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ORIGINAL ARTICLE

Kynurenic acid in human renal cell carcinoma: its antiproliferative and antimigrative action on Caki-2 cells

Katarzyna Walczak · Małgorzata Żurawska · Jacek Kiś · Radosław Starownik · Wojciech Zgrajka · Krzysztof Bar · Waldemar A. Turski · Wojciech Rzeski

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Abstract Kidneys possess a complex enzyme system which plays a major role in tryptophan metabolism. Taking into account a considerably high concentration of one of the tryptophan metabolites, kynurenic acid (KYNA) in this organ and previously reported antiproliferative activity against colon cancer cells in vitro, we measured its content in human normal and tumour kidney tissue. KYNA concentration was considerably higher in normal renal tissue (379.7 ± 39.7 pmol/g wet weight) than in renal cell carcinomas (115.5 ± 20.8 pmol/g wet weight). In in vitro experiments, KYNA in higher micro- and millimolar concentrations significantly inhibited proliferation, DNA synthesis and migration of renal cancer Caki-2 cells. Our results suggest that KYNA may affect cell cycle regulators and signalling pathways through overexpression of p21 Waf1/Cip1 and inhibition of phosphorylation of Rb protein and p38 MAPK. In conclusion, KYNA may be suggested as an endogenous agent, controlling the growth of tumour, or a chemopreventive agent.

Keywords Kynurenic acid · Renal cell carcinoma · Proliferation · Migration

K. Walczak (✉) · W. Rzeski
Department of Medical Biology, Institute of Agricultural Medicine, Jaczewskiego 2, 20-950 Lublin, Poland
e-mail: walczak.ka@gmail.com

K. Walczak
Department of Pharmacology, Medical University, Chodźki 4a, 20-093 Lublin, Poland

M. Żurawska · W. Rzeski
Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria-Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland

J. Kiś
Human Anatomy Department, Medical University, Jaczewskiego 4, 20-090 Lublin, Poland

J. Kiś · R. Starownik · K. Bar
Department of Urology and Urological Oncology, Medical University, Jaczewskiego 8, 20-954 Lublin, Poland

W. Zgrajka · W. A. Turski
Department of Toxicology, Institute of Agricultural Medicine, Jaczewskiego 2, 20-950 Lublin, Poland

W. A. Turski
Department of Experimental and Clinical Pharmacology, Medical University, Jaczewskiego 8, 20-090 Lublin, Poland

Introduction

Recently, the antiproliferative properties of kynurenic acid (KYNA), an endogenous metabolite of tryptophan formed along the kynurenine pathway, were reported. It was found that KYNA significantly inhibited the proliferation of colon adenocarcinoma cells Caco-2, HT-29 and LS-180 in vitro (Walczak et al. 2011); however, the receptor mechanism of its antiproliferative activity still needs to be elucidated. KYNA is a broad-spectrum antagonist of all types of ionotropic glutamate receptors (Perkins and Stone 1982), and previous studies revealed that antagonists of glutamate receptors inhibit proliferation and migration of various cancer cells (Stepulak et al. 2005; Rzeski et al. 2001). KYNA is also a competitive antagonist of the strychnine-insensitive glycine co-agonist site of the *N*-methyl-D-aspartate (NMDA) receptor (Birch et al. 1988) and a non-competitive inhibitor of the α-7 nicotinic acetylcholine receptor (Hilmas et al. 2001). Recently, it was reported that KYNA is an agonist of G-protein-coupled receptor (GPR35) (Wang et al. 2006), and an agonist of aryl hydrocarbon receptor (DiNatale et al. 2010).

Moreover, recent studies revealed that KYNA inhibited substrate-specific uptake by two important efflux pumps, multidrug resistance protein 4 (MRP4) and breast cancer resistance protein (BCRP) (Mutsaers et al. 2011), which is expressed in a broad spectrum of tissues (including stem cells, placenta, liver, small intestine, colon, lung, kidney) (Maliepaard et al. 2001). Despite their important physiological functions in detoxification of xenobiotics, they are also involved in drug resistance in cancer. Low efficacy of chemotherapeutic drugs and the clinical development of resistance to chemotherapy is a serious problem in cancer therapy (Isaac et al. 2011).

