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Inhibition of hydrogen peroxide-induced necrotic cell death with 3-amino-2-indolylmaleimide derivatives

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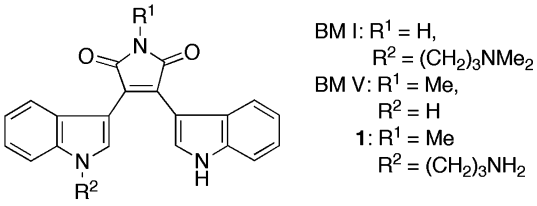
This paper is dedicated to Professor Iwao Ojima on his 60th birthday

Abstract—Novel analogs of indolylmaleimide derivatives (IM derivatives) were synthesized and tested for cell death-inhibitory activity. 2-(1*H*-Indol-3-yl)-3-pentylamino-maleimide IM-54 was the most effective cell death inhibitor among the compounds tested. IM-54 inhibited necrotic cell death induced by H_2O_2, but not apoptotic cell death induced by etoposide. These results indicated that this novel cell death inhibitor is distinct from the well-known caspase inhibitor, Z-VAD, which can block apoptotic cell death, but not necrotic cell death. IM-54 is expected to be a powerful bioprobe for clarifying the unique signaling pathway of necrotic cell death.

In the previous paper,[1] we reported the structure–activity relationship of bisindolylmaleimide derivatives as inhibitors of H_2O_2-induced necrotic cell death, and established that compound **1** is a more potent cell death inhibitor than the previously reported compound, BM I[2] (Fig. 1). Although **1** was much less toxic than BM I, it still had some toxicity at a very high concentration. Kinase inhibition profiling suggested that this toxicity may be due to the inhibition of S6K1. Thus, more specific analogs lacking S6K1-inhibitory activity are desirable for mechanistic studies. Here we report a novel indolylmaleimide derivative (IM-54) with a strong cell death-inhibitory activity but without inhibitory activity for S6K1 or any examined subtype of PKCs.



BM I: R^1 = H, R^2 = $(CH_2)_3NMe_2$
BM V: R^1 = Me, R^2 = H
1: R^1 = Me, R^2 = $(CH_2)_3NH_2$

Figure 1. Structures of BM I, BM V, and **1**.

Based on the SAR of the bisindolylmaleimides reported in the previous paper,[1] we hypothesized that the coplanarity of one indole ring with the maleimide ring is important for the activity, and we expected that removal of the second indole ring would favor such a coplanar conformation. Thus, the mono-indolylmaleimide, IM-1, was synthesized from **2**[3] according to the reported procedure[4] (Scheme 1), and tested as an inhibitor of necrotic cell death induced by H_2O_2 (100 μM) with the assay system using HL60 cells described in the previous paper.[1] Contrary to our expectation, IM-1 (3 μM) showed negligible inhibition, being similar in this respect to the corresponding succinimide derivative **2** (Fig. 2a). Interestingly, however, the *N*-methylated derivative IM-2 showed a slightly greater inhibition (Fig. 2). Similar activities were observed with 3-halo-2-indolylmaleimides IM-3 and IM-4, although the 3-bromo derivative, IM-4, showed strong cytotoxicity at 10 μM even in the absence of H_2O_2 (Fig. 2b), probably due to its electrophilic nature. Next, various mono-indolylmaleimide (IM)

