Biochemical and Biophysical Research Communications 425 (2012) 613–618

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Triparanol suppresses human tumor growth in vitro and in vivo

Xinyu Bi [a,1], Xingpeng Han [b,1], Fang Zhang [c,1], Miao He [d], Yi Zhang [e], Xiu-Yi Zhi [e,*], Hong Zhao [a,*]

[a] Department of Abdominal Surgical Oncology, Lab of Abdominal Surgical Oncology, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China
[b] Department of Pathology, Tianjin Chest Hospital, Tianjin 300051, China
[c] Zhejiang Provincial Key Laboratory of Applied Enzymology, Yangtze Delta Region Institute of Tsinghua University, Jiaxing 314006, Zhejiang, China
[d] Life Sciences School, Sun Yat-sen University, Guangzhou 510275, China
[e] Department of Thoracic Surgery, Xuanwu Hospital, Capital Medical University, Beijing 100053, China

ARTICLE INFO

Article history:
Received 20 July 2012
Available online 1 August 2012

Keywords:
Triparanol
Tumor suppression
Hedgehog signaling

ABSTRACT

Despite the improved contemporary multidisciplinary regimens treating cancer, majority of cancer patients still suffer from adverse effects and relapse, therefore posing a significant challenge to uncover more efficacious molecular therapeutics targeting signaling pathways central to tumorigenesis. Here, our study have demonstrated that Triparanol, a cholesterol synthesis inhibitor, can block proliferation and induce apoptosis in multiple human cancer cells including lung, breast, liver, pancreatic, prostate cancer and melanoma cells, and growth inhibition can be rescued by exogenous addition of cholesterol. Remarkably, we have proved Triparanol can significantly repress Hedgehog pathway signaling in these human cancer cells. Furthermore, study in a mouse xenograft model of human lung cancer has validated that Triparanol can impede tumor growth in vivo. We have therefore uncovered Triparanol as potential new cancer therapeutic in treating multiple types of human cancers with deregulated Hedgehog signaling.

1. Introduction

The hallmarks of most deadly cancers include the development of drug resistance, cancer relapse and poor prognosis. Despite the improved contemporary tumor debulking surgery, multidisciplinary regimens including radio- and chemotherapy, the majority of cancer patients not only experience adverse effects, but also eventually relapse [1–3]. This poses a significant challenge for cancer drug development to uncover more efficacious molecular therapeutics. One such approach is the development of molecularly targeted therapies that block signaling pathways central to tumorigenesis. Clinical trials of new agents using this research paradigm have been encouraging [4].

Deregulated Hedgehog (Hh) pathway activation plays essential roles in various types of human cancers including glioma, basal cell carcinoma, medulloblastoma, lung, breast, pancreatic and gastric cancers [5–10]. The Hh family of proteins controls multiple cellular functions, including cell growth, survival and outcome, as well as body patterning and organ morphogenesis during embryonic development [5,9–12]. Hh signaling is controlled by two transmembrane proteins, Patched (Ptch1) and Smoothened (SMO). In the absence of the Hh ligand, PTCH1 inhibits SMO, causing cleavage of GLI1 to the N-terminal repressor form. Once Hh binds to PTCH1, the inhibitory effect on SMO is released, causing active full-length GLI1 to transport into the nucleus and activate transcription of the Hh target genes in a context- and cell-type specific manner, including GLI1, PTCH1, HHIP and C-MYC, thereby translating into defined transcriptional programs [9–12]. Targeted inhibition of aberrant Hh signaling leads to suppression of cancer stem cells awakened and propelled by inappropriate Hh signaling [6–7,12].

Triparanol (structure shown in Fig. 1A), a known cholesterol biosynthesis inhibitor blocking the 24-dehydrocholesterol reductase (24-DHCR) [13], can interfere with posttranslational modification of Hedgehog signaling molecules as well as the sterol sensing domain of its receptor PTCH1, leading to down-regulation of Hh signaling [14–16]. Therefore, we investigated the potential of Triparanol in the treatment of several types of human cancer cells with aberrantly activated Hh signaling, including lung, breast, liver, pancreatic and prostate cancer, as well as melanoma [6–7].

