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Isolation, Structure Determination, *In Vivo/Vitro* Assay and Docking Study of a Xanthone with antitumor activity from Fungus *Penicillium oxalicum*

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Abstract: Investigation of EtOAc extract from the fermentation broth of the fungus *Penicillium oxalicum* led to the isolation of a xanthone, secalonic acid D, which showed good antitumor activity both *in vivo* and *in vitro*. The docking study revealed that secalonic acid D could block the active pocket of topoisomerase I with seven hydrogen bonds between secalonic acid D and ARG488, LYS532, ASP533.

Keywords: Xanthone; antitumor; docking.

1. Introduction

In our research to find anti-tumor leading compounds from marine microorganisims, a bioassay-guided separation led to the isolation of secalonic acid D (SAD) (**1**) [1], 2-(4-hydroxybenzyl)quinazolin-4(3*H*)-one (**2**) [2], oxaline (**3**) [3] and penipanoid C (**4**) [4] from the fermentation broth of fungus *Penicillium oxalicum* obtain from Taiwan. SAD was reported to be able to inhibit topoisomerase I as an inhibitor recently [5]. Whereas the *in-vivo* anti-tumor activity and the mechanism how it interacts with topoisomerase I are still unknown.

In order to evaluate its *in-vivo* antitumor activity and explore the inhibiting mechanism of topoisomerase I, the growth inhibiting activity against H22 cells in mice was evaluated and a docking study between SAD and topoisomerase I was carried out.

2. Materials and Methods

2.1. Microorganism Material

The Strain TW01-P1 was isolated from the soil of obtained from Xitou mountain in Taiwan. The strain TW01-P1 was identified as *Penicillium oxalicum* by comparing the ITSrDNA in the GenBank

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and has been deposited in School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University.

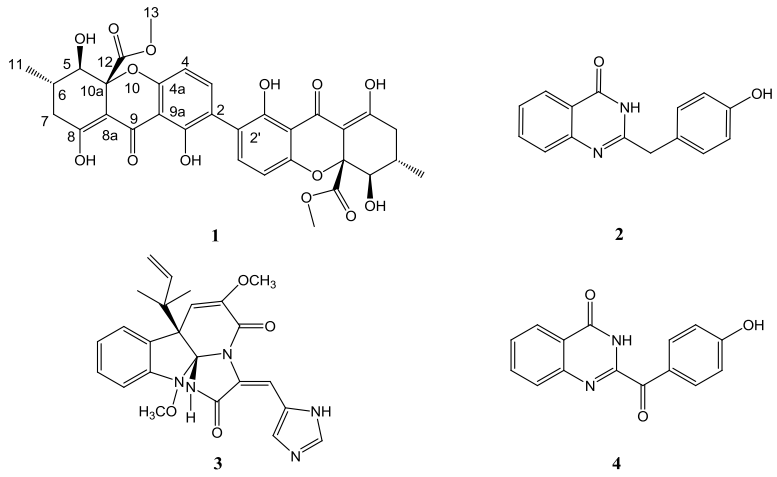

Figure 1. Structures of compounds **1**-**4**

2.2. Fermentation and Isolation

The strain TW01-P1 was incubated in liquid medium (2% glucose, 2% maltose, 1% monosodium glutamate, 0.05% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.3% yeast extract, tap water, pH 6.5), on rotary shaker at 28 °C at 180 rpm for 8 days

The fermentation broth of the strain TW01-P1 (about 80 L) was concentrated and extracted with ethyl acetate and *n*-butanol, successively. The EtOAc crude extract (18 g) was applied on a silica gel column, eluted with $CHCl_3$-CH_3OH gradient (from 100:1 to 0:1) to afford 12 fractions. Fr. 9 was re-crystallized with $CHCl_3$-CH_3OH to afford compound **1** (500 mg). Fr. 7 was further separated by HPLC eluted with MeOH 70% (v/v) to give compound **2** (10 mg), **3**(15 mg) and **4** (8 mg).

2.3. In vivo assay

The mice were housed individually in metabolic cages with 12h:12h light:dark cycle and free water intake. The H22 tumor cell was diluted with physiological saline to the cell density of 4.65×10^7 /ml^{-1}. After well mixed, the H22 tumor cell was injected into the mice at armpit with 0.2 ml.

Twenty-four hours after transplanted with tumor cell of H22, the mice were divided into administration group, 5-fluorouracil group and control group randomly according to their body weight. The dosage was 15 mg/kg of secalonic acid D for administration group and 20 mg/kg of 5-fluorouracil for the 5-fluorouracil group. The administration group and 5-fluorouracil group were intragastric administrated one time each day. The same volume of distilled water was intragastric administrated for the control group.

The mice were sacrificed on day 12 and all tumors were separated from the bodies to weigh and calculate the tumor inhibiting rate (TIR). The TIR were calculated by the following formula: TIR =100%× (mean weight of H22 tumors of control group - mean weight of H22 tumors of administration group) / mean weight of H22 tumors of administration group.

