

## Original Research Article

# Quinolinol-platinum (II) complex suppresses survival and invasion of laryngeal cancer cells via targeting YES-associated protein expression

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Sent for review: 18 January 2020

Revised accepted: 22 July 2020

### Abstract

**Purpose:** To investigate the anti-proliferative potential of quinolinol-platinum (II) complex {QN-Pt (II)} in laryngeal cancer cells.

**Methods:** The inhibitory potential of QN-Pt (II) on the proliferation of laryngeal cancer cells was determined using 2H-tetrazolium, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl bromide (MTT) and colony formation assays. Inhibition of cell migration was determined using wound-healing assay, while changes in LC3-II and p62 expressions were assessed by western blotting.

**Results:** QN-Pt (II) inhibited the viability of TU212 and M4e laryngeal cancer cells in the concentration range of 2 to 10  $\mu$ M ( $p < 0.05$ ). Moreover, it suppressed the colony formation potential and migration of TU212 and M4e cells. The QN-Pt (II) treatment increased the proportion of TU212 and M4e cells in G1/G0 phase of the cell cycle, but decreased the cell proportion in S and G2/M phase ( $p < 0.05$ ). Treatment with 10  $\mu$ M QN-Pt (II) increased the expression of LC3-II, but downregulated P62 expression in TU212 and M4e cells. The expression of Yes-associated protein was inhibited in TU212 and M4e cells on treatment with QN-Pt (II). However, transfection of YAP-cDNA into TU212 and M4e cells reversed the inhibitory effect of QN-Pt (II) on cell proliferation ( $p < 0.05$ ). Moreover, YAP-cDNA transfection suppressed LC3II expression, inhibited YAP phosphorylation and promoted P62 expression in QN-Pt (II)-treated TU212 and M4e cells ( $p < 0.05$ ).

**Conclusion:** QN-Pt (II) suppresses laryngeal cancer cell viability via arrest of cell cycle and activation of apoptosis. Moreover, QN-Pt (II) targets Yes-associated protein in laryngeal cancer cells. Thus, QN-Pt (II) is a potential therapeutic agent for laryngeal cancer.

**Keywords:** Laryngeal cancer, YAP-protein, Apoptosis, Phosphorylation, Therapeutic agent

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## INTRODUCTION

Laryngeal cancer comprises squamous cell carcinomas which originate from the skin of the larynx [1]. The prognosis of laryngeal cancer

depends on its location in the larynx [2]. Laryngeal cancer is treated using chemotherapy, radiotherapy and surgery, either separately or as combination therapy [3]. The specific treatment for laryngeal cancer is decided based on its

location, stage and category [4]. Severely-affected laryngeal cancer patients are subjected to laryngectomy, in which the vocal cords are removed completely or partially [3]. Mortality due to laryngeal cancer has shown marked increases since 1990 [5]. Moreover, the average survival of patients with laryngeal cancer has also decreased, when compared to patients with other kinds of cancers [2]. Therefore, there is need for effective and novel therapeutic strategies for laryngeal cancer.

The treatment of various disorders/diseases using a variety of drugs is aimed at targeting different signaling pathways. Effective treatments result from accurate targeting signaling pathways by therapeutic molecules. The Hippo signaling pathways plays major role in maintaining the size of various organs, and in development of tumors [6]. A downstream factor of the Hippo pathway is Yes-associated protein (YAP) which is a cancer-related oncogene [7]. It has been reported that YAP accelerates proliferation, suppresses apoptosis and enhances transformation of carcinoma cells [7]. The level of YAP protein is increased in carcinoma cells, including hepatic cancer cells [8].

Organic compounds linked to metal ions as metal-complexes have shown promising anti-tumor properties, but their use is limited by undesirable side effects [9]. This has led clinicians to replace the metal ion with organic compounds with known anti-cancer effects [10]. Novel complexes synthesized from metal ions by linkage to organic compounds have produced effective anti-tumor activities [11,12].

In the present study, the anti-proliferative potential of quinolinol-platinum (II) complex [QN-Pt (II)] in laryngeal cancer cells was investigated for the first time.

## EXPERIMENTAL

### Cell culture

Cancer cell lines (TU212 and M4e) and normal cell line (HBe) were obtained from the American Type Culture Collection (ATCC; Manassas, VA USA). The cell lines were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) mixed with 10 % fetal bovine serum (FBS; Gibco).

