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Original Research Article

N-(4-hydroxyphenyl) retinamide inhibits migration of renal carcinoma cells and promotes autophagy via MAPK p38 pathway

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Abstract

Purpose: *To investigate the effect of N-(4-hydroxyphenyl) retinamide (4HPR) on autophagy and migration of renal carcinoma cells.*
Methods: *Renal cancer cell lines were treated with various concentrations of 4HPR. Proliferation of the cells was studied using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltrazolium bromide (MTT), while apoptosis and cell cycle arrest were determined by flow cytometry.*
Results: *Treatment of RCCs with 30 µM 4HPR caused significant inhibition of viability. In 786-O and OS-RC-2 cell lines, 4HPR reduced colony formation by 39 and 43 %, respectively. In addition, 4HPR increased the percentage of 786-O cells in G1 phase from 58.79 ± 3.43 to 71.68 ± 4.47 % ($p < 0.05$). It also decreased the percentage of cells in the S-phase from 21.98 ± 2.78 to 09.17 ± 1.43 %, and enhanced the activation of p38 and JNK in 786-O cells at 48 h. Western blot assay showed that the activation of p38 and JNK by 4HPR was inhibited on pre-treatment with SB203580 (inhibitor of p38) and SP600125 (inhibitor of JNK), respectively. Reduction of 786-O cell viability by 4HPR treatment was also significantly inhibited by pre-treatment with sp203580 and sp600125 ($p < 0.05$). Furthermore, the inhibitors also reversed the effect of 4HPR on the expressions of Bax and Bcl-2 in 786-O cells.*
Conclusion: *These results indicate that 4HPR inhibits the growth of renal cancer cells via activation of MAPK signalling pathway. Thus, 4HPR is a potential drug target for management of renal cancer.*

Keywords: *Retinamide, Renal cancer, Autophagy, MAPK signalling, Cell proliferation, N-terminal kinase*

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INTRODUCTION

Renal cell carcinoma (RCC), a neoplasm in majority of adult kidneys, is a tumour of epithelial origin [1,2]. In USA alone, more than 60,000 new cases of RCC (3.6 % of all cancers) were detected in 2015 [3]. Renal cell carcinoma treatment at the metastatic stage is a challenge to urologists because of its low response to various therapeutic strategies including chemotherapy and radiotherapy [4]. Some of the targeted molecular approaches have been found

to be effective to some extent but the overall response rates are unsatisfactory [4]. The resistance of RCC to limited number of available chemotherapy and radio-therapy approaches requires immediate steps to be taken towards developing new and more effective treatment strategies [5,6]. Indeed, the development of effective treatment approach for renal cell carcinoma has continued to be a challenging task for clinicians.

A semi-synthetic member of the retinoid family, 4HPR) has shown promising activity against various types of tumours [7]. In ovarian carcinoma cells, treatment with 4HPR led to inhibition of proliferation through induction of cell apoptosis [8,9]. Investigation of its mechanism of action has revealed that 4HPR treatment either activates retinoic acid receptor (RAR) pathway, or induces cell apoptosis without activation of RAR pathway [9-11]. Furthermore, 4HPR-induced cell apoptosis has also been found to activate c-Jun N-terminal kinase, generate reactive oxygen species (ROS) or promote production of ceramide [11-14]. The inhibitory effect of 4HPR against neuroblastoma and ovary cancer involves release of mitochondrial cytochrome c [11,15-17].

In the present study, the effect of 4HPR on renal carcinoma cells, and the probable molecular mechanism of action involved, were investigated.

EXPERIMENTAL

Cell culture and reagents

Renal cancer cell lines 786-O and OS-RC-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Utah, hyClone, UT, USA) at 37 ˚C in an atmosphere of 5 % CO_2. The medium contained 10 % fetal bovine serum (FBS; Gibco Life Technologies, Carlsbad, CA, USA) and streptomycin/penicillin (100 U/ml).

Assay of cell viability

Cell viability was assayed in 786-O and OS-RC-2 renal carcinoma cells using MTT assay. The cells were treated with 5, 10, 15, 20, 25 and 30 µM of 4HPR, or with medium alone (control) for 48 h, and seeded at a density of 1 x 10^5 cells per well in 96-well plates which were incubated in DMEM medium containing 10 % FBS at 37 ^oC in an atmosphere of 5 % CO_2. The incubation was followed by the addition of MTT reagent (20 µl, 5 mg/mL) to each well of the plate. The plates were again incubated for 4 h under the same conditions. After 4 h, dimethyl sulphoxide was added to the wells of plates and left for 5 min to dissolve any formazan crystals. The absorbance of the formazan solution in each well was read at 490 nm in an enzyme immunoassay plate reader (Bio-Rad, Hercules, CA, USA). The experiments were carried out in triplicate.

