Tropical Journal of Pharmaceutical Research June 2022; 21 (6): 1215-1220
**ISSN:** 1596-5996 (print); 1596-9827 (electronic)


Available online at http://www.tjpr.org
**http://dx.doi.org/10.4314/tjpr.v21i6.12**

**Original Research Article**

# Oxobedfordia acid reduces colon cancer cell viability through apoptosis induction and inhibits colon cancer growth in mice model

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*Sent for review: 2 October 2021* *Revised accepted: 31 May 2022*

## Abstract

***Purpose:*** *Colon cancer is amongst the most commonly diagnosed carcinoma globally and ranks 3rd highest of all the kinds of tumors. In the present study effect of oxobedfordia acid on colon cancer cell viability and colorectal tumor growth in vivo was investigated.*
***Methods:*** *Cytotoxicity of oxobedfordia acid in SW480, HCT116, and FHC cells was evaluated by MTT assay. Colon cancer in the mice was induced by implanting subcutaneously 2 x $10^6$ HT-29 cells/mouse in the right flank. Various parameters, including cell viability, tumor growth and expression levels of cancer factors, were also assessed.*
***Results:*** *Treatment with oxobedfordia acid significantly reduced viability in SW480 and HCT116 cells ($p < 0.05$). Furthermore, oxobedfordia acid caused increased miR-331-3p levels in cells. Moreover, oxobedfordia acid caused a significant reduction in NRP2 expression and increased apoptosis induction in SW480 and HCT116 cells. Oxobedfordia acid treatment for 48 h significantly increased p53 and p-c-Jun levels, but reduced Bcl-2 expression in cells ($p < 0.05$). In the mouse model of colon cancer, oxobedfordia acid significantly retarded tumor growth. Furthermore, in oxobedfordia acid-treated mice, expression of miR-331-3p was elevated while NRP2 level was lowered when compared with control group ($p < 0.05$).*
***Conclusion:*** *Oxobedfordia acid treatment suppresses colon cancer cell viability and inhibits tumor growth in mice through enhancement of miR-331-3p and reduction in NRP2 expression. Hence, oxobedfordia acid can potentially be developed as an agent for the management of colorectal cancer.*

***Keywords:*** *Oxobedfordia acid, Colorectal carcinoma, Chemotherapy, Neuropilin-2*



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## INTRODUCTION

Colon cancer is amongst the most commonly diagnosed carcinoma globally and ranks 3rd highest of all the kinds of tumors [1,2]. Each year around 8 lakh new cases of colon cancer are diagnosed and 20% of such patients are detected at advanced stage [1,2]. Presently available treatment for colorectal cancer involves surgical intervention along with chemotherapy consisting of fluoropyrimidines followed by radiotherapy [3]. Studies have demonstrated that

administration of chemotherapeutic agents to colorectal cancer patients effectively suppresses tumor metastasis, inhibits tumor growth and alleviate clinical symptoms [4]. Unfortunately, development of resistance to many of the available therapeutic agents has been reported in colorectal cancer patients. Therefore, studies need to be intensified to explore the molecular targets for inhibition of colorectal cancer and evaluation of natural products against the tumor growth.

Expression of microRNA (miRNA/miR) has been shown to be abnormally down-regulated in colorectal cancer cells and plays important role in tumor development [5]. In various vital biological processes these small non-coding RNAs commonly known as miRNAs play key role [6]. Many studies have been performed to understand the role of miRNAs because of their involvement in different carcinomas such as breast cancer, osteosarcoma and glioma [7].

The miRNAs have been found to be closely associated with the tumor development and are believed to be of therapeutic significance to regulate the cancer progression. Neuropilin-2 (NRP2) is one of the members of NRPs family that acts as non-tyrosine kinase transmembrane glycoprotein [8]. In cell membranes, NRP2 functions as receptor for vascular semaphorin (SEMA) as well as endothelial growth factors (VEGF) [9]. Overexpression of NRP2 has been demonstrated in various kinds of cancer cells such as breast, pulmonary, cervical carcinoma cells [10].

Impaired apoptotic pathway is the major factor responsible for tumor growth because undesired cells from the body are not eliminated. Cell death through apoptotic pathway is mainly regulated by the expression of proteins like Bcl-2 and Bcl-XL [11]. Tumor suppressive effect of p53 is associated with suppression of cellular proliferation through apoptotic induction and cell cycle arrest [12]. JNK is a member of superfamily of MAPKs and has been demonstrated to promote phosphorylation of c-Jun. JNK activation induces c-Jun phosphorylation and subsequently elevates Bcl-2 and p53 expression to inhibit apoptosis of cancer cells [13]. However, it has been revealed that ovarian cancer cells develop resistance towards cisplatin drug following JNK1 inactivation [14].

