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Inhibitory activities against topoisomerase I & II by polyhydroxybenzoyl amide derivatives and their structure–activity relationship

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Abstract—*o*-, *m*-, *p*-Phenylenediamines having 2,3,4-trihydroxy, 3,4 dihydroxy, and 4-hydroxybenzoyl moieties were prepared and their inhibitory activities were measured against topoisomerase I and II. More hydroxy groups on two aromatic rings increased the activities. Bis(trihydroxybenzoyl)-*o*-phenylenediamide showed $IC_{50} = 0.90$ and 0.09 μM against topoisomerase I and II, respectively. Compounds with hydroxy groups protected by acetyl moiety still had the activities. Less hydroxy groups decreased their activities. Benzothiazole derivatives also indicated the activities.

1. Introduction

DNA topoisomerases (topo) are ubiquitous enzymes that perform essential cellular functions involved in replication, recombination, and packaging and unfolding of DNA in chromatin.[1] Topo I and II inhibitors which have various structures with different side chains have been reported.[2–11] Clinically potent anticancer agents, topo inhibitors like camptothecin,[12] etoposide,[13] and doxorubicin[14] bind to the cleavable complex formed between topo and DNA, and keep it from going back the original DNA. This action is associated with severe side effects as well as other anticancer agents targeted at DNA.[15,16] Now agents directly inhibiting topo are urgently being requested. Suzuki et al.[17] reported isoaurostatin isolated from *Thermomonospora alba* showed topo I inhibition ($IC_{50} = 307$ μM), and they proved that this compound directly inhibited topo I. Then they tried to prepare their modified compounds which have hydroxyphenyl-, dihydroxyphenyl-, and trihydroxyphenyl groups on benzofuran-2-one, and one of the prepared compounds[18] having trihydroxyphenyl group improved topo I inhibition to 3.0 μM (IC_{50}). In this short communication we prepared *o*-, *m*-, *p*-phenylenediamines and benzo-thiazoles having more hydroxy groups on phenyl moiety in order to enhance topo inhibition.

2. Chemistry

Reactions of *o*-, *m*-, *p*-phenylenediamine (**1a–c**) with 2,3,4-triacetyloxy, 3,4-diacetyloxy, and 4-acetyloxybenzoyl chlorides (**2a–c**) gave the corresponding amides (**3a–i**), which were subjected to deacetylation with hydrazine hydrate yielding the corresponding compounds (**4a–i**). Benzothiazole derivatives (**6a–c**) were also prepared by the same method.

3. Inhibitory activity

Inhibition against relaxation activity of topo was measured by detecting the conversion of supercoiled pBR322 DNA to its relaxed form.[19,20] As shown in Table 1, *o*-compound **4a** showed most potent topo I

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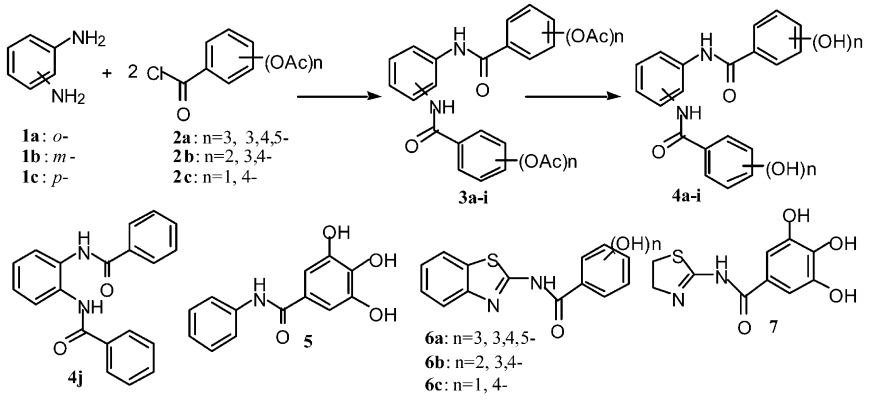

Scheme 1.

inhibitory activity with IC_{50} 0.9 μM. *m*- and *p*-Compounds (**4b**,**c**) indicated IC_{50} 1.6 and 1.4 μM, respectively. Their acetyl compounds (**3a–c**) weakened the activities showing IC_{50} 4.7, 11.2, and 8.6 μM. Among 3,4-dihydroxy derivatives (**4d–f**), *p*-compound **4d** showed the activity IC_{50} 2.6 μM. Their acetyl compounds did not show the activity except *p*-compound **3e** with IC_{50} 43.2 μM. For monohydroxybenzoyl (**4g–i**) and their acetyl (**3g–i**) derivatives no activity were observed (Scheme 1).

