Pro-apoptotic and anticancer properties of *Thapring* – A Tibetan herbal formulation

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**A R T I C L E   I N F O**

**Article history:**
Received 8 March 2011
Received in revised form 3 May 2011
Accepted 25 May 2011
Available online 31 May 2011

**Keywords:**
Traditional Tibetan Medicine
Herbal formulation
HCC
VEGF
Apoptosis
Cytochrome c
ΔΨm

**A B S T R A C T**

Aim of the study: To evaluate the pro-apoptotic and anti-tumorigenic properties of *Thapring* – a Traditional Tibetan Medicine – in hepatoma cells and in a transgenic mouse model of hepatocellular carcinoma.

**Material and methods:** The pro-apoptotic action and growth inhibition property of *Thapring* were assessed in Huh7, HepG2 and A549 cell lines using flow cytometry and MTT assay, respectively. Confocal microscopy for colocalization of cytochrome c and mitochondria was done using dsRed mitotracker in Huh7 cells. The activation of p38 MAP kinase and p53 pathway was evaluated by Western blotting. Serological studies for liver function, vascular endothelial growth factor and superoxide dismutase were assessed in the serum of X15-myc transgenic mice. Immuno-histochemical studies for Bcl2 and p21Waf1 expression were also carried out in the liver section of the above mice.

**Results:** Treatment with *Thapring* inhibited proliferation and accumulation of hepatoma cells in G1 phase. There was increased cytochrome c release from mitochondria and decreased Bcl2 levels – the key markers of apoptotic cell death. Besides activation of p38 MAP kinase and increased p53 expression were also observed. Oral administration of *Thapring* in transgenic mice lowered serum VEGF levels and conferred hepatoprotection as evident from normal serum ALT levels. Further, immunohistochemical analysis of the liver samples revealed reduced expression of anti-apoptotic protein Bcl2 and over-expression of cell cycle regulator p21Waf1.

**Conclusions:** The ability of *Thapring* to impose growth arrest and trigger pro-apoptotic death in cell culture as well as ameliorative effects in vivo provides scientific basis for its usefulness as traditional medicine and its clinical application in adjunct/combination therapy along with other known anticancer drugs.

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**1. Introduction**

The Traditional Tibetan Medicine (TTM) is an age-old legacy of herbal and spiritual healing evolved around 7th century and is an assimilation of healing methods from India, China, Greece and Persia (Dunkenberger, 2000). Here each treatment is codified in the form of sacred texts or pharmacopeia that is interpreted with Buddhist understanding of herbal cures (Lobsang and Dakpa, 2001). TTM is known to offer cure for chronic diseases like cancer and atherosclerosis (Melzer et al., 2006). *Thapring* is one herbal formulation that has been in use since the beginning of twentieth century for the treatment of a variety of cancers (Dawa, 2003). *Thapring* is a multicomponent herbal formulation composed of *Terminalia chebula* (Rey) (family: Combretaceae), *Saussurea lappa* (C.B. Clarke) (family: Asteraceae), *Acorus calamus* (L) (family: Araceae), *Aconitum ferox* (Wall. ex Ser) (family: Ranunculaceae), *Oxytropis microphylla* (Pall) (family: Fabaceae), *Commiphora mukul* (Hook) (family: Burseraceae), *Acacia catechu* (L.F. Willd) (family: Fabaceae), *Delphinium brunoninum* (Royale) (family: Ranunculaceae) and a mineral ingredient (Dawa, 2003). Keeping in view the tumor regression property of *Thapring* and its clinical application as an alternative chemopreventive/chemotherapeutic medicine for cancer, here we have scientifically evaluated the growth inhibitory and pro-apoptotic action of *Thapring* in cell culture model as well as its
anti-tumorigenic action in a genetic mouse model of hepatocellular carcinoma.

2. Material and methods

2.1. Chemicals

Propidium iodide, Silibinin, DMSO, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) and methanol were purchased from Sigma Chemical Co. (St. Louis, MO) and JCI dye from Molecular probe. Dulbecco modified eagle medium (DMEM), fetal bovine serum (FBS), streptomycin and penicillin was purchased from Gibco BRL, USA. VEGF quantikine ELISA kit was procured from R&D system (Minneapolis, USA) and Fugene 6 was from Roche diagnostics. Immunohistochemistry kit was procured from DAKO and DAB, hematoxylin from Sigma and DPX from BDH.