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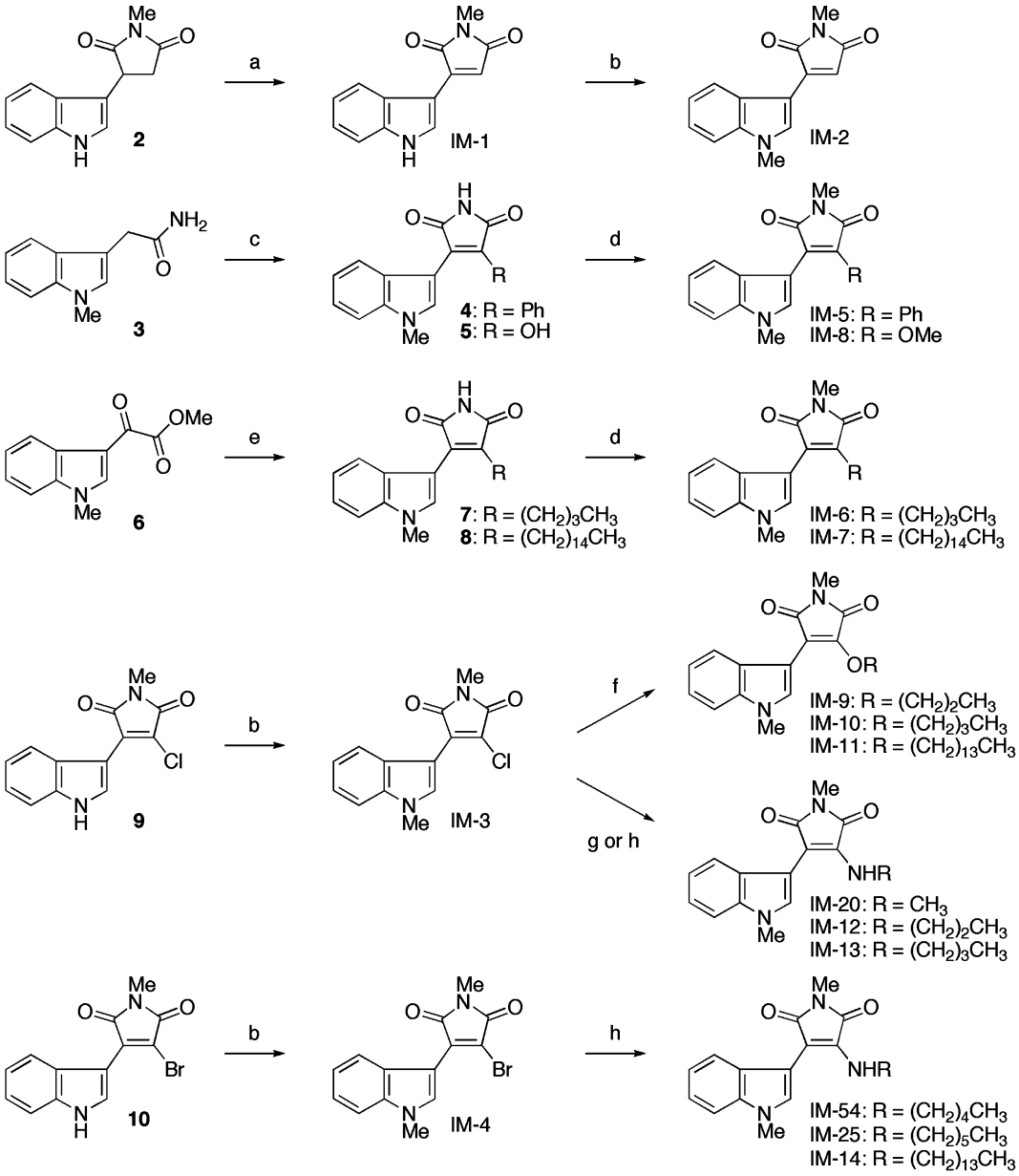

Scheme 1. Synthesis of indolylmaleimide derivatives. Reagents: (a) DDQ, dioxane (45%); (b) MeI, K_2CO_3, DMF (IM-2: 98%, IM-3: 88%, and IM-4: 95%); (c) $PhCOCO_2Et$ or $(CO_2Me)_2$, *t*-BuOK, DMF (**4**: 88% and **5**: 90%); (d) MeI, NaH, DMF (IM-5: 97%, IM-6: 84%, IM-7: 90%, and IM-8: 16%); (e) RCH_2CONH_2, *t*-BuOK, DMF (**7**: 77% and **8**: 61%); (f) ROH, NaH, THF (IM-9: 90%, IM-10: 91%, and IM-11: 93%); (g) 40%$MeNH_2$aq/THF (2/1) (IM-20: 28%); (h) RNH_2, CH_2Cl_2 (IM-12: 82%, IM-13: 76%, and IM-14: 88%) or THF (IM-54: 59% and IM-25: 83%).