In the present study oxobedfordia acid was investigated as anti-proliferation agent and as tumor growth inhibitor in mice.

## EXPERIMENTAL

### Cell culture

Colorectal cancer cell lines SW480 and HCT116 and normal cell line FHC were obtained from the Chinese Academy of Sciences. Cell lines were grown in RPMI-1640 medium in an incubator.

### MTT assay

Cells were grown into 96 well plates (1 x $10^6$ cells/well) for 24 h. Medium mixed with 2 to 10 µM concentrations of oxobedfordia acid was added for 48 h. After incubation was over, medium was removed from the wells, and 20 µl MTT reagent (5 mg/ml; Gibco, USA) was added. At the end of experiment, MTT (Sigma) stock solution of 5 mg/mL concentration and volume 100 µL was supplemented to cells with 4 h of incubation. The formazan crystals then produced are dissolved with the use of DMSO and finally absorbance was calculated at 540 nm.

### Animals and treatment

Seventy Male CD1 nude mice (5-7 week old) were supplied by Wenzhou Medical University (Wenzhou, China). They were kept in the animal house (in cages) under regulated temperature (23± 1$^o$C) and humidity (60%). All the mice were exposed to 12 h light/dark cycles and provided free access to tap water and diet *ad libitum*. Following acclimatization to laboratory conditions for 7-days the mice randomly assigned to seven groups (10 mice/group): Control, model (tumor), oxobedfordia acid **+** saline and four oxobedfordia acid treatment groups (at 5, 10, 15 and 20 mg/kg doses). Mice in model and four oxobedfordia acid treatment groups were implanted subcutaneously 2 x $10^6$ HT-29 cells/mouse in the right flank (35). On day 7 of HT-29 cell implantation, the mice in treatment groups were intragastrically given 5, 10, 15 and 20 mg/kg doses oxobedfordia acid in physiological saline. Experimental protocols on the mice were performed by following the international guidelines for animal studies. Approval for the studies was provided by the institutional ethics committee.

### Tumor volume measurement in mice

Digital calipers were used for the measurement of tumor size formed in mice after every week of the study. Following completion of the study (5 weeks), mice were sacrificed using sevoflurane anaesthesia to extract the tumors which were dissected and subsequently stored at -70 ˚C.

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNAs extraction from cells or tissues was performed using the TRIzol reagent. Reverse transcription of the RNA samples was carried out for cDNA synthesis using the Prime Script RT reagent kit in conditions like 37 ˚C for 20 min and 85 ˚C for 8 s. Samples were then subjected to RT-PCR using the iQ SYBR Green supermix. The samples were amplified and detected using S1000 Thermal cycler real-time PCR system (Bio-Rad). Primers used for specific amplification of miR-331-3p, NRP2 and GAPDH were designed by Primer 5.0. The conditions for qRT-PCR includes: an initial step of 10 minute in 95 °C, and then 38 cycles of amplification, which includes 95 °C for 10 s, 59 °C for 15 s and 72 °C for 25 s. Quantification was determined by $2^{-\Delta\Delta CT}$ method.

### Immunoblotting

Cellular and tissue proteins were obtained using lysis buffer, followed by protein concentration determination using a BCA protein quantification kit (Pierce, 23225). From each sample, 50 µg of proteins were run on SDS-PAGE gels which were processed for blotting to PVDF membranes. Blocking of PVDF membrane with 5% non-fat dry milk was done for 2 h. Furthermore, incubation with primary antibodies against c-Jun, -P-c-Jun, Bcl-2, p53 and β-actin was carried out. Next day, after washing with PBS thrice, and membranes were treated with HRP-linked secondary antibody for 2 h. Finally, ECL chromogenic substrate was added for colour-reaction.

### Statistical analysis

For data analysis, SPSS 21.0 software was used and data were expressed as mean ± standard deviation (SD). In addition, t-test was utilized for comparison between two groups. $P < 0.05$ was considered statistically significant.

## RESULTS

### Impact of oxobedfordia acid on viability of colorectal cells

Oxobedfordia acid treatment significantly lowered SW480 and HCT116 cell viability compared to untreated cells (Figure 1). Treatment with 10 µM oxobedfordia acid decreased SW480 and HCT116 cell viability to 23 and 19 %, respectively at 48 h. In FHC cells no significant changes in viability were observed on treatment with oxobedfordia acid.