2.2. Plant material

_Thapring_ was procured from the Tibetan Medical and Astrological Institute (TMAI), Dharamsala, India. All the herbs used in this formulation were identified by Dr. Tsering Norbu (Menrampa). The voucher numbers of plant specimens used in _Thapring_ are: _Terminalia chebula_ (TMAI/A-1), _Sassurea lappa_ (TMAI/R-7), _Acorus calamus_ (TMAI/Sha-3), _Aconitum ferox_ (TMAI/G-5), _Oxytropis microphylla_ (TMAI/Nga-5), _Commiphora molmol_ (TMAI/G-1), _Acacia catechu_ (TMAI/S-10), and _Delphinium brunonianum_ (TMAI/B-10).

2.3. Cell culture and transfection

Human hepatoma cells Huh7 was a kind gift from Dr. A. Siddiqui (University of Colorado, Denver), HepG2 cells (HB-8065), AML12 cells (immortalized mouse hepatocytes, CRL 2254) HEK293 (CRL-1573) cells and A549 (CCL-185) cells were purchased from ATCC. All cultures were grown in DMEM supplemented with 10% FBS, penicillin 100 μg/ml and streptomycin (100 μg/ml) incubated at 37°C in a humidified chamber and 5% CO₂ atmosphere. Cells were seeded at a density of 0.6 million per 60 mm dish and transfected using Fugene 6 (Roche) as per manufacturer’s protocol. For tracking cytochrome c localization, pEGFP-cytochrome c (1 μg) was cotransfected with pBRed Mitotracker (1 μg) and analyzed by confocal microscopy (Nikon A1R).

2.4. Cell viability assay

MTT assays were performed as described earlier (Choedon et al., 2006). Cells were seeded as above, and after 24 h treated with increasing concentrations (10, 50, 100, and 200 μg/ml) of _Thapring_ for 48 h. Then cells were incubated with MTT (200 μg/ml) for 45 min at 37°C in dark. Formazan crystals were solubilised in DMSO and absorbance measured at 560 nm. Untreated cells were used as control of cell viability (100%). The mean absorbance value of three experiments was expressed as percentage of cell viability as compared to control.

2.5. Animal tumor model

Development of the transgenic mice model (X15-myc) of hepatocellular carcinoma (HCC) has been described earlier (Lakhhtakia et al., 2003). The transgene positive of mice were identified by PCR using tail DNA biopsies and used for evaluating the anticancer property of _Thapring_. Necessary ethical clearance was taken from the Institutional Animal Ethics Committee for doing animal experiments. The animals were maintained under standard conditions, treated humanely and provided pellet diet and water _ad libitum_. The experimental mice were divided into three groups (n = 5 per group), viz., treated, untreated and positive control and administered daily with _Thapring_ (400 mg/kg) through oral route for 12 months as described earlier (Choedon et al., 2006). Silibinin (100 mg/kg) was used as positive control. Mice were housed in humidity (30–70%) and temperature (74 ± 2°F). The tissue samples (liver) were removed surgically and fixed in 10% buffered formalin. Treatment of mice for the histology and immunohistochemistry work lasted for 3 months.

2.6. SOD estimation

The SOD activity was determined spectrophotometrically in serum samples by measuring the autoxidation of epinephrine for 3 min at 480 nm (Misra, 1987). The assay mixture contained 50 mM glycine buffer pH 10.4 with epinephrine (20 μg/ml).

2.7. Assessment of liver functions

For liver function test, blood samples from the experimental mice were collected by retro-orbital bleeding at 0, 2, 4, 6, 8, 10 months post treatment. The serum alanine aminotransferase (ALT) levels were measured by autoanalyzer while the VEGF levels were measured as per manufacturer’s protocol (R&D system, Minneapolis).