derivatives having a methyl group on the indole nitrogen were synthesized as shown in Scheme 1, based on the reported procedure.[5] The 3-phenyl derivative, IM-5, was less active than IM-2, but the 3-butyl derivative, IM-6, showed a comparable activity to that of IM-2. IM-7, having a long hydrophobic chain, was less effective. Finally we were pleased to find that alkyloxy and alkylamino derivatives showed a much stronger cell death inhibition. No cytotoxicity was observed for these compounds even at 10 μM. At the same length of methylene chain, alkylamino derivatives IM-20, IM-12, and IM-13 showed a slightly higher activity than the alkyloxy derivatives, IM-8–IM-10. Among the aminoalkyl derivatives, the best activity was obtained with IM-54, having the C_5 alkyl chain. The derivatives having a long hydrophobic chain, IM-11 and IM-14, had a very weak activity. The structure–activity relationship clearly suggests that heteroatom substitution at the maleimide C3 position would be important, so that the lone-pair electrons can be delocalized on the maleimide ring. In BM V, the lone pair of the indole nitrogen may also be distributed partly on the maleimide ring.

The dose–response curves of BM I, BM V, compound **1**, and IM-54 for ability to improve the viability of HL60 cells treated with H_2O_2 are shown in Figure 3a. The cytoprotective effect of compound **1** plateaued at 3 μM, and a slight decrease in cell viability was observed at 10 μM. In contrast, dose-dependent cell death inhibition was observed up to 10 μM for IM-54. Furthermore,