Dibenzoyl-*o*-phenylene diamine (**4j**) and 2,3,4-trihydroxy-benzoyl anilide and 2,3,4-trihydroxy-benzoyl anilide (**5**) had also no activity. 2-(2,3,4-Trihydroxy-benzoyl)-2-aminobenzothiazole (**6a**) and dihydroxy derivaive (**6b**) showed IC_{50} 8.2 and 16.9 μM, but monohydroxy derivatives (**6c**) had no activity. 2-(2,3,4-Trihydroxy-benzoyl)-2-aminothiazole (**7**) with no phenyl group showed weak inhibition. Moreover, topo II inhibitory activities of compounds (**4a–c**) were observed to show IC_{50} 0.09, 0.22, and 0.15 μM, respectively. From these results the following three essential factors are required for the inhibitory activity. 1: two trihydroxybenzoyl (galloyl) groups. 2: a hydrogen bond of carbonyl or enolic hydoxy group with another amide or nitrogen group. 3: benzene ring.

Table 1. Inhibition of topoisomerase I by polyhydroxyphenyl derivatives

Compd		*n*	R	Position	Topo I inhibition (IC_{50}, μM)
3a	*o*	3	CH_3CO	3,4,5-	4.7
3b	*m*	3	CH_3CO	3,4,5-	11.2
3c	*p*	3	CH_3CO	3,4,5-	8.6
4a	*o*	3	H	3,4,5-	0.9
4b	*m*	3	H	3,4,5-	1.6
4c	*p*	3	H	3,4,5-	1.4
3d	*o*	2	CH_3CO	3,4-	88.0
3e	*m*	2	CH_3CO	3,4-	43.2
3f	*p*	2	CH_3CO	3,4-	> 100
4d	*o*	2	H	3,4-	6.4
4e	*m*	2	H	3,4-	10.0
4f	*p*	2	H	3,4-	2.6
3g	*o*	1	CH_3CO	4-	> 100
3h	*m*	1	CH_3CO	4-	> 100
3i	*p*	1	CH_3CO	4-	> 100
4g	*o*	1	H	4-	> 100
4h	*m*	1	H	4-	> 100
4i	*p*	1	H	4-	> 100
4j	*o*	0			> 100
5					> 100
6a	H	3		3,4,5-	8.2
6b	H	2		3,4-	16.9
6c	H	1		4-	> 100
7					34.4

4. Inhibitory property

Inhibitory properties of **4a** were examined as the following four methods.

4.1. Inhibitory manner of 4a against topo I and II

The type of inhibition was determined by Lineweaver–Burk plots[21] of substrate concentrations against the rate of relaxation of supercoiled pBR322 DNA by topo I and II in the presence or absence of **4a**. As shown in Figure 1, **4a** inhibited the relaxation activities of topo I and II noncompetitively with respect to pBR322 DNA exhibiting K_i values of 0.4 μM and 74.1 nM, respectively.

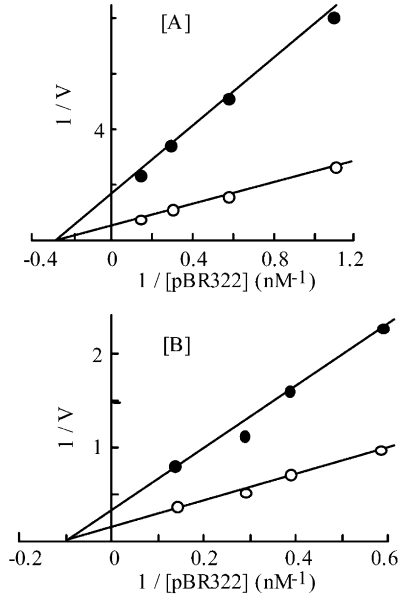

Figure 1. Lineweaver–Burk plots of pBR322 DNA concentrations against rate of relaxation by topo I [A] and topo II [B] with (○) and without **4a** (●).

The Michaelis constants (K_m values) of topo I and II were 3.7 nM and 10.5 nM, respectively. In view of inhibitory potency (K_i/K_m) against DNA relaxation by topo I and II, **4a** was 15-fold potent against topo II than topo I. From these results, **4a** was considered to bind with a different site from the binding site of the substrate DNA in the enzyme molecule.