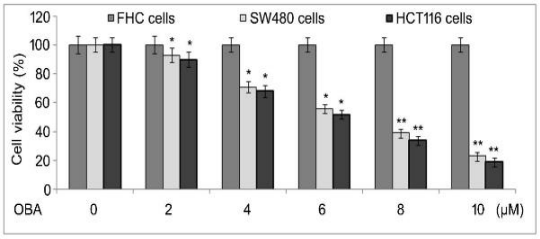


**Figure 1:** Oxobedfordia acid treatment reduce cellular viability. Treatment of the cells with 2 to 10 µM oxobedfordia acid for 48 h was followed by measurement of changes in cell viability by MTT assay. *$P < 0.05$ and **$p < 0.01$ vs. control

### Impact of oxobedfordia acid on miR-331-3p and NRP2

Treatment of cells with 4 and 10 µM oxobedfordia acid significantly elevated miR-331-3p expression (Figure 2). Moreover, oxobedfordia acid treatment at 4 and 10 µM caused a remarkable decrease in NRP2 expression. Increased miR-331-3p level and decreased NRP2 level by oxobedfordia acid was more in HCT116 cells than SW480 cells.

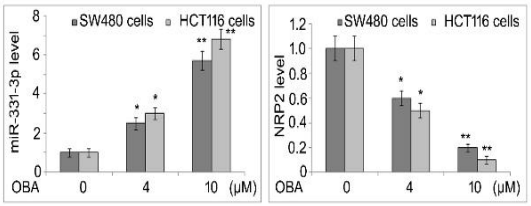


**Figure 2:** Effect of oxobedfordia acid on expression of miR-331-3p and NRP2. The SW480 and HCT116 cells were incubated with oxobedfordia acid or left untreated. *$P < 0.05$ and **$p < 0.01$ vs. control

### Impact of oxobedfordia acid on apoptotic proteins

Oxobedfordia acid treatment at 4 and 10 µM for 48 h caused a prominent increase in c-Jun and p53 expression (Figure 3). P53 and c-Jun expression was increased after oxobedfordia acid treatment in dose dependent manner. Oxobedfordia acid treatment caused significant reduction in Bcl-2 protein level at 4 and 10 µM doses.

### Effect of oxobedfordia acid on tumor growth in mice

Effect of oxobedfordia acid treatment on tumor growth was determined during 5-weeks of treatment (Figure 4). Growth of tumors was inhibited significantly in 5, 10, 15 and 20 mg/kg oxobedfordia acid treated mice than control

group from 2nd week. Tumor growth reduction by oxobedfordia acid treatment was significant ($p < 0.05$) on 2nd, 3rd, 4th and 5th weeks than control group. However, oxobedfordia acid treatment of the mice for five weeks didn't impact body weight (Figure 4 B).

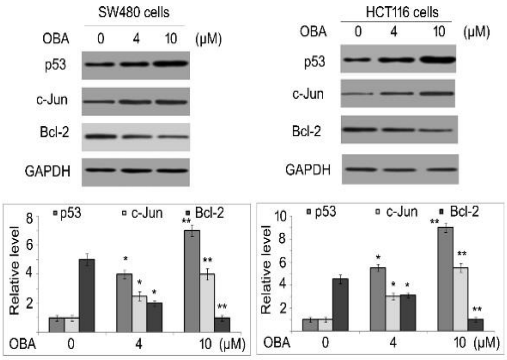


**Figure 3:** Oxobedfordia acid treatment promotes anti-apoptotic protein expression. Cells were treated with oxobedfordia acid or left untreated. *$P < 0.05$ and **$p < 0.01$ vs. control

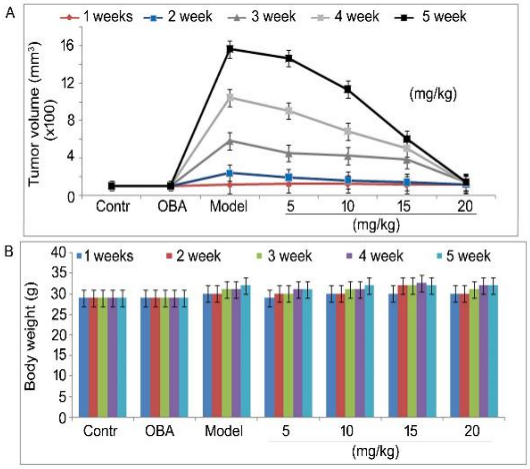


**Figure 4:** Oxobedfordia acid treatment inhibited tumor growth. Tumor volume (A) body weight (B) determination for 5-weeks following oxobedfordia acid treatment was performed. *$P < 0.05$ and **$p < 0.01$ vs. control

### Effect of oxobedfordia acid on miR-331-3p and NRP2 expression in mice

Tumor samples from the oxobedfordia acid treated mice showed significantly ($P<0.05$) raised level of miR-331-3p than control group (Figure 5). Increased miR-331-3p expression was higher in 10 mg/kg treated mice group compared to the 5 mg/kg group. Moreover, NRP2 expression in oxobedfordia acid treated mice tumors was significantly lowered than control.

