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Structure–activity relationship of *N*-methyl-bisindolylmaleimide derivatives as cell death inhibitors

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This paper is dedicated to Prof. Iwao Ojima on his 60th birthday

Abstract—A series of *N*-methyl-bisindolylmaleimide derivatives was synthesized and evaluated as cell death inhibitors. *N*-Methyl-2-[1-(3-aminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide (**21**) was the most potent inhibitor of H_2O_2-induced necrotic death of human leukemia HL60 cells among them.

Cell death signaling is currently one of the hottest topics in biological research. A great deal of knowledge has been accumulated on apoptosis, a naturally occurring process of cell suicide, which plays a crucial role in the development and maintenance of multicellular organisms.[1] Signals of typical physiological apoptosis are mediated by a cascade of proteases, called caspases, and the use of low-molecular-weight caspase inhibitors has made a major contribution to apoptosis research.[1b,1c] Recently, evidence for the existence of caspase-independent cell death signaling pathways has been accumulating.[2] Such caspase-independent pathways are suggested to be involved in some types of necrosis, which were classically regarded as passive, unregulated cell death.[3] The molecular basis of caspase-independent cell death pathways, however, remains to be clarified, despite its importance, for example, in ischemia-reperfusion injury and neurodegenerative disorders.[4] Small molecules, which specifically inhibit caspase-independent cell death pathways would be powerful biological probes. Furthermore, a specific cell death inhibitor that did not affect physiologically essential apoptosis could be a lead compound for developing therapeutic agents for the treatment of diseases such as stroke and neurodegenerative disorders.[5] Recently, Asakai et al. reported that bisindolylmaleimide I (BM I or GF109203X) inhibited necrotic cell death induced by oxidative stress, such as H_2O_2 or sodium nitroprusside (SNP, in vivo NO donor), in a variety of primary-cultured cells (Fig. 1).[6] They also reported that necrotic cell death induced by H_2O_2 was inhibited by a well-known chemical antioxidant, *N*-acetylcysteine (NAC), but not by a caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD). Since BM I was originally reported as a strong protein kinase C (PKC) inhibitor,[7] PKC inhibition was considered likely to be a mechanism of action. However, the experimental fact that bisindolylmaleimide V (BM V), a bisindolylmaleimide derivative that has no PKC-inhibitory activity, showed weaker but significant inhibition of cell death, suggested that PKC inhibition is not essential for the cytoprotective effect. Moreover BM I and V did not inhibit caspase-dependent apoptotic cell death. Thus, it is likely that these compounds inhibit a novel

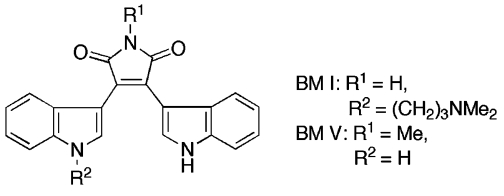

Figure 1. Structures of BM I and BM V.

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caspase-independent signaling pathway, and it is of interest to clarify their molecular mechanism of action. BM I itself is, however, not suitable for this purpose, because it inhibits not only PKC, but also several other kinases,[8] and is cytotoxic at high concentration. BM V has a negligible inhibitory effect on several kinases including PKC,[8] but its cell death-inhibitory activity is not potent enough for mechanistic studies. Therefore, a highly specific and potent derivative is required. Herein, we report the structure–activity relationship of bisindolylmaleimide derivatives and the development of a novel cell death inhibitor which is more potent than BM I and lacks strong PKC-inhibitory activity.

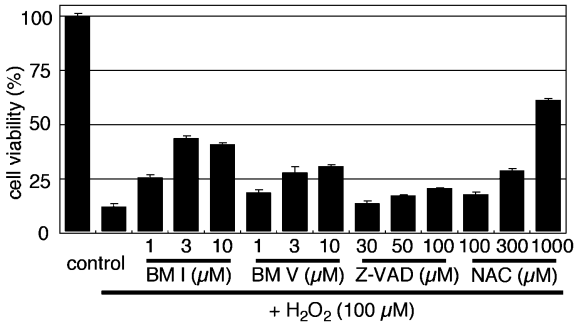


Figure 3. Cell death inhibition by BM I, BM V, Z-VAD, and NAC. Cell viability was determined by AlamarBlue™ assay.