2.8. Determination of mitochondrial membrane integrity

For analysis of changes in mitochondrial transmembrane potential, Huh7 cells were incubated with JCI (100 nm) for 15 min at 37°C in dark and then washed with phosphate buffered saline and analyzed in fluorescence activated cell sorter (FACS)-FL1 green and red FL2 channel.

2.9. Cell cycle analysis

Cell cycle analysis was performed by the method previously described (Mukherji et al., 2007). Briefly, Huh7 cells were fixed in 70% ethanol and stained with Propidium iodide (50 μg/ml) and subjected to apoptosis analysis in a FACSscan (Becton Dickinson, San Jose, CA). Based on propidium iodide staining, the percentage of cell cycle distribution was determined using FlowJo software.

2.10. Western blot analysis

Western blotting was done according to Khatte and Kumar (2010). Briefly, Huh7 cells were directly lysed in 2 × sample loading buffer (100 mM Tris–Cl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue and 20% glycerol) and protein concentrations were determined by Bradford method. The samples were electrophoresed and protein bands were visualized by enhanced chemiluminescence western blotting detection system (Santa Cruz, USA).

2.11. Immunohistochemistry studies

Paraffin sections of embedded liver tissues of 4–5 μm thickness were cut, dewaxed and then treated with proteinase K (20 mg/ml) for 20 min at 37°C and kept at room temperature for 10 min. After three quick washing with PBS, the slides were blocked in 0.3% hydrogen peroxide followed by 2 washes in PBS for 5 min each. The slides were then incubated with primary antibodies (1:150 dilutions) in PBS for 2 h at RT. After a quick rinse with PBS, slides were overlaid with secondary antibody (DAKO) and developed using DAB. Slides were then counterstained with hematoxylin, dehy-
Mitochondria is an integral part of apoptotic machinery and events such as disruption of electron transport, loss of mitochondrial membrane potential, release of cytochrome c resulting in activation of caspases are classical evidence for apoptosis (Green and Reed, 2000; Martinou, 2000). To study the mitochondrial membrane integrity, cells were treated with Thapring and stained with JC1 followed by FACS analysis. Treated cells showed marginal shift in ΔΨm (Fig. 2A). However, confocal microscopy of these cells showed disruption of ΔΨm and a significant decrease in cell viability as evident from increased monomeric form of JC1 dye upon treatment with Thapring (more green fluorescence) as opposed to untreated cells (more red fluorescence due to aggregated dye) (Fig. 2B and C).

To confirm whether cytochrome c was released from mitochondria following Thapring treatment, we expressed dsRED mitotracker and cytochrome c GFP in hepatoma cells. We observed a significant increase in cytochrome c release with increasing concentration of Thapring along with a concomitant decrease in level of colocalization of cytochrome c and mitochondria (Fig. 3A and B). To support this observation, we also looked into the canonical markers of anti-apoptosis such as Bcl2 and pro-apoptotic protein such as Bid (Youle and Strasser, 2008). As expected Bcl2 levels decreased with increasing drug dose while Bid levels increased only marginally (Fig. 3C). Further, a decline in Bcl2 expression in the liver tissue of Thapring-treated transgenic mice (Fig. 3D), reconfirmed the pro-apoptotic action of the herbal drug that was mitochondria-dependent.

3.2. The proapoptotic action of Thapring is mediated by mitochondria

Emerging preclinical evidence continues to shed light on important questions in angiogenesis research, including how to optimally target critical angiogenic growth factors. Increased angiogenesis is considered as hallmark of cancer. Several factors are responsible for increased vascularisation. Among these factors, VEGF has been identified as the most predominant. VEGF expression in hypervascular tumor such as HCC has been considered to be associated with size and grade of tumor (Yamaguchi et al., 1998). Interest-
ingly, the proangiogenic VEGF levels were markedly reduced in the Thapring treated transgenic mice (Fig. 4C).

3.5. Activation of p38 MAPK and induction of p53 by Thapring

Since p38 mitogen-activated protein kinase (MAPK) pathway is well known to play a mediatory role in pro-apoptotic action (Padhan et al., 2008; Cuadrado and Nebreda, 2010), we next examined the levels of phosphorylated p38 in cells treated with Thapring. As shown in Fig. 5A, there was a marked increase in the levels of phosphorylated p38 and its downstream target ATF2 suggesting that p38 MAPK is necessary for Thapring-mediated apoptosis.