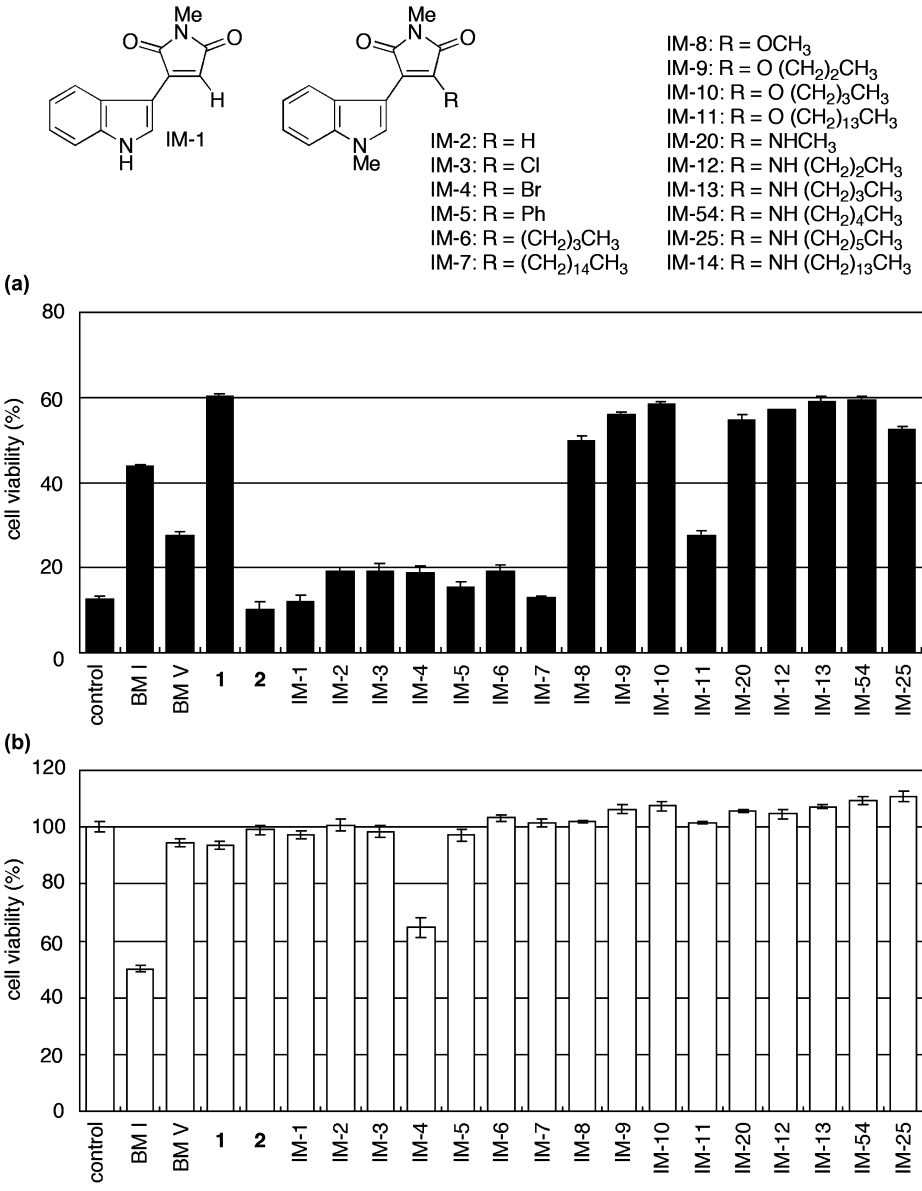

Figure 2. Structure and activities of various indolylmaleimide derivatives. (a) Cell death inhibition: viability of HL60 cells treated with H_2O_2 (100 μM, 3 h) in the absence (control) or the presence of 3 μM indolylmaleimide derivative. (b) Cytotoxicity: viability of HL60 cells treated with 10 μM indolylmaleimide derivative.

we examined the inhibitory activity of IM-54 toward S6K1 (Table 1).[1] As expected, IM-54 showed no inhibition of S6K1 even at 50 μM, in clear contrast to compound **1**. We also tested the inhibitory activity of IM-54 and **1** at a high concentration (50 μM) with all subtypes of PKCs. Significant inhibition of PKCε was observed with the bisindolylmaleimide derivative **1**, but IM-54 did not inhibit any of the subtypes of PKC. These results imply that the toxicity observed for **1** at high concentrations may be caused by the inhibition of kinases such as S6K1 and PKCε.

Finally we compared IM-54 with well-known cell death inhibitors in two different models of cell death. As described in the previous paper,[1] H_2O_2-induced necrotic cell death of HL60 cells was inhibited by the well-known antioxidant *N*-acetylcysteine (NAC), but not by the general caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD) (Figs. 4b and 5a). The suicide-type chemical antioxidant NAC had to be used at a much higher concentration (1 mM) than H_2O_2 (100 μM), whereas IM-54 strongly inhibited necrotic cell death at much lower concentrations (~10 μM). These experimental results clearly indicate that IM-54 is not a suicide-type antioxidant. In fact, IM-54 was chemically stable upon treatment with H_2O_2. We next examined the effect of IM-54 on the typical apoptotic cell death induced by etoposide (Figs. 4c and 5b).[6] The apoptotic cell death induced by etoposide was completely inhibited by Z-VAD, indicating that this apoptosis is caspase-dependent, whereas NAC was not effective against this type of cell death.[7] In contrast to its strong inhibition of necrotic cell death, IM-54 was not effective against etoposide-induced apoptotic cell

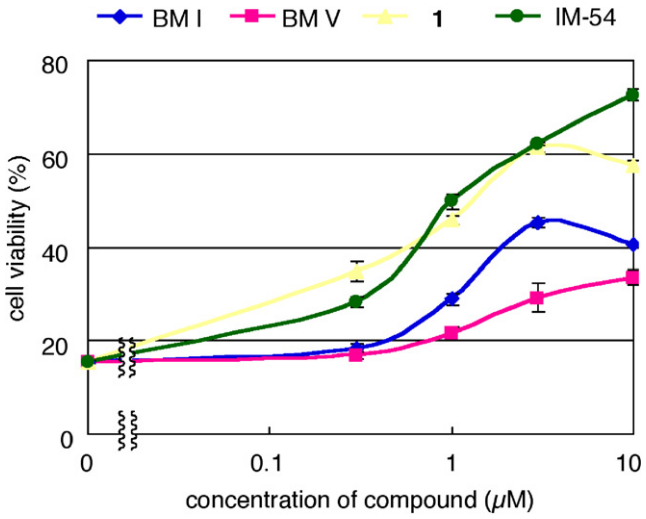

Figure 3. Dose–response curves of indolylmaleimide derivative for its ability to improve the viability of HL60 cells treated with H_2O_2 (100 μM, 3 h).

Table 1. Kinase inhibition by **1** and IM-54

Kinase		Activity (% of control)	
		1 (50 μM)	IM-54 (50 μM)
S6K1		28 ± 0	96 ± 15
PKC	α	66 ± 3	95 ± 1
	β1	78 ± 1	84 ± 2
	β2	84 ± 3	106 ± 0
	γ	85 ± 7	115 ± 4
	δ	82 ± 5	108 ± 8
	ε	10 ± 1	95 ± 1
	η	55 ± 3	98 ± 1
	ι	87 ± 1	96 ± 2
	μ	88 ± 2	97 ± 0
	θ	78 ± 3	100 ± 3
	ζ	85 ± 6	100 ± 1

death. These results suggest that IM-54 does not affect this caspase-dependent pathway, and has significant selectivity for necrotic cell death. Overall, IM-54 has sufficient potency and selectivity to be useful as a tool for the further mechanistic study of this novel type of necrotic cell death inhibition.

