Review Article
Natural compound Alternol as a novel therapeutic for prostate cancer treatment

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Abstract: Alternol is a monomeric compound purified from the fermentation of a microbial strain obtained from the bark of the yew tree. Recent studies have confirmed that it has specific anti-prostate cancer efficacy *in vitro* and *in vivo*. In *in vitro* cell culture experiments Alternol inhibits prostate cancer cell proliferation by causing cell cycle arrest, reduces the expression of Bcl-2 and other pro-survival proteins, increases the level of radical oxygen species by activating xanthine dehydrogenase, blunts mitochondrial aerobic respiration and ATP production, and triggers autophagy flux. However, there is no significant adverse effect on benign prostatic cells. Animal experiments demonstrated that Alternol significantly inhibits the growth of prostate cancer xenografts without obvious adverse effect on normal tissues and organs. Therefore, Alternol is expected to be developed as a new anti-prostate cancer therapy.

Keywords: Alternol, prostate cancer, apoptosis, cell cycle, radical oxygen species

Introduction

Prostate cancer is the most common cancer in males [1]. At present, clinical treatment is still mainly based on surgery, followed by radiation therapy, hormone therapy and chemotherapy [2]. However, the treatment of castration-resistant prostate cancer (CRPC) is a major obstacle in clinic. The first-line chemo-drugs are extremely limited. Therefore, it is urgent to seek new and effective therapy for castration-resistant prostate cancer [3].

Alternol is a small molecule compound purified from the fermentation product of a mutant fungus. Early research has been applied to a variety of tumor cell lines, including gastric cancer, liver cancer, lymphocytic leukemia and melanoma [4-6] and confirmed that Alternol selectively kills prostate cancer cells without significant side effect on benign prostate epithelial cells, indicating a promising future as a new prostate cancer therapy.

Anti-cancer efficacy in vitro

One of the main objects of anti-tumor drugs is to induce apoptosis and kill tumor cells. Current research reports showed that most of the prostate cancer cell lines exhibited cell death response after treatment with Alternol (5~10 μM) [6, 7]. It has a killing effect in a 45%, 40%, and 35% reduction of survival rate of C4-2, PC-3, and DU145 cells, respectively, while only less than 20% in benign prostate epithelial RWPE-1 cells, suggesting a selective effect on malignant cells [8]. Further studies *in vitro* showed that Alternol-induced death effect is an apoptotic reaction in prostate cancer cell lines, including androgen-responsive LNCaP, castration-resistant C4-2 and 22RV1, and androgen receptor-negative PC-3, but it does not induce apoptosis in normal prostate RWPE-1 and BPH1 cells [9]. Apoptosis reaction was confirmed by in-depth analysis of caspase-3 processing, PARP cleavage, flow cytometry-based Annexin V/ Propidium iodide staining, and mitochondrial membrane potential.

Anti-tumor effect in vivo

The xenograft model of human tumor cells established subcutaneously or *in situ* in nude mice is one of the effective methods for preclinical evaluation of anti-tumor therapeutics. Animal studies reported that Alternol has a sig-

nificant inhibitory effect on the growth of prostate cancer xenografts in nude mice [9]. In a subcutaneous xenografts model in nude mice established with prostate cancer PC-3 cells, intraperitoneal injection of Alternol (10-20 mg/kg bodyweight) significantly inhibited the xenograft tumor growth compared with the solvent control group. The tumor wet weight was also significantly lower than the control group, but no obvious side effects and weight loss were observed to the animals [9, 10]. Our unpublished preclinical studies revealed that Alternol was well-tolerated in mice with a maximal tolerance dose (MTD) at 665 mg/kg bodyweight and a LD_{50} at 953 mg/kg bodyweight, which is more than 30-fold higher over the effective dose of 20 mg/kg bodyweight to suppress xenograft tumor growth.

