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Indomethacin Analogues that Enhance Doxorubicin Cytotoxicity in Multidrug Resistant Cells without Cox Inhibitory Activity

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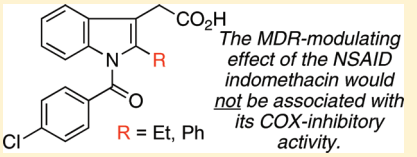
Supporting Information

ABSTRACT: Conformationally restricted indomethacin analogues were designed and prepared from the corresponding 2-substituted indoles, which were synthesized by a one-pot isomerization/enamide-ene metathesis as the key reaction. Conformational analysis by calculations, NMR studies, and X-ray crystallography suggested that these analogues were conformationally restricted in the *s-cis* or the *s-trans* form due to the 2-substituent as expected. Their biological activities on cyclooxygenase-1 (COX-1) inhibition, cyclooxygenase-2 (COX-2) inhibition, and modulation of MRP-1-mediated multidrug resistance (MDR) are described. Some of these indomethacin analogues enhanced doxorubicin cytotoxicity, although they do not have any COX inhibitory activity, which suggests that the MDR-modulating effect of an NSAID can be unassociated with its COX-inhibitory activity. This may be an entry into the combination chemotherapy of doxorubicin with a MDR modulator.



KEYWORDS: Cancer, conformation analysis, cytotoxicity, drug design, nitrogen heterocycles

Multidrug resistance (MDR) continues to be a serious problem in cancer therapy and is one of the limiting factors in the development and application of antitumor drugs. Doxorubicin is a chemotherapeutic drug widely used for the treatment of various solid tumors. However, its efficacy is often limited by the development of MDR, which has been linked to the up-regulation of P-glycoprotein (P-gp) in cancer cells.[1] It is known that tumor progression often is accompanied by increased COX-2 expression, and selective COX-2 inhibitors prevent the formation of multiple tumor types in experimental animals.[2] In fact, it is also known that MDR caused by multidrug resistance associated protein 1 (MRP-1) is modulated by nonsteroidal antiinflammatory drugs (NSAIDs), nonselective COX inhibitors such as indomethacin **1a**, or COX-2 selective inhibitors.[3,4] Mazzanti reported that the MDR phenotype might be associated with the expression of COX-2 in a human hepatocellular carcinoma cell line.[5] Cho showed a mechanism by which COX inhibitors exerted an enhancing effect on the cytotoxic effect of doxorubicin via direct inhibition of P-gpATPase activity in human esophageal squamous cell carcinoma cell lines.[6] However, it is less certain whether COX-1 and/or COX-2 activity is directly related to the MDR property of cancer cells.[2]

Indomethacin (**1a**, Figure 1) is a clinically useful NSAID with an indole structure, which inhibits both COX-1 and COX-2. In indomethacin, the two stable forms, the *s-trans* form and the *s-cis* form, around the amide bond are known (Figure 1). The COX inhibitory activities are closely related to the conformation of indomethacin itself. It has been reported that the bioactive conformations of indomethacin in complexes with COX-1 and COX-2 are the *s-trans* form[7] and the *s-cis* form,[8] respectively (4COX.pdb and 1PGG.pdb). We thought that the introduction of an alkyl substituent other than a methyl group at the 2-position of indomethacin might restrict its conformation in the *s-cis* or *s-trans* form, respectively, due to steric repulsion or $\pi-\pi$ stacking interaction by the introduced substituent with the *N*-4-chlorobenzoyl side chain. When the substituent at the 2-position (R) is an alkyl group bulkier than methyl, the *s-cis* form can be more stable than the *s-trans* form due to steric repulsion. However, introduction of a phenyl substituent at the 2-position may make the *s-trans* form more stable due to $\pi-\pi$ stacking (Figure 2). That these conformationally restricted analogues may be selectively active toward COX-1 or COX-2 offers some insight into the relationship between the COX-1 and/or COX-2 inhibitory effect and the MDR property of cancer cells. Since we have just developed a novel synthetic method for various substituted indoles using a combination of isomerization and enamide-ene metathesis,[9,10] consequently we decided to design and synthesize the indomethacin analogues **1b**−**d** substituted at the 2-position.[11]

