Hypericum sampsonii induces apoptosis and nuclear export of retinoid X receptor-alpha

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Natural products derived from plants provide a rich source for development of new anticancer drugs. Recent studies suggest that modulation of subcellular localization of retinoid X receptor-alpha (RXRα) represents a potential approach for inducing cancer cell apoptosis. In this study, we screened a herbal library for inducing translocation of RXRα from the nucleus to the cytoplasm. Our results revealed that the extract of Hypericum sampsonii, a member of the genus Hypericum, had remarkable effect on RXRα subcellular localization in various cancer cells. Treatment of NIH-H460 human lung cancer cells with H.sampsonii extract resulted in relocalization of RXRα from the nucleus to the cytoplasm. Cytoplasmic RXRα induced by H.sampsonii was associated with mitochondria, accompanied with cytochrome c release and apoptosis. H.sampsonii extract effectively inhibited the growth of various cancer cell lines, including NIH-H460 lung cancer, MGC-803 stomach cancer and SMMC7721 liver cancer cells. The growth inhibitory effect of H.sampsonii extract depended on levels of RXRα, as it failed to inhibit the growth of CV-1 cells lacking detectable RXRα, whereas transfection of RXRα into CV-1 cells restored its apoptotic response to H.sampsonii. Furthermore, the apoptotic effect of H.sampsonii was significantly enhanced when RXRα was overexpressed in NIH-H460 cells. Together, our results demonstrate that H.sampsonii contains ingredient(s) that induce apoptosis of cancer cells by modulating subcellular localization of RXRα.

Introduction

Herbal medications have been widely practiced for centuries by various cultures throughout history. In China, the prevalence of traditional Chinese medicine (TCM) can be dated back to two thousand years ago. Specific herbal extracts and their combinations have been devised to treat specific diseases including cancers (1,2). On the basis of well-documented efficacy in clinic, natural products of plant provide excellent and reliable sources for the development of new drugs (1–3). In Western medicine, one of the challenges in searching for an effective cancer treatment is that in vitro activity does not always lead to human efficacy. In contrast, TCM, which is effective in humans, is often without a known molecular target. Recent studies have provided various screening approaches for identifying novel leads from herbal extracts for drug discovery (4). Among them, mechanism-based screening is one of the most valuable methods, as it offers opportunity of optimizing the leads (5,6).

Nuclear receptors (NRs) represent the largest family of eukaryotic transcriptional factors, which plays a critical role in the regulation of cell growth, proliferation and differentiation, metabolism, immune response and apoptosis (7–11). They are activated by the binding of ligands such as vitamins, steroid hormones and fatty acids (7,8,12,13). Ligand-binding promotes a conformational change of the ligand-binding domain (LBD) that affects dimerization, binding of accessory proteins and cross-talk with other signaling pathways (7,8,14). NRs are frequent biological targets of active compounds contained in herbal remedies (5,9–11). Several diseases related to malfunction of NRs, such as cancers, osteoporosis, diabetes and obesity, are currently treated with NR-targeted drugs (10,11).

Among NRs, retinoid X receptor (RXR) subtypes (α, β and γ) are unique in both structure and diatomic functions (12,13,15–19). They heterodimerize with many members of the NR superfamily, including retinoid acid receptor (RAR), vitamin D receptor, peroxisome proliferator-activated receptor and thyroid hormone receptor, as well as several orphan receptors (12,13,15–19). RXRs, therefore, play an essential role in the regulation of multiple nuclear hormone-signaling pathways. Genetic disruption of RXRα targeted at the prostatic epithelium results in intraepithelial neoplasia (20), whereas targeted disruption of RXRα in the skin leads to various skin dysfunctions (21,22). Diminished RXRα protein expression is frequently observed in cancer cells, suggesting its role in the development of human cancer (23). Owing to the promiscuity of the RXR ligand-binding pocket (LBP) (24), a number of natural and synthetic compounds with diverse structures, such as 9-cis-retinoic acid (9-cis-RA) (25), dietary fatty acids (26–28), Targretin/bexarotene (29), phytanic acid (30,31) and non-steroidal anti-inflammatory drug (NSAID)—R-etodolac (32), have been shown to bind RXRs and act as RXR ligands. Some of them are used or are being evaluated in various models for the prevention and treatment of cancers and diseases.

The mechanisms by which RXRs exert their biological effects have been the subject of intensive study. RXRs bind specific DNA response elements either as heterodimers or homodimers to positively or negatively regulate transcription.

Abbreviations: CAT, chloramphenicol acetyltransferase; DAPI, 4′,6′-diamidino-2-phenylindole; EtOH, ethanol; GFP, green fluorescence protein; Hsp60, heat shock protein 60; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NES, nuclear export sequence; NRs, Nuclear receptors; 9-cis-RA, 9-cis-retinoic acid; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; RXR, retinoid X receptor.