The mechanism of Alternol action in anti-prostate cancer

Effect on cell cycle evolution

The normal cell cycle is composed of G_1-S-G_2-M, during which there are two important regulatory points G_1/S and G_2/M, and the loss of its regulatory function leads to tumor occurrence [11]. In vitro culture condition, the infinite proliferation characteristics of malignant tumors are related to the uncontrolled cell proliferation cycle. Limiting the cell cycle evolution is the key to inhibit the growth and proliferation of tumor cells [12]. Most chemotherapy drugs can cause cell cycle arrest and achieve anti-tumor effects. However, when performing cell cycle analysis, the drug concentration needs to be controlled within the non-lethal range to avoid pseudo cell cycle arrest, because cells in different cycles have different lethal responses to drugs. Flow cytometry cell cycle analysis revealed that after Alternol treatment with sub-lethal concentration (0.5 µM) of Alternol, C4-2 cells accumulated significantly in G_2/M phase cells, indicating that Alternol has a blocking effect on the cell cycle evolution of prostate cancer [8].

Effect on intracellular radical oxygen species

Tumor cells often accumulate radical oxygen species (ROS) due to rapid proliferation, but excessive ROS accumulation, which exceeds the cell's compensatory capacity, leads to a super oxidative stress reaction and causes cell death. Thus, promoting ROS accumulation is a way to develop anti-tumor drugs [13]. In vitro studies have found that Alternol induces a significant ROS accumulation in prostate cancer cells, while benign cell did not show an obvious ROS accumulation. Quantitative analysis revealed that Alternol-induced ROS accumulation gradually increased and peaked at 4 hours after addition of the drug. A pretreatment with ROS scavengers n-acetylcysteine (N-Ac) and dihydrolipoic acid (DHLA) significantly reduced the ROS level and cell death [9].

Typical apoptotic reactions include chromosomal DNA fragmentation, PARP cleavage, caspase-3 processing and changes in mitochondrial membrane permeability [14]. Studies have shown that N-Ac or DHLA pretreatment eliminates Alternol-induced caspase-3 processing, PARP cleavage and DNA fragmentation and significantly reduces the degree of mitochondrial damage and pro-apoptotic Bax activation [9]. These data demonstrate that Alternol induces prostate cancer cell apoptosis through an oxidative stress-dependent mechanism.

ROS including reactive nitrogen species (RNS) are the most basic reactive molecules of biological organisms, which are composed of various molecular forms, including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and nitric oxide ($NO^{\bullet-}$), active hydroxyl ($OH^{\bullet-}$), peroxynitrite ($ONOO^-$) and lipid peroxyl radical ($LOO^{\bullet-}$) [13]. There are multiple mechanisms for producing ROS in mammalian cells. The main participants are the mitochondrial respiratory chain, membrane-bound nicotinamide adenine dinucleotide phosphate oxidase (NOX), cytoplasmic xanthine dehydrogenase (XDH), and nitric oxide nitrogen synthase (NOS) [15]. In a recent research report, the researchers conducted a very comprehensive investigation to clarify the main source of Alternol-induced ROS response [16]. The results of the study showed that although Alternol treatment increased the rate of mitochondrial superoxide formation, it was significantly lower than the total ROS-positive cell population. The pretreatment of cells with several mitochondrial specific antioxidant MitoQ, and NOX or NOS-specific inhibitors had no protective effect on Alternol-induced ROS accumulation and cell death, suggesting that the mitochondrial-produced ROS, or NOX and NOS activities are not the main cause of Alternol-induced cancer death. Fur-

ther analysis revealed that after treatment with Alternol, the levels of uric acid and H_2O_2, the final products of xanthine oxidase (XDH), were significantly increased, and XDH activity was also significantly enhanced in parallel. Consistently, XDH inhibitors allopurinol [17] and febuxostat [18], gene-specific small interfering RNA-mediated silencing of XDH gene all significantly reduced cellular ROS content and greatly decreased apoptosis induced by Alternol in prostate cancer cells. These data indicate that XDH plays a key role in Alternol-induced ROS accumulation and apoptosis. In addition, compared with the malignant cells treated with Alternol, the activities of superoxidase dismutase (SOD) and hydrogen peroxide hydrolase are significantly enhanced in benign cells, which is presumed to be related to the relative tolerance of benign cells to Alternol [16]. Cell-based protein-ligand binding and bioinformatics simulation analysis show that Alternol interacts with XDH protein at its catalytic domain, indicating that Alternol can directly act on XDH to enhance its oxidative activity, leading to a large amount of ROS accumulation and subsequent apoptosis [16].