First, we established an assay system not involving primary-cultured cells, which are unsuitable for mechanistic study. After several trials, human leukemia HL60 cells were found to be suitable.[9] As shown in Figure 2, morphologically typical necrotic cell death was induced by 100 μM H_2O_2 in HL60 cells. As in the case of the cultured cells reported by Asakai et al., a high concentration of NAC strongly inhibited the H_2O_2-induced death of HL60 cells, whereas the effect of a general caspase inhibitor, Z-VAD, was quite limited even at a high concentration (Fig. 3). BM I and V significantly inhibited cell death (Fig. 3) even at 1 μM.[10] These results imply that the cell death induced in this assay system is not mediated by caspases, but rather, similar molecular mechanisms to those of the necrotic cell death of primary-cultured cells reported by Asakai may be involved. However, the effects of BM I and BM V were limited in this system. At lower concentrations, dose-dependent improvement of the viability of HL60 cells was observed, but at higher concentrations the viability reached a plateau (Fig. 3), probably due to cytotoxicity at these concentrations. In fact BM I showed some cytotoxicity to HL60 cells (with 10 μM BM I, viability was reduced to 81% without H_2O_2 treatment). The observed cytotoxicity may be induced by the inhibition of kinases. Therefore, by using this assay system, both cytoprotective and cytotoxic activities of the bisindolylmaleimide derivatives could be evaluated in terms of the viability of H_2O_2=treated and untreated HL60 cells.

First, compounds **1**–**4**, BM V derivatives modified on the maleimide ring, were synthesized[11] (Scheme 1) and examined for cell death inhibitory activity and cytotoxicity at 3 μM (Fig. 4). Succinimide derivatives **1** and **2** showed negligible cell death inhibition. The maleic anhydride derivative **3** and amide derivative **4** exhibited some inhibition, but were much less potent than BM V. These results suggest that the maleimide ring is essential for cell death inhibition. Next, we examined the effects of various substituents on the indole rings. Since the maleimide N–H group of bisindolylmaleimide derivatives and indolocarbazole derivatives such as staurosporine is critical for the inhibition of various kinases,[12] we decided to retain the *N*-methyl-maleimide structure to avoid undesired interaction with PKC and other kinases. As shown in Table 1, the *N*-methyl bisindolylmaleimide derivatives **6**–**14** were synthesized from **5a** or **5b** in good yields.[11a,13] Figure 4a and b shows the cell death-inhibitory activity and cytotoxicity of these compounds at 3 μM, respectively. Among the methyl substituted derivatives **6**–**10**, the 2- and 4-substituted derivatives **6** and **7** had very weak activity, whereas the 5-, 6-, and 7-methyl derivatives **8**–**10** inhibited cell death much more potently than did BM V. BM V (and also **8**–**10**) can take a conformation in which one of the indole rings is coplanar with the maleimide ring and the π-electrons can be delocalized over the indole and maleimide rings. However, the 2- and 4-substituted derivatives **6** and **7** cannot readily take such a coplanar conformation due to the severe steric repulsion between the methyl group and the carbonyl oxygen or the other indole ring. The observed drastic difference depending on the position of the substituent may suggest the

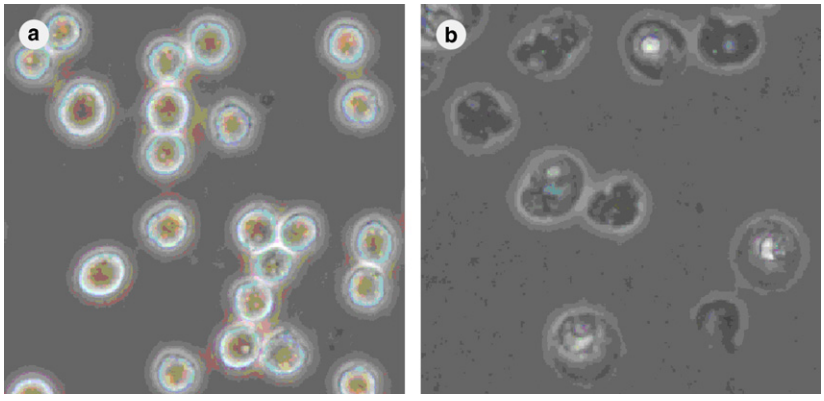
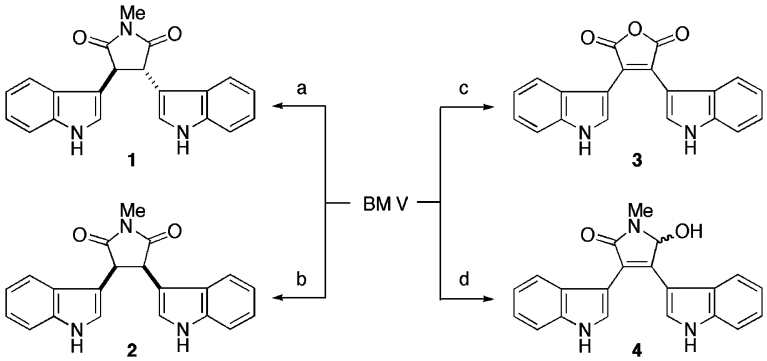