The present study was carried out to investigate whether the COX activity of a series of our conformationally restricted

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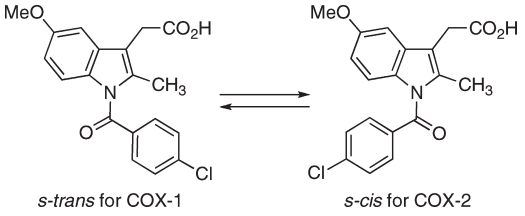

Figure 1. Structure of indomethacin (**1a**).

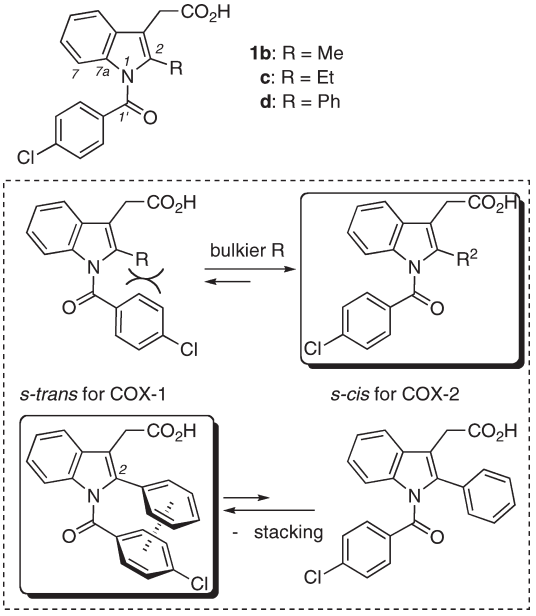

Figure 2. Designed indomethacin derivatives (**1b**−**d**).

indomethacin analogues was related to their doxorubicin cytotoxicity using MDR cell lines.

RESULTS AND DISCUSSION

Conformational Analysis by Calculations. Since conformational restriction is an important point in this research project, we decided to investigate the conformational stability of the designed indomethacin derivatives by theoretical calculations.

Thus, we first investigated the stable conformation of compounds **1a**−**d** by molecular field calculations using Macromodel. As a result, it appeared that **1a**−**c** would favor the *s-cis* form (Figure 3) while **1d** would favor the *s-trans* form (Figure 4). Energy differences between the two forms in these compounds were quite high, as indicated in the figures.[12]

Next, the rotational barrier energy around the C7a−N1−C1′−O dihedral angle of the model compounds **A**−**D**, in which the carboxymethyl group at the 3-position of the indomethacin analogues is replaced with a methyl group (Figure 5), was calculated on the basis of density functional theory (DFT). The dihedral angle was rotated from 0° to 360° at intervals of 10°, and the single point energies of the optimized forms were calculated with B3LYP/6-31G* to obtain the energy profile. The dihedral angle defines the *s-cis* form at 180° and the *s-trans* form at 0°, respectively. As shown in Figure 5, in **D** (the model of **1d**), the *s-trans* form was relatively more stable than the *s-cis* form while in **A**, **B**, and **C** (the models of **1a**, **1b**, and **1c**), the *s-cis* form