The tumor suppressor p53 protein is a critical mediator of many cellular functions including the response to genotoxic stress, differentiation, senescence and apoptosis (Mendez et al., 2010). Inactivation of p53 is commonly seen in tumors (Goh et al., 2011). p53 has become the focus on most intensive cancer based research. Interestingly, we observed an increase in the levels of p53 upon treatment with Thapring along with increased phosphorylation of its negative regulator mdm2 which is indicative of its inactivation (Fig. 5B). The increased p53 level also coincided with increase in its downstream target p21Waf1 (Fig. 5B). Further our immunohistochemistry studies in the liver of X15-myc transgenic mice showed elevated levels of p21Waf1 upon treatment as compared to control (Fig. 5C). This observation further validated our above observations in cell culture that Thapring activates apoptotic pathway in cancer cells.

4. Discussion

Herbal medicines and remedies were the most commonly used complementary alternative medicine (CAM) therapies against cancer. The majority of cancer patients use CAM to increase the body’s ability to fight cancer or improve physical and emotional well-being, and many seemed to have benefitted from its usage. Tibetan formulations are mostly seen in the context of CAM. The dosages of individual components in a herbal formulation are very low and are apparently without side-effects. It is believed that when several herbs or herbal extracts are formulated together, it results in highly effective recipe against some disease and such formulations are considered integral to the therapy. In terms of modern medical research, one such formulation best known as Padma 28 – a multicomponent Tibetan herbal formulation – with wound healing property (Aslam et al., 2010), anti-inflammatory activity (Moeslinger et al., 2000) and used in the treatment of some circulatory disorders (Melzer et al., 2006) and chronic hepatitis B infection (Gladysz et al., 1993). Padma Lax is a related formulation that is reported to have anti-proliferative/anti-cancer property (Hofbauer et al., 2006). In the present study, we have used another Tibetan herbal formulation called Thapring that has been effectively used against a variety of cancer (Dawa, 2003). Here, we provide the pharmacological basis of its beneficial effects using cell culture and preclinical mouse models.

Induction of apoptosis is considered as one of the key mechanisms for the targeted therapy of various cancers. Cancer drugs are believed to kill target cells either by inducing death receptor
Fig. 3. Intracellular localization of cytochrome c in the presence of Thapring. Huh7 cells were co-transfected with plasmids pEGFP-Cyt c, pDsRed-mito. After 36 h, cells were treated further for 24 h with indicated concentrations of Thapring and analyzed by confocal microscopy (A) for the distribution of DsRed-Mitracker (Red) and pEGFP-cytochrome c (Green). (B) Quantitative data of the confocal images showing post treatment per cent colocalization of the mitochondrial marker and cytochrome c. Level of significance: *, p < 0.05; **, p < 0.01. (C) Levels of pro-apoptotic Bid and anti-apoptotic Bcl2 in cells treated with Thapring. C, control, Sn, silibinin. (D) Immunohistochemistry (IHC) for Bcl2 expression in the liver of untreated (control) and Thapring-treated X15-myc (at 400×).

pathway or interfering with mitochondrial pathways (Constantini et al., 2000). Our cell cycle analysis of Thapring-treated cells showed selective killing of hepatoma cells and their arrest in G1 phase (Fig. 1). The induction of apoptosis was accompanied by stimulation of p38 MAPK signaling pathway and its effector molecule ATF2 (Fig. 5A) that is well known to mediate apoptotic cell death (Cuadrado and Nebreda, 2010). Our subsequent studies indicated that Thapring can also promote the intrinsic death pathway by changing the permeability of mitochondrial outer membrane in order to facilitate the release of cytochrome c (Fig. 2). As expected, we observed the release of key apoptogenic factor cytochrome c from mitochondria (Fig. 3A and B) which is essential for the activation of caspases and induction of apoptosis (Green and Reed, 2000; Martinou, 2000). The Thapring-treated cells also showed decreased expression of canonical anti-apoptotic markers like Bcl2. It is well known that the Bcl-2 family includes both pro-apoptotic (Bad, Bak and Bax) and anti-apoptotic (Bcl-2 and Bcl-XL) proteins that are critical modulators of the intrinsic death pathway (Youle and Strasser, 2008).