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of target genes (12,13,15–19). On binding ligands, the receptors undergo conformational changes to release coressor and recruit coactivators, permitting the multiprotein transcriptional machinery to initiate transcription (7,8,14). Recent studies have revealed an RXR non-genotropic signal transduction pathway, which appears to play a role in development, differentiation and apoptosis. Like many nuclear proteins, RXR shuttles between the cytoplasm and the nucleus (33,34). Cytoplasmic localization of RXR may play a role in postnatal testicular development (35,36). In response to nerve growth factor (NGF) treatment in PC12 pheochromocytoma cells, RXR translocates from the nucleus to the cytoplasm, resulting in co-migration of orphan NR NGFI-B (also known as Nur77 and TR3) and differentiation (37), whereas RXR co-migrates with thyroid hormone receptor to mitochondria to regulate mitochondrial transcription (38). Similarly, translocation of RXRα/Nur77 heterodimer from the nucleus to the cytoplasm (34,39,40) leads to apoptosis of cancer cells, which is mediated by mitochondrial targeting of RXRα/Nur77 heterodimer and their regulation of Bcl-2 activity (39,41). Such a translocation of RXRα/Nur77 heterodimer between the nucleus and the cytoplasm can be regulated by RXRα ligand (34,39) and epidermal growth factor (42).

RXRα translocation to the cytoplasm is regulated by its dimerization and ligand binding (39). Previous studies (39) demonstrated that certain RXRα transcription agonists, such as 9-cis-RA, inhibited the RXRα translocation. However, RXRα ligands that induce the translocation remain to be identified. In this study, we hypothesized that certain Chinese herbs could inhibit cancer cell growth through their modulation of RXRα subcellular localization. After screening >500 kinds of crude extracts from a herbal library, we found that extract of Hypericum sampsonii (also referred to as Yuan Bao Cao in China) (43–45) contains active components that induce RXRα nuclear export and apoptosis in cancer cells. H. sampsonii is a member of the genus Hypericum, which also includes Hypericum perforatum (commonly known as St John’s wort). St John’s wort is widely used for the treatment of depression and a range of other ailments, including bacterial and viral infection, peptic ulcers and inflammation, burns and skin disease (46,47). Lipophilic extracts of St John’s wort also show anti-neoplastic activity in vitro and in vivo (48,49). H. sampsonii has been traditionally used in China for the treatment of hematemesis, epistaxis, menstrual irregularity, external traumatic injury and swellings (44). It also exhibits anticancer activity (44,45), and has been used as a promising anticancer herb in Taiwan (44). Our present results demonstrated that H. sampsonii extract was able to induce the translocation of RXRα from the nucleus to the cytoplasm where it targeted mitochondria. The effect of H. sampsonii on RXRα translocation was associated with extensive RXRα-dependent apoptosis of cancer cells. This finding demonstrates for the first time that a Chinese herb exerts its anticancer activity through its modulation of RXRα subcellular localization. Our results provide a mechanistic rationale for the identification of an active component(s) in H. sampsonii and the development of RXRα-based herbal medicine.

Materials and methods

Reagents
Lipofectamine PLUS reagents from Invitrogen (Carlsbad, CA), enhanced chemiluminescence (ECL) reagents and anti-mouse IgG conjugated with Cy3 from Amersham Pharmacia Biotech (Piscataway, NJ), polyclonal anti-RXRα (D20), anti-Hsp60, anti-PARP (sc-7150), anti-Flag (mouse), and goat anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-β-actin antibody and fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG from Sigma (St Louis, MO), monoclonal anti-cytochrome c antibody from Pharmingen (San Diego, CA) were used in this study. All other chemicals used were commercial products of analytical grade obtained from Sigma.

Herb material and extraction
H. sampsonii was collected from Nan-ning, Guangxi Province, China, in June, 2004, and authenticated by Professor Changqi Hu (School of Pharmacy, Medical Center of Fudan University, Shanghai, China). The dried whole herb was finely ground and macerated for 5 h twice at 50°C with a 5-fold amount of 95% ethanol (EtOH). The combined EtOH extracts were evaporated under reduced pressure to give a residue. Usually, extracting by this way generated some 5% solids in the residue. The residue was macerated in water and extracted repeatedly with petroleum ether, chloroform and ethyl acetate. The chloroform partition was purified by flash chromatography on a silica gel using EtOH gradient (0–100%). The elute was concentrated to dryness. Water extracts (control) were obtained by refluxing at 100°C for 3 h and were freeze-dried. All dried extracts were stored at −80°C until use.

