-8-5-11 cells to doxorubicin. The resistance of the KB 8-5 cells to taxol was completely reversed by Ro 32-2241, and that of the KB-8-5-11 cells was partially reversed. Merritt et al. [46] examined also the effects of Ro 32-2241 on resistant cells *in vivo*. Surprisingly, they were not able to demonstrate a significant increase in the doxorubicin toxicity on tumor mass under conditions where the plasma concentration of Ro 32-2241 should be capable of reversing P-gp activity as based on *in vitro* studies.

## Bisindolylmaleimides induce intrinsic apoptosis in cancer cells

The most promising mechanism of bisindolylmaleimides action in cancer cells is the ability to initiate apoptosis. It is noteworthy, that almost all results which demonstrated Bis-mediated apoptosis induction were performed with Bis-IX (Ro 31-8220) [9, 47–49]. According to Zhu et al. [47] Ro 31-8220 inhibited the growth of gastric cancer cells (AGS), arrested cells at the $G_0/G_1$ phase, and induced cell death by apoptosis. Further evidences were provided by their previous studies, where PKC activation by 12-*o*-tetradecanoylphorbol (TPA) rescued the apoptosis of AGS cells induced by nonsteroidal anti-inflammatory drugs (NSAIDs) [50]. The analysis of apoptosis-related genes showed that Bis-IX increased both p53 and $p21^{waf1/cip1}$ proteins expression, indicating that p53 may be involved in Bis-IX-induced apoptosis as well as cell cycle kinetics and apoptosis in gastric cells. The PKC activation by TPA caused the up-regulation of c-myc expression from its pol II promoter [50]. On the other hand, Xu et al. [51] demonstrated PKC-dependent inhibition of c-myc transcription from the pol III promoter. Ro 31-8220 also increased the protein level of c-myc [47] and according to the authors conclusions, serve as a crucial step in apoptosis of gastric cancer cells. Further, the evaluation of *bcl-2* gene family, showed that Bis-IX induced overexpression of bax, but had no effect on the expression of bcl-2. The authors did not consider the presence of other than PKC-mediated molecular mechanism of Bis-IX action. Moreover, a key role of PKC in Bis-IX-induced apoptosis was contested by other authors [9, 48, 49].

In 2000, Han et al. [9] investigated the effects of several bisindolylmaleimide derivatives: Bis-I, Bis-II, Bis-III, Bis-V and Bis-IX on proliferation, differentiation or apoptosis of HL-60 human promyelocytic leukemia cells. First of all, the role of PKC in tested bisindolylmaleimide derivatives action was verified and the effects of several Bis on c-Raf-1 phosphorylation, a known PKC family target, were examined. Treatment of HL-60 cells with 100 nM phorbol-12-myristate acetate (PMA) caused the hyperphosphorylation of c-Raf-1.

However, pretreatment of the cells with Bis-I, Bis-II, Bis-III or Bis-IX, but not Bis-V, completely blocked this phosphorylation. In contrast, none of these compounds showed significant inhibitory effect on the phosphorylation status of proteins in HL-60 cells stimulated by serum. Thus, these results confirmed the *in vitro* data obtained previously by Davis et al. [52, 53] that Bis-I, Bis-II, Bis-III and Bis-IX were relatively selective and potent inhibitors of PKC. Next, the biological effects of these compounds on HL-60 cells were investigated. Treatment for 24 hours of HL-60 cells with Bis-IX, but not other Bis compounds induced apoptosis. It was demonstrated, that Bis-IX induced chromatin condensation, cleavage of NUMA (a nuclear target of caspases during apoptosis), efflux of mitochondrial cytochrome *c* to the cytosol, followed by caspase 3 activation [9]. It was concluded that apoptotic effect of Bis-IX on HL-60 cells was mediated by a well-characterized transduction process of apoptotic signal. Prolonged treatment of HL-60 cells with other Bis derivatives for up to 4 days failed to induce apoptosis. Moreover, Bis-I, Bis-III and Bis-V did not have observable effects on cell proliferation, whereas Bis-III profoundly inhibited proliferation in cell growth assay. These experiments clearly demonstrate that the inhibition of PKC was a separable, distinct and unrelated event from apoptotic induction and cell proliferation. To experimentally test this hypothesis, Han et al. [9] pretreated HL-60 cells with Bis-I or Bis-II or Bis-III or Bis-IV or Bis-V and then 10 μM of Bis-IX was added to the culture and incubation continued for 20 h. The ability of Bis-IX to induce apoptosis was not affected by the presence of any Bis analog. Intrinsic apoptotic pathway initiated by Bis-IX was inhibited by Bcl-2 overexpression, whereas it failed to prevent its inhibitory effect on cell proliferation. To explain the different biological effects of Bis derivatives Han et al. [9] analyzed their chemical structure and found that N-2 thioamidine prosthetic group was largely responsible for the potent apoptotic activity of Bis-IX. Further, the substitution of this group with an amine group (Ro 31-7549) reduced its proapoptotic activity. However, it is also interesting 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. For example, Bis-III is an N-3 de-methylated version of Ro 31-7549, and Bis-III completely lacked apoptotic activity. Therefore, Han et al. [9] suggested that as far as the apoptotic activity of Ro 31-7549 is concerned, the presence of the N-3 methyl group is required. They also predict that N-3 methyl group is required for the apoptotic action of Bis-IX and its absence reduced the proapoptotic potential. Finally, it was concluded that the potent apoptotic activity of Bis-IX was due to the presence of both the N-2 thioamidine and N-3 methyl groups.