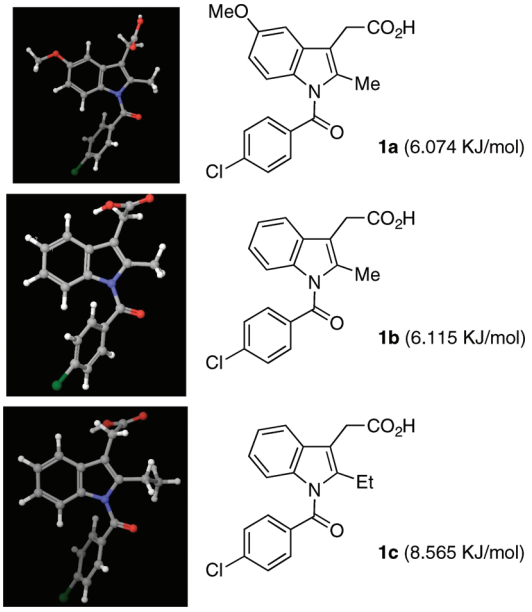

Figure 3. Compounds stable in the *s-cis* conformer by molecular field calculation. Numbers in parentheses indicate energy differences between the two comformers.

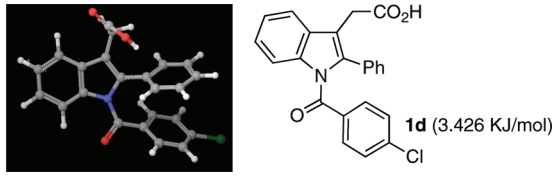

Figure 4. Compounds stable in the *s-trans* conformer by molecular field calculation. Numbers in parentheses indicate energy differences between the two comformers.

was more stable than the *s-trans* form. In **C**, the model of **1c**, a huge energy increase was observed at 330°, probably due to the steric bulk of the ethyl.

Hence, both molecular field and density functional theory calculations led to almost the same results, which indicated that the substituents at the 2-position on the indole ring can affect the conformation of indomethacin derivatives, as expected.

Chemistry. Most of the known indomethacin analogues are modified at the carboxylic acid moiety or at the 5-position of the indole ring, which were prepared by the traditional Fisher indole synthesis.[13−15] However, the designed target compounds (especially **1c** and **1d**) could not be prepared by the traditional methods. The synthetic route used to construct the target compounds (**1a**−**d**) is depicted in Scheme 1. The substituted indoles (**3a**−**d**), the key synthetic compounds, were obtained from the corresponding *N*-allyl-*N*-toluenesulfonyl-2-vinylaniline derivatives by a one-pot isomerization/enamide-ene metathesis and subsequent removal of the *p*-toluenesulfonyl group on the nitrogen. The introduction of a substituent at the 3-position on the indole ring was accomplished by a conventional 3-alkylation or Mannich reaction to yield **4a**−**d**. 4-Chlorobenzylation of **4a**−**d** and subsequent hydrogenation led to **1a**−**d**, which have not been prepared so far except for **1b**,[16] probably due to limitations in the traditional synthetic methods as described above.