2. Materials and methods

2.1. Cell lines and cell culture

We obtained all human cell lines from American Type Culture Collections (ATCC). These cell lines include: lung cancer A549; breast cancer MCF7; hepatocellular carcinoma (liver cancer) HepG2; pancreatic cancer cell line bxpc3; prostate cancer cell line

* Corresponding authors.
E-mail addresses: xiuyizhi@yahoo.com.cn (X.-Y. Zhi), zhaohong9@sina.com (H. Zhao).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.07.136

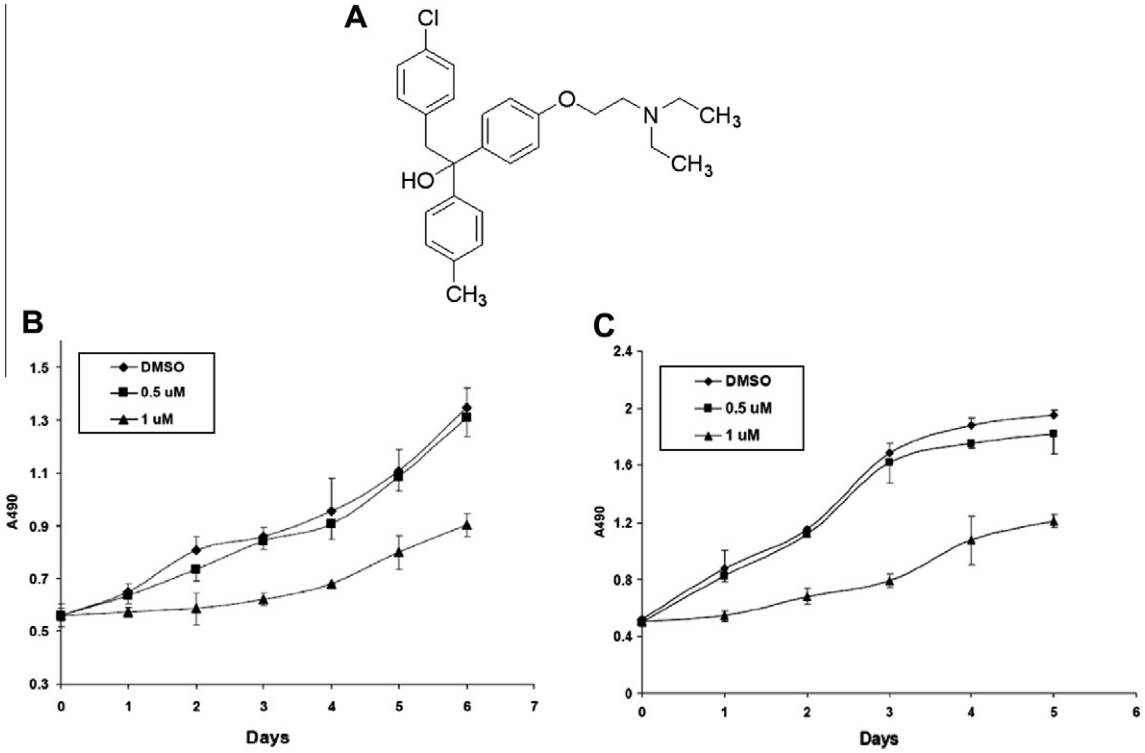

Fig. 1. Triparanol suppresses the proliferation of human lung and breast cancer cells. (A) Structure of Triparanol. (B and C) MTS cell proliferation assay in A549 human lung cancer cells in (B) and MCF7 human breast cancer cells in (C). DMSO was used as control, Triparanol was used in 0.5 or 1 μM as indicated.

LnCAP; melanoma G361; and normal fibroblast. Cell lines are routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml), at 37 °C in a humid incubator with 5% CO_2.

2.2. Incubation of Triparanol and cholesterol with cells

Compounds was purchased from Sigma, and dissolved in DMSO. One day before treatment, cells (5×10^4) will be plated in 96-wells in growth medium without antibiotics and with reduced serum (2.5%). Cells will be incubated with Triparanol at different concentrations as indicated with or without cholesterol (5 μM) in the same medium until ready for further analysis.

2.3. Semi-quantitative RT-PCR

Total RNA from cell lines or tissue samples was isolated using Qiagen RNeasy Mini Kit. RT-PCR was performed in GeneAmp PCR system 2700 using One-step RT-PCR Kit from Invitrogen Life Technologies. Primer sequences for amplifying different genes in Hedgehog signaling pathway are listed in the table below (Table 1). GAPDH was used as internal control.