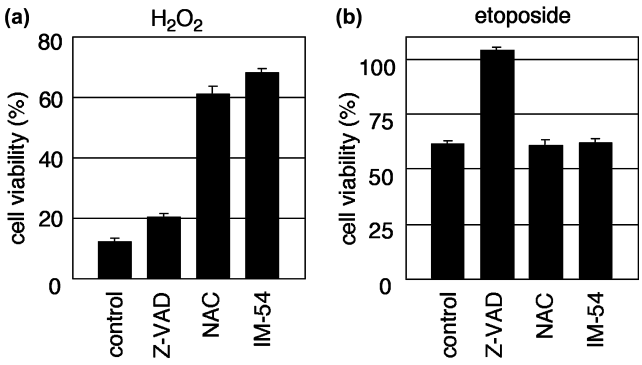

Figure 5. Comparison of the effects of Z-VAD (100 μM), NAC (1 mM), and IM-54 (10 μM) on different types of cell death. (a) Necrotic cell death induced by H_2O_2 (100 μM, 3 h); (b) apoptotic cell death induced by etoposide (100 μM, 4 h).

Apoptosis and necrosis were classically distinguished on the basis of morphological changes,[8] and, until recently, only apoptosis was believed to be a regulated cell death process, whereas necrosis was taken to be a passive process caused by overwhelming cell damage.[8] Recent studies, however, indicate the existence of signaling pathways for necrotic cell death, and necrosis is now suggested to be a kind of programmed cell death.[9] Moreover, necrosis was found to play an important role in pathological cell death, for example, in ischemia-reperfusion injury[9,10] and neurodegenerative disorders.[9,11] In contrast to our extensive knowledge of apoptosis,[12] however, the molecular basis of necrosis remains to be elucidated. Since IM-54 effectively blocks necrotic cell death at low concentrations, it is likely that this compound selectively affects a signaling pathway, which plays a critical role in necrotic cell death. Therefore clarification of the molecular mechanism of action of the novel cell death inhibitor IM-54 should contribute to future necrosis research. Furthermore, IM-54 is expected to be a useful probe for the classification of cell death type. IM-54 could be a lead compound for developing therapeutic agents for treatment of diseases such as stroke and neurodegenerative disorders.

In conclusion, we have succeeded in developing a novel cell death inhibitor, which has unique activity compared with other cell death inhibitors. The low inhibitory

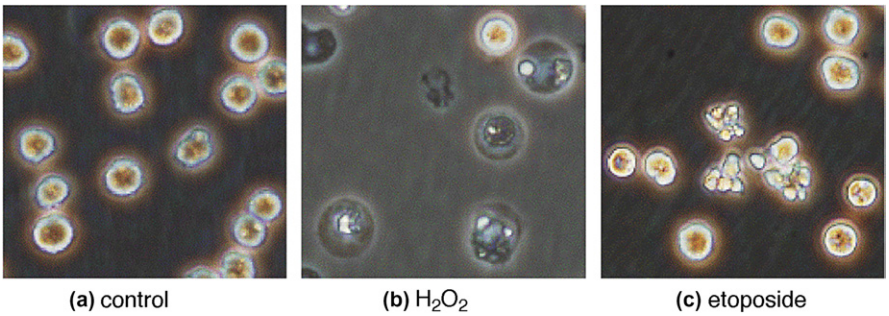

Figure 4. Phase-contrast micrographs of HL60 cells. (a) Intact cells; (b) typical necrotic cell death induced by H_2O_2 (100 μM, 3 h); (c) typical apoptotic cell death induced by etoposide (100 μM, 4 h).

activity toward kinases and the absence of a plateau of cytoprotective effect up to 10 μM suggested that our novel IM derivatives are much more suitable for mechanistic studies than previously reported bisindolylmaleimide derivatives as regards potency and selectivity. Biological studies to identify the target molecule of IM-54 are in progress.

Acknowledgments

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