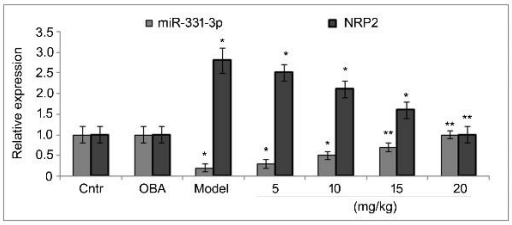


**Figure 5:** Oxobedfordia acid promotes miR-331-3p expression and suppresses NRP2 level. Mice were treated with 5 and 10 mg/kg oxobedfordia acid or left untreated (model group). *$P < 0.05$ and **$p < 0.01$ vs. control

## DISCUSSION

In colorectal cancer patient's advancement in therapeutic strategies has led to increase in survival rate but development of resistance to available drugs is a challenge to clinicians [15]. Therefore, identification of therapeutic molecular targets and discovery of more potential drugs is immediately required to inhibit the colorectal cancer growth. Colorectal tumor pathogenesis has been found to be closely linked with miRNAs expression [16]. These miRNAs play important role in colorectal cancer progression by regulating multiple gene expression and related activities [17]. MiR-331-3p is an independent prognostic factor which is the member of miRNAs family and is involved in regulation of cancer progression [18].

Growth of prostate cancer has been found to be inversely related with miR-331-3p expression and its presence in the serum of hepatocellular carcinoma patients plays a key in tumorigenicity and metastatic potential of oesophageal carcinoma cells [20]. There are reports that NRP2 expression also regulates pancreatic adenocarcinoma proliferation and melanoma progression and angiogenesis [21].

In the present study *in vitro* data revealed that oxobedfordia acid treatment significantly lowered SW480 and HCT116 cell viability. However, in FHC cells no significant changes in viability were observed on treatment with oxobedfordia acid. Moreover, oxobedfordia acid treatment significantly elevated miR-331-3p expression compared to the control cells. Additionally, oxobedfordia acid treatment caused a remarkable decrease in NRP2 levels. Thus, initial *in vitro* findings revealed that oxobedfordia acid treatment effectively raised miR-331-3p levels and targeted NRP2 level to inhibit cell proliferation.

Therapeutic agents usually regulate tumor growth by activation of apoptotic pathway and

elevation of pro-apoptotic proteins. It is reported that apoptosis is mainly regulated by the expression of genes belonging to anti-apoptotic Bcl-2 family [13]. Tumor suppressor gene, p53 after mutation leads to impairment in apoptotic pathway and promotes tumor progression [13].

Overexpression of mutated form of p53 gene indicates unfavourable prognosis in many types of cancers including colorectal cancer cells [22]. Oxobedfordia acid caused a prominent increase in p53 and c-Jun expression. Oxobedfordia acid treatment caused a significant reduction in Bcl-2 protein expression. In vivo studies demonstrated that growth of tumors was suppressed significantly in oxobedfordia acid treated mice than control group from 2nd week. Tumor growth reduction by oxobedfordia acid treatment was significant ($P<0.05$) on 2nd, 3rd, 4th and 5th week than control group. Tumor samples from the oxobedfordia acid treated mice showed significantly ($P<0.05$) raised level of miR-331-3p than control group. Moreover, NRP2 expression in oxobedfordia acid treated mice tumors was significantly suppressed than control group.

## CONCLUSION

Oxobedfordia acid treatment inhibits proliferation of colorectal cancer cells without affecting normal cell growth. It also enhances miR-331-3p levels and suppresses NRP2 level in colorectal cancer cells. Moreover, colorectal tumor growth in mice model is suppressed by oxobedfordia acid treatment by inhibiting miR-331-3p levels and reducing NRP2 level. Thus, oxobedfordia acid can potentially be developed as a therapeutic agent for the management of colorectal cancer.

## DECLARATIONS

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Songbo Ma and Zhi Yu contributed equally to this work.

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