Table 1
Primer sequences of Semi-quantitative RT-PCR.

Gene	Primer sequences	Size (bp)
GLI1	F: 5′-TACTCACGCCTCGAAAACCT-3′ R: 5′-GTCTGCTTTCCTCCCTGATG-3′	340
PTCH1	F: 5′-CGCCTATGCCTGTCTAACCATGC-3′ R: 5′-TAAATCCATGCTGAGAATTGCA-3′	448
GAPDH	F: 5′-ATCAGCAATGCCTCCTGCAC-3′ R: 5′-TTCACCACCTTCTTGATGTC-3′	350

2.4. Flow cytometry analysis of apoptosis

Apoptosis was analyzed by using an Annexin V FITC Apoptosis Detection Kit (Oncogene), according to the manufacturer's protocol. After treatment with compound at various concentrations for 2 days, cells were harvested by trypsinization, stained and immediately analyzed by flow cytometry (FACScan; Decton Dickinson, Franklin Lake, New Jersey). Early apoptotic cells with exposed phosphatidylserine but intact cell membranes bind to Annexin V-FITC, but not propidium iodide (PI). Cells in necrotic or late apoptotic stages are labeled with both Annexin V-FITC and PI.

2.5. Cell proliferation assays

Cells will be treated with Triparanol in 96-well plates for 6–7 days. Cell proliferation was assayed by MTS assay (Promega) according to the manufacturer's protocol. The MTS reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron-coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion may be accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. Data are representative of at least three independent experiments with similar results.

2.6. Mouse xenograft model study with Triparanol

We tested Triparanol in vivo in the mouse xenograft model bearing human lung cancer cells A549. Briefly, female athymic