Figure 2. Phase-contrast micrographs of HL60 cells. (a) Intact cells; (b) typical necrotic cell death induced by H_2O_2 (100 μM, 3 h).



Scheme 1. Conversion of maleimide ring. Reagents and conditions: (a) Mg, MeOH (84%); (b) H_2, Pd/C, DMF (92%); (c) 10% KOHaq, reflux, then 2 N HClaq (71%); (d) $LiAlH_4$, THF (60%).

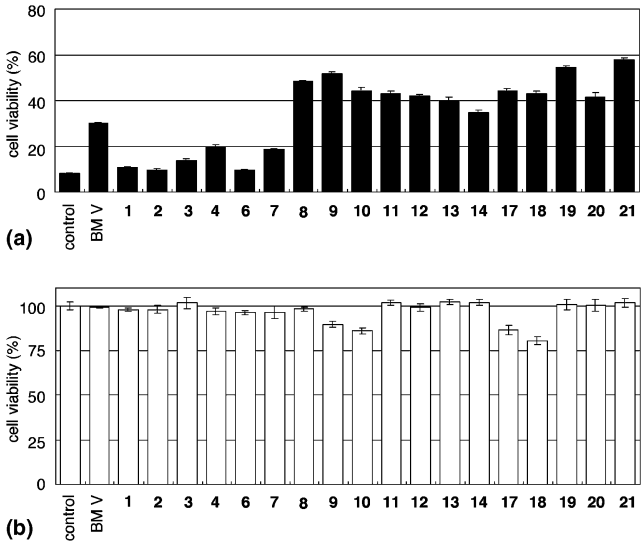
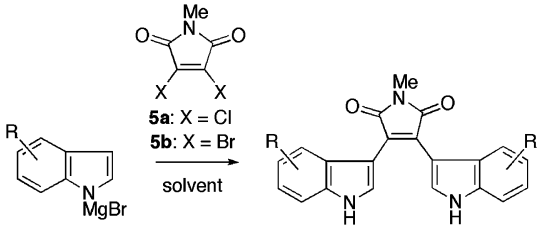


Figure 4. Activities of various bisindolylmaleimide derivatives. (a) Cell death inhibition. Viability of HL60 cells treated with H_2O_2 (100 μM, 3 h) in the absence (control) or presence of 3 μM bisindolylmaleimide derivative. (b) Cytotoxicity. Viability of HL60 cells treated with 3 μM bisindolylmaleimide derivative.

