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Structure-activity relationships of 1′-acetoxychavicol acetate homologues as new nuclear export signal inhibitors

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Bioassay-guided separation use of the fission yeast expressing NES of Rev, a HIV-1 viral regulatory protein, resulted in isolation of 1′-acetoxychavicol acetate (ACA) from *Alpinia galanga* as a new Rev-transport inhibitor from the nucleus to cytoplasm. Rational design and synthesis of eleven ACA derivatives containing systematic chemical variations were made, biological evaluation of inhibitory activities of these analogues provides the basis to formulate the structure-activity relationship (SAR). The key elements observed were: (1) The *para* substitution of the acetoxyl and 1′-acetoxypropenyl groups at the benzene ring was essential, (2) linear ethyl and propyl chain carbonates were more active than branching chain carbonates, (3) the substitution of acetoxyl groups with alkyl carbamate groups lost or reduced the activities. This study revealed a new salient pharmacophore features as potential drug leads against the HIV virus.

1. Introduction

The current strategy for the treatment of HIV infection is called highly active antiretroviral therapy (HAART) and involves the use of agents that target the viral entry step and the reverse transcriptase and protease enzymes (Pani et al. 2002; Witvrouw et al. 2004; Ye and Li 2006). This therapy has changed the course of HIV infection dramatically. However, the rapid development of drug resistance has led to the emergence of HIV strains that are resistant to multiple anti-AIDS drugs (St Clair et al. 1991; Menéndez-Arias 2002). Furthermore, these drugs have limited or transient benefits due to their adverse effects (Tözsér 2001). Therefore, the discovery and characterization of new anti-HIV agents is required.

Replication of HIV-1 entails an ordered pattern of the viral gene expression, which is dependent upon the viral regulatory protein Rev. Rev acts to increase cytoplasmic accumulation of the viral mRNAs, which encodes the viral structural proteins, through the transport from the nucleus to cytoplasm. As Rev is critical for viral replication, inhibition of the function of Rev is an attractive strategy for therapeutic intervention. Recently, the transport of Rev was shown to be mediated by the receptor protein, chromosomal region maintenance 1 (CRM1), through the direct binding to the nuclear export signal (NES) of Rev (Murakami et al. 2002; Daly et al. 1989; Kjems and Askjaer 2000; Kudo et al. 1998). Leptomycin B (Kobayashi et al. 1998a) has been shown to inhibit the binding of the NES of Rev (RevNES) to CRM1 and exhibits potent inhibitory effect on the proliferation of HIV-1 virus. Leptomycin B was recognized to specifically bind chromosome maintenance region 1 (CRM1) protein and inhibited nuclear export signal (NES)-mediated transport of Rev and U snRNA protein (Kudo et al. 1998; Wolff et al. 1997). Furthermore, the analogous polyketide, callystatin A, isolated from a marine sponge also inhibited the Rev transport from the nucleus to cytoplasm (Murakami et al. 2002). In spite of the potent *in vitro* biological activity, the significant toxic feature of the two polyketides limited their use as chemotherapeutics. A new Rev-transport inhibitor was isolated from the nucleus to cytoplasm with appropriate lipophilicity as medicinal leads, valtrate, from Valerianae radix according to bioassay-guided separation using fission yeast expressing the fusion proteins of GST-NLS-GFP-RevNES (Murakami et al. 2002).

2. Investigations, results and discussion

In order to find new Rev-transport inhibitors, we utilized fission yeast *Schizosaccharomyces pombe* (Kudo et al. 1998), which expressed a fusion protein consisting of glutathione *S*-transferase (GST), SV40 T antigen nuclear localization signal (NLS), green fluorescent protein (GFP), and RevNES. Among about 800 extracts of medicinal plants, the extract of the rhizomes s of *Alpinia galanga*

showed Rev-transport inhibitory activity. Bioassay-guided separation of the extract disclosed 1′-acetoxychavicol acetate (ACA) as a new Rev-transport inhibitor. ACA (**1**) completely inhibited the transport from the nucleus to cytoplasm of the fused protein of *S. pombe* at the concentration of 1 μg/mL.

The Rev protein was an essential factor for HIV-1 replication and promoted the export of unspliced or partially spliced mRNA responsible for the production of the viral structural proteins (Malim et al. 1989). This protein was a potential target for the development of antiviral therapies. Currently reported Rev inhibitors target mainly the interaction between Rev and the Rev-responsive element (Ratmeyer et al. 1996; Zapp et al. 1997). In this paper, it was shown that the low-molecular-mass compound ACA, isolated from the rhizomes of *A. galanga*, inhibited Rev transport completely in an *in vitro* yeast model. ACA may inhibit HIV-1 replication by blocking Rev transport via the CRM1 pathway.