It cannot be excluded that antiproliferative properties of KYNA are associated with its interaction with mitogen-activated protein kinases (MAPK) members, which are involved in several cellular processes such as motility, invasion, proliferation and survival (Chan-Hui and Weaver 1998). Mutational activation of the MAPK pathway is a frequent event in human cancer, thus, several strategies for inhibiting MAPK signalling are now being tested as cancer therapies (Halilovic and Solit 2008). The MAPK family includes extracellular signal-regulated kinase (ERK), the c-Jun NH_2-terminal kinase (JNK) and p38 (Robinson and Cobb 1997). p38 MAPK is involved not only in the control of cell cycle regulating the expression of various cytokines, transcriptional factors and receptors (Zarubin and Han 2005), but also in cellular processes such as apoptosis, differentiation, growth and inflammatory responses (Holloway and Coulson 2006; Kyriakis and Avruch 2001). Intensified activity of all MAPK in various renal cell carcinoma (RCC) cell lines was reported (Huang et al. 2008).

Cell proliferation is under strict control of cell cycle regulators including cyclins, cyclin-dependent kinases (CDK), CDK inhibitors and tumour suppressor proteins (p53, Rb). Disruption of the normal regulation of cell cycle progression is crucial in the development of cancer (reviewed in Meeran and Katiyar 2008).

Renal cell carcinoma represents the third leading cause of death among genitourinary malignancies. RCC is extremely resistant to chemotherapy or radiotherapy; therefore, many patients with advanced disease can be offered only palliative therapy. RCC in the metastatic form is associated with a very poor prognosis. The 5-year survival rate does not exceed 9.5% (Petard et al. 2008). Although RCC is among the heterogenous group of diseases, clear-cell RCC represents about 75% of all human renal epithelial neoplasms (Weiss and Lin 2006).

KYNA and other products of tryptophan degradation are eliminated from the body mainly by the kidneys. Moreover, this organ contains several enzymes involved in tryptophan metabolism, mainly via the kynurenine pathway, such as indoleamine 2,3-dioxygenase, kynurenine aminotransferases, kynureninase, kynurenine 3-monooxygenase and 3-hydroxyanthranilate-3,4-dioxygenase (Pawlak et al. 2002; Bertazzo et al. 2001; Okuno and Kido 1991). Previous studies revealed that KYNA content in rat kidneys is considerably higher than in the other organs, including the liver, lungs or spleen (Moroni et al. 1991; Pawlak et al. 2003; Turski et al. 2009). Taking these results into consideration, we decided to measure the KYNA content in human normal kidney and kidney tumour tissue. Moreover, we investigated the biological properties of KYNA on clear-cell RCC in vitro. We investigated whether KYNA affects proliferation, DNA synthesis and the migration of clear-cell RCC Caki-2 cells. The molecular mechanism of antiproliferative activity of KYNA was also determined.

Materials and methods

KYNA determination in human kidney samples

The research study was approved by the Institutional Ethics Committee in Lublin, Poland. A total of 15 patients (11 men, 4 women; mean age 60.79 ± 16.25) with histologically confirmed RCC were included in the analysis. All patients underwent radical nephrectomy. Resected RCC and normal kidney tissue samples were immediately frozen and stored at −80°C until further analysis. Samples were weighed (wet weight) and sonicated (1:5 wt/vol) in distilled water. The resulting homogenate was centrifuged. The supernatant was acidified with 14 µl of 50% trichloroacetic acid and centrifuged. Next, supernatant was applied on cation-exchange resin (Dowex 50 W+, Sigma). Eluted KYNA was subjected to the HPLC (Hewlett Packard 1050 HPLC system: ESA catecholamine HR-80, 3 µm, C_{18} reverse-phase column) and quantified fluorometrically (Hewlett Packard 1046A fluorescence detector: excitation 344 nm, emission 398 nm).

Cell culture

The clear-cell RCC Caki-2 cells were obtained from ECACC (European Collection of Cell Cultures, Centre for Applied Microbiology and Research, Salisbury, UK). Caki-2 cells were grown in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All reagents were obtained from Sigma (Sigma Chemicals, St. Louis, MO, USA).