Effect on apoptosis regulating factors

Bcl-2 protein is the main anti-apoptotic factor in cells and Bax is a pro-apoptotic factor of Bcl-2 family members, both are key molecules of Bcl-2 family to regulate apoptosis. After activation of Bax protein, a multi-step cascade reaction occurs, migrating from the cytoplasm to the outer mitochondrial membrane to form a dimer channel. When Bax forms an oligomer and colonizes on the mitochondrial membrane, it will form an apoptosis-inducing channel and release cytochrome C, causing apoptotic cell death [19, 20]. It was reported that the anti-apoptotic protein Bcl-2 protein level was significantly reduced after Alternol treatment in prostate cancer PC-3 and C4-2 cells, while the other anti-apoptotic factor Bcl-X$_L$ did not significantly decrease. The pro-apoptotic factor Bif-1 protein was increased slightly after Alternol treatment, and Bif-1 can interact with Bax to firm a dimer, participating in apoptosis induction. These studies suggest that Bcl-2 family proteins are involved in the apoptosis reaction after Alternol treatment [9].

Although it has been reported that the pancreatic cancer (PANC-1 and BxPC3) cells treated with Alternol have slightly increased levels of the DNA damage factor p53 protein, but in prostate cancer LNCaP and C4-2 cells with wild-type p53 protein, or p53 gene deficient prostate cancer PC-3, Alternol treatment induces apoptosis, indicating that p53 is not a key factor in the apoptosis response caused by Alternol. However, DUl45 cells are lacking the pro-apoptotic protein Bax and showed no apoptotic response to Alternol, suggesting that the Bax protein is the key mechanism of Alternol-induced apoptosis. Animal experiments also showed that compared with the control group, TUNEL-positive cells and processed caspase-3 protein were significantly increased in prostate cancer PC-3 cell-derived xenografts treated with Alternol, while DU145-derived xenografts treated with Alternol had no significant increase of TUNEL index or processed caspase-3 protein [9]. These data further indicate that Alternol induces apoptosis through Bax-dependent rather than p53-dependent mechanism to inhibit tumor growth.

Impact on cancer cell metabolism

The tricarboxylic acid (TCA) cycle is the main mechanism by which cell mitochondria use glucose aerobic metabolism to generate energy ATP and other biological macromolecular intermediates. Among these metabolites, pyruvate dehydrogenase (PDH) and its two coenzymes dihydrolipoyllysine acetyltransferase (DLAT) and dihydrolipidamide dehydrogenase (DLD) forms a complex that converts the glycolytic metabolite pyruvate to acetyl-CoA (Acetyl-CoA). Another complex of kento-oxoglutarate dehydrogenase (OGDH) with dihydrofatty acid amide S-succinyl (DLST) and DLD is also a key step in catalyzing the metabolic intermediate succinate [21]. In a recent report, using biotin labeling method combined with Mass-Spectrometry proteomic technology, 14 cellular proteins were identified and confirmed to bind to Alternol in prostate cancer cells, four of which are the main TCA enzymes, including DLAT, DLST, fumarate hydratase (FH) and malate dehydrogenase-2 (MDH2) [10]. Cellular enzyme activity analysis showed that the basic activity of the two complexes of PDH and OGDH in malignant PC-3 cells was significantly higher than that of benign BPH1 cells, indicating a higher metabolic TCA activity in malignant cells. After Alternol treatment, PDH and OGDH activities in malignant PC-3 cells were decreased signifi-

cantly, but the activity of FH or MDH2 was increased significantly. Conversely, the activities of these enzymes in benign BPH1 cells did not change significantly after Alternol treatment [10]. Oxygen consumption analysis revealed that Alternol treatment caused a great reduction in aerobic respiration and ATP production in cancer cells. Meanwhile, the ATP content in xenograft tumors dissected from animals received Alternol treatment was also significantly lower than that of control treatment. However, Alternol treatment has no significant inhibitory effect on ATP content in normal liver tissue of nude mice [10].