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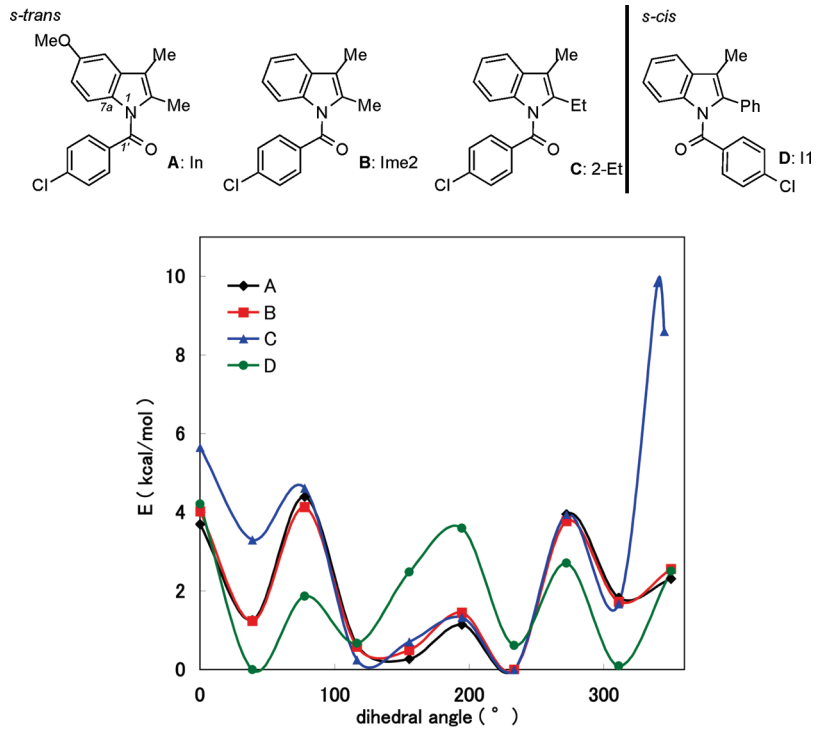
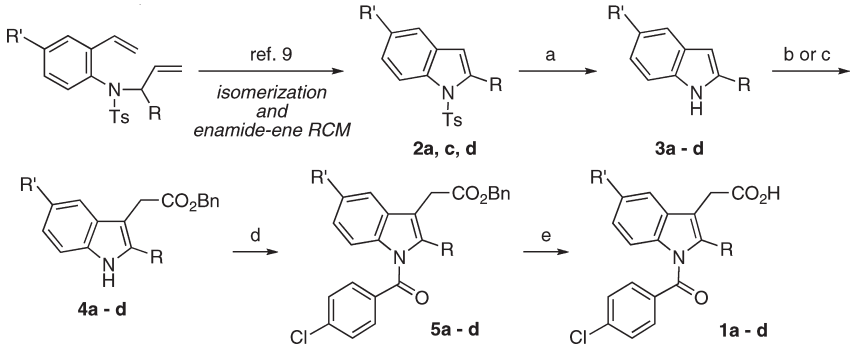


Figure 5. DFT calculation.

Scheme 1. Synthesis of Indomethacin 1a and Its Derivatives (1b−1d)[a]



[a] Conditions: (a) KOH, EtOH, THF, reflux, 78−98%. (b) ICH_2CO_2Bn, *n*BuLi, $ZnCl_2$, THF, 0 °C to rt, 30−88%. (c) (i) Me_2NH, HCHO, AcOH. (ii) MeI, toluene. (iii) KCN, $MeCN/H_2O$ = 1:3, reflux. (iv) *c*HCl, dioxane, reflux. (v) BnBr, $CsCO_3$, DMF, 0 °C, rt, 76% (5 steps). (d) 4-ClC_6H_4COCl, *t*BuOK, THF, −78 °C, 69−95%. (e) 10% Pd/C, H_2, AcOEt, rt or −15 °C, 76−95%.

Conformational Analysis by NMR. NOE experiments for **1a** and **1c** were carried out to investigate the stable conformation of the compounds in solution. Irradiation of the aromatic methine proton at the *ortho*-position of the benzoyl carbonyl of **1a** led to an NOE with the methine proton at the 7 position of the indole ring of 1.7% and with the methyl proton at the 2 position of 0.54% (Figure 6). These results suggest that the aromatic methine proton at the *ortho*-position of the benzoyl carbonyl and the methine proton at the 7 position of the indole ring are close to each other. Thus, **1a** would be restricted in the *s-cis* form, at least to some extent, as speculated. Furthermore, as shown in Figure 6, the NOE in **1c** was observed only at the 7-position when the same aromatic methine proton was irradiated, which suggested that **1c** would be significantly restricted in the *s-cis* form. The ethyl group appeared to be more effective in restricting the conformation than the methyl group (**1a** vs **1c**). These results support our hypothesis that the introduction of a bulkier substituent at the 2-position of indomethacin can restrict its conformation in

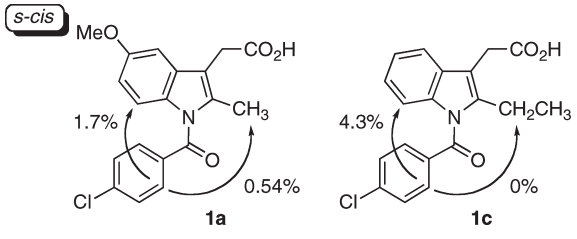


Figure 6. NOE correlations of **1a** and **c**. Arrows start from the equivalent methine.