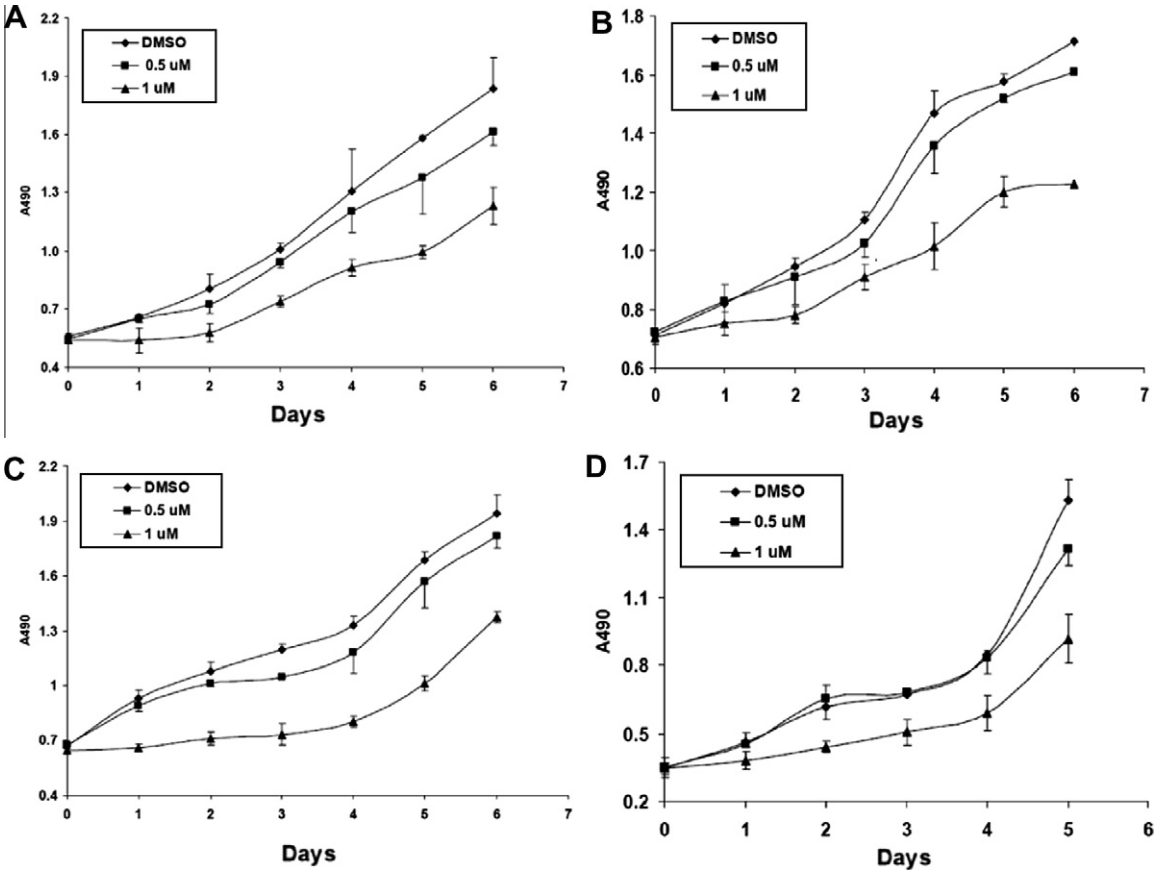

Fig. 2. Triparanol inhibits proliferation of multiple types of human cancer cells. (A–D) MTS cell proliferation assay in HeG2 human liver cancer cells in (A), bxpc3 human pancreatic cancer cells in (B), LnCAP human prostate cancer cells in (C), and human melanoma cells in (D). DMSO was used as control, Triparanol was used in 0.5 or 1 μM as indicated.

nude mice were maintained in pathogen-free conditions. Seven mice were used in each group and injected s.c. with 5×10^6 A549 lung cancer cells in the dorsal area in a volume of 100 μl. After inoculation, human lung cancer cells were allowed to grow in mice for 5 days to become visible tumor nodules. Animals were then injected with Triparanol at a dose of 50 mg/kg body weight (1 mg/mouse per day). DMSO alone was used as control. The compound and control were adjusted in 50 ml volume for i.p. injection in the abdomens of the mice. Injections were performed daily around the same time for 21 days. Tumors were allowed to grow for additional 2 weeks after completion of the compound treatment. Tumor size was measured every five days, and tumor volumes were calculated using width (x) and length (y) ($x^2y/2$, where $x < y$) [19]. Forty days after the initial tumor inoculation, tumors were dissected from the mice of each group and tumor weights were measured using a scale. Data was presented as mean values (+S.D.).

2.7. Histological examination for toxicity evaluation

After the in vivo studies were completed, different organs were resected from the mice. These organs include liver, lung, heart, kidney, intestines, ovary, brain, spleen, skin and muscle. The specimens were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned and histologically analyzed by hematoxylin and eosin staining. The HE stained slides were examined by a mouse pathologist for toxicity evidence from all the organs tested as compared with the DMSO controls.

2.8. Mouse serum cholesterol assay

Cholesterol assay in the mouse serum was performed according to the manufacturer's menu (the amplex red cholesterol assay from Molecular Probes) [20].

2.9. Statistical analysis

Data shown represent mean values (+S.D.). Paired T-Test in the Excel was used for comparing different treatments and cell lines. A p value of 0.05 or less was considered to be significant.

3. Results

We first examined the effect of Triparanol on the proliferation of human lung cancer cells, A549. Cells were plated in 96-well plates and incubated with 0.5 or 1 μM of the compound for the time as indicated, MTS assay was employed to measure cell proliferation at each time points (Fig. 1B, $p < 0.05$). We found that Triparanol significantly inhibited proliferation of the human lung cancer cells at the dose of 1 μM (Fig. 1B).

We further assessed whether Triparanol could suppress proliferation of human breast cancer cells, MCF7. In consistency with the A549 result, Triparanol also significantly suppressed the proliferation of human breast cancer cells MCF7 at 1 μM (Fig. 1C, $p < 0.05$).

We further tested the effect of Triparanol on a wider spectrum of human cancer cells, including human liver, pancreatic and