Analysis of colony formation

The renal carcinoma cells (786-O and OS-RC-2) were seeded into 6-well culture plates at a density of 4 x 10^3 cells per well. The cells were treated with 30 µM of 4HPR either in DMEM or in medium alone (control) for 2 weeks. Thereafter, the cells were subjected to crystal violet staining for the calculation of number of colonies. The colony-forming growth rate was determined as the ratio of number of colonies to the number of cells seeded, expressed as a percentage.

Determination of apoptosis

Flow cytometry was performed for determination of the effect of 4HPR on cell apoptosis. The 786-O cells were seeded at a density of 2 x 10^6 cells per well in 6-well plates and incubated with 4HPR either in DMEM or in medium alone (control) for 48 h. After harvesting, the cells were treated with HEPES binding buffer, and stained at room temperature with annexin V□fluorescein isothiocyanate (FITC; BD Biosciences, San Jose, CA, USA) and propidium iodide (PI; BD) for 20 min. Induction of apoptosis was determined in the cell samples using FACSCanto™ flow cytometer (BD Biosciences) according to the instructions of the manufacturer.

Analysis of cell cycle

Cell cycle progression in 786-O cells was evaluated by flow cytometric analysis. The cells were seeded into 6-well plates at a density of 2 x 10^6 cells per well and incubated with 4HPR either in DMEM medium or in medium alone (control) for 48 h. The cells were then harvested and fixed for 24 h by washing twice with ethyl alcohol (70 %) at -20 ^oC. Thereafter, cell washing was performed twice and the cells were subsequently incubated for 45 min with PI (50 µg/mL) and RNAase A (50 µg/mL) at room temperature.

The distribution of cells in various phases of the cell cycle was analysed using FACS Calibur system with CELL quest software version 3.3 (both from Becton Dickinson, San Jose, CA,

USA) and ModFit LT software (version 3.0; Verity Software House, Topsham, ME, USA) in accordance with manufacturer's instructions.

Cell migration assay

Transwell chamber was used for analysis of 786-O cell migration. The cells at a density of 3 x 10^5 per 400 µL were seeded in the upper chambers of 24-well Transwell chamber with 8 µm pore size (Millipore), and incubated with 4HPR either in DMEM or in medium alone (control) for 48 h. The bottom chamber was provided with 500 µl/well of DMEM supplemented with 10 % FBS. After incubation, 786-O cells that migrated into the lower side of the filter were fixed in methyl alcohol and stained with crystal violet. Cell counting was performed using bright field microscopy.

Western blot assay

The 786-O renal cancer cells were treated with 4HPR for 48 h and then subjected to lysis using RIPA buffer. The lysate obtained was centrifuged at 4 ^oC for 45 min and the supernatant was collected. The protein content of the cell lysate was determined using BCA assay. The cell extract was heated in loading buffer for 15 min and subjected to separation using 10 % SDS-PAGE.

The protein samples were then transferred to PVDF membranes and blocked with 5 % bovine serum albumin (BSA, Solarbio) in TBST. The membranes were incubated with primary antibodies overnight at 4 ^oC, and washed twice with PBS prior to incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies at 37 ^oC. The resultant protein bands were quantified with super signal West pico Chemiluminescent Substrate kit.

Assay of inhibitions of p38 and JNK

The cells were incubated with 10 µM SB203580 for 30 min prior to treatment with 4HPR for 48 h. Inhibition of JNK was achieved by incubation with 20 µM of SP600125 for 30 min before treatment with 4HPR for 48 h. The inhibitions of p38 and JNK were analysed using western blot.

Statistical analysis

Data are expressed as mean ± standard deviation (SD, n = 3). Significant differences between various groups were determined with one-way analysis of variance (ANOVA) using SPSS 17.0. Statistically significant difference was set at $p < 0.05$.

RESULTS

4HPR inhibited 786-O and OS-RC-2 renal carcinoma cell viability

The effect of 4HPR on viability of renal carcinoma cells was investigated at concentrations of 5, 10, 15, 20, 25 and 30 µM by MTT assay. At 30 µM, 4HPR caused significant inhibitions in the viabilities of 786-O ($p < 0.05$) and OS-RC-2 ($p < 0.02$) cells, when compared to the control cells. At 30 µM of 4HPR, the viabilities of 786-O and OS-RC-2 cells were reduced to 22 and 20 %, respectively at 48 h (Figure 1).

