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From the traditional Chinese medicine plant *Schisandra chinensis* new scaffolds effective on HIV-1 reverse transcriptase resistant to non-nucleoside inhibitors

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HIV-1 reverse transcriptase (RT) is still an extremely attractive pharmaceutical target for the identification of new inhibitors possibly active on drug resistant strains. Medicinal plants are a rich source of chemical diversity and can be used to identify novel scaffolds to be further developed by chemical modifications. We investigated the ability of the main lignans from *Schisandra chinensis* (Turcz.) Baill. fruits, commonly used in Traditional Chinese Medicine, to affect HIV-1 RT functions. We purified 6 lignans from *Schisandra chinensis* fruits and assayed their effects on HIV-1 RT and viral replication. Among the *S. chinensis* fruit lignans, Schisandrin B and Deoxyschizandrin selectively inhibited the HIV-1 RT-associated DNA polymerase activity. Structure activity relationship revealed the importance of cyclooctadiene ring substituents for efficacy. In addition, Schisandrin B was also able to impair HIV-1 RT drug resistant mutants and the early phases of viral replication. We identified Schisandrin B and Deoxyschizandrin as new scaffold for the further development of novel HIV-1 RT inhibitors.

Keywords: *Schisandra chinensis*, HIV-1, reverse transcriptase, plant extracts, plant diversity, HIV resistance

Introduction

Despite the approval of 25 drugs for Human Immunodeficiency Virus type 1 (HIV-1) therapy, emergence of drug resistance strains and side effects due to chronic drug administration require the identification of new HIV-1 inhibitors (Mehellou De Clercq, 2010). For this purpose, HIV-1 reverse transcriptase (RT) is still an extremely attractive drug target since it is a viral-coded protein characterized by multifunctional activities essential for different and fundamental steps in the viral retrotranscription process (Tramontano and Di Santo, 2010; Distinto *et al.*, 2012; Esposito *et al.*, 2012; Corona *et al.*, 2013). In particular, HIV-1 RT has two main enzymatic functions: an RNA-Dependent DNA Polymerase (RDDP) activity, responsible for the formation of the RNA:DNA intermediate, and a Ribonuclease H (RNase H) activity, responsible for the hydrolytic cleavage of the RNA strand of the RNA:DNA hybrid (Esposito *et al.*, 2012). According to their different mechanism of action, RT Inhibitors (RTIs), all selectively inhibiting the DNA polymerase function, are divided into i) Nucleoside RT inhibitors (NRTIs), that structurally resemble and compete with natural nucleotides, and ii) Non-Nucleoside RT Inhibitors (NNRTIs) that are structurally different allosteric agents and bind to a hydrophobic pocket near to the DNA polymerase active site (Esposito *et al.*, 2012). Selection of drug resistant HIV-1 strains represents one of the major therapeutic problems. In particular, the HIV-1 RT Y181C and Y188L point mutations are important for the development of drug resistance to first-generation NNRTIs such as Nevirapine (Mellors *et al.*, 1992, 1993; Ren and Stammer, 2008), while the RT K103N mutation plays a crucial role in drug resistance to second-generation NNRTIs such as Efavirenz (Maga *et al.*, 1997; Domoal and Demeter, 2004).

Plants are a source of chemical diversity and can be used to identify new scaffolds to be further developed by chemical modifications (Li and Vederas, 2009). Despite this, only a limited number of plant extracts have been actually searched for their efficacy on HIV-1 replication and no natural compound has been developed up to clinical approval for HIV treatment (Yu *et al.*, 2007; Cos *et al.*, 2008). Traditional Chinese Medicine (TCM) is the major ancient therapeutic system in China and its herbal component is the most important (Pan *et al.*, 2013). Until now only very few plants used in TCM have been explored for antiviral efficacy, despite the fact that screening for active lead compounds from TCM extracts is considered more efficient than a screening from a standard combinatorial chemical library. In fact, considering the long TCM history, a great number of compounds (hits) are likely to be discovered and to be better suited for lead development given their higher degree of drug-like properties (Li and Peng, 2013).