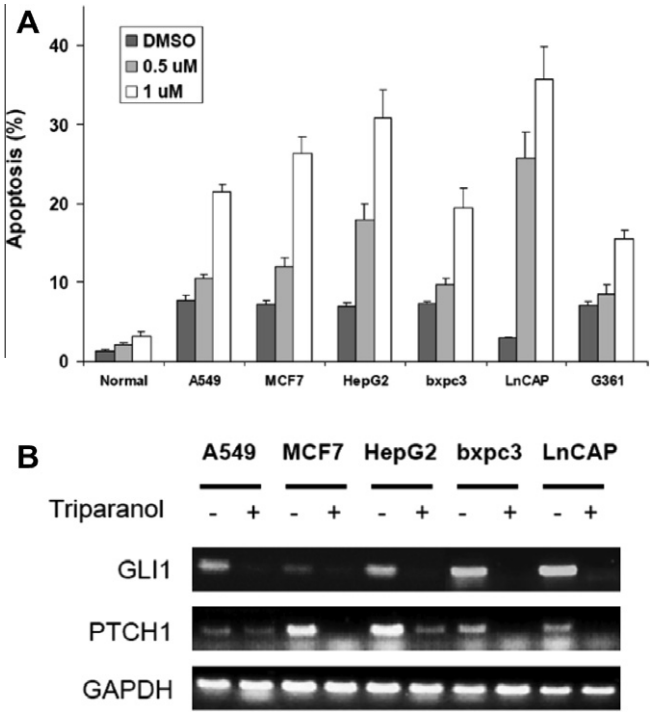

Fig. 3. Triparanol induces apoptosis in human cancer but not normal cells, and it represses Hh signaling in human cancer cells. (A) Apoptosis assay in multiple types of human cancer cells and normal control cells treated with Triparanol at indicated doses. (B) Effect of Triparanol on Hh target gene expression by semi-quantitative RT-PCR. GAPDH was used as a loading control.

prostate cancer cells and melanoma cells. In line with the A549 and MCF7 data, we observed that Triparanol significantly inhibited the proliferation of the human liver, pancreatic and prostate cancer cells in dose-dependent manner (Fig. 2A–C, $p < 0.05$). Triparanol showed remarkable inhibition of melanoma cells at 1 μM for all the time points examined, and it significantly suppressed melanoma proliferation on day 5 at a lower dose of 0.5 μM (Fig. 2D).

To determine whether Triparanol specifically suppresses cancer cells, we examined the effect of Triparanol on the proliferation of normal human fibroblast cells. We found that Triparanol did not affect the viability of the normal cells even after 6-day treatment at 1 or 2 μM (Supplementary Fig. 1), suggesting that the Triparanol specifically inhibits cancer cells.

To determine whether the inhibition of cancer cell proliferation results in induction of apoptosis, we assayed apoptosis in multiple cancer cells following Triparanol treatment. Apoptosis was assessed by flow cytometry after the treatment of Triparanol at different doses (0.5 and 1 μM) for 4 days in several types of human cancer cell lines (including lung cancer A549; breast cancer MCF7; hepatocellular carcinoma HepG2; pancreatic cancer cell line bxpc3; prostate cancer cell line LnCAP and melanoma G361). Triparanol induced significant level of apoptosis in all the cell lines tested and showed dose dependence (Fig. 3A, $p < 0.05$). As a control, no noticeable apoptosis induction was observed in the normal fibroblast cells following Triparanol treatment (Fig. 3A, Normal).

As aberrant Hh pathway signaling represents as the major signaling pathway that contributes to tumorigenesis in multiple types of human cancers [5–10], we further evaluated whether Triparanol could repress Hh pathway signaling in these human cancer cells. Human cancer cells including A549, MCF7, HepG2, bxpc3, LnCAP and G361) were plated in 6-well plates and incubated with 1 μM Triparanol for 1 day before isolation of total RNA for semi-quantitative RT-PCR analysis. GAPDH served as loading control. Key components and direct target genes of the Hh pathway (e.g. PTCH1 and GLI1) were dramatically down-regulated by Triparanol treatment (Fig. 3B). These results suggested that Triparanol can inhibit Hh