Cell lines and culture
NIH-H460 lung cancer cells [American Type Culture Collection (ATCC)] were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), SMMC-7721 liver cancer (ATCC) and MGC-803 gastric cancer cells (41), and CV-1 African green monkey kidney cells were maintained in Dulbecco’s modified Eagle`s medium (DMEM) supplemented with 10% FBS. All cultured cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. Cell lines were sub-cultured according to their individual growth profiles in order to ensure exponential growth throughout the experiments.

MTT assay
Cells were seeded in a 96-well plate and treated the following day with H. sampsonii extracts dissolved in 0.2% dimethyl sulfoxide (DMSO) at serial concentrations (5, 10, 20, 40, 60 and 80 µg/ml). Control group received 0.2% DMSO. After 3 days, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (4.14 mg/ml) was added to each well and incubated at 37°C for 4 h. The formed formazan crystals were dissolved in 100 µl DMSO for 10 min with shaking. Each plate was read immediately on a microplate reader (Bio-Rad, USA) at a wavelength of 490 nm. Three independent experiments were performed in triplicate. Results are expressed as the concentration of the extract required to inhibit cellular growth 50% (IC50) ± standard deviation (SD).

Apoptosis assays
Cells cultured in six-well plates in 0.5% FBS medium were incubated with vehicle or different amounts of extract of H. sampsonii for 24 h. After incubation, detached and attached cells were collected and centrifuged. The cells were resuspended in phosphate-buffered saline (PBS) containing 50 µg/ml 4′-6-diamidino-2-phenylindole (DAPI) and 100 µg/ml DNase-free RNase A, and incubated for 20 min at 37°C with protection from light. Fluorescence microscope (Olympus) was used to visualize the nuclei. Apoptotic cells were identified as typical morphology of shrinkage of the cytoplasm, membrane blebbing and nuclear condensation and/or fragmentation (24,41). At least 1000 cells from >10 random microscopic fields were counted by two investigators.

Cell lysis and fraction
Control and treated cells were rinsed with ice-cold PBS and harvested in a lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 2 µg/ml pepstatin A and 2 µg/ml leupeptin] with proteinase inhibitors and homogenized. The cell extracts were centrifuged at 800 × g for 10 min. The pellet containing nuclei was resuspended in 200 µl of 1.6 M sucrose in hypotonic buffer plus protease inhibitors and laid over 1 ml of 2.0 M sucrose in the same buffer and then centrifuged at 150 000 × g for 90 min at 4°C to obtain the nuclear fraction. The supernatant was purified by centrifuging at 10 000 × g for 30 min at 4°C to obtain cytosolic fractions. Nuclear fractions were resuspended in 100 µl of...
Western blotting
The total lysates or fractions were electrophoresed on 8% sodium dodecyl
sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred
tonitrocellulose membranes. Blots were blocked in 5% non-fat milk in TBST
[20 mM Tris–HCl (pH 7.4), 137 mM NaCl and 0.05% Tween-20] overnight at
4°C. After two additional washes in TBST, the blots were incubated with
various primary antibodies overnight at 4°C, followed by peroxidase-con-
jugated secondary antibody for another 1 h at room temperature
(24:41). The blots were developed by using ECL system according to proposed
protocol. The blots were reprobed with anti-β-actin antibody to confirm equal
loading of proteins in each lane.

Immunohistochemistry
The immunostaining method was described previously (24,39,41) and was
adopted with modification. For initial herbal screening, cells were cultured
on 96-well plates or glass slides in 24-well plates. Control and treated cells
were fixed with cold 4% polyformaldehyde in PBS for 30 min. The fixed cells
were washed twice in PBS, and then incubated in cold permeabilization solu-
tions (0.1% Triton X-100 and 0.1% sodium citrate) for 10 min. Cells were
stained with polyclonal anti-RXRα antibody (1 : 500) followed by anti-goat
IgG conjugated with Cy3 (1 : 1000), and then re-stained with DAPI. For
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antibody (1 : 500) followed by anti-goat
IgG conjugated with Cy3 (1 : 1000), and then re-stained with DAPI. For
confoal study, NIH-H460 cells were co-stained with anti-RXRα antibody
and anti-Hsp60 goat IgG (1 : 500) to determine whether RXRα targeted at
mitochondria. FITC-labeled anti-rabbit IgG (1 : 500) and anti-goat IgG conju-
gated with Cy3 (1 : 1000) were used to recognize RXRα and Hsp60, respecti-
vately. To determine whether RXRα nuclear export was associated with
cytochrome c release and apoptosis, cells were immunostained with anti-
cytochrome c (mouse) and anti-RXRα (rabbit) followed by FITC-conjugated
anti-mouse IgG and Cy3-conjugated anti-rabbit IgG. Cells were stained with
DAPI. Alternatively, CV-1 cells transfected with or without Flag-tagged
RXRα were exposed to H.sampsonii, Cells were then immunostained with
anti-Flag (mouse) followed by Cy3-conjugated anti-mouse IgG and co-stained
with DAPI. The images were taken under a fluorescent microscopy (Olympus)
or an LSM-510 confocal laser scanning microscope system (Carl Zeiss,
Oberkochen, Germany).