4.2. Stabilization of topo-cleavable complex by 4a

Topo inhibitors of the cleavable complex-forming type such as camptothecin and etoposide stabilize the cleavable complex (topo–DNA reaction intermediate) and inhibit the DNA rejoining reaction of topo, which is the inhibitory mechanism of the inhibitors, therefore the inhibitors induce nicked or linearized DNA in the cleavage assay.[22,23] To determine whether **4a** is an inhibitor of the cleavable complex-forming type or not, cleavage assays were carried out. Camptothecin and etoposide were used as the controls of cleavable complex-forming inhibitors against topo I and II, respectively. As shown in Figure 2[A], camptothecin induced nicked DNA with increasing concentrations. Unlike camptothecin, **4a** could not induce the nicked DNA even at 100 μM. The results for the stabilization of topo II-cleavable complex are shown in Figure 2[B]. Etoposide induced the linearized DNA, but **4a** failed to linearize DNA even at 1000 μM. These results suggest that **4a** is an inhibitor of the cleavable-nonforming type. **4a** may directly act on topo I and II molecules in earlier step than the formation of the topo–DNA complex and inhibit the DNA breaking and rejoining reactions by the enzymes.

4.3. DNA interaction by 4a

Some topo inhibitors such as doxorubicin and amsacrine are DNA intercalators. To determine whether **4a** has the ability to intercalate into DNA strands, CD (circular dichroism) spectral change of DNA by addition of **4a** was measured, because the spectrum is sensitive to the conformation changes of DNA by intercalators.[24,25] Doxorubicin was used as control of intercalator at the same concentration. The spectrum of DNA changed greatly with increasing concentrations of doxorubicin. On the other hand, the spectral changes by **4a** did not occur, therefore, it is clear that **4a** has no ability to intercalate into DNA. Thus, **4a** is different from inhibitors causing DNA damage such as cleavable complex-forming inhibitors and DNA intercalators.

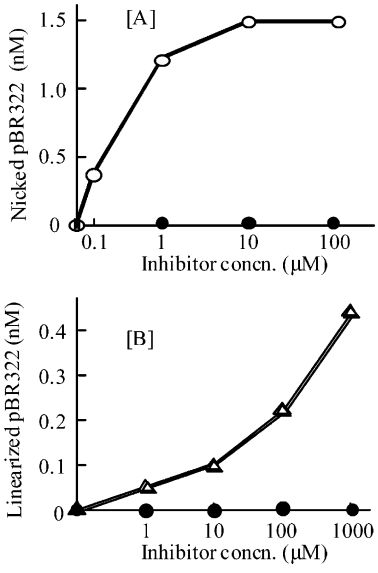

Figure 2. Stabilization of topo I [A] and topo II [B]-cleavable complexes by **4a** (●), camptothecin (○) and etoposide (△).

4.4. Effect of 4a on the growth and cell cycle of HeLa cells

The cell growth inhibition of **4a** was determined in HeLa cells by Alamar Blue assay.[26] The values of cell growth inhibition (GI_{50}) of **4a**, camptothecin and etoposide were 30, 0.6 and 40 μM, respectively. Camptothecin and etoposide promote the accumulation of damaged DNA by stabilization of cleavable complex in the cells, therefore the inhibitors arrest the cell cycle progression. The cell cycle progression was analyzed with a flow cytometer (Becton Dickinson FACS Calibur) using the ModFit LT which is a software to determine the percentage of cells in G0/G1, S and G2/M phases.[27] HeLa cells were arrested at S phase and G2/M phase when cultured with 0.1 μM camptothecin and 2 μM etoposide, respectively. On the other hand, **4a** did not affect on the cell cycle even at an extremely high concentration (100 μM). The results suggest that the cytotoxicity of **4a** is clearly different from that of camptothecin and etoposide.

5. Materials and general experimental procedures

o-, *m-*, *p*-Phenylenediamines (**1a–c**) were purchased from Tokyo Kasei. Polyacetyloxybenzoyl chlorides (**2a–c**) were prepared according to Gazit's method.[28]

5.1. Bis(polyacetyloxybenzolyl)-*o*-, *m*-, *p*-phenylene-diamides (3a–i)

To a solution of **1a–c** (0.22 g, 2 mmol) and triethylamine (0.70 mL, 5 mmol) in CH_2Cl_2 (10 mL) was added dropwise a solution of **2a–b** (4 mmol) in CH_2Cl_2 (10 mL) under cooling with ice and water. The reaction mixture was allowed to stir for 2 h at room temperature. The CH_2Cl_2 layer was washed with water (10 mL×2), dried over $MgSO_4$, and evaporated to dryness. The residue was recrystallized from MeOH. Yield: 68–81%.

5.2. Dipolyhydroxybenzolyl *o*-, *m*-, *p*-phenylenediamines (4a–i)

Hydrazine hydrate (0.35 g, 7 mmol) was added to a solution of **3a–i** (1 mmol) in CH_2Cl_2 (10 mL). The reaction mixture stirred for 2 h at room temperature, washed with water (10 mL), dried over $MgSO_4$, and evaporated to dryness. The residue was recrystallized from MeOH. Yield: 61–71%.

Compounds (**4j**, **5**, **6a–c**, **7**) were prepared by the same method described above. Structures of each compound were determined by spectral data and elemental analyses.

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