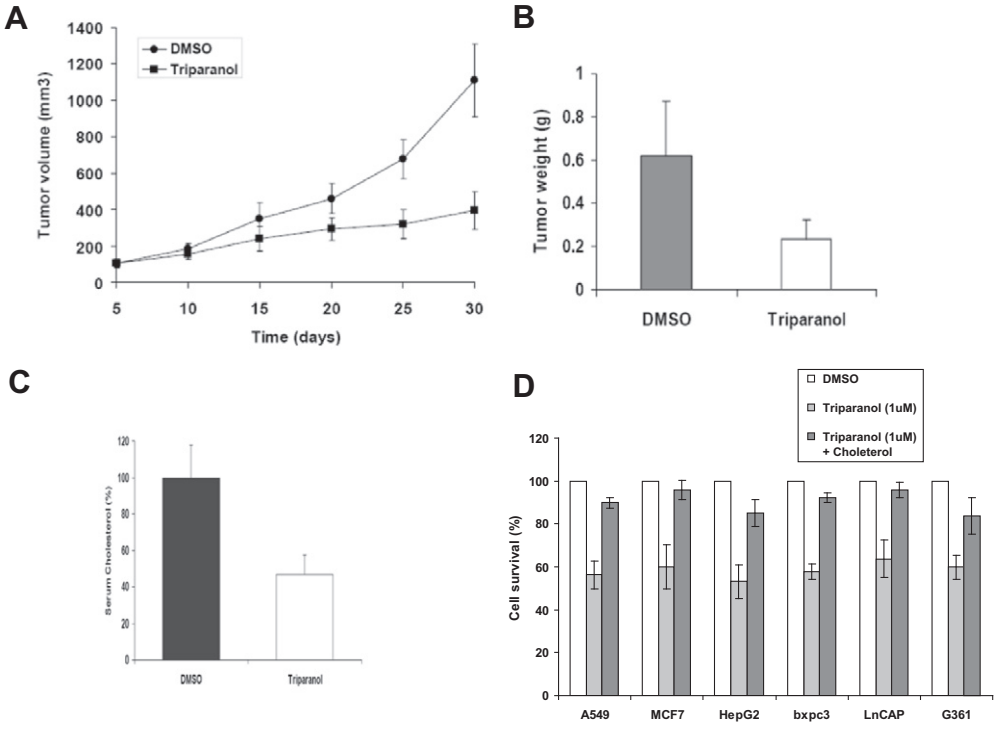

Fig. 4. Triparanol suppresses tumor growth in a human lung cancer Mouse Xenograft Model. (A) Tumor size measured in mice treated with either Triparanol or DMSO control. (B) Tumor weight measured in day 40 after tumors were dissected from mice treated with Triparanol or DMSO. (C) Serum cholesterol levels in mice treated with Triparanol or DMSO. (D) Addition of Cholesterol rescued the growth inhibitory effect of Triparanol (1 μM) in multiple cancer cell lines. Data represented are mean values ± SD (error bar).

signaling pathway, contributing to growth retardation in these human cancer cells.

To validate whether Triparanol can suppress tumor growth in vivo, we examined the effects of Triparanol in a human lung cancer mouse xenograft model. SCID mice were injected s.c. with 5×10^6 A549 human lung cancer cells in the dorsal area in a volume of 100 μl. Five days after inoculation, the mice started receiving treatment with the compound at a dose of 50 mg/kg body weight daily for 21 days ($n = 7$). DMSO alone was used as control ($n = 7$). Remarkably, tumor growth was significantly abrogated after Triparanol treatment compared with DMSO control (Fig. 4A, $p = 0.02$). Tumor weight also significantly decreased after the treatment with Triparanol (Fig. 4B, $p = 0.03$). These in vivo results are consistent with our in vitro data, demonstrating that Triparanol can suppress tumor growth both in vitro and in vivo. To assess the toxicity of the Triparanol treatment, we examined the effect of Triparal on multiple mouse organ systems using hematoxylin and eosin (HE) staining. No toxic effect were observed in all the organs examined, including the liver, lung, heart, kidney, intestines, ovary, brain, spleen, skin and muscle (data not shown). This result suggests that Triparanol is therapeutically safe in cancer treatment.

To validate Triparanol was absorbed and exerted its biological effect of inhibiting cholesterol synthesis, we further assessed the serum cholesterol levels following Triparanol treatment. We showed that serum cholesterol level of mice treated with Triparanol was almost half of control (Fig. 4C), indicating that Triparanol was properly absorbed and exerted its biological function. To verify that the growth inhibitory action of Triparanol is mainly due to inhibition of cholesterol synthesis, exogenously added cholesterol was shown to effectively reverse the in vitro growth inhibitory action of 1 μM of Triparanol in multiple cancer cell lines tested (Fig. 4D, $p < 0.05$).