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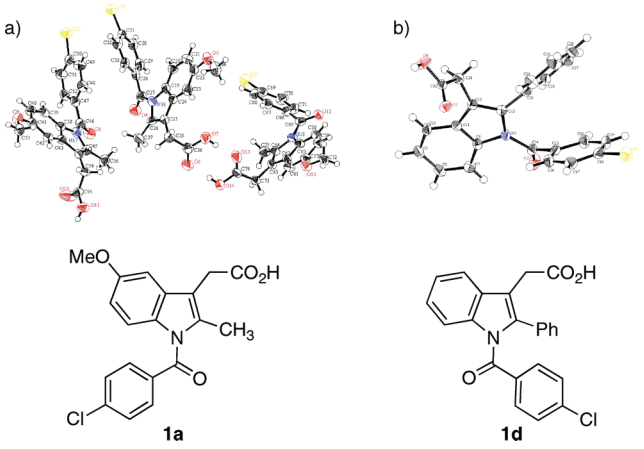

Figure 7. ORTEP drawings of (a) **1a** and (b) **1d**.

Table 1. SAR Studies on the COXs Inhibition by 1a−d ($n = 3$)

	COX-1		COX-2	
compound	ihibition (100 μM, %)	IC_{50} (μM)	ihibition (100 μM, %)	IC_{50} (μM)
1a (synthesized)	81 ± 11	0.01	89 ± 0.7	18
1b[a]	79 ± 6.4	0.01	76 ± 1.4	39
1c	<0	>400	14 ± 0.9	>400
1d	<0	>400	13 ± 2.0	>400
1a (purchased)	110 ± 11	0.12	83 ± 0.45	20
aspirin	38 ± 3.1	>100	2 ± 1.5	>400

[a] These data are in accord with those reported previously.[20]

the *s-cis* form due to steric repulsion of the substituent at the 2-position with the *N*-acyl side chain.[17]

Conformational Analysis by X-ray Crystallography. The crystal structures of indomethacin **1a** and its derivative **1d** were determined by single-crystal X-ray diffraction methods (Figure 7).[18] Crystals of **1a** and **1d** suitable for X-ray analysis were grown in 50% aqueous ethanol and in ethyl acetate, respectively. The analysis showed that a crystal of **1a** consists of units of the *s-cis* and the *s-trans* forms in a ratio of 2:1, which is in accord with the previously reported α type polymorphism of indomethacin.[14,19] On the other hand, compound **1d** crystallized only in the *s-trans* form, as we had hypothesized, which was identical with the stable form suggested by the theoretical calculations.

Biological Activity. *COX Inhibition.* Table 1 shows the COX-1 and COX-2 inhibitory activities of indomethacin **1a** and its derivatives **1b**−**d** at a concentration of 100 μM and also their IC_{50} values. Although **1a** and **1b** showed potent but nonselective COX-inhibitory activity, compounds **1c** and **1d** were almost inactive versus COX-1 and COX-2. It is possible that the 2-substituent introduced in **1c** and **1d** might affect the binding to COX-1 and COX-2 due to steric hindrance.

Effect on Multidrug Resistance (MDR). The development of potent and nontoxic agents, which have minimal side effects, to prevent MRP-1-induced MDR is one of the major aims of medical and pharmacological research. Thus, according to the reported procedure,[21] compounds **1a**−**d** were evaluated in a cell biological cytotoxicity assay employing the MRP-1 expressing human glioblastoma cell line T98G as a model system of MDR tumors at a concentration of 50 μM with or without 0.3, 1, or 3 μM doxorubicin.