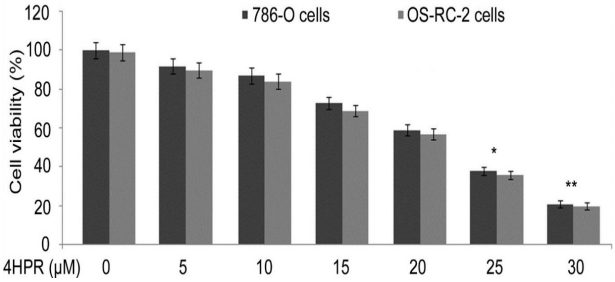

Figure 1: Effect of 4HPR on the proliferation of 786-O and OS-RC-2 cells. Both cell lines were treated with indicated concentration of 4HPR for 48 h and then subjected to MTT assay for analysis of proliferation rate. Values are mean absorbance ± SD (n = 3). $**p < 0.05$ and $*p < 0.02$, compared to untreated cells

4HPR inhibited 786-O and OS-RC-2 renal carcinoma cell colony formation

Treatment of 786-O and OS-RC-2 cells with 4HPR (30 µM) reduced colony formation by 39 and 43 %, respectively after 2 weeks, relative to control cells (Figure 2).

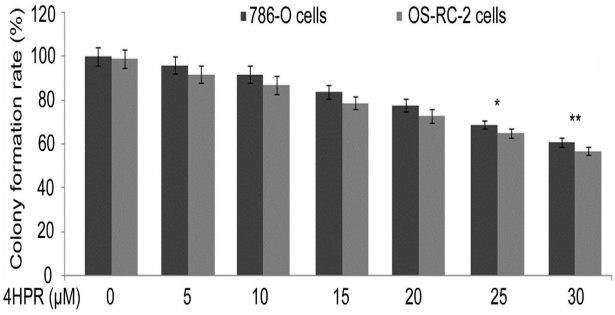

Figure 2: Effect of 4HPR on colony formation in 786-O and OS-RC-2 cells. 4HPR treatment inhibited colony forming potential of both cell lines significantly, when compared to control cells. Colony forming experiments were performed thrice independently to determine the mean values. $**p < 0.01$ and $*p < 0.02$, compared to untreated control cells

4HPR induced cell cycle arrest in 786-O renal carcinoma cells

Changes in the cell cycle of 786-O cells on treatment with 30 µM of 4HPR for 48 h were studied by flow cytometric assay. On treatment with 4HPR, the percentage of cells in G1 phase increased from 58.79 ± 3.43 to 71.68 ± 4.47 % ($p < 0.05$). In the S-phase of cell cycle, the percentage of 786-O cells decreased from 21.98 ± 2.78 to 09.17 ± 1.43% on treatment with 30 µM 4HPR. There was no significant change in the proportion of cells in G2/M phase on treatment with 4HPR. The proportions of 786-O cells in G2/M phase in untreated cultures, and in cultures treated with 4HPR were 18.75 ± 2.34 and 15.36 ± 2.24 %, respectively (Figure 3).

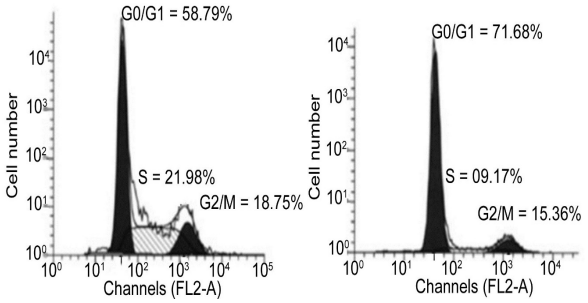

Figure 3: Effect of 4HPR treatment on cell cycle arrest in 786-O renal carcinoma cells. The 786-O cells, after incubation for 48 h with 4HPR were examined for cell cycle distribution by flow cytometry. The values are expressed as mean ± SD of 3 independent experiments

4HPR increased apoptosis in 786-O renal carcinoma cells

Flow cytometric analysis showed that apoptosis in 786-O cells increased significantly at 48 h ($p < 0.05$) on treatment with 4HPR. Treatment of the cells with 30 µM 4HPR increased the percentage of apoptosis from 2.61 ± 1.09 to 21.67 ± 3.33 at 48 h (Figure 4).