Another interesting observation in this study included the up-regulation of p53 gene and its target and cell cycle inhibitor p21Waf1 and inactivation of its negative regulator Mdm2 (Fig. 5B). p53 is considered as guardian of cells owing to its tumor suppressor property (Menendez et al., 2010). The mutational inactivation or down-regulation of p53 is a common scene in many cancers (Goh et al., 2011). Therefore, sustained elevated levels of p53 along with increased phosphorylation of its negative regulator Mdm2 upon treatment with Thapring are good indicators of its growth inhibition properties.

Interestingly, our cell culture studies were adequately supported by in vivo studies in a genetic mouse model of HCC. Oral treatment of X15-myc mice with Thapring resulted in a significant reduction in the serum levels of ALT (Fig. 4A) which is considered as a hallmark of hepatotoxicity (Ozer et al., 2008). Suppression of ALT levels indicated that Thapring also confers hepatoprotection. The herbal formulation also exhibited a strong anti-oxidative property by preventing oxidative damage to liver as seen in Fig. 4B. More importantly, there was a significant reduction in the serum VEGF levels of the oncomice treated with Thapring (Fig. 4C) which is considered an important arm in the management of cancer (Greenberg and Chereshe, 2009; Kaseb et al., 2009). Besides, there was a dramatic reduction in the tissue expression of anti-apoptotic protein Bcl2 (Fig. 3D) and improvement in the expression of growth inhibitory p21Waf1 (Fig. 5C) in support of the pro-apoptotic action of Thapring in vivo.

Synergistic interactions are documented for constituents within a total extract of a single herb, as well as between different herbs in a formulation like Thapring. The concept, that a whole or
Fig. 4. Normalization of serum parameters by Thapring. The X15-myc onc mice of different age groups were treated orally with Thapring as described under Section 2 and their sera were analyzed for the levels of ALT (A), VEGF (B) and SOD (C). Data are shown as mean ± SD (n = 4). PB, pre-bleed serum. Level of significance: *, p < 0.02; **, p < 0.03.

partially purified extract of a herb offers advantages over a single isolated ingredient, also underpins the philosophy of herbal medicine. Therefore, multi-target drug design combined with a network-dependent approach is a promising concept to combat multifactorial diseases like cancer. The control of a complex disease system should involve simultaneous disruption of multiple targets located in distant cellular networks (Keith et al., 2005). Thus, Thapring as a TTM promises to offer all that for treating cancer.

Fig. 5. Activation of apoptotic markers in the presence of Thapring. Huh7 cells were treated with indicated concentrations of Thapring for 24 h, and analyzed for the levels of: (A) phosphorylated p38 and ATF2 and (B) p21^{WAF1}, p53 and phosphorylated and total mdm2. (C) Immunohistochemical (IHC) analysis of p21^{WAF1} expression in the liver of untreated (control) and Thapring-treated X15-myc (at 400×).
5. Conclusions

Thappring is used as an anticancer and hepatoprotective agent in TTM. The present investigation provides scientific basis in support of its therapeutic applications. The present study suggests that Thappring possesses a strong anti-cancer activity (growth inhibition, cell cycle arrest, pro-apoptotic activity) in hepatoma cells and shows minimal cytotoxic effect on non-hepatoma cells and non-transformed AML12 hepatocytes. These results warranted an in vivo study in a genetic mouse model of HCC. Oral administration of the drug in mice revealed a clear hepatoprotection and conferred anti-carcinogenic and anti-angiogenic effects. Thus, Thappring appears to be a good and reliable candidate as CAM for clinical application as adjunct/combination therapy in cancer.

Acknowledgements

We thank Dr. S. Jameel for kindly providing pE GFP-cytochrome c and pDsRed-Mitotracker plasmids. This work was supported by the core fund of ICGEB, New Delhi.

References