Proliferation assay (MTT assay)

Caki-2 cells were plated on 96-well microplates (Nunc) at a density of 2.5×10^4. KYNA was dissolved in 1 N NaOH, and then the PBS was added to achieve the stock solution

of KYNA (0.5 M). Next day, the culture medium was removed and Caki-2 cells were exposed to fresh medium (control) or serial dilutions of KYNA (0.001–10 mM) in fresh medium. pH of the final concentrations of KYNA was controlled. Cell proliferation was assessed after 96 h by using the MTT method in which the yellow tetrazolium salt (MTT) is metabolized by viable cells to purple formazan crystals. Tumour cells were incubated for 3 h with MTT solution (5 mg/ml). Formazan crystals were solubilized overnight in SDS (sodium dodecyl sulphate) buffer (10% SDS in 0.01 N HCl), and the product quantified spectrophotometrically by measuring absorbance at 570 nm wavelength using E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

BrdU assay

BrdU assay is an immunoassay for the quantification of BrdU incorporation into newly synthesized DNA of actively proliferating cells. Caki-2 cells were plated on 96-well microplates (Nunc) at a density of 4×10^4 cells/ml. Next day, the culture medium was removed and the cells exposed to fresh medium (control) or serial dilutions of KYNA (0.001–10 mM) in fresh medium. Cell proliferation was quantified after 48 h by measurement of 5′-bromo-2′-deoxy-uridine (BrdU) incorporation during DNA synthesis, according to the manufacturer's procedure (Cell Proliferation ELISA BrdU, Roche Diagnostics GmbH, Penzberg, Germany).

Cell migration assessment

Tumour cell motility was assessed in the wound assay model as previously described (Rzeski et al. 2006). Caki-2 cells were plated at 2.5×10^5 cells on 3 cm culture dishes (Nunc). On the following day, the cell monolayer was scratched by pipet tip (P300), the medium and dislodged cells were aspirated and the plates rinsed twice with PBS. Next, the fresh culture medium (supplemented with 2% FBS) was applied and the number of cells migrated into the wound area after 24 h was determined in control, and the cultures treated with KYNA (2.5 mM). The plates were then stained with the May-Grünwald-Giemsa method and observation performed by an Olympus BX51 System Microscope. Micrographs were prepared in analySIS® software. Cells migrated to the wound area were counted on micrographs and the results expressed as a mean cell number migrated to the selected 40 fields taken from 4 micrographs.

Western blot

Caki-2 cells were lysed in RIPA buffer [1% NP40 (Tergitol), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM NaF, 0.5 mM DTT, 1 mM PMSF, protease inhibitor mixture in PBS, pH 7.4] and centrifuged at $14{,}000 \times g$ for 10 min. Protein content in supernatants was determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA). Supernatants were solubilized in sample buffer (30% glycerol, 10% SDS, 0.5 M Tris–HCl, pH 6.8, 0.012% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min. For Western blotting, equal amounts of proteins were electrophoresed on 10% SDS-PAGE and transferred to PVDF membrane. After blocking for 1 h at room temperature with 5% nonfat dry milk in TBS-0.1% Tween 20 (TBS-T), the membranes were probed at 4°C overnight with primary antibodies (anti-phospho-p38, anti-p21 Waf1/Cip1, anti-cyclin D1, anti-phospho-Rb, 1:1,000; anti-β-actin, 1:2,000; Cell Signaling Technology Inc., Danvers, MA, USA). The membranes were then washed in TBS-T buffer and incubated with secondary antibody coupled to horseradish peroxidase (1:2,000 in 5% nonfat milk in TBS-T) for 1 h at room temperature, and visualized by using enhanced chemiluminescence (Pierce Biotechnology). Serial exposures were made on Kodak BioMax Light film (Eastman Kodak Company, Rochester, NY, USA).

Data analysis

Data were presented as the mean value and standard error of the mean (SEM). KYNA determination in human kidney samples and cell migration assessment were statistically analyzed using paired t test. Statistical analysis of proliferation data was performed using one-way ANOVA with Tukey post hoc test. $P < 0.05$ was considered to be statistically significant. The IC_{50} value (the concentration of drug necessary to induce 50% inhibition), together with confidence limits, was calculated using computerized linear regression analysis of quantal log dose-probit functions, according to the method of Litchfield and Wilcoxon (1949).

Results

HPLC analysis of cell lysates revealed the presence of KYNA in the samples of normal kidney tissue, as well as in the renal tumours. KYNA concentration was significantly higher in normal renal tissue (379.7 ± 39.7 pmol/g wet weight) than in RCC (115.5 ± 20.8 pmol/g wet weight) (Fig. 1).

In order to investigate the influence of KYNA on cell proliferation, cells were exposed to serial dilutions of the tested compound for 96 h. KYNA significantly inhibited proliferation of Caki-2 cells exposed to KYNA (0.001–10 mM) with $IC_{50} = 0.04$ mM (Fig. 2a). Moreover,

BrdU assay revealed that KYNA decreased DNA synthesis in Caki-2 cells with $IC_{50} = 2.13$ mM (Fig. 2b).