Reporter gene assay
CV-1 cells were seeded at a concentration of 5.0 × 104 cells per well in 48-well
plates. Lipofectamine transfection reagent was used for transient transfection
according to manufacturer’s instruction. Reporter constructs, (TREpal)-2-tk-
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confocal study, NIH-H460 cells were co-stained with anti-RXRα
IgG conjugated with Cy3 (1 : 1000), and then re-stained with DAPI. For
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Reporter gene assay
CV-1 cells were seeded at a concentration of 5.0 × 104 cells per well in 48-well
plates. Lipofectamine transfection reagent was used for transient transfection
according to manufacturer’s instruction. Reporter constructs, (TREpal)-2-tk-
CAT and (RARE-κ-CAT, have been described previously (15,16). To assay
RXRα homodimer transactivation, (TREpal)-1-tk-CAT (50 ng) and RXRα
expression vector (10 ng) were co-transfected into cells. To assay
RXRα/Nur77 heterodimer transactivation, J&RARE-κ-CAT (100 ng), RXRα
(5 ng) and Nur77 (20 ng) were co-transfected. Each transfection also contained
50 ng pCMV-β-gal plasmid along with carrier DNA PBuescript to give
to 1000 ng of total DNA/well. Twenty-four hours after transfection, cells
were treated with SR11237 and/or various concentrations of H.sampsonii
extracts for another 20 h. Cells were then harvested for measuring
β-galactosidase activity and CAT activity as described (15,16). Relative
CAT activity was normalized to β-galactosidase value to correct transfection
efficiency. Values are the means ± SD of three independent experiments.

Statistical analysis
Statistical significance of differences between groups was analyzed by using
the Student’s t-test. Values of P<0.05 were considered significant.

Results
H.sampsonii induces RXRα nuclear export
It has been demonstrated that translocation of RXRα from the nucleus to the cytoplasm represents a unique pathway in the inhibition of cell growth in various cancer cells
(34,39,40,42). To identify RXRα candidate ligands that induce RXRα nuclear export and apoptosis, we screened a herbal library by immunostaining using anti-RXRα antibody
(39). NIH-H460 lung cancer cells were chosen for this study
because RXRα is highly expressed in these cells and is known to translocate from the nucleus to the cytoplasm in response to certain apoptotic stimuli (39). Cells were treated
for 3 h with vehicle or various herbal extracts at concentrations
that induced apoptosis on the basis of MTT assays (data
not shown). In some experiments, time-course was also
determined. After treatment, cells were fixed with 4% poly-
formaldehyde and subjected to immunostaining using anti-
RXRα antibody. The subcellular distribution of RXRα was
visualized via fluorescent microscopy. Analysis of the effects
of extracts from our herbal library revealed that a crude alcoholic extract of H.sampsonii, one of the genus Hypericum (43–45), could significantly induce RXRα nuclear export.
Endogenous RXRα in NIH-H460 cells was mainly found in the nucleus before treatment. Treatment of cells with vehicle control did not show any effect on RXRα nuclear
localization. However, when cells were treated with H.sampsonii
extract, a majority of RXRα was found in the cyto-
plasm (Figure 1A). Induction of RXRα nuclear export by
H.sampsonii was confirmed by our cellular fractionation
approach (39). Immunoblotting showed that accumulation
of RXRα in the cytosolic fraction was significantly enhanced
when cells were treated with H.sampsonii, whereas RXRα
remained in the nuclear fraction in vehicle control
(Figure 1B). To further explore the effect of H.sampsonii
extract on RXRα subcellular localization, an expression vec-
tor encoding RXRα fused with green fluorescence protein
(GFP) was transfected into NIH-H460 cells. Transfected
GFP–RXRα mainly resided in the nucleus. Upon treatment of
cells with H.sampsonii extract, GFP–RXRα was found
predominantly in the cytoplasm in a significant amount of
transfected cells (Figure 1C).

Induction of RXRα nuclear export was H.sampsonii dose-
dependent and again seen in certain fractions of the herb. We observed that about 30% of NIH-H460 cells displayed RXRα cytoplasmic localization when cells were treated
with 20 μg/ml H.sampsonii extract, while treatment with
40 μg/ml of the extract caused RXRα export in >90% cells.
When H.sampsonii alcoholic extract was partitioned with
petroleum ether, chloroform and ethyl acetate, the active com-
ponents that induced RXRα nuclear export were mainly
retained in the chloroform fraction. RXRα translocation
was recognized again in gradient EtOH elute from the chlo-
roform fractions, strongly in 70% aqueous EtOH, weakly in
30% aqueous EtOH and undetectable in water elute
(Figure 1D).