A. galanga is widely cultivated in India, China and Southeast Asian countries, such as Thailand, Indonesia, and Philippines. The rhizomes of this plant were extensively used as spice or ginger substitutes for flavoring food, and also in traditional medicine for several purposes, such as stomachic in China, or for carminative, antiflatulent, antifungal, and anti-itching in Thailand. ACA was first isolated from the rhizomes of *A. galanga* and found to prevent the growth of various fungi (Janssen and Scheffer 1985). In chemical and pharmacological studies of *A. galanga*, the pungent principal compound, 1′-acetoxychavicol acetate, was reported to possess anti-inflammatory, pungency, antifungal, gastroprotective, xanthine oxidase inhibitory activities, antiallergic and inhibition of nitric oxide (NO) production in lipopolysaccharide-activated mouse peritoneal macrophages (Itokawa et al. 1987; Kondo et al. 1993; Moffatt et al. 2000; Zheng et al. 2002; Nakamura et al. 1998; Yang and Eilerman 1999; Janssen and Scheffer 1985; Matsuda et al. 2003a and b; Noro et al. 1998; Matsuda et al. 2005). It was also reported that ACA showed antituberculosis and anti-allergy activity (Palittapongarnpim et al. 2002; Matsuda et al. 2003; Yoshikawa et al. 2004). Furthermore, numerous studies have demonstrated that ACA suppresses the development of many tumors, such as skin cancer, oral cancer, colon cancer, liver cancer, bile-duct cancer and oesophageal cancer *in vivo* (Murakami et al. 1996; Ohnishi et al. 1996; Tanaka et al. 1997a and b; Kobayashi et al. 1998b; Kawabata et al. 2000; Miyauchi et al. 2000), but the mechanism was less well understood. Recently, ACA was found to inhibit beta interferon mRNA expression and nuclear factor kB activation in lipopolysaccharide-activated mouse peritoneal macrophages, resulting in inhibition of the production of nitric oxide (Ando et al. 2005).

In this study, we have discovered a new function of ACA. ACA was able to compete with LMB, an inhibitor of Rev transport, in binding to CRM1, suggesting that ACA, similar to LMB, probably blocks Rev transport via CRM1-mediated export pathway. As a Rev transport inhibitor that targets a different stage of the HIV-1 replication cycle, ACA may produce favorable interactions with other agents to overcome these issues. Development of the low-cost, low cytotoxicity Rev transport inhibitor ACA is a promising approach towards novel antiretroviral therapies.

Eleven new derivatives of ACA were designed and synthesized. Bioassay results reveal salient features of the structure-activity relationship of these series: Introduction of a *N*-substituent on the ACA nucleus decreases the bioactivity of the derivatives; introduction of an *O*-substituent on ACA nucleus can be tolerated. To clarify the structure-activity relationships, a variety of synthetic ACA analogues were tested to see how they inhibited the transport from the nucleus to cytoplasm (Table), ipr ester (**2**), Et (**5**), Pr carbonate (**6**) showed similar or higher activity to ACA, these structures all have the acetoxyl and 1′-acetoxypropenyl groups at the benzene ring, so these findings suggested that acetoxyl and 1′-acetoxypropenyl groups were essential for strong activity. tBu ester (**3**), iPr (**7**), tBu carbonate (**8**) did not show activity in these concentration. While Et (**5**) and Pr carbonate (**6**) showed strong activity. Thus, it is suggested that the head group preference carboxyl or carbonate group connect linear short chain without branch. Pr (**10**) and iPr carbamate (**11**) of ACA showed very weak activity, while Et (**9**) and tBu carbamate (**12**) of ACA did not show activity even in 2 mM concentration. Introduction of carbamate group reduced the activity over 20 fold. These findings suggested the substitution of acetoxyl groups with alkyl carbamate groups lost or reduced the activity.

Table: Effect of ACA analogues on fission yeast *Schizosaccharomyces pombe*

Compd.	2 mM	1 mM	0.5 mM	0.2 mM	0.1 mM	0.05 mM
1	+−	+−	+	+	+−	−
2	+−	+−	+−	−	−	−
3	−	−	−	−	−	−
4	−	−	−	−	−	−
5	+	+	+−	−	−	−
6	+	++	+	+	+	+−
7	−	−	−	−	−	−
8	−	−	−	−	−	−
9	−	−	−	−	−	−
10	+−	−	−	−	−	−
11	+−	−	−	−	−	−
12	−	−	−	−	−	−

The results are the evaluation of three independent experiments, concentration from 0.05–2 mM, positive control used valtrate at concentration 20, 10, 3 μg/mL. The yeasts were scored by this system: $0 < - < 10\% < +- < 40\% < + < 60\% < ++ < 80\%$