Table 1. Synthesis of bisindolylmaleimide derivatives



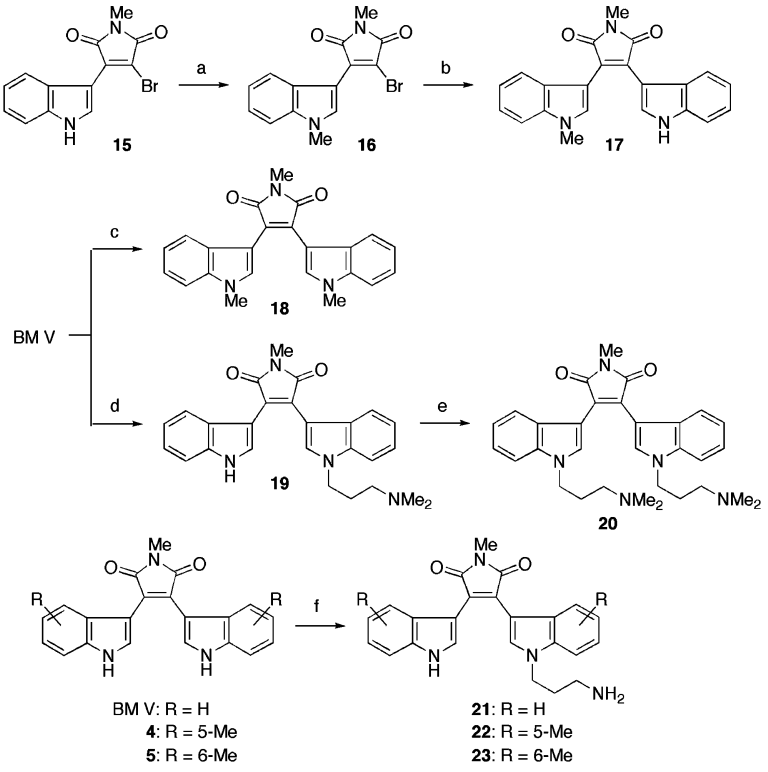
Compound	X	R	Solvent	Yield (%)
BMV	Cl	H	Toluene	98
6	Br	2-Me	THF	62
7	Cl	4-Me	Toluene	92
8	Cl	5-Me	Toluene	92
9	Br	6-Me	Toluene	88
10	Br	7-Me	Toluene	97
11	Cl	5-F	Toluene	85
12	Cl	5-CI	Toluene	91
13	Cl	5-Br	Toluene	82
14	Cl	5-OMe	Toluene	91

importance of such a coplanar structure for the cell death-inhibitory activity. Among the methyl-substituted derivatives, the 6-methyl derivative **9** had the strongest activity, but unfortunately it showed some cytotoxicity. Cytotoxicity was also observed for the 7-methyl derivative **10**, but the 5-methyl substituted derivative **8** had strong activity without cytotoxicity. Thus, we examined other 5-substituted derivatives **11**–**14**, but none of them were superior to **8**.

Next, we examined the effects of the substitution at the indole nitrogen. Compounds **17**–**20** were synthesized[7,14] from BM V or **15** (Scheme 2) and tested for activity (Fig. 4). Methyl-substituted derivatives **17** and **18** showed not only improved cell death inhibition, but also showed cytotoxicity. Introduction of the dimethylaminopropyl group(s), which is the same substituent as in BM I, increased the inhibitory activity, and the mono-substituted derivative **19** was more potent than the di-substituted derivative **20**. Finally, the mono-aminopropyl derivative **21** was found to be the best compound. It has the highest cell death-inhibitory activity among the bisindolylmaleimide derivatives synthesized and showed no cytotoxicity.

The dose–response curve of **21** is shown in Figure 5. Although, as with BM I and BM V, the activity curve reaches a plateau at high concentration, **21** is much more potent than BM I and BM V under our assay conditions. Finally, we also synthesized compounds **22** and **23**, which have an aminopropyl group on one indole nitrogen and methyl groups at the 5- or 6-position of the two indole rings, in the expectation that both substituents would contribute to improve the cell death inhibition (Scheme 2). However, greatly decreased cell viability was observed at 10 μM for these compounds (Fig. 5).

Finally, we examined the inhibitory activities of **21** toward several kinases, which are reported to be inhibited by BM I,[8] to confirm that *N*-methylation of the maleimide ring indeed completely suppresses the kinase inhibitory activity. Table 2 summarizes the activities of various kinases in the presence of 1 μM BM I (data cited from Ref. 8) or 50 μM **21**.[15] Generally, as expected, the *N*-methyl derivative **21** did not inhibit the kinases, except



Scheme 2. Synthesis of *N*-substituted bisindolylmaleimide derivatives. Reagents and conditions: (a) MeI, K_2CO_3, DMF (98%); (b) indolyl–MgBr, toluene (93%); (c) MeI, K_2CO_3, DMF (99%); (d) NaH, $Cl(CH_2)_3NMe_2{\cdot}HCl$, DMF (80%); (e) NaH, $Cl(CH_2)_3NMe_2{\cdot}HCl$, DMF (23%); (f) NaH, $Cl(CH_2)_3NH_2{\cdot}HCl$, DMF (**21**: 69%, **22**: 81%, **23**: 10%).