4. Discussion

In the present study, we have demonstrated that the cholesterol synthesis inhibitor Triparanol can significantly inhibit tumor growth both in vitro and in vivo (Figs. 1–4). It has been previously reported that Triparanol could inhibit growth of cultured rat hepatoma cells [17], and it also impeded tumor growth in mouse chondrosarcoma xenografts [18]. Our data are consistent with these previous results, and we have further investigated the effect of Triparanol in an expanded spectrum of human cancer cells, including human lung, breast, liver, pancreatic and prostate cancer cells, as well as human melanoma cells (Figs. 1 and 2). In addition, we have assessed the effect of Triparanol in a human lung cancer mouse xenograft model, and we observed that Tripanarol significantly abrogated tumor growth in vivo (Fig. 4), further demonstrating the tumor suppression function of Triparanol both in vitro and in vivo.

We have further investigated the mechanisms underlying the effect of Triparanol on tumor suppression by apoptosis assay. Notably, Triparanol significantly induced apoptosis in multiple cancer cell lines tested but not in normal control cells (Fig. 3A), suggesting Triparanol inhibits tumor growth through induction of apoptosis. As aberrant Hh signaling plays pivotal roles in tumorigenesis of multiple human cancers [5–10,12], we assessed whether Triparanol could affect Hh signaling in these human cancer cells. Indeed we proved Triparanol could remarkably repress Hh signaling, as shown by decreased expression of Hh downstream target genes GLI1 and PTCH1 (Fig. 3B). Furthermore, we showed that Triparanol could significantly reduces serum level of cholesterol in vivo, validating that Triparanol was properly absorbed and exerted its biological effect of inhibiting cholesterol synthesis (Fig. 4C). We further demonstrated that exogenously added cholesterol could effectively rescue the in vitro growth inhibitory action of Triparanol at 1 μM in multiple cancer cell lines tested (Fig. 4D), verifying that the growth inhibitory action of Triparanol is mainly due to it inhibition of cholesterol synthesis. These data are in accordance with the emerging evidence indicating cholesterol modification of Hh family of tissue patterning factors plays essential roles in Hh signal transduction [15–16,19–22]. Our results thus unraveled new function of Triparanol in tumor suppression in addition to its effects in embryonic development.

We have therefore uncovered Triparanol as a potential new cancer therapeutic in treating multiple types of human cancers with deregulated Hh signaling. Recently developed small molecule inhibitors of the Hh pathway antagonizing Smo have proved promising in clinical trials [23–24], though mutations in Smo have recently been reported to confer resistance to such inhibitors by disrupting drug binding [25–26]. Thus development of additional Hh inhibitors blocking upstream cholesterol modification thereby bypassing such refractory Smo mutations will open up a more effective avenue in abrogating Hh signaling in human cancers.

Acknowledgment

This work has been supported by a Zhejiang Provincial Natural Science Foundation Grant to F. Zhang (Y2110030).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.136.