H.sampsonii-induced RXRα nuclear export was also
observed in several other human cancer cell lines, including
SMMC-7721 liver cancer and MGC-803 gastric cancer cells.
Treatment with H.sampsonii extract (40 μg/ml) resulted in
RXRα nuclear export in ~62% SMMC-7721 and 55%
MGC-803 cells (Figure 1E). Time-course analysis demon-
strated that induction of RXRα translocation by H.sampsonii
extract in different cancer cells occurred as early as 3 h
post-treatment and lasted until 16 h when extensive apoptosis
occurred (data not shown).

Previous studies showed that RXRα transactivation against
SR11237 inhibited RXRα nuclear export by silencing RXRα
nuclear export sequence (NES) (39). We, therefore, examined
whether SR11237 could modulate the effect of H.sampsonii on
RXRα subcellular localization. Treatment of NIH-H460 cells
with SR11237 did not significantly alter RXRα nuclear
localization (Figure 1F). However, when cells were treated
with SR11237 and *H. sampsonii*, *H. sampsonii*-induced RXRα migration was significantly inhibited by SR11237 (Figure 1F). Thus, *H. sampsonii*-induced RXRα nuclear export is probably regulated by RXRα ligand binding.

**H. sampsonii inhibits RXRα transactivation**

Our observation that *H. sampsonii* induced RXRα nuclear export suggested that it might inhibit RXRα transcriptional function. We, therefore, examined whether *H. sampsonii*...
regulated transactivation of RXRα homodimers and heterodimers. To determine its effect on RXRα homodimer activity, a plasmid containing two copies of RXRα homodimer-responsive element (TREpal) (16) fused with the thymidine kinase (tk) minimal promoter and chloramphenicol acetyltransferase (CAT) reporter gene, (TREpal)-tk-CAT, was co-transfected with RXRα expression vector into CV-1 cells. Transfected cells were exposed to RXRα ligand SR11237 for 20 h. As shown in Figure 2A, SR11237 strongly induced RXRα heterodimers. To determine its effect on RXRα heterodimer transactivation, we evaluated its effect on transactivation of RXRα/Nur77 heterodimer on βRARE (β retinoic acid responsive element) derived from the RARβ promoter (50). RXRα/Nur77 heterodimer is known to bind to the βRARE and activate the response element in response to RXRα ligands (51). Co-transfection of RXRα and Nur77 expression vectors strongly activated a reporter containing the βRARE (βRARE-tk-CAT) (51) when CV-1 cells were treated with SR11237. Similar to its inhibitory effect on RXRα homodimer, H. sampsonii dose-dependently suppressed RXRα/Nur77 transactivation (Figure 2B). Together, H. sampsonii inhibits RXRα transactivation probably by inducing its nuclear export.

**H. sampsonii induces RXRα mitochondrial targeting, cytochrome c release and poly (ADP-ribose) polymerase (PARP) cleavage**

Once in the cytoplasm, RXRα may associate with mitochondria, an event that is known to induce cytochrome c release and apoptosis (39). To determine whether cytoplasmic RXRα induced by *H. sampsonii* associated with mitochondria, NIH-H460 cells were treated with or without *H. sampsonii*, and immunostained with anti-RXRα antibody and an antibody against heat shock protein 60 (Hsp60), a mitochondria-specific protein (24). Confocal microscopy analysis showed that the distribution of RXRα overlapped extensively with that of Hsp60 when cells were treated with *H. sampsonii* (Figure 3A), suggesting an association of cytoplasmic RXRα with mitochondria. Thus, in response to *H. sampsonii*, RXRα migrated from the nucleus to the cytoplasm where it targeted mitochondria.

It has been widely accepted that mitochondria plays an important role in many critical apoptotic pathways (24,52). We previously reported that mitochondrial localization of RXRα resulted in extensive apoptosis (39). Induction of cytochrome c release from mitochondria is a key step to initiate apoptosis, which normally occurs before nuclear fragmentation and represents an early event in apoptosis. To determine whether *H. sampsonii*-induced RXRα mitochondrial targeting was associated with cytochrome c release, NIH-H460 cells were treated with or without *H. sampsonii* for 12 h. Cells were immunostained with anti-cytochrome c and anti-RXRα. In the absence of *H. sampsonii* treatment, cytochrome c showed punctate distribution in NIH-H460 cells, demonstrating its localization in mitochondria. However, cytochrome c was diffusely distributed in a majority of NIH-H460 cells when they were treated with *H. sampsonii*, indicating cytochrome c release from mitochondria (Figure 3B). As PARP cleavage is another sensitive apoptotic marker, which occurs early in the apoptotic response as a result of caspase-3 activity, we further analyzed the cleavage of PARP in response to *H. sampsonii* in NIH-H460 cells. *H. sampsonii* treatment resulted in cleavage of PARP, producing an 85 kDa fragment, which was visible at 12 h post-treatment. The amount of the 85 kDa PARP fragment increased when cells were treated with *H. sampsonii* for prolonged times (Figure 3C).