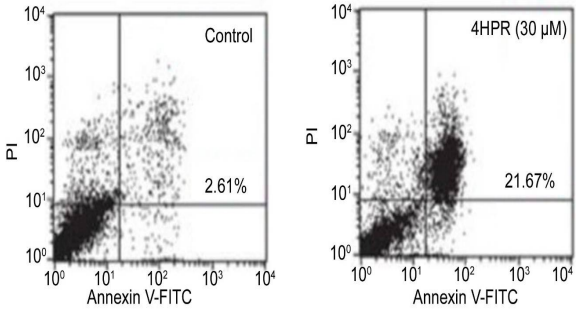

Figure 4: Effect of 4HPR treatment on apoptosis in 786-O cells. 786-O cells were treated with 4HPR for 48 h and then analysed for apoptosis by flow cytometry. Values are presented as mean ± SD (n = 3)

Effect of expressions of 4HPR on Bax and Bcl-2 in 786-O cells

In 786-O cells, treatment with 4HPR markedly promoted the expression of Bax, when compared to the control cells. However, the expression of Bcl-2 was downregulated by 4HPR in 786-O cells at 48 h of treatment, when compared to control cells (Figure 5).

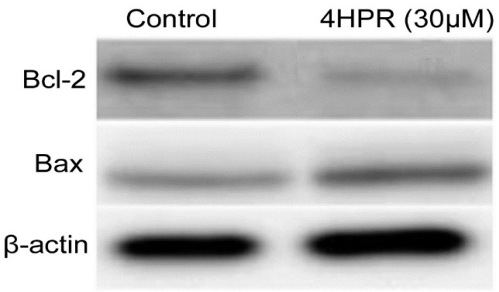

Figure 5: Effect of 4HPR on the expressions of Bax and Bcl-2 in 786-O cells. The 786-O cells were treated with 4HPR for 48 h and subsequently subjected to western blot assay for analysis of expressions of apoptosis-related proteins

4HPR inhibited the migration of 786-O renal carcinoma cells

Treatment of 786-O cells with 4HPR for 48 h was followed by analysis of cell migration by Transwell migration assay. As shown in Figure 6, the migration of 786-O cells was significantly inhibited by 4HPR treatment for 48 h, relative to untreated cells ($p < 0.05$).

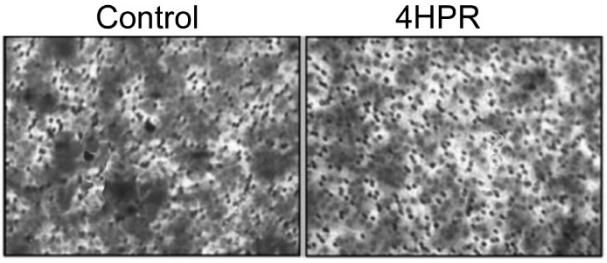

Figure 6: Effect of 4HPR treatment on the migration of 786-O cells at 48 h. Following treatment with 4HPR for 48 h, the cells were subjected to Transwell migration assay, and the images were captured at magnification, x200

4HPR treatment caused activation of MAPK signalling pathway in 786-O renal carcinoma cells

In the analysis of effect of 4HPR on MAPK signalling pathway in 786-O cells, western blot assay was used to assay MAPKs phosphorylation. It was found that 4HPR treatment enhanced the activation of p38 and JNK in 786-O cells at 48 h (Figure **7**). This

suggests that 4HPR treatment activates MAPK signalling pathway in renal carcinoma cells.

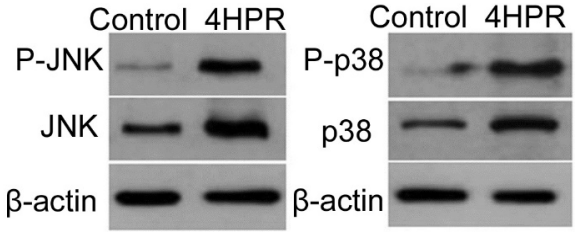

Figure 7: Effect of 4HPR treatment on p38 and JNK activation in renal carcinoma cells. The 786-O cells were treated for 48 h with 4HPR or incubated in medium alone (control). The effect of 4HPR on p38 and JNK activation was analysed by assaying their phosphorylation using western blot

Effect of inhibition of MAPK signalling on 4HPR-mediated viability suppression in 786-O cells

The 786-O cells were incubated with SB203580, an inhibitor of p38, and SP600125, an inhibitor of JNK prior to treatment with 4HPR. Western blot assay showed that the activations of p38 and JNK by 4HPR were inhibited on pre-treatment with SB203580 and SP600125, respectively in 786-O cells (Figure 8 a). Reduction of 786-O cell viability by 4HPR treatment was also significantly suppressed by SB203580 and SP600125 pre-treatments ($p < 0.05$; Figure 8 b). Furthermore, inhibitors of p38 and JNK also reversed the effect of 4HPR on the expressions of Bax and Bcl-2 in 786-O cells (Figure 8 c).