References

[1] P.A. Jänne, E.H. Baldini, Patterns of failure following surgical resection for maligant pleural mesothelioma, Thorac. Surg. Clin. 14 (2004) 567–573.
[2] A.M. Gonzalez-Angulo, F. Morales-Vasquez, G.N. Hortobagyi, Overview of resistance to systemic therapy in patients with breast cancer, Adv. Exp. Med. Biol. 608 (2007) 1–22.
[3] D. Hiss, Optimizing molecular-targeted therapies in ovarian cancer: the renewed surge of interest in ovarian cancer biomarkers and cell signaling pathways, J. Oncol. (2012) 737981.
[4] K.A. Johnson, P.H. Brown, Drug development for cancer chemoprevention: focus on molecular targets, Semin. Oncol. 37 (2010) 345–358.
[5] M. Varjosalo, J. Taipale, Hedgehog: functions and mechanisms, Genes Dev. 22 (2008) 2454–2472.
[6] L. Yang, G. Xie, Q. Fan, J. Xie, Activation of the hedgehog-signaling pathway in human cancer and the clinical implications, Oncogene 29 (2010) 469–481.
[7] S.J. Scales, F.J. de Sauvage, Mechanisms of Hedgehog pathway activation in cancer and implications for therapy, Trends Pharmacol. Sci. 30 (2009) 303–312.
[8] X. Ma, K. Chen, S. Huang, et al., Frequent activation of the hedgehog pathway in advanced gastric adenocarcinomas, Carcinogenesis 26 (2005) 1698–1705.
[9] J.E. Hooper, M.P. Scott, Communicating with Hedgehogs, Nat. Rev. Mol. Cell Biol. 6 (2005) 306–317.
[10] D. Huangfu, K.V. Anderson, Signaling from Smo to Ci/ Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates, Development 133 (2006) 3–14.
[11] T. Osterlund, P. Kogerman, Hedgehog signalling: how to go from Smo to Ci and Gli, Trends Cell Biol. 16 (2006) 176–180.
[12] A. Ruiz i Altaba, P. Sanchez, N. Dahmane, Gli and hedgehog in cancer: tumors, embryos and stem cells, Nat. Rev. Cancer 2 (2002) 361–372.
[13] J. Avigan, D. Steinberg, H.E. Vroman, M.J. Thompson, E. Mosettig, Studies of cholesterol biosynthesis. I. The identification of desmosterol in serum and tissues of animals and man treated with MER-29, J. Biol. Chem. 235 (1960) 3123–3126.
[14] M.K. Cooper, J.A. Porter, K.E. Young, P.A. Beachy, Teratogen-mediated inhibition of target tissue response to Shh signaling, Science 280 (1998) 1603–1607.
[15] C. Roux, C. Wolf, N. Mulliez, W. Gaoua, V. Cormier, F. Chevy, D. Citadelle, Role of cholesterol in embryonic development, Am. J. Clin. Nutr. 71 (2000) 1270S–1279S.
[16] F. Gofflot, C. Hars, F. Illien, F. Chevy, C. Wolf, J.J. Picard, C. Roux, Molecular mechanisms underlying limb anomalies associated with cholesterol deficiency during gestation: implications of Hedgehog signaling, Hum. Mol. Genet. 12 (2003) 1187–1198.

[17] G. Popják, A. Meenan, E.J. Parish, W.D. Nes, Inhibition of cholesterol synthesis and cell growth by 24(R, S),clinph and triparanol in cultured rat hepatoma cells, J. Biol. Chem. 264 (1989) 6230–6238.
[18] T.D. Tiet, S. Hopyan, P. Nadesan, N. Gokgoz, R. Poon, A.C. Lin, T. Yan, I.L. Andrulis, B.A. Alman, J.S. Wunder, Constitutive hedgehog signaling in chondrosarcoma up-regulates tumor cell proliferation, Am. J. Pathol. 168 (2006) 321–330.
[19] J.A. Porter, K.E. Young, P.A. Beachy, Cholesterol modification of hedgehog signaling proteins in animal development, Science 274 (1996) 255–259.
[20] R.K. Mann, P.A. Beachy, Cholesterol modification of proteins, Biochim. Biophys. Acta 1529 (2000) 188–202.
[21] M.K. Cooper, C.A. Wassif, P.A. Krakowiak, J. Taipale, R. Gong, R.I. Kelley, F.D. Porter, P.A. Beachy, A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis, Nat. Genet. 33 (2003) 508–513.
[22] R.K. Mann, P.A. Beachy, Novel lipid modifications of secreted protein signals, Annu. Rev. Biochem. 73 (2004) 891–923.
[23] E. De Smaele, E. Ferretti, A. Gulino, Vismodegib, a small-molecule inhibitor of the hedgehog pathway for the treatment of advanced cancers, Curr. Opin. Investig. Drugs 11 (2010) 707–718.
[24] J.A. Low, F.J. de Sauvage, Clinical experience with Hedgehog pathway inhibitors, J. Clin. Oncol. 28 (2010) 5321–5326.
[25] R.L. Yauch, G.J. Dijkgraaf, B. Alicke, T. Januario, C.P. Ahn, T. Holcomb, K. Pujara, J. Stinson, C.A. Callahan, T. Tang, J.F. Bazan, Z. Kan, S. Seshagiri, C.L. Hann, S.E. Gould, J.A. Low, C.M. Rudin, F.J. de Sauvage, Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma, Science 326 (2009) 572–574.
[26] G.J. Dijkgraaf, B. Alicke, L. Weinmann, T. Januario, K. West, Z. Modrusan, D. Burdick, R. Goldsmith, K. Robarge, D. Sutherlin, S.J. Scales, S.E. Gould, R.L. Yauch, F.J. de Sauvage, Small molecule inhibition of GDC-0449 refractory smoothened mutants and down-stream mechanisms of drug resistance, Cancer Res. 71 (2011) 435–444.