**H. sampsonii-induced apoptosis and growth inhibition is associated with levels of RXRα protein**

We next evaluated the effect of *H. sampsonii* on growth and apoptosis in a number of cancer cell lines. Using MTT assays, we found that NIH-H460 lung cancer cells were very sensitive to *H. sampsonii* with an IC₅₀ of 38 μg/ml, followed by SMMC7721 liver cancer and MGC-803 gastric cancer cells, with an IC₅₀ of 49 and 52 μg/ml, respectively (Table I). Consistently, DAPI staining demonstrated that *H. sampsonii* induced apoptosis in 45% NIH-H460, followed by SMMC7721 (31%) and MGC-803 (24%) (Figure 4A and B). Together, these results demonstrate that *H. sampsonii* is a potent apoptosis inducer in cancer cells.

To determine whether *H. sampsonii*-induced growth inhibition was associated with RXRα protein expression levels, NIH-H460 cells were stably transfected with RXRα. The resulting stable clones (NIH-H460/RXRα) expressed significantly higher levels of RXRα protein, as compared with their parental NIH-H460 cell line (data not shown). RXRα stable clone and its parental cells were subjected to *H. sampsonii* treatment. Treatment of *H. sampsonii* dose-dependently inhibited the growth of both cell lines. However, the RXRα stable cells were more sensitive to growth inhibition by *H. sampsonii* than its parental cells (Figure 4C). DAPI staining revealed that *H. sampsonii* caused significant morphological changes in nuclear chromatin that represented typical apoptosis in both parental and the RXRα stable lines in a dose-dependent manner (Figure 4D). However, *H. sampsonii* was much more effective in the RXRα stable line than in its parental line, when two concentrations (20 and 40 μg/ml) of *H. sampsonii* were used (Figure 4E). In response to 40 μg/ml *H. sampsonii*, 92% RXRα stable cells underwent apoptosis, while only 42% parental NIH-H460 cells were apoptotic. At 20 μg/ml, *H. sampsonii* induced ~54% cell death in the RXRα stable line, whereas it had much reduced effect (~14%) in the NIH-H460 parental cells.

CV-1 cells lacking detectable RXRα protein showed significant resistance to *H. sampsonii*. Their growth was not clearly affected even when high concentration of *H. sampsonii* (80 μg/ml) was used (Table I). To further determine whether levels of RXRα protein regulated the apoptotic effect of *H. sampsonii*, CV-1 cells were transfected with Flag-RXRα and subjected to vehicle or *H. sampsonii* treatment. Flag-RXRα transfection did not have any effect on apoptosis of CV-1 cells. However, when cells were treated with *H. sampsonii*, cells transfected with Flag-RXRα underwent extensive apoptosis indicated by nuclear condensation, whereas non-transfected CV-1 cells were not apoptotic even though they were treated with *H. sampsonii* (Figure 4F). Collectively, these results demonstrate that the apoptotic effect of *H. sampsonii* depends on RXRα levels.
Discussion

Since St John’s wort is widely used for the treatment of mild and moderate depression (38,39), attention has also been attracted to determine pharmacological value of several other species of this genus. *H. sampsonii* is the closely related species of St John’s wort in China, which has been of scientific interest for many years owing to its widespread use in folk medicine for a range of ailments (44,45). Recent studies have shown that *H. sampsonii* possesses anticancer activities (44,45). However, how *H. sampsonii* exerts its various biological effects remains virtually unknown. Here, we report that...
were treated with that RXR nucleus to the cytoplasm. RXR extract could induce rapid migration of RXR from the nucleus to the cytoplasm. RXR immunostaining revealed that RXR resided mainly in the cytoplasm when cells were treated with H.sampsonii extract (Figure 1A–E). Confocal microscopy analysis showed that the cytoplasmic RXR was associated with mitochondria (Figure 3A). Consistently, H.sampsonii extract antagonized SR11237-induced RXR nuclear export. Evidence has been accumulating to demonstrate that non-genotropic action commonly exists for a number of different NRs (44,53–56). The redistribution of NRs between nucleus and cytoplasm is an important event for the regulation of their activities and the execution of their functions. Translocation of RXR was previously observed in cells treated with a number of structurally different agents, such as NGF (37), 12-O-tetradecanoylphorbol-13-acetate (TPA) and those related to retinoïd-derived compound AHPN/CD437 (39). To our knowledge, our finding is the first report that a natural Chinese herbal medicine modulates RXR subcellular distribution. Since the cytoplasmic localization of RXRs is associated with the regulation of important biological processes, such as development (35,36), differentiation (37) and apoptosis (34,39,40,42), our results will provide impetus for further characterization of bioactive components in H.sampsonii, which modulate RXRs subcellular localization. The identification and characterization of such bioactive components will certainly add to our knowledge on the mechanism of H.sampsonii action and may provide important leads for developing new RXR-based modern medicine. In this regard, St John’s wort is known to bind pregnane X receptor (also known as steroid X receptor) plays a role in the regulation of diverse endocrine signal transduction pathways (12,13,15–19), our findings suggest that RXR may be an important mediator of the biological activities of H.sampsonii.