The medium contained penicillin (100 U/mL) and streptomycin (100 mg/mL). The cell culture was performed in an incubator in a 5 % CO<sub>2</sub> atmosphere at 37 °C.

### Proliferation assay

The TU212, M4e and HBe cells were seeded in 96-well plates at a density of  $2 \times 10^5$  cells per well, and cultured for 24 h. Then, fresh medium mixed with 1, 2, 4, 6, 8 or 10  $\mu$ M QN-Pt (II) was added to the plates, and incubation was continued for 48 h. Incubation with QN-Pt (II) was followed by addition of MTT solution (5 mg/mL) and further incubation for 4 h at 37°C. The resultant formazan crystals in the plates were dissolved by adding 120  $\mu$ L of DMSO to each well of the plate. The optical density of each formazan solution was read in a Multimode Reader (Varioskan Flash; Thermo Fisher Scientific, Inc.) at 576 nm. All assays were done in triplicate.

### Colony formation assay

The colony forming potential of TU212, M4e and HBe cells was observed using optical light microscopy. The cells were seeded in 6-well plates at a density of  $1 \times 10^5$  cells per well, and treated with 8 and 10  $\mu$ M QN-Pt (II) for 48 h. Then, they were stained with crystal violet dye (Beyotime Institute) for 45 min at room temperature, and the stained cells were observed under optical light microscope (IX70; Olympus Corporation) for colony formation.

### Cell migration assay

The TU212 and M4e cells were seeded in 60-mm dishes at a density of  $1 \times 10^5$  cells per dish, and were cultured to 100 % confluence. A wound was created in the center of each dish by scratching the cellular monolayer with a 200- $\mu$ L pipette tip. The scratched cells were cleared by washing the dishes with PBS to remove any cell debris. This was followed by addition of serum-free medium. The cells were treated with 8 and 10  $\mu$ M QN-Pt (II), or normal saline (control) for 48 h. Wound healing was observed under an inverted microscope (Leica Microsystems, Wetzlar, Germany), and images were taken at x4 magnification using a digital camera.

### Determination of cell cycle progression

The effect of QN-Pt (II) on cell cycle distribution in TU212 and M4e cells was determined using flow cytometry after treatment of the cells with 8 and 10  $\mu$ M QN-Pt (II) for 48 h. Cells from each cell line were seeded in 6-well plates at a density of  $2 \times 10^5$  cells per well in RPMI-1640 medium containing 8 and 10  $\mu$ M QN-Pt (II). Following treatment for 48 h, the cells were washed in PBS after harvesting, and then fixed for 3 h in 70 % ethyl alcohol. Thereafter, the cells were

centrifuged for 5 min, washed with PBS and resuspended in 320  $\mu$ L buffer containing RNase (10  $\mu$ L) and of PI (25  $\mu$ L). The cells were incubated in the dark for 20 min at room temperature. Then, the cellular DNA content was analyzed in a flow cytometer (Beckman Coulter Inc.) for determination of cell cycle distribution.

### Transfection of cells

The TU212 and M4e cells were grown in 6-well plates, each at a density of  $2 \times 10^5$  cells per well, for 24 h at 37 °C. Then, YAP-cDNA, small silencer (si)RNA or vector was transfected into the cells using lipofectamine® 3000 in accordance with the manufacturer's instructions. The sequence of YAP siRNA used was: forward: 5'-GGU GAU ACU AUC AAC CAA ATT-3'; and backward: 5'-UUU GGU UGA UAG UAU CAC CTT-3'. The cells were then separately treated with 8 or 10  $\mu$ M QN-Pt (II) for 48 h.

### Western blot analysis

Following QN-Pt (II) treatment, the TU212 and M4e cells were washed in ice-cold PBS and then lysed using modified RIPA buffer [50 mM Tris-HCl, pH 7.4 containing NP-40 (1.1 %), Na-deoxycholate (0.3 %), sodium chloride (150 mM),  $\text{Na}_3\text{VO}_4$  (1.1 mM) sodium fluoride (1.1 mM) and protease inhibitors]. The lysate was centrifuged at 4 °C for 25 min at 12,000 g and the protein content of the supernatant was estimated using BCA method. The proteins were resolved using electrophoresis on 12 % SDS-PAGE, and subsequently transferred to PVDF membranes (EMD Millipore). The membranes were blocked by incubation with 5 % skimmed milk at 37 °C for 2.5 h. Then, the proteins were probed by overnight-incubation of the membranes with anti-GAPDH, anti-LC3, anti-P62, anti-YAP and anti-p-YAP primary antibodies at 4 °C. Thereafter, the membranes were washed with PBS and subjected to incubation for 2 h with horseradish peroxidase-conjugated secondary antibody at room temperature. The signals were visualized using SuperSignal® West Pico Trial kit (Pierce; Thermo Fisher Scientific, Inc.), and were captured using ImageJ 1.43U system.