Metabolomic analysis using gas chromatography combined with small molecule mass spectrometry (GC-MS) revealed that the basic levels of citric acid, succinic acid, fumaric acid, and malic acid in malignant PC-3 cells were significantly higher than those of benign BPH1 cells, indicating a higher metabolic activity in malignant cells. Alternol treatment drastically reduced the levels of succinic acid, fumaric acid, malic acid, and isocitrate in PC-3 cells, however, there is no change in BPH1 cells [10]. These studies indicate that Alternol reduces the excessive TCA activity in malignant cells to a level comparable to benign cells by simultaneously targeting various TCA enzymes, resulting in reduced aerobic respiration and reduced ATP production, thereby inhibiting tumor growth.

Effect on prostate cancer cell autophagy

Autophagy occurs when cells are starved and under energy stress, part of the cytoplasm and organelles are wrapped into autophagosomes with a double membrane structure, which are then fused with lysosome to form autophagolysosomes, whose contents are degraded into small molecules, providing nutrients for cells to cope with metabolism and energy stress [22]. It has been shown that autophagy is closely related to the occurrence and development of tumors. On the one hand, tumor cells can use autophagy to respond to the energy stress caused by the rapid proliferation of tumor cells during the initial stage of tumor development. Cancer treatment either kill tumor cells by triggering excessive autophagy reaction, or eliminate damaged mitochondria by activating autophagy, resulting in reduced apoptosis of tumor cells [22]. LKB1-AMPK pa-

thway is a cellular energy sensing machinery and plays an important role in mediating cell decision to enter autophagy or apoptosis by regulating p27 phosphorylation [23]. After activation, LKB1-AMPK pathway induces p27 phosphorylation and enhances its protein stability, leading to enhanced autophagy and cell survival from apoptosis or necrosis induced by various factors. Consistently, knocking down p27 protein expression results in apoptosis under cellular stress condition [23]. In prostate cancer C4-2 cells, Alternol treatment reduces p27 phosphorylation, while in benign cell RWPE-1, p27 phosphorylation was significantly increased after Alternol treatment [8]. Consequently, Alternol treatment significantly reduced autophagy response in C4-2 cells, while the autophagy response was enhanced in RWPE-1 cells. It is speculated that this phenomenon may be related to the selective killing of prostate cancer cells by Alternol [8]. However, Alternol concentration used in this study was significantly lower than the lethal concentration reported in other studies (5-10 μM). Therefore, the relationship between Alternol-induced autophagy and its anti-tumor effect needs to be further explored.

Conclusion

Small molecular compound Alternol was derived from a mutant fungal and has specific anti-tumor effect *in vitro* and *in vivo*. Alternol inhibits cancer cell proliferation and xenograft tumor growth. It triggers ROS accumulation in tumor cells by activating XDH enzyme, leading to a ROS-dependent apoptosis. Alternol attenuates the expression of Bcl-2 family proteins in tumor cells, leading to apoptosis. Alternol also binds to multiple TCA enzymes and disrupts energy homeostasis in prostate cancer cell-derived xenografts and subsequently tumor suppression.

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69: 7-34.

[2] Miller K, Nogueira L, Mariotto A, Rowland J, Yabroff K, Alfano C, Jemal A, Kramer J and Siegel R. Cancer treatment and survivorship statistics, 2019. CA Cancer J Clin 2019; 69: 363-385.

[3] Dong L, Zieren R, Xue W, de Reijke T and Pienta K. Metastatic prostate cancer remains incurable, why? Asian J Urol 2019; 6: 26-41.

[4] Liu Z, Zhu J, Sun B, Liu S, Geng S, Liu X and Li C. Alternol inhibits proliferation and induces apoptosis in mouse lymphocyte leukemia (L1210) cells. Mol Cell Biochem 2007; 306: 115-22.