2.4. In vitro assay

The *in-vitro* bioassay was done as the procedure described before against A549, Colo205, MDA-MB-435 cell lines with IC_{50} values of 0.97, 0.89, 1.30 μm/mL, respectively [6].

2.5. Docking

The protein was prepared by discovery studio 3.0 package with default setting. The complex of human topoisomerase I and DNA (PDB ID: 1A35) was used for docking. The stereo-configuration of ligand, secalonic acid D, was achieved from its X-crystal structure. The CDOCKER protocols were setup using the programs default settings. The generated poses were ranked using -CDOCKER interaction energy.

3. Results and Discussion

3.1. Structural elucidation

Compound **1** was obtained as yellow crystal with $[a]_{,D}^{25}$ + 85 (*c* 0.12, $CHCl_3$). EI-MS gave the $[M+H]^+$ at *m/z* 639.2. All the ^1H and ^{13}C data were assigned by the HSQC and HMBC spectrum and was shown in Table 1. The NMR data assigned were similar with those of SAD in the literature [1]. The absolute configuration of C-5, 6, 10 and C-5′, 6′, 10′ were determined by the X-ray diffraction analyses and the absolute configurations were determined to be R for C-5, 5′, 10, 10′ and S for C-6, 6′, respectively.

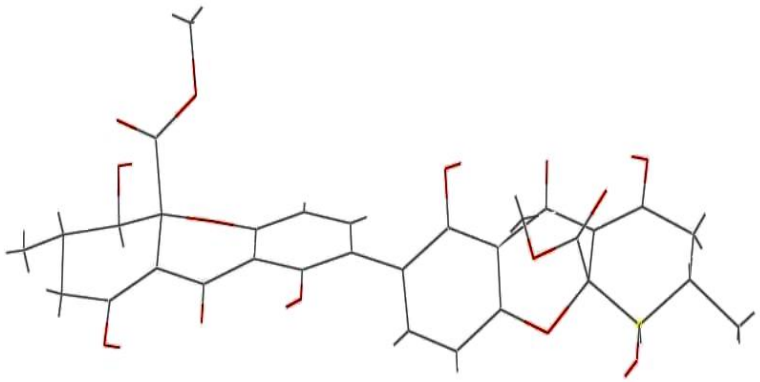

Figure 2. X-crystal structure of secalonic acid D

3.2. In vivo/vitro assay

In-vivo assay against H22 cell line revealed that secalonic acid D could inhibit the growth of H22 tumor cell on mice with inhibiting rate of 42.48 % (Table 2), which was a little better than those of the positive drug 5-fluorouracil (37.96%). Compared with the control group, the weight of the mice for the secalonic acid D group did not changed significantly, suggesting the relatively low toxicity of secalonic acid D.

Table 2. The tumor inhibiting activity of secalonic acid D on mice

	dosage ($mg \cdot kg^{-1}$)	weight (g) 0day/12day	tumor weight (g)	inhibiting rate
control	---	25.63/30.01	1.49 ± 0.56	---
5-fluorouracil	20	26.03/30.02	0.93 ± 0.32	37.96
secalonic acid D	15	25.85/28.44	0.86 ± 0.37	42.48

The results of the *in-vitro* bioassay against A549, Colo205, MDA-MB-435 cell lines indicated that secalonic acid D could inhibit the growth of these three tumor cell lines with IC_{50} values of 0.97, 0.89, 1.30 μm/mL, respectively.

Table 1. NMR spectral data for Secalonic acid D (at 600 MHz in $CDCl_3$ for ^1H NMR and 150 MHz in $CDCl_3$ for ^{13}C NMR, δ in ppm, J in Hz)

Position	δc	δ_H (mult.; J in Hz)	HMBC (H→C)
1 and 1'	159.4	-	-
2 and 2'	118.2	-	-
3 and 3'	140.2	7.45 (1H, d, 8.4)	C-2, 4a
4 and 4'	107.6	6.63 (1H, d, 8.4)	C-2, 4a, 9, 9a
5 and 5'	76.6	3.93 (1H, dd, 10.8, 2.7)	C-10a, 12
6 and 6'	29.2	2.41 (1H,m)	C-7, 8
7a and 7a'	36.3	2.75 (1H, dd, 18.3, 5.3)	C-5, 6, 8, 8a
7b and 7b'	36.3	2.27 (1H, dd, 18.3,10.5)	C-5, 6, 8, 8a
8 and 8'	177.5	-	-
9 and 9'	187.2	-	-
10 and 10'	-	-	-
11 and 11'	18.0	1.17 (3H, d, 6.3)	C-5, 6, 7
12 and 12'	170.3	-	-
13 and 13'	53.3	3.73 (3H, s)	C-12
4a and 4a'	158.3	-	-
8a and 8a'	101.5	-	-
9a and 9a'	106.9	-	-
10a and10a'	84.7	-	-
1-OH and 1'-OH	-	11.75 (1H, s)	C-1, 2, 9a
5-OH and 5'-OH	-	2.81 (1H, d, 2.7)	-
8-OH and 8'-OH	-	13.78 (1H, s)	C-7, 8, 8a