Eleven new derivatives of ACA containing systematic chemical variations were rationally designed under the guidance of molecular modeling and successfully synthesized. The bioassay data obtained from these compounds demonstrate that the *para* substitution of the acetoxyl and 1′-acetoxypropenyl groups at the benzene ring was essential, were key determinants of the bioactivity of this type of inhibitors. Our data provide new insights into the essential interactions of ACA, thus leading to a legible pharmacophore of NES inhibitors that will aid future inhibitor design. In conclusion, the structural requirements of ACA analogues were clarified as follows: (1) The *para* substitution of the acetoxyl and 1′-acetoxypropenyl groups at the benzene ring was essential, (2) linear ethyl and propyl chain carbonates are more active than branching chain carbonates, (3) the substitution of acetoxyl groups with alkyl carbamate groups prevented or reduced the activities.

3. Experimental

3.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on a JNM-GX-500 (JEOL) spectrometer. Chemical shifts were reported with reference to the respective residual solvent peaks (δ_H 3.30 and δ_C 49.0 for CD_3OD). FABMS data were obtained on a JMS SX-102 (JEOL) instrument using *m*-nitrobenzyl alcohol as the matrix. For flash column chromatography, silica gel (BW-200, 400–500 mesh, Fuji Sylisia) was used, whereas TLC and HPTLC

analysis were carried out on precoated plates (Merck, Kieselgel $60F_{254}$, 0.25 mm, and RP-18 WF_{254}, respectively). Spots were visualized under UV 254 and 365 nm, and 1% $Ce(SO_4)_2$/10% H_2SO_4, *p*-anisaldehyde/H_2SO_4 (AcOH 5 mL, *c*-H_2SO_4 25 mL, EtOH 425 mL, water 25 mL) spray reagents. Reversed-phase HPLC was performed on a semipreparative Cosmosil C18-AR-II column (250 × 10 mm, 4 μm, 80 Å), using a Shimadzu SPD-10A vp UV-VIS detector.

3.2. Plant material

A. galanga was collected in Thai, July 2001. A voucher specimen was deposited in the Medicinal Plants Source Exploration Laboratory, Graduate School of Pharmaceutical Science, Osaka University, Japan.

3.3. Extraction and isolation

Air-dried *A. galanga* (300 g) was cut into small pieces, and successively extracted three times with MeOH at room temperature. The extracts were combined and concentrated in vacuum to obtain a residue. A part of this extract (18 g) was partitioned between water and EtOAc, to yield an active organic fraction (9 g) that was then subjected to normal-phase flash chromatography (Hexane : EtOAc, 10 : 1, Hexane : EtOAc, 3 : 1, and MeOH elution), to afford four fractions (A1–A4). Fraction A3 showed 90% growth inhibition of NES at a concentration of 5 μg/mL. The active fraction A3 was submitted to RP-18 HPLC (MeCN : H_2O, 70 : 30), and yielded pure compound (2.5 g). The chemical structure of this compound was identified as ACA by comparison of the spectral data of mass and NMR with those of reported (De Pooter et al. 1985; Morita and Itokawa 1988).

3.4. Screening assay

A fission yeast, *S. pombe*, that expresses a fusion protein consisting of glutathione *S*-transferase (GST), the simian virus 40 T-antigen nuclear-localization signal (NLS), green fluorescent protein (GFP) and the Rev nuclear-export signal (RevNES) was utilized to perform a screening assay for Rev transport inhibition as described by Kudo et al. (1998). The practical assay protocol was as follows. After inducing the fusion protein of *S. pombe* in thiamine-free medium for 24 h at 37 °C, the cells were seeded in 96-well plates along with test samples in the medium containing 1% DMSO and incubated at 37 °C for further 3 h. The distribution of the GST-NLS-GFP-RevNES-fused protein was monitored by fluorescence microscope.

3.5. Screening for Rev transport inhibitors

More than 800 medicina plants from China, Thai, Congo, Kenyan and Brazil were extracted with methanol and screening assays were performed to select Rev transport inhibitors. The extract obtained from *A. galanga* inhibited transport of the fusion protein of *S. pombe* from the nucleus to the cytoplasm at a concentration of 5 μg/ml. In order to determine the substances accounting for this inhibition of Rev transport, the methanol extract of *A. galanga* was fractionated further and the different fractions were tested. A dose-dependent inhibitory effect was observed when incubated with A3 fraction, whilst other fractions were not obviously active. Therefore, A3 fraction was purified further to obtain the bioactive compound ACA. This compound completely inhibited the export of the RevNES fusion protein from the nucleus at 1 μg/mL in comparison with the DMSO control.

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