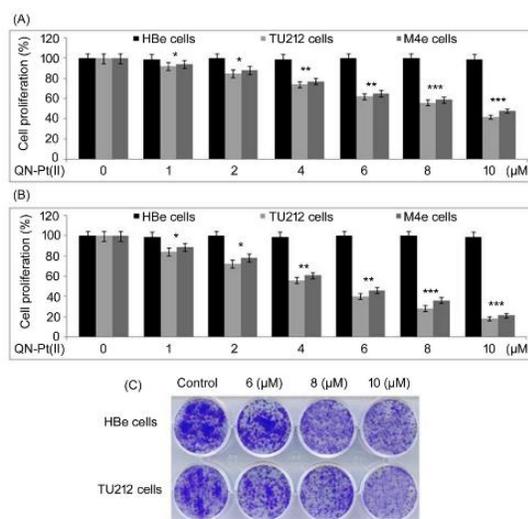
### Statistical analysis

Data are expressed as mean  $\pm$  SEM ( $n = 3$ ), and were compared amongst groups using GraphPad Prism (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). Data analysis was made with one-way ANOVA and Dunn's least significant difference tests. Differences amongst groups were taken as significant at  $p < 0.05$ .

## RESULTS

### QN-Pt (II) decreased the viability of U212 and M4e cells

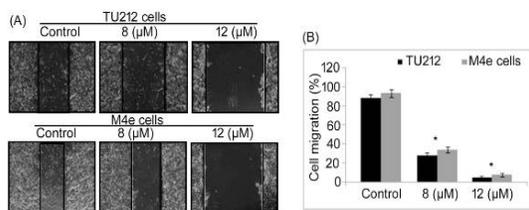
Treatment of TU212 and M4e cancer cells with QN-Pt (II) in the concentration range of 1-10  $\mu$ M suppressed their viability without affecting normal HBe cells (Figure 1 A). The viabilities of TU212 and M4e cells were reduced to 18 and 21, respectively on treatment with 10  $\mu$ M QN-Pt (II) for 48 h. Increase in duration of QN-Pt (II) treatment from 24 to 48 h caused more decreases in viabilities of TU212 and M4e cells (Figures 1 A and B). The colony-forming potential of TU212 and M4e cells were also reduced on treatment with QN-Pt (II), when compared to control (Figure 1 C).



**Figure 1:** Effect of QN-Pt (II) on cell growth. A & B: Cells treated with QN-Pt (II) for 24 and 48 h were subjected to measurement of changes in proliferation using MTT assay. C: Colony formation potential of TU212 and M4e cells treated with QN-Pt (II), as determined under an optical light microscope after staining with crystal violet. \* $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.01$ , vs. control cells

### QN-Pt (II) inhibited the migration of TU212 and M4e cells

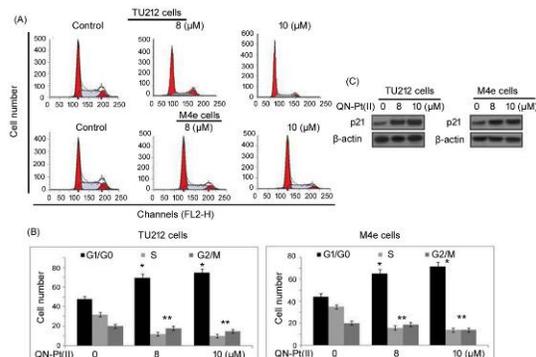
As shown in Figure 2, treatment with 10  $\mu$ M QN-Pt (II) for 48 h resulted in suppression of wound healing in TU212 and M4e cells. However, in control TU212 and M4e cells, the degrees of wound healing were 88 and 93 %, respectively, at 48 h, relative to 5 and 8 % wound healing in TU212 and M4e cells, respectively, on treatment with 10  $\mu$ M QN-Pt (II).