Bis-IX appeared as the most potent apoptosis inducer among bisindolylmaleimide derivatives in chronic lymphocytic leukaemia (CLL) cells [48]. According to Snowden et al. [48] Bis-IX-induced cell death occurred primarily by perturbation of mitochondria and activation of the intrinsic apoptotic pathway, consistently with previous results. Similarly to HL-60 cell line, Bis-IX caused the release of the proapoptotic mediators cytochrome *c*, Smac/DIABLO and Omi from mitochondria of CLL cells. Moreover, it was demonstrated that Bis-IX activated caspase 9 as the initiator caspase. Interestingly, on exposure to Bis-IX, processing of both the initiator caspase 8 and 9 and the effector caspase 3 and 7 was observed, making it difficult to distinguish between both pathways. However, more detailed studies showed that caspase 8 was activated as a downstream caspase, as described previously in Jurkat cells [54]. Snowden et al. [48] focused also on Bcl-2 family proteins and their role in Bis-IX-induced intrinsic apoptosis. It was found that the Bis-IX-mediated apoptosis was accompanied by a conformational change and translocation of cytosolic Bax to mitochondria. At the same time, the Mcl-1 protein cleavage occurred. It was one of the first reports showing that cleavage of Mcl-1 protein occurs during apoptosis execution. Mcl-1 is commonly expressed in CLL cells, and its high levels strongly correlate with a failure to achieve complete remission of leukaemia. In addition, elevated levels of Mcl-1 were found at time of relapse, in cells from patients with acute myelogenous and acute lympocytic leukaemia [55]. Hitherto, the Mcl-1 overexpression is known to be the antiapoptotic factor in many cancer types, such as melanoma, hepatocellular carcinoma and cholangiocarcinoma, and Mcl-1 elimination correlates positively with cell death induction [56-58]. Thus, the Bis-IX-mediated Mcl-1 cleavage might facilitate a feed-forward amplification loop in drug-induced apoptosis.

The potent-Bis-IX proapoptotic action was also demonstrated in our research performed on colon adenocarcinoma COLO 205 cells [49]. We found that Bis-IX caused intrinsic apoptotic pathway activation, represented by cytochrome *c* efflux and caspase 9 cleavage. The Bcl-2 protein delivery into COLO 205 cells rescued cells from Bis-IX-induced cell death. Interestingly, we found that Bis-IX was also able to facilitate extrinsic apoptotic pathway triggered by death ligand TNF-α. This property of Bis-IX was also reported previously by other authors and indicates another, less known molecular mechanism of its action.