To evaluate whether KYNA affects the migration of RCC, cell migration assessment was applied. KYNA 2.5 mM significantly inhibited migration of Caki-2 cells exposed to tested compound for 24 h to 47% in comparison to control (Fig. 3).

To determine the potential molecular mechanism of antiproliferative activity of KYNA, Caki-2 cells were exposed to 2.5 mM KYNA for 5 min to 48 h. Western blot analysis revealed that KYNA significantly decreased the phosphorylation of p38 MAPK starting at 15 min of exposition to the tested compound. Moreover, analysis of protein expression and phosphorylation status of key cell cycle regulators were carried out. Expression of CDK inhibitor p21 Waf1/Cip1 protein was enhanced by KYNA at 15 min after exposition to tested compound. This effect gradually increased up to 48 h. KYNA did not affect the expression of cyclin D1. Phosphorylation status of Rb protein was considerable decreased after 24 and 48 h (Fig. 4).

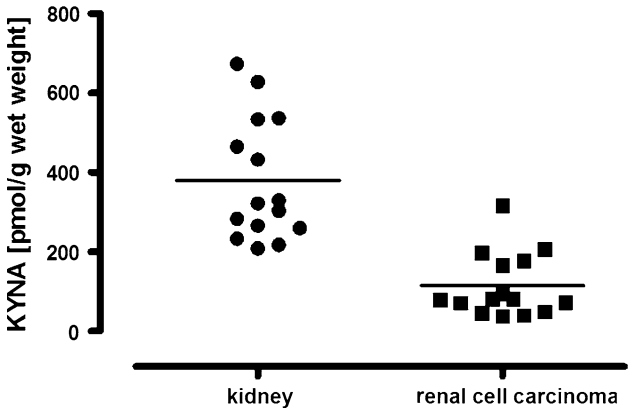


Fig. 1 Scatter plots of kynurenic acid (KYNA) concentration in human kidney and RCC ($N = 15$). Samples were collected from patients who underwent radical nephrectomy. Results are expressed as pmol KYNA/g wet weight. *Horizontal bar* represents the mean value. *N* number of subjects

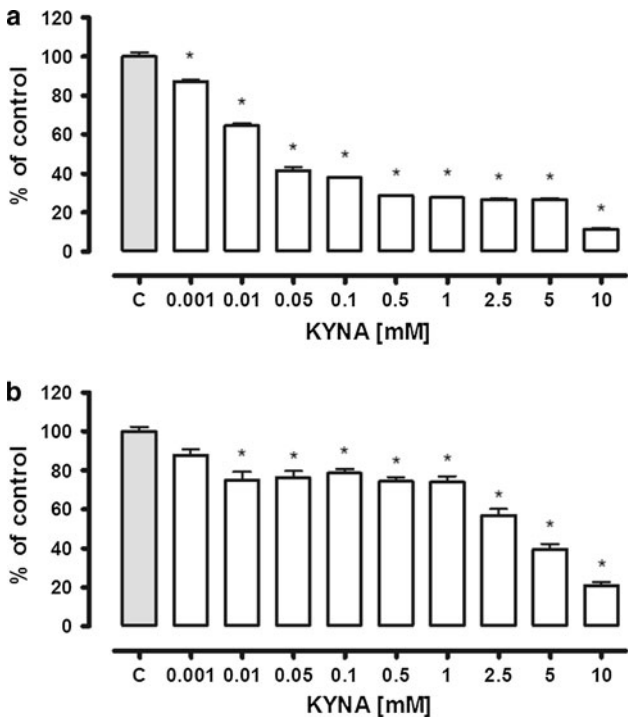
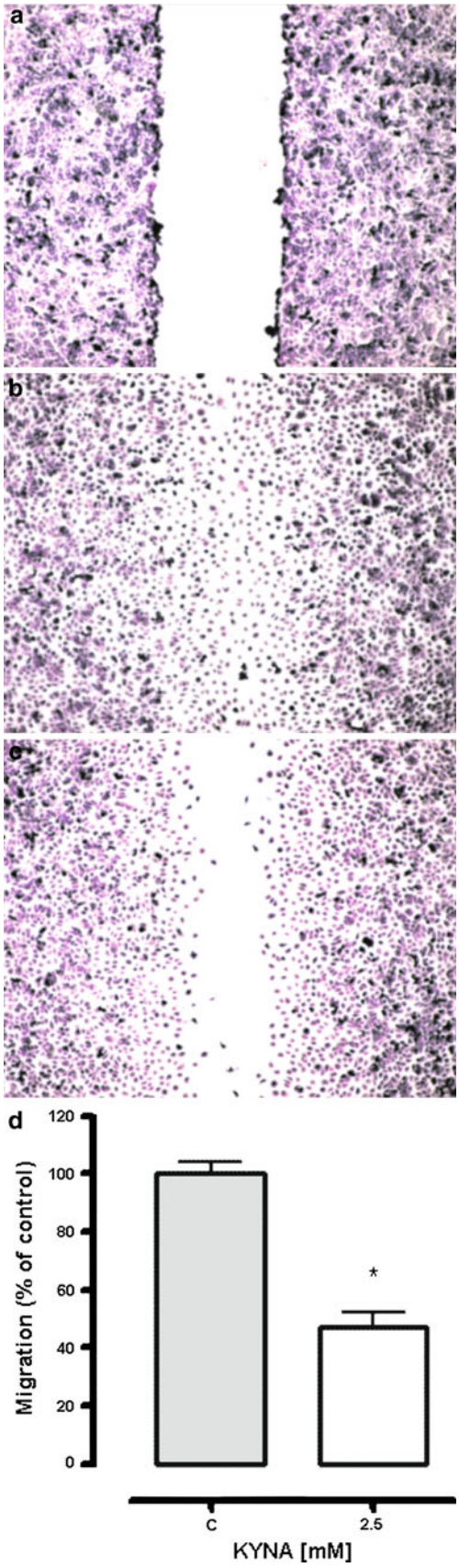