In TCM, the fruit of *Schisandra chinensis* (Turcz.) Baill. is commonly known as Wu-Wei-Zi with thousands years of history. It is recorded in the "Shen Nong's herbal classic" as

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a top grade material that helps in cough and prevents asthma and it is officially listed in the Chinese Pharmacopoeia as a tonic, sedative and astringent agent (National Pharmacopoeia Committee, 2010). In the past decades, Wu-Wei-Zi has been developed as an alternative medicine for the treatment of various liver diseases (Pao *et al.*, 1975; Liu *et al.*, 1985). Chemical investigations on Wu-Wei-Zi revealed that the mainly components are lignans, especially dibenzo[a,c]cyclooctadiene lignans, which showed antihepatotoxic, antioxidant, anticancer activities, as well as effects on the central nervous system (Liu and Lesca, 1982; Hancke *et al.*, 1999; Opletal *et al.*, 2004; Panossian and Wikman, 2008). Particularly, components such as Schisandrol A and Schisandrol B showed the capacity to lowering elevate transaminase levels in mice protecting liver by damages and oxidative stress (Pu *et al.*, 2012), while other components such as Schisantherin A and Deoxyschizandrin were reported to show liver-protective, anti-tumor and anti-oxidant activities and showed also cardioprotective effects in rats (Xu *et al.*, 2005; Pan *et al.*, 2011; Pu *et al.*, 2012; Chang *et al.*, 2013). Furthermore, another component, Schisandrin B, showed a generalized protective effect against tissues oxidation, particularly in neuronal cells, and was able to selectively inhibit ATR kinase activity in DNA damage response (Nishida *et al.*, 2009; Lam and Ko, 2012; Chen *et al.*, 2013).

In addition, single components of other members of the *Schisandraceae* family such as the *S. sphaerandra* and *S. rubriflora* have been reported to inhibit the HIV-1 RT activity as well as viral replication, respectively (Sun *et al.*, 1996; Chen *et al.*, 2006; Xiao *et al.*, 2010).

Hence, in the present work, we investigated the effect of six dibenzo[a,c]cyclooctadiene lignans extracted and purified from the *S. chinensis* fruits on both HIV-1 RDDP and RNase H RT functions.

In biochemical assays two components, Schisandrin B and Deoxyschizandrin, selectively inhibited the HIV-1 RT-associated DNA polymerase activity in the micromolar range. Schisandrin B was able to impair the early phases of HIV-1 replication in cell-based assays and, importantly, it was effective also on HIV-1 K103N, Y181C, and Y188L single RT mutants in biochemical assays.

Materials and Methods

Compound purification

Briefly, six lignans including Schisandrol A, Schisandrol B, Schisantherin A, Deoxyschizandrin, Schisandrin B, and Schisandrin C were isolated and purified by repeated open-column chromatography including SiO_2, Sephadex LH-20 and ODS column from the petroleum ether and EtOAc part of *S. chinensis* (Hu *et al.*, 2013). The chemical structure was confirmed based on 1H NMR and 13C NMR data. The purities (higher than 97%) of those 6 compounds were determined by HPLC- DAD.

Protein expression and purification

HIV-1 RT gene subcloned into the p6HRT_prot plasmid was kindly provided by Stuart Le Grice (NCI). Protein expression and purification was performed in M15 *E. coli* cells as described (Suchaud *et al.*, 2010). HIV-1 RT K103N, Y108C and Y188L mutants were produced by site-directed mutagenesis using the Stratagene kit according manufacturer's indication.

HIV-1 RNase H polymerase-independent cleavage assay

The HIV-1 RT-associated RNase H activity was measured as previously described (Corona *et al.*, 2014).

HIV-1 RDDP assay

The HIV-1 RT-associated RDDP activity was measured using the Enz-Check Reverse Transcriptase Assay Kit (Invitrogen), as previously described (Esposito *et al.*, 2013).

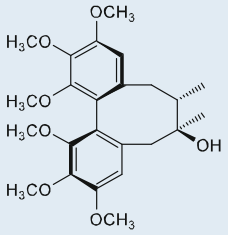
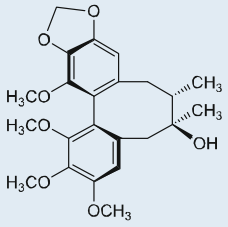
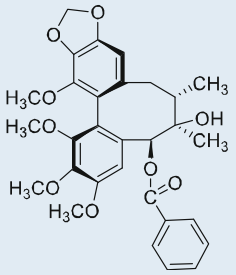
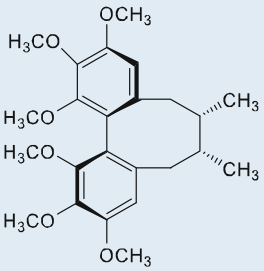
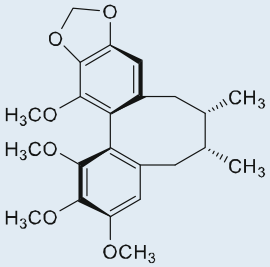
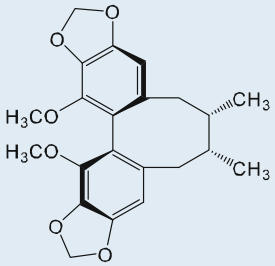
Cell culture assay