An important finding reported here is that H.sampsonii extract could induce rapid migration of RXR from the nucleus to the cytoplasm. RXR immunostaining revealed that RXR resided mainly in the cytoplasm when cells were treated with H.sampsonii extract (Figure 1A–E). Confocal microscopy analysis showed that the cytoplasmic RXR was associated with mitochondria (Figure 3A). Consistently, H.sampsonii extract antagonized SR11237-induced RXR nuclear export. Evidence has been accumulating to demonstrate that non-genotropic action commonly exists for a number of different NRs (44,53–56). The redistribution of NRs between nucleus and cytoplasm is an important event for the regulation of their activities and the execution of their functions. Translocation of RXR was previously observed in cells treated with a number of structurally different agents, such as NGF (37), 12-O-tetradecanoylphorbol-13-acetate (TPA) and those related to retinoïd-derived compound AHPN/CD437 (39). To our knowledge, our finding is the first report that a natural Chinese herbal medicine modulates RXR subcellular distribution. Since the cytoplasmic localization of RXRs is associated with the regulation of important biological processes, such as development (35,36), differentiation (37) and apoptosis (34,39,40,42), our results will provide impetus for further characterization of bioactive components in H.sampsonii, which modulate RXRs subcellular localization. The identification and characterization of such bioactive components will certainly add to our knowledge on the mechanism of H.sampsonii action and may provide important leads for developing new RXR-based modern medicine. In this regard, St John’s wort is known to bind pregnane X receptor (also known as steroid X receptor) plays a role in the regulation of diverse endocrine signal transduction pathways (12,13,15–19), our findings suggest that RXR may be an important mediator of the biological activities of H.sampsonii.

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**Table 1. Growth inhibitory effect of H.sampsonii crude extracts and fractions in different cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Alcoholic extract of H.sampsonii IC₅₀ (µg/ml)</th>
<th>Chloroform fractions</th>
<th>Chloroform fractions</th>
<th>Chloroform fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude</td>
<td>70% EtOH</td>
<td>30% EtOH</td>
<td>H₂O</td>
</tr>
<tr>
<td>NIH-460</td>
<td>38 ± 3</td>
<td>30 ± 4</td>
<td>36 ± 4</td>
<td>54 ± 6</td>
</tr>
<tr>
<td>SMMC-7721</td>
<td>49 ± 6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MGC-803</td>
<td>52 ± 4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CV-1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = IC₅₀ not obtained since highest concentration 80 µg/ml showed <50% inhibition of growth. n = 5 determinations using the same extract. Null entries indicate ‘without examination’. The indicated cells were seeded in 96-well plates and incubated for 24 h. The cells were treated with ~5–100 µg/ml of H.sampsonii extract and its fractions for 72 h. The growth inhibitory effect of H.sampsonii was assayed by MTT method as indicated in Materials and methods. Results are expressed as the concentration of the extract required to inhibit cellular growth 50% (IC₅₀) ± SD. Each assay was repeated in triplicate in three independent experiments.
**Fig. 4.** *H. sampsonii* induces apoptosis in RXRα-dependent manner. (A and B) Apoptotic effect of *H. sampsonii* on various cancer cells. NIH-H460 lung cancer, SMMC-7721 liver cancer and MGC-803 stomach cancer cells were cultured in 6-well plates in 0.5% serum medium in the presence of 40 μg/ml *H. sampsonii* for 24 h. Detached and attached cells were collected and subjected to DAPI staining as indicated in Materials and methods. Representative apoptotic morphology for different cancer cells was indicated in (A). The number of apoptotic cells (B) were independently counted by two observers from at least 1000 cells in 10 random microscopic fields. The data were expressed as mean ± SD from three independent experiments. Each concentration was conducted in triplicate. (C, D and E) The effect of RXRα expression levels on *H. sampsonii*-induced growth inhibition and apoptosis. NIH-H460 and its RXRα stable line (NIH-H460/RXRα, see Materials and methods) were used to determine the effect of RXRα expression levels on *H. sampsonii* activities. NIH-H460/RXRα showed more sensitivity than its parental NIH-H460 to *H. sampsonii*, as demonstrated by MTT methods during a time-course of 72-h treatment (C) and by DAPI staining when cells were treated with vehicle or various concentrations of *H. sampsonii* in 0.5% serum medium for 24 h (D). The number of apoptotic cells (E) were independently counted by two observers from at least 1000 cells in 10 random microscopic fields. Results are expressed as mean ± SD. Each assay was repeated in triplicate in three independent experiments. (F) The role of RXRα in the apoptosis induced by *H. sampsonii*. CV-1 cells were transfected with or without Flag-tagged RXRα before treatment of *H. sampsonii* (10 μg/ml) for 24 h. Cells were immunostained with anti-Flag (mouse) followed by Cy3-conjugated anti-mouse IgG and co-stained with DAPI.
RXRα in the nucleus (39). It is not impossible that certain agents induce RXRα nuclear export by directly binding to RXRα, resulting in an RXRα conformation that activates its NES. It remains to be seen if an active component in *H. sampsonii* induces RXRα migration by directly binding to RXRα. In support of this hypothesis, RXRα has been shown to bind to natural compounds with diverse structures (25–28,30–32), such as 9-cis-RA and various fatty acids, owing to the promiscuity of its LBP (56,58,59).