**Figure 2:** Effect of QN-Pt (II) migration of the cells. The TU212 and M4e cells were treated with 10 μM QN-Pt (II) for 48 h, and migration was determined using wound healing assay. \* $P < 0.05$ , \*\* $p < 0.01$  vs. control cells (x200)

### QN-Pt (II) induced cell cycle arrest in TU212 and M4e cells

Flow cytometric analysis of QN-Pt (II)-treated TU212 and M4e cells showed increased counts of cells in the G1/G0 phase of the cell cycle. The increase was concentration-dependent (Figures 3 A and B). In TU212 cells, treatment with 8 and 10 μM QN-Pt (II) increased G1/G0 phase cell count to  $69.43 \pm 4.66$  and  $74.37 \pm 4.82$  %, respectively, relative to  $47.76 \pm 2.19$  % in control. In M4e cells, QN-Pt (II) treatment at doses of 8 and 10 μM increased G1/G0 phase cells to  $64.91 \pm 4.87$  and  $71.42 \pm 5.11$  %, respectively, relative to  $44.21 \pm 2.45$  % in control cells. In contrast, the populations of TU212 and M4e cells in S and G2/M phases were decreased by treatment with QN-Pt (II). The expression of p21 protein was enhanced in TU212 and M4e cells by QN-Pt (II) (Figure 3 C).

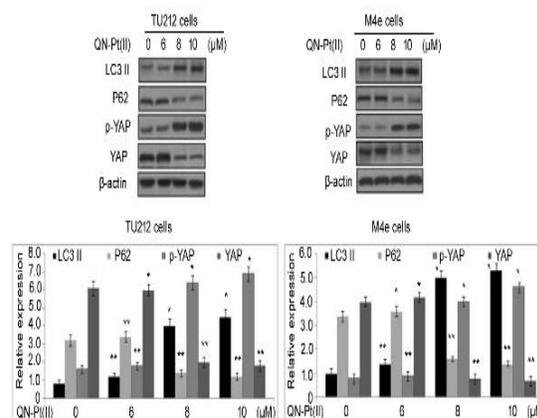


**Figure 3:** Effect of QN-Pt (II) on cell cycle distribution. A & B: Cell cycle arrest in TU212 and M4e cells treated with 4, 6 and 10 μM QN-Pt (II) for 48 h, followed by flow cytometric analysis after PI staining. C: Results of immunoblot assay for the protein expression of p21 in TU212 and M4e cells treated with QN-Pt (II). \* $P < 0.05$ , \*\* $p < 0.01$ , vs. control cells

### QN-Pt (II) promoted autophagy and activated YAP in TU212 and M4e cells

As shown in Figure 4, treatment with QN-Pt (II) at doses of 4, 6 and 10 μM upregulated the

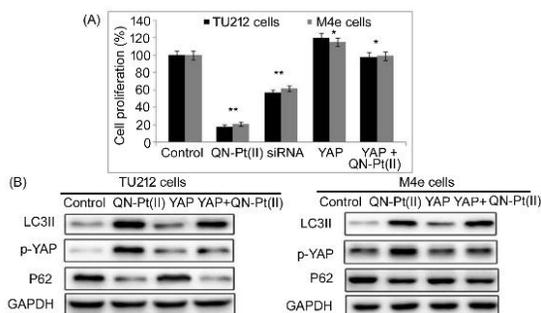
expressions of LC3-II in TU212 and M4e cells in a concentration-dependent manner. The elevation of LC3-II expression was maximum in TU212 and M4e cells on treatment with 10 μM QN-Pt (II) for 48 h. In contrast, treatment with QN-Pt (II) increased P62 degradation in TU212 and M4e cells in a concentration-based manner. The P62 degradation was markedly higher in TU212 and M4e cells treated with 4, 6 and 10 μM QN-Pt (II) for 48 h. Total YAP expressions in TU212 and M4e cells were suppressed on treatment with 4, 6 and 10 μM QN-Pt (II). However, p-YAP levels were markedly enhanced in both cell lines by QN-Pt (II) treatment.