## Bisindolylmaleimides sensitize cancer cells to death ligand-induced apoptosis

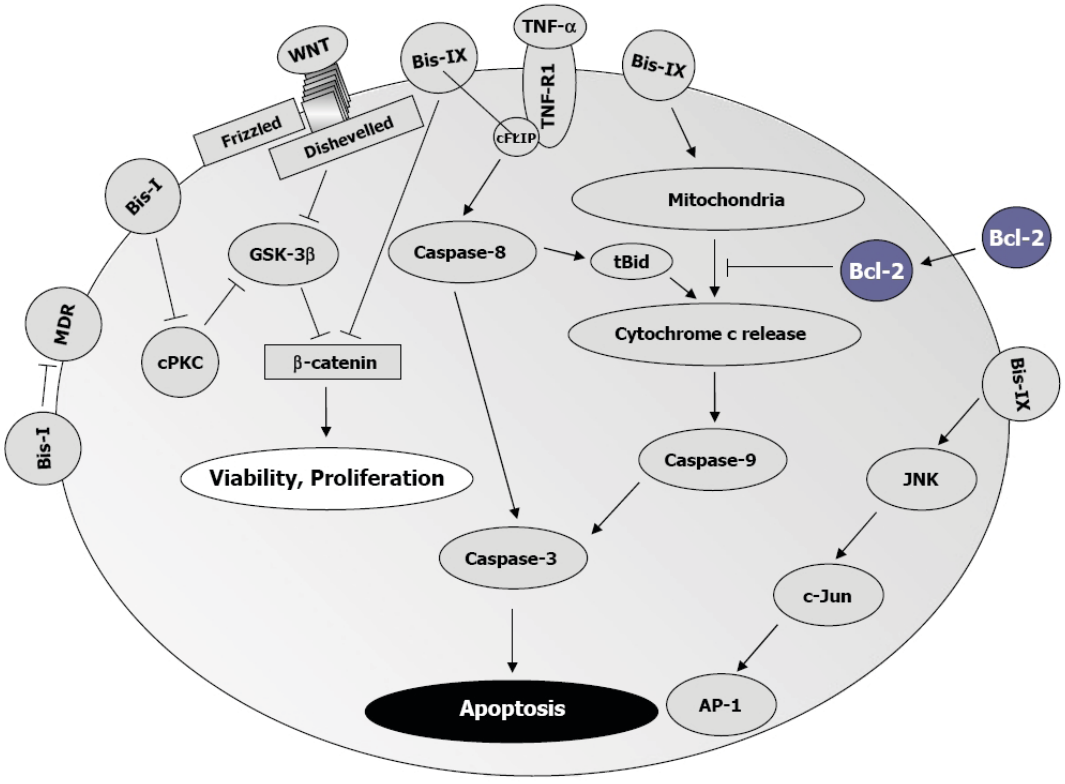
In contrast to chemotherapy and irradiation, death receptors of TNF-α family induce apoptosis in a p53-independent manner and thus represent attractive therapeutic tool for treatment or co-treatment of cancer. Ligand-induced trimerization of these receptors triggers apoptosis via the recruitment of adaptor protein FADD, which subsequently helps in the recruitment and activation of procaspase 8 (FLICE). Activated caspase 8 leads to activation of downstream effector caspases either directly (Type I cells) or via an amplification loop involving truncated Bid-mediated release of cytochrome *c* from the mitochondria (Type II cells) [59]. However, a great deal of cancers is resistant to death ligand-induced apoptosis. There are different cancer type-specific strategies of apoptotic resistance, which in general are determined by expression of antiapoptotic and/

or down-regulation/mutation of proapoptotic molecules [60]. Improving the anti-tumor activity of death ligands by use of metabolic inhibitors, such as bisindolylmaleimide derivatives is an ongoing area of investigation. The first report, showing that bisindolylmaleimides restore the sensitivity of transformed cells to death ligand-induced cell deletion was published by Zhou et al. [61]. Examination of cells that were stimulated with anti-Fas agonistic antibody alone or with Bis-VIII showed that Bis-VIII converted human astrocytoma 123N1 cells from being entirely resistant to Fas-mediated apoptosis to being highly sensitive to it. Although 132N1 cells express functional Fas receptors on the cell surface they are relatively resistant to apoptosis induced by anti-Fas antibody. Bis-VIII induced highly significant increase in apoptotic cells in the presence of anti-Fas antibody was dose-dependent. Thus, with 100 ng/mL anti-Fas antibody the potentiating effect was clearly evident at a concentration of Bis-VIII as low as 1 μM and nearly complete apoptosis was obtained with 3 μM of Bis-VIII. The potentiation by Bis-VIII was also dependent on the dose of anti-Fas antibody. Example, with 3 μM Bis-VIII, apoptosis was induced in more than 70% of 123N1 cells after addition of 50 ng/mL anti-Fas antibody [61]. Moreover, it was demonstrated that Bis-VIII also enhanced apoptosis induced by TNF-α, which given individually at 5-20 ng/mL TNF-α caused no substantial increase in apoptosis. Incubation with Bis-VIII facilitated apoptosis induced by TNF-α in a concentration-dependent manner, with 20 ng/mL TNF-α-inducing apoptosis in over 60% of cells in the presence of 10 μM Bis-VIII. In contrast, Bis-VIII had little effect on apoptosis of 123N1 cells treated with dexamethasone or irradiation. These results indicate that Bis-VIII selectively facilitates signal transduction mechanisms of apoptosis induced by activation of TNFR family. Among eight other tested bisindolylmaleimide derivatives only Bis-IX was able to restore the susceptibility of 123N1 cells to proapoptotic action of death ligands. Interestingly, this effect was not achieved when Bis-I or Bis-II or Bis-IV was administrated. On the other hand, Bis-III, Bis-X and Bis-XI produced intermediate potentiation [61]. Zhou et al. [61] postulated that Bis-VIII-mediated apoptosis of 123N1 cells is PKC-independent. Three other PKC inhibitors, structurally unrelated to bisindolylmaleimides, such as H7, calphostin and chelerythrine chloride, were tested and all of them failed to potentiate Fas-induced apoptosis. The authors concluded that inhibition of PKC cannot account for the potentiation by Bis-VIII and Bis-IX of Fas-mediated apoptosis. These observations are in accordance with results of Snowden et al. [48], Han et al. [9] and our own [49].