The cytotoxicity of the compounds for the human T lymphoblastoid cell line Jurkat Clone E6-1 (ATCC® TIB-152) was assessed using the MTT method (Mossmann *et al.*, 1983). The ability of active compounds to inhibit the viral replication was performed using a single round of infection which measures the efficiency of the early events of the virus life cycle as previously described (Helseth *et al.*, 1990). Compound concentration required to inhibit early phases of HIV-1 replication by 50% (EC_{50}) was calculated by nonlinear regression analysis with Sigma Plot (Jandel Scientific). Only results within a linear range (HIV-1 LTR-driven reporter CAT gene expression, i.e. conversion of chloramphenicol to acetyl chloramphenicol above 50%) were elaborated.

Results

With the aim of identifying new chemical scaffolds that could be used to develop novel anti-HIV-1 agents, we assayed the fruit components of the TCM plant *Schisandra chinensis* (Turcz.) Baill. fruits, also taking into account that other compounds extracted from plants belonging to the *Schisandraceae* family have been shown to inhibit both HIV-1 RT-associated RDDP activity and virus replication (Sun *et al.*, 1996; Xiao *et al.*, 2010). Starting from *S. chinensis* fruit extracts, we extracted and purified six dibenzocyclooctadiene lignans: Schisandrol A, Schisandrol B, Schisantherin A, Deoxyschizandrin, Schisandrin B, and Schisandrin C. All these six compounds show a twist boat-chair configuration with a linked biphenyl cyclohexadiene system and each ring is substituted with various groups. Schisantherin A is a *S*-biphenyl while other five lignans are *R*-biphenyl configuration. The six compounds were first tested for their effects on both HIV-1 RT associated functions, RDDP and RNase H activities, using Efavirenz and RDS1643 (Tramontano *et al.*, 2005) as positive controls (Table 1). Results showed that lignans Deoxyschizandrin and Schisandrin B inhibited the RT-associated RDDP activity in the micromolar range (IC_{50} values around 30 μM concentration), lignans Schisandrol B and Schisandrin C slightly inhibited the HIV-1 RT-associated RDDP activity (IC_{50} values around 100 μM concentration), while lignans Schisandrol A and Schisantherrin A were ineffective. Differently, none of the six lignans was able to inhibit the RT-asso-

Table 1. **Effect of *S. chinensis* components on the HIV-1 RT-associated activities**

Compound	Chemical structure	RDDP IC_{50} (μM)[a]	RNase H IC_{50} (μM)[b]
Schisandrol A		>100 (92%)[c]	>100 (100%)
Schisandrol B		100 ± 5.0	>100 (100%)
Schisantherrin A		>100 (67%)	>100 (92%)
Deoxyschisandrin		34.5 ± 4.5	>100 (100%)
Schizandrin B		29.0 ± 1.0	>100 (100%)
Schizandrin C		100 ± 5.0	>100 (82%)
RDS1643		> 50	8.6 ± 1.1
Efavirenz		0.023± 0.004	> 10

[a] Compound concentration required to reduce HIV-1 RT-associated RDDP activity by 50%.
[b] Compound concentration required to reduce HIV-1 RT-associated RNase H activity by 50%.
[c] Percentage of control activity in the presence of 100 μM compound concentration.

Table 2. **Effect of deoxyschisandrin and Schizandrin B on HIV-1 replication**

Compound	EC_{50} (μM)[a]	CC_{50} (μM)[b]	SI[c]
Deoxyschisandrin	> 20	20	-
Schizandrin B	15	> 100	> 6

[a] Compound concentration required to inhibit early phases of HIV-1 replication by 50%.
[b] Compound concentration required to reduce Jurkat cell viability by 50%.
[c] Selective index.

ciated RH function even at 100 μM concentration (Table 1).

In order to assess the potential of the new identified scaffolds, we tested the ability of the two most active compounds, Deoxyschizandrin and Schisandrin B, to inhibit the early phase of the HIV-1 replication using an *env* complementation system capable of one single round of infection (Helseth *et al*., 1990) previously used to examine early events, i.e. entry, reverse transcription and LTR-driven gene expression in the infection process (Parolin *et al*., 2003). Results showed that Schisandrin B holds an interesting antiviral activity and impair the early steps of HIV-1 replication with a selective index > 6, while Deoxyschizandrin was ineffective due to cell toxicity (Table 2).