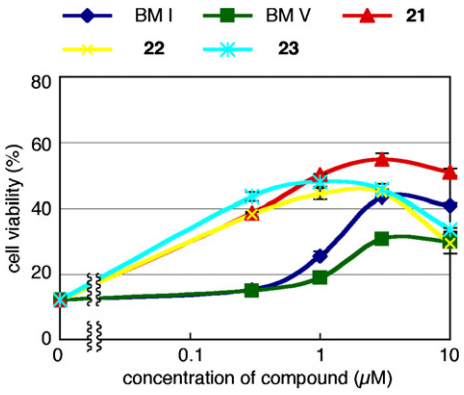


Figure 5. Dose–response curves of bisindolylmaleimide derivative for ability to improve the viability of HL60 cells treated with H_2O_2 (100 μM, 3 h).

Table 2. Kinase inhibition by bisindolylmaleimide derivatives

Kinase	Activity (% of control)	
	BMI[a] (1 μM)	**21** (50 μM)
PKCα	4 ± 0	66 ± 3
RSK2	9 ± 2	106 ± 1
MSK1	21 ± 1	90 ± 6
S6K1	32 ± 8	28 ± 0
GSK3β	50 ± 5	94 ± 8
AMPK	54 ± 0	88 ± 1
CHK1	60 ± 2	93 ± 5
SGK	63 ± 6	109 ± 14

[a] Data cited from Ref. 8.

for PKCα and S6K1. Considering the high dose of **21** compared to BM I, the weak inhibition of PKCα by **21** should have had little effect on the activity in our assay system. However, the activity of S6K1 was reduced to 28% in the presence of 50 μM **21**, and IC_{50} value of **21** for this kinase was determined to be 25 μM. Since S6K1 is suggested to be one of the prosurvival and proliferative kinases,[16] inhibition of this enzyme may contribute to the cytotoxicity at high concentrations of **21**.

In conclusion, we have synthesized various *N*-methylbisindolylmaleimide derivatives and elucidated their basic structure–activity relationships as cell death inhibitors. The data should provide critical information for the future design of more potent and specific cell death inhibitors. Among these compounds, **21** was more potent than BM I as a cell death inhibitor, with lower toxicity. Inhibition of various kinases by **21** was also tested. In contrast to BM I, **21** showed negligible inhibition of the tested kinases, except for S6K1, even at high concentration. Removal of this S6K1 inhibitory activity may be the key to the development of more advanced analogs.

Acknowledgments

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9. Cell culture: HL60 cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in a humidified incubator at 37 °C under 5% CO_2/95% air.
10. AlamarBlue™ assay: HL60 cells (4×10^4 cells/well) were suspended in fresh medium in a 96-well plate (95.5 μL/well). After 2 h of incubation, the cells were treated with test compounds (DMSO solution, 0.5 μL/well) for 1 h and then H_2O_2 (in medium, 4 μL/well, 100 μM at final concentration) was added (final volume 100 μL/well). In all experiments, the final DMSO concentration was the same (0.5%). After 3 h, 10 μL of AlamarBlue™ (Biosource International) was added to each well. The cell viability was determined based on the increase of fluorescence (excitation 560 nm/emission 590 nm) during 4–5 h of incubation. The cytotoxicity of test compounds was determined according to the same procedure, but without H_2O_2 treatment.
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15. KinaseProfiler™ (Upstate USA, Inc.): Protein kinase inhibition assays were performed using the KinaseProfiler™ service (Upstate USA, Inc.). Briefly, protein kinases were assayed for their ability to phosphorylate the appropriate peptide/protein substrates in the presence of 50 μM compound **21** and 100 μM ATP. Activities are given as mean percentages of those in control incubations (averages of duplicate determinations). Abbreviations of kinase are as follows: AMPK, AMP-activated protein kinase; CHK1, checkpoint kinase 1; GSK3β, glycogen synthase kinase 3β; MSK1, mitogen- and stress-activated protein kinase 1; PKC, protein kinase C; RSK2, ribosomal S6 kinase 2; SGK, serum- and glucocorticoid-induced kinase; S6K1, p70 ribosomal protein S6 kinase 1.
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