[5] Liu Z, Chen J, Zhao S and Li C. [Apoptosis-inducing effect of alternol on mouse lymphocyte leukemia cells and its mechanism]. Yao Xue Xue Bao 2007; 42: 1259-65.

[6] Zaffaroni N, Marco C, Villa R, Riboldi S, Daidone M and Double J. Cell growth inhibition, G2M cell cycle arrest and apoptosis induced by the imidazoacridinone C1311 in human tumour cell lines. Eur J Cancer 2001; 37: 1953-62.

[7] Cong P, Qu Y, Chen J, Duan L, Lin C, Zhu X, Li-Ling J and Zhang M. Growth inhibition and apoptosis induction by alternol in pancreatic carcinoma cells. World J Gastroenterol 2015; 21: 4526-35.

[8] Yeung E, Morrison A, Plumeri D, Wang J, Tong C, Yan X and Li J. Alternol exerts prostate-selective antitumor effects through modulations of the AMPK signaling pathway. Prostate 2012; 72: 165-72.

[9] Tang Y, Chen R, Huang Y, Li G, Huang Y, Chen J, Duan L, Zhu B, Thrasher J, Zhang X and Li B. Natural compound Alternol induces oxidative stress-dependent apoptotic cell death preferentially in prostate cancer cells. Mol Cancer Ther 2014; 13: 1526-36.

[10] Li C, He C, Xu Y, Xu H, Tang Y, Chavan H, Duan S, Artigues A, Forrest M, Krishnamurthy P, Han S, Holzbeierlein J and Li B. Alternol eliminates excessive ATP production by disturbing Krebs cycle in prostate cancer. Prostate 2019; 79: 628-639.

[11] Dalton S. Linking the cell cycle to cell fate decisions. Trends Cell Biol 2015; 25: 592-600.

[12] Yao Y, Zhang B, Chen H, Chen N, Liu L, Yishan W, Li C and Zheng Q. Alteronol inhibits proliferation in HeLa cells through inducing a G1-phase arrest. J Pharm Pharmacol 2012; 64: 101-7.

[13] Moloney J and Cotter T. ROS signalling in the biology of cancer. Semin Cell Dev Biol 2018; 80: 50-64.

[14] D'Arcy M. Cell death: a review of the major forms of apoptosis, necrosis and autophagy. Cell Biol Int 2019; 43: 582-592.

[15] Moloney J and Cotter T. ROS signalling in the biology of cancer. Semin Cell Dev Biol 2018; 80: 50-64.

[16] Xu H, Li C, Mozziconacci O, Zhu R, Xu Y, Tang Y, Chen R, Huang Y, Holzbeierlein J, Schöneich C, Huang J and Li B. Xanthine oxidase-mediated oxidative stress promotes cancer cell-specific apoptosis. Free Radic Biol Med 2019; 139: 70-79.

[17] Delgado JN, Cosgrove FP and Isaacson EI. Allopurinol: xanthine oxidase inhibitor. Tex Med 1966; 62: 100-101.

[18] Osada Y, Tsuchimoto M, Fukushima M, Takahashi K, Kondo S, Hasegawa M and Komoriya K. Hypouricemic effect of the novel xanthine oxidase inhibitor, TEI-6720, in rodents. Eur J Pharmacol 1993; 241: 183-188.

[19] Zheng C, Liu T, Liu H and Wang J. BCL-2 family proteins in apoptosis and its regulation. Curr Protein Pept Sci 2019.

[20] Cosentino K and García-Sáez A. Bax and bak pores: are we closing the circle? Trends Cell Biol 2017; 27: 266-275.

[21] Murphy M and O'Neill L. Krebs Cycle Reimagined: the emerging roles of succinate and itaconate as signal transducers. Cell 2018; 174: 780-784.

[22] Saha S, Panigrahi D, Patil S and Bhutia S. Autophagy in health and disease: a comprehensive review. Biomed Pharmacother 2018; 104: 485-495.

[23] Liang J, Shao S, Xu Z, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont D, Gutterman J, Walker C, Slingerland J and Mills G. The energy sensing LKB1-AMPK pathway regulates p27-kip1 phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol 2007; 9: 218-24.