To further assess the Schisandrin B scaffold potential for drug development, we evaluated its ability to inhibit the HIV-1 RT-associated RDDP activity of enzymes bearing some of the most common, clinically relevant, single mutations involved in NNRTI resistance: K103N, Y181C, and Y188L RTs (Table 3). Interestingly, Schisandrin B confirmed its ability to inhibit the HIV-1 RT-associated RDDP function showing only a 2-fold increase in IC_{50} value when tested on the Y181C RT mutant and a 3-fold increase when tested on the K103N and Y188L RT mutants.

Discussion

TCM is a millenary therapeutic system based on a holistic approach in which the pathological manifestations are the result of an overall body disequilibrium, while western medicine is characterized by a more analytic approach. Although these methodologies can appear so different, TCM constitutes an ancient and rich source of knowledge, with a great application potential also in an analytical drug discovery process. In fact, since TCM extracts have been experienced for their medical properties for such a long time, their use in screening processes allows for more efficient identification of hits compounds with drug-like characteristics (Li and Peng, 2013).

In the present study we investigated the anti-HIV-1 therapeutic potential of components of the fruits from *S. chinensis*, a plant belonging to *Schisandraceae* family whose other members have already been shown to have constituents with anti-HIV-1 activity (Sun *et al*., 1996; Xiao *et al*., 2010), and whose main components are a group of lignans known in TCM also for their drug-like properties (Pao *et al*., 1975; Liu and Lesca, 1982; Liu *et al*., 1985; Hancke *et al*., 1999).

The structure activity relationship (SAR) study of the effects of the six tested lignans on the HIV-1 RT-associated RDDP activity demonstrates the importance of the substituents on the cyclooctadiene ring for RT inhibition. In particular, the two methyl substituents in position 6 and 7 of the cyclooctadiene ring appear to be essential for RT inhibition by Schisandrin C, Deoxyschizandrin, and Schisandrin B, as showed also for some structurally-related lignans extracted from the fruits of *S. rubiflora*, another plant belonging to the same taxonomic family of *S. chinensis* (Chen *et al*., 2006). The importance of these substituents is also demonstrated by the fact that, on the one side, the addition of an hydroxy moiety in position 7 of the cyclooctadiene ring reduces RT inhibition (compare Schisandrin B to Schisandrol B), on the other side their substitution with hydrogen atoms abolishes RT inhibition (compare Deoxyschizandrin to Schisandrol A). Active compounds differ for the absence (Deoxyschizandrin) or the presence of one (Schisandrin B and Schisandrol A) or two (Schisandrin C) dioxolane rings as cyclooctadiene ring substituents. While the presence of two dioxolane rings reduces the potency of RT inhibition, hence suggesting the importance of the three-hydroxymethyl substituted phenyl ring for Deoxyschizandrin and Schisandrin B interaction with RT, the presence of only one dioxolane ring clearly reduces the compound cytotoxicity leading Schisandrin B to exert an anti-viral effect in cell-based assays. It is worth to note that also the modification of the methoxy substituents in positions 1 and 13 could probably be important in enhancing the Schisandrin B inhibition potency, as suggested by two similar molecules extracted from the fruits of *S. rubiflora*, where the replacement of the two methoxy moieties with hydroxyl groups showed a significant increase in the compounds anti-HIV-1 activity (Chen *et al*., 2006).

In the effort of assessing the Schisandrin B relevance as new scaffold for drug development, we evaluated its effects on the single HIV-1 RT K103N, Y181C, and Y188L mutants, that are relevant for NNRTI resistance in clinical practice. Interestingly, Schisandrin B showed only a 2–3 fold reduction in its potency of RT inhibition against all three mutant RTs, while Efavirenz showed a 7–10 fold IC_{50} increase on K103N and Y188L RT mutants.

In conclusion, results confirmed that TCM is a valuable source of compounds with drug-like properties and demonstrated that Schisandrin B is a promising scaffold for the further development of novel HIV-1 RT inhibitors that can be effective also on NNRTI resistant strains.

Table 3. **Effect of Schizandrin B on HIV-1 RT mutants**

Compound	IC_{50} (μM)[a]		
	K103N RT	Y181C RT	Y188L RT
Schizandrin B	90 ± 7	55 ± 4	95 ± 5
Efavirenz	0.176 ± 0.025	0.050 ± 0.009	0.198 ± 0.062

[a] Compound concentration required to reduce HIV-1 RT-associated RDDP activity by 50%.

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