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

Jin-Zhang Zeng1,*, De-Fu Sun1,*, Li Wang1, Xihua Cao3, Jian-Bin Qi2, Ting Yang1, Chang-Qi Hu2, Wen Liu3 and Xiao-Kun Zhang1,3

1Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, 2School of Pharmacy, Medical Center of Fudan University, Shanghai, China and 3Cancer Center, Burnham Institute for Medical Research, La Jolla, CA, USA

To whom correspondence should be addressed: The Samost Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 500 Caobao Road, Shanghai 200233, China. Tel: +86 21 54481877; Fax: +86 21 54971085; Email: jzzeng@sibs.ac.cn

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 RXRαs, 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 RXRαs 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 functional dimensions (12,13,15–19). They heterodimerize with many members of the NR superfamily, including retinoic 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.

*These authors contributed equally to this work.

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of target genes (12,13,15–19). On binding ligands, the receptors undergo conformational changes to release corepressors 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 (commonly known as St John’s wort) has been traditionally used in China for the treatment of skin disease (46,47). Lipophilic extracts of St John’s wort also showed anti-neoplastic activity in vivo (48,49).

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.

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 peptatin A and 2 μg/ml leupeptin) for 20 min on ice. The whole cell lysates were purified by centrifuging at 12 000·g for 10 min. Subcellular fractionation was performed as described with minor modifications (39). Briefly, cells were suspended for 5 min on ice in 0.5 ml of hypotonic buffer (250 mM sucrose, 20 mM HEPES–KOH (pH 7.4), 10 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 1.5 mM EDTA (pH 8.0), 1 mM dithiothreitol (DTT)) 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

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% solid content. The residue was suspended 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.

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Western blotting

The total lysates or fractions were electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose 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-conjugated 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 with 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 solution (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 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 confocal 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 conjugated with Cy3 (1 : 1000) were used to recognize RXRα and Hsp60, respectively. 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 alcoholic extract, GFP–RXRα and transfected reportedly. When 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 alcoholic extract, GFP–RXRα and transfected reportedly. When

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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)2-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 reporter gene activity, whereas H.sampsonii did not. When cells were treated with SR11237 and H.sampsonii, SR11237-induced RXRα homodimer transactivation was significantly reduced in a H.sampsonii concentration-dependent manner.

To determine whether H.sampsonii regulated 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 SR11237-induced 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/mg, followed by SMMC7721 liver cancer and MGC-803 gastric cancer cells, with an IC50 of 49 and 52 μg/mg, 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). An 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 apoptotic features 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

cal microscopy analysis showed that the cytoplasmic RXR

extract could induce rapid migration of RXR

was treated with vehicle or

H.sampsonii

that may be an important mediator of the biological activities of

H.sampsonii.

Fig. 3. H.sampsonii induces RXRα mitochondrial targeting and apoptosis. (A) RXRα targeting at mitochondria by H.sampsonii treatment. NIH-H460 cells plated on slides were treated with vehicle or H.sampsonii (40 μg/ml) for 6 h. Distribution of RXRα was examined by immunostaining using anti-RXRα antibody. RXRα images overlaid with those of mitochondria protein Hsp60 by co-staining with anti-Hsp60 antibody were visualized using confocal microscopy. (B) Association of cytochrome c release with translocation of RXRα. NIH-H460 cells were treated with or without H.sampsonii (20 μg/ml) for 12 h. 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. (C) Induction of PARP cleavage by H.sampsonii. NIH-H460 cells were treated with 40 μg/ml of H.sampsonii for the indicated time. Control cells were collected before drug administration (C₀) and 24 h post-treatment with vehicle (C₂₄). PARP and its fragment were detected by immunoblotting using anti-PARP antibody.

H.sampsonii contains components that modulate transactivation and subcellular localization of RXRα. In addition, our results demonstrated that the effect of H.sampsonii was associated with growth inhibition and apoptosis of cancer cells in an RXRα-dependent manner. Given the fact that RXRα plays a role in the regulation of diverse endocrine signal transduction pathways (12,13,15–19), our findings suggest that RXRα 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 RXRα from the nucleus to the cytoplasm. RXRα immunostaining revealed that RXRα resided mainly in the cytoplasm when cells were treated with H.sampsonii extract (Figure 1A–E). Confocal microscopy analysis showed that the cytoplasmic RXRα

was associated with mitochondria (Figure 3A). Consistently, H.sampsonii extract antagonized SR11237-induced RXRα transactivation (Figure 2A and B), presumably owing to its induction of RXRα 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 RXRα 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 retinoid-derived compound AHPN/CD437 (39). To our knowledge, our finding is the first report that a natural Chinese herbal medicine modulates RXRα subcellular distribution. Since the cytoplasmic localization of RXRα 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 RXRα 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 RXRα-based modern medicine. In this regard, St John’s wort is known to bind pregnane X receptor (also known as steroid X receptor) (53,54), a finding that provides an important explanation for its interaction with a variety of drugs.

How H.sampsonii induces RXRα nuclear export remains to be determined. Induction of RXRα nuclear export by AHPN/CD437 did not require its binding to RXRα (39). Instead, the effect of AHPN/CD437 and TPA was mediated by its induction of RXRα heterodimerization partner, Nur77, and their post-translational modifications (39,55,57). H.sampsonii extract may indirectly induce RXRα nuclear export in a fashion similar to that utilized by AHPN/CD437. We recently reported that RXRα nuclear export was mediated by an NES, which was highly regulated by RXRα conformation induced by RXRα dimerization and ligand binding (39). RXRα transcriptional agonists such as SK11237 and 9-cis-RA have been demonstrated to silence the RXRα NES activity and retain

<table>
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<th>Cell line</th>
<th>Alcoholic extract of H.sampsonii IC₅₀ (µg/ml)</th>
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<td>49 ± 6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NA</td>
</tr>
<tr>
<td>MGC-803</td>
<td>52 ± 4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NA</td>
</tr>
<tr>
<td>CV-1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</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**