**Figure 4:** Effect of QN-Pt (II) on expressions of autophagy markers. The TU212 and M4e cells were treated with QN-Pt (II) at doses of 4, 6 and 10 μM for 48 h, followed by determination of the expression levels of LC3-II, p62, p-YAP and YAP with Western blotting; \* $p < 0.05$ , \*\* $p < 0.01$ , vs. control cells. (YAP = yes-associated protein)

### YAP up-regulation antagonized Pt (II)-induced inhibition of cell proliferation

Transfection of YAP-cDNA into TU212 and M4e cells reversed the inhibitory effect of QN-Pt (II) on cell proliferation (Figure 5 A). The proliferations of TU212 and M4e cells were significantly increased by YAP-cDNA transfection. At a dose of 10 μM, QN-Pt (II) decreased the proliferations of TU212 and M4e cells to 18 and 21 %, respectively, after 48 h. However, transfection with YAP-cDNA increased the proliferation of QN-Pt (II)-treated TU212 and M4e cells to 98 and 99 %, respectively. This suggests that YAP-cDNA transfection reversed the inhibitory effect of QN-Pt (II) on the proliferative potential of TU212 and M4e cells. Moreover, YAP-cDNA transfection suppressed LC3II expression, inhibited YAP phosphorylation and promoted P62 expression in QN-Pt (II)-treated TU212 and M4e cells (Figure 5 B).



**Figure 5:** Effect of YAP-cDNA transfection on QN-Pt (II)-induced inhibition of cell growth. The TU212 and M4e cells transfected with YAP-cDNA were treated with 10  $\mu$ M QN-Pt (II) for 48 h. A: Changes in TU212 and M4e cell proliferations, as measured using MTT assay. B: Changes in expressions of LC3II, p-YAP and P62, as assayed using Western blotting; \* $p < 0.05$ , \*\* $p < 0.01$ , vs. control cells

## DISCUSSION

Platinum-containing drugs have been used extensively in anti-tumor chemotherapeutic strategies in the past few decades [13]. The use of a drug such as cisplatin is limited because of development of resistance and various harmful side effects in cells [14]. To eliminate this limitation, metal ion complexes are formed with organic compounds with potential anticancer properties [15]. Complexes derived from hydroxyquinoline and many metals have shown promising anti-cancer effects *in vitro* and *in vivo* [15]. The present study investigated the anti-cancer potential of quinolinol-platinum (II) complex [QN-Pt (II)] in TU212 and M4e laryngeal cancer cells. The results showed that QN-Pt (II) effectively inhibited cell growth in TU212 and M4e cell lines, without any effect on the viability of HBe normal cells. These preliminary data indicate the anti-cancer potential of QN-Pt (II) against TU212 and M4e laryngeal carcinoma cells. Moreover, QN-Pt (II) inhibited wound healing potential of TU212 and M4e cells, and arrested their cell cycle at the G0/G1 phase, as was evident in increases in G1/G0 phase cell counts. It has been reported that autophagy is critical in inducing cell death, and is of importance in tumor growth [16]. The initiation of autophagy is characterized by cleavage of C-terminal LC3 to generate LC3II protein which is subsequently translocated into the autophagosomes [17]. Thus, LC3-II plays a vital role in the production of autophagosomes during autophagy [18].

Autophagy is accurately indicated by the degradation of p62 sequestosome [18]. In the present study, it was found that QN-Pt (II) promoted LC3-II expression and enhanced p62

degradation in TU212 and M4e cells. These findings suggest inhibitory effect of QN-Pt (II) on TU212 and M4e cells through induction of autophagy. A downstream factor of Hippo-YAP pathway, YAP is associated with inhibition of excessive proliferation of cells [19].

Suppression of YAP expression in cancer cells is associated with autophagy induction and cell differentiation [20]. In pulmonary and hepatic cancer cells, phosphorylation of YAP is important in the development of chemotherapeutic agents [21]. Therefore, YAP expression is considered a marker for development of effective treatment strategy for cancers [22]. It has been reported that phosphorylated YAP is unable to penetrate the nucleus, such that its activity is inhibited [23]. In the present study, QN-Pt (II) promoted YAP phosphorylation in TU212 and M4e cells. This suggests that QN-Pt (II) induces autophagy in TU212 and M4e cells via phosphorylation of YAP.

## CONCLUSION

The results obtained in this study indicate that QN-Pt (II) effectively inhibits laryngeal carcinoma cell proliferation and migration, arrests cell cycle and activates autophagy. Moreover, QN-Pt (II) targets YAP expression, promotes expression of LC3-II and increases p62 degradation in laryngeal cancer cells. Therefore, QN-Pt (II) can potentially be used in the development of an effective and novel treatment for laryngeal cancer.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Tao Li - conceived and designed the study; Baotong Xie and Tao Li - collected, analyzed the data and wrote the manuscript. Both the authors read and approved the manuscript for publication.

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