Another interesting finding reported here is that *H. sampsonii* potently induced apoptosis in various cancer cells, which was associated with its induction of RXRα nuclear export. The apoptotic effect of *H. sampsonii* was demonstrated by several independent assays, including DAPI staining (Figure 4F), cytochrome c release (Figure 3B) and PARP cleavage (Figure 3C). The ability of *H. sampsonii* to induce apoptosis of tumor cells may explain the potential anti-neoplastic activity of this herb. Interestingly, recent studies have also demonstrated that St John’s wort exerts anti-neoplastic effect *in vitro* and *in vivo*, owing to the potent apoptotic effect of its ingredients, hypericin and hyperforin, in cancer cells (48,49). However, when examining the effect of both compounds on inducing RXRα translocation in different cancer cells such as NIH-H460 lung cancer cells and MGC-803 gastric cancer cells, we did not observe any effect of these compounds at concentrations between ~0.01 and 1.0 μM on RXRα subcellular localization examined by either immunostaining or immunoblotting (data not shown). These results suggest that ingredients other than hypericin and hyperforin function to modulate RXRα cellular distribution.

The apoptotic effect of *H. sampsonii* could be observed in several cancer cell lines derived from lung, stomach and liver (Figure 4A). Among different cancer cell lines, NIH-H460 lung cancer cells were the most sensitive to *H. sampsonii*, probably reflecting different levels of RXRα expressed in these cells (data not shown), different cellular environment or different levels of factors, such as Nur77. Under the same cellular environment, levels of RXRα determined the efficacy of *H. sampsonii*, as overexpression of RXRα in NIH-H460 cells enhanced the apoptotic effect of *H. sampsonii* (Figure 4C–E), whereas *H. sampsonii* exhibited limited activity in CV-1 cells that express undetectable levels of RXRα (Table 1). When CV-1 cells were transfected with Flag-RXRα, cells expressing transfected Flag-RXRα were highly responsive to *H. sampsonii*, displaying extensive apoptosis (Figure 4F). Thus, RXRα is an important mediator of the apoptotic effect of *H. sampsonii*. The apoptotic effect of *H. sampsonii* appears to be resulting from its induction of RXRα cytoplasmic localization, as both events are closely associated (Figure 3B). This is consistent with previous observations that cytoplasmic localization of RXRα induced by several apoptotic stimuli resulted in apoptosis (34,39,40,42). Our observation that cytoplasmic RXRα was able to target mitochondria (Figure 3A) suggests that RXRα mitochondrial targeting may be responsible for its apoptosis induction.

Scientific investigations of herbal and alternative therapies represent a potentially important source for developing new modern medicine (1–3). Integrating ancient knowledge and modern technology may facilitate the process. The central role that RXRα plays in the regulation of diverse endocrine signal transduction pathways through its exceptional dimerization function makes it an attractive molecular target for drug development (19). The complexity in RXR signaling also offers an excellent opportunity to develop RXR ligands that selectively regulate a specific RXR-signaling pathway, which is clearly therapeutically advantageous by reducing adverse effects caused by interaction with other receptors. The results presented here demonstrate that *H. sampsonii* contains component(s) that induce migration of RXRα from the nucleus to the cytoplasm, an important RXRα pathway that regulates apoptosis and differentiation (34,37,39,40,42). It remains to be seen whether other Chinese herbal medicines exert their effects, such as modulation of growth, differentiation, apoptosis, immune response, by targeting various RXRα pathways.

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**References**

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