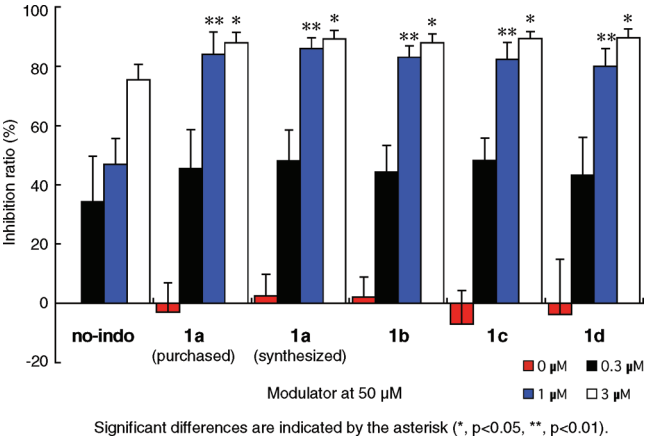

Figure 8. Influence of the indole compounds **1a**−**d** on the cytotoxicity of doxorubicin. T98G cells were pretreated with the compounds [50 μM] for 2 h and then cotreated with doxorubicin [0, 0.3, 1, or 3 μM] for 3 days. Survival was measured by crystal violet staining, as described in the Supporting Information. The data shown are the mean percentages ± SD for a minimum of three experiments.

As shown in Figure 8, doxorubicin showed moderate cytotoxicity in the cell line because of the MRP-1 expression, e.g., about 40% inhibition of the cell growth at 1 μM. In accord with the previously reported results,[21] the coadministration of indomethacin (**1a**) effectively enhanced the cytotoxicity of doxorubicin by about two times at 1 μM. In this system, **1c** and **1d**, having only insignificant COX-1 and COX-2 inhibitory activity (IC_{50} > 400 μM), surprisingly enhanced the cytotoxicity of doxorubicin, as was the case with **1a**. Also, **1c** and **1d** without doxorubicin did not show any cytotoxicity in this cell line. The previous biological studies suggested that the COX inhibitory effect of the NSAIDs, including indomethacin **1a**, may be related to the MDR of cancer cells.[3,4,6] Accordingly, these results, demonstrating that the MDR-modulating effect of the NSAID indomethacin would not be associated with its COX-inhibitory activity, are of significant importance.

CONCLUSION

We designed and synthesized the conformationally restricted indomethacin analogues **1c** and **1d**, whose conformations were restricted due to the effect of the 2-substituent as expected. Although these analogues were inactive versus COX-1 and COX-2, they surprisingly enhanced the cytotoxicity of doxorubicin in spite of their non-COX inhibitory activities. These compounds may become a prototype for developing a useful MDR modulator without cytotoxicity.

ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.A., Y.K., and S.S. contributed to the design, synthesis, calculation by Macromodel and study on COX-inhibitory activity. T.O. and T.S. contributed to the study on modulation of MRP-1 mediated multidrug resistance. M.I., H.A., and Y.I. contributed to the DFT calculation. A.Y. contributed to X-ray crystallography analysis. M.A. and S.S. contributed to writing of the manuscript, and are also the corresponding authors.

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(11) Demethoxy indomethacine **1b** would provide some information concerning an effect of the methoxy group.

(12) Energy difference of **1d** is lower than those of **1a**−**c**, which is enough to restrict the conformation. Energy difference of **1c** is higher than that of **1b**, probably due to the influence of the steric bulk of the ethyl.

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(16) Compound **1b** is a known.

(17) An NOE experiment of **1d** could not performed, since the aromatic proton signals of the 4-chlorobenzoyl and the 2-phenyl groups were overlapped and could not be assigned.

(18) CCDC 814524 and CCDC 814525 contain the supplementary crystallographic data for **1a** and **1d**, respectively. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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