Recently, we have published results showing Bis-IX mediates sensitization of cancer cells to death ligand-induced apoptosis Rokhlin et al. [62, 63] observed similar effect of Bis-IX in prostate carcinoma cancer cells (LNCaP, PC3 cell lines). Search for the molecular mechanisms of bisindolylmaleimides actions suggest inhibition of antiapoptotic proteins involved in extrinsic apoptotic signal. Up to date, several studies including our own suggested the importance of an anti-apoptotic protein FLIP (FLICE-inhibitory protein) in the death ligand-induced signal repression. Cellular FLIP exists as numerous splice variants at the mRNA level, but only two forms, called $FLIP_L$ and $FLIP_S$ exist at the protein level [64]. cFLIP competes with procaspase 8 and limits or totally blocks its autoactivation. Elimination or down-regulation of cFLIP has been shown to restore the sensitivity to cell death induction by death receptor ligands [65, 66]. Importantly, Willems et al. [67] and Carroll et al. [68] found that Bis-III and Bis-VIII reduced cFLIP cellular level thus allowing the activation of caspase 8 in T cells. We examined COLO 205 for apoptosis initiated by TNF-α and Bis-IX co-treatment. We demonstrated that when these factors were used simultaneously they evoked apoptosis accompanied by down-regulation of $cFLIP_L$ [49]. In our previous studies we showed, however, that cycloheximide (translation inhibitor), but not actinomycin D (transcription inhibitor) sensitized COLO 205 cells to TNF-α-induced apoptosis [65]. Thus, we suggest that Bis-IX more likely modulates the translation process. Nevertheless, Rokhlin et al. [62] claimed that transcription inhibition belongs to the biological effects of Bis-IX. To test this hypothesis Rokhlin et al. [62] treated LNCaP cells with Bis-IX (4 μM), actinomycin D (4 μM) and cycloheximide (10 μg/mL) and examined the levels of p53, $p21^{WAF1}$, and Mdm2 proteins. Western blot analysis gave evidence that both actinomycin D and Bis-IX induced accumulation of p53 to the same level whereas it decreased the levels of $p21^{WAF1}$ and Mdm2.

Cycloheximide did not induce accumulation of p53, but levels of $p21^{WAF1}$ and Mdm2 were markedly decreased after cycloheximide treatment. Additionally, to examine whether Bis-IX is a general inhibitor of transcription, Rokhlin et al. [63] transiently transfected HeLa cells with pcDNA3.1-Hygro vector. Then, cells were treated with actinomycin D and Bis-IX and the expression of hygromycin gene was investigated. It was found that both compounds inhibited the expression of the gene. Moreover, the fluorescence analysis of Bis-IX binding to DNA confirmed its ability to interact with DNA and actinomycin D prevented the binding of Bis-IX to DNA. These data suggested that actinomycin D and Bis-IX interact by overlapping at the identical DNA binding sites. It remains unclear why in COLO 205 cells actinomycin D was not able to sensitize COLO 205 cells to apoptosis induction. A possible explanation is the antiapoptotic role of cFLIP protein. cFLIP was characterized as a short half-live protein, hence the inhibition of translation could be sufficient to restore the sensitivity of cancer cells to TNF-α-mediated apoptosis. In LNCaP cells the apoptosis resistance could result from the increased expression of several antiapoptotic proteins, which have to be eliminated in order to allow cells to die. Regardless of detailed molecular mechanisms, it became apparent that Bis-IX could be useful as an sensitizing agent and in support of immunotherapy.

***Figure 1.*** **Schematic illustration of bisindolylmaleimides action on intracellular signaling. The pro- and antiapoptotic effects are indicated. Bis-I and Bis-IX were selected as they are the most potent metabolic inhibitors among a variety of Bis derivatives.**




## CONCLUSION

Herein, the various molecular mechanisms of pro- and antiapoptotic effects of bisindolylmaleimides action in cancer cells are described in *Fig. 1*. Inhibition of PKC and/or MDR activity, modulation of Wnt signaling pathway and β-catenin stabilization, as well as the elimination of antiapoptotic proteins, such as cFLIP, and finally, activation of intrinsic apoptotic pathway are well documented cellular targets of bisindolylmaleimides. All these cellular changes help to restore the natural process of cancer cell deletion. Thus, bisindolylmaleimide derivatives could be seriously considered as candidates in anticancer treatment or as adjuvants in novel immunotherapy.

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