3.3. Docking

Previous research indicated that secalonic acid D could inhibit the topoisomerase I with the same activity as the positive drug camptothecin. However, camptothecin acted as a topoisomerase I poison and secalonic acid D could inhibit the binding between DNA and topoisomerase I, suggesting that secalonic acid D was a topoisomerase I inhibitor [5]. But the specific mechanism how secalonic acid D interacted with topoisomerase I was still unknown.

The complex of human topoisomerase I and DNA (PDB ID: 1A35) was used for docking for its relatively high resolution of 2.5 Å. For a non-intercalative inhibitor of topoisomerase I, the active pocket composed of catalytic residues ARG488, LYS532, ARG590, HIS632, TYR723 was defined as binding site as shown in Figure 2 (A). During topoisomerase I-catalyzed DNA supercoiling, Tyr 723 is involved in reversible formation of a 3′-*O*-phospho-tyrosine bond between the active tyrosine site and the cleaved DNA backbone[7, 8]. Lys532 makes a minor groove contact with the strongly preferred thymidine residue at the site of covalent attachment (-1 position) and functions as a general acid during cleavage to protonate the leaving 5′-oxygen [9]. ARG488, 590 and HIS632 play an important role in stabilizing the catalytic intermediate [7, 10]. So a molecular could function as an inhibitor by either occupies these five residues or blocks it from interacting with DNA molecules.

Our docking results confirmed that secalonic acid D was a topoisomerase I inhibitor rather than a poison. Secalonic acid D could form five hydrogen bonds with two catalytic residues ARG488, LYS532 and three other residues ARG364, GLN633, LYS587, respectively. Furthermore, the binding pose as shown in Figure 2 (B) could block the other two catalytic residues ARG590, TYR 723, so that it could enhance the inhibiting activity of human topo-I. The ΔG_{bind} of the secalonic acid D and human

topoisomerase I complex was -31.81 kcal·mol^{-1} according to the MM-GBSA programme, confirming the strong binding affinity. It was reported that some triterpenoid derivatives were synthesized to function as non-intercalative topoisomerase I inhibitor, which also bounded at the same binding site. However, these triterpenoids itself showed no topo-I inhibiting activity because the short length as the docking results and some of the triterpenoid derivatives synthesized with longer molecular length showed strong topo-I inhibiting activity [11]. Secalonic acid D is a natural dimer of xanthone with long molecular length, which makes it able to form hydrogen bonds extensively at the central region of topoisomerase I through two carbonyl groups and hydroxyls at both end of the molecule, so that it could inhibit DNA molecules from binding with the catalytic residues or even the catalytic region.

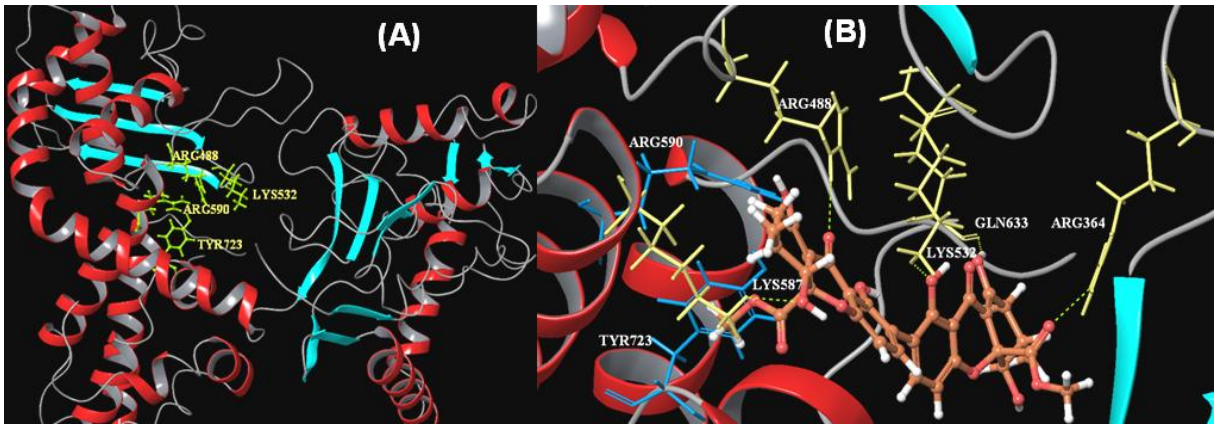

Figure 3. Docking site (A) and docking results (B) of secalonic acid D with human topoisomerase I.

Acknowledgements

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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