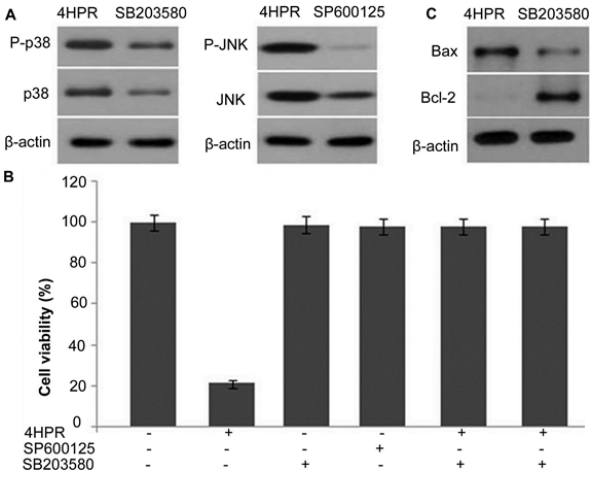

Figure 8: Effect of SB203580 and SP600125 on 4HPR-induced activation of p38/MAPK and JNK/MAPK in 786-O cells. (A) After incubation with SB203580 and SP600125, the cells were treated for 48 h with 4HPR and then analysed by western blot analysis. *$p < 0.05$, compared to control cells. (B) Both SB203580 and SP600125 reversed the effect of 4HPR on viability of 786-O cells. Values presented are mean absorbance ± SD (n = 3); *$p < 0.05$, compared to cells treated with SB203580 and SP600125. (C) Both SB203580 and SP600125 reversed the effect of 4HPR on expressions of Bax and Bcl-2. Cells incubated with SB203580 and SP600125 were treated with 4HPR and then examined by western blot assay

DISCUSSION

The development and metastasis of tumour are greatly facilitated by the microenvironment of the tumour which enhances the rate of cell proliferation, migration, invasion and apoptosis [18]. The present study investigated the effect of 4HPR on renal carcinoma cell proliferation, apoptosis and migration with the aim of developing novel strategy for efficient treatment of renal carcinoma. The proliferation of ovarian cancer cells is inhibited *in vitro* by 4HPR through induction of apoptosis [8,9].

In the present study, 4HPR exhibited inhibitory effects on the proliferations of 786-O and OS-RC-2 renal carcinoma cell lines. Inhibition of carcinoma cell proliferation can be induced by chemotherapeutic agents either through arrest of cell cycle or by cell apoptosis. It is known that G1 phase of cell cycle is associated with the accumulation of various materials required for replication of DNA and transformation into S phase [19]. Thus, a defect in the G1 phase will lead to failure of DNA replication which consequently causes inhibition of cell proliferation [19].

The results of this study showed that treatment of 786-O cells with 4HPR promoted induction of cell apoptosis, and induced cell cycle arrest in G1 phase. The levels of expression of Bax and Bcl-2 are considered as indicators of the degree of induction of apoptosis [20, 21]. In the present study, 4HPR treatment increased the expression of Bax in 786-O cells, relative to control cells, and decreased the expression of Bcl-2. These findings confirm that 4HPR induces apoptosis in renal carcinoma cells. The two pathways (p38/MapK and JNK/MapK) have been found to be associated with the induction of cell apoptosis in malignant tumours [22].

Studies have revealed that the MAPK signalling pathway after activation by chemotherapeutic agents, inhibits growth and induces apoptosis in carcinoma cells [23,24]. Analysis of the effect of 4HPR on MAPK signalling pathway demonstrated that it activated JNK and MAPK by phosphorylation at p38 in 786-O cells. However, pre-treatment of 786-O cells with SB203580 and SP600125 prevented activation of p38 and JNK by 4HPR. These results suggest that 4HPR exhibits its inhibitory effect on cell proliferation

by activation of MAPK signalling pathway. In 786-O cells, treatment with 4HPR caused a marked reduction in migration potential, when compared to control cells.

CONCLUSION

The present study demonstrates that 4HPR treatment has inhibitory effect on proliferation of renal carcinoma cells by inducing apoptosis and suppressing migration potential. It exhibits these effects through activation of p38/MAPK and JNK/MAPK pathway. Therefore, 4HPR has potential for inhibiting renal carcinoma growth. Thus, it may be a suitable drug candidate for the development of a treatment strategy for the management of renal cancer.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors 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 them. Jianguo Gao, Jianer Tang, Yu Chen, Junwen Shen and Ning Wang, performed experiments. Zhihai Fang, Guiqin Shen and Fan Ren carried out literature survey and compiled the data. Rongjiang Wang designed the study and wrote the manuscript.

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