Fig. 2 The antiproliferative effect of KYNA on clear-cell RCC Caki-2 cells. Cells were exposed to fresh medium (control, C) or KYNA (0.001–10 mM). Proliferation was measured by means of MTT assay after 96 h (**a**) or BrdU assay after 48 h of incubation (**b**). Data represent a mean value (% of control) ± SEM of six independent experiments. Values significant in comparison to control (100%) at least with $^*P < 0.05$ (one-way ANOVA with Tukey post hoc test)

Discussion

Taking into consideration that the kidneys possess a complex enzyme system, which plays a major role in tryptophan metabolism (Pawlak et al. 2002; Bertazzo et al. 2001; Okuno and Kido 1991) and a significantly high KYNA concentration in this organ (Moroni et al. 1991; Pawlak et al. 2003; Turski et al. 2009), we studied the effect of KYNA on clear-cell RCC Caki-2 cells. We revealed that KYNA, in concentrations which were non-toxic for normal fibroblasts (data not shown), significantly inhibited proliferation and DNA synthesis in Caki-2 cells. Similarly, the antiproliferative action that KYNA exerted on colon adenocarcinoma cells has been reported recently (Walczak et al. 2011). It is noteworthy that KYNA inhibited the proliferation of renal cancer cells Caki-2 ($IC_{50} = 0.04$ mM) more efficiently than colon adenocarcinoma cells Caco-2 ($IC_{50} = 1.2$ mM), HT-29 ($IC_{50} = 0.9$ mM) and LS-180 ($IC_{50} = 0.2$ mM) (Walczak et al. 2011). The antiproliferative effect of KYNA in high concentration ($IC_{50} = 5.9$ mM) was previously reported in synoviocytes (Parada-Turska et al. 2006). In contrast, Di Serio et al. (2005) reported the stimulatory effect of KYNA on the proliferation rate of mouse microglia and human glioblastoma cells in vitro. However, we did not observe enhanced proliferation of Caki-2 cells in the range of KYNA concentrations studied by Di Serio et al. (2005). Furthermore, we found that KYNA significantly decreased Caki-2 migration, which suggests that it may inhibit renal cancer metastases. The exact molecular mechanism of KYNA antiproliferative and antimigratory activity is unknown. KYNA is an antagonist of glutamate receptors (Perkins and Stone 1982). In previous reports, it has been shown that glutamate antagonists inhibit the proliferation and migration of various cancer cells in vitro (Stepulak et al. 2005; Rzeski et al. 2001). Thus, the



◀ **Fig. 3** Effect of KYNA on renal cancer cell motility. Scratched monolayers of Caki-2 cells were incubated for 24 h alone (control) or in the presence of KYNA (2.5 mM). Micrographs show **a** wound, **b** cell migration after 24 h in control culture and **c** following exposure to KYNA 2.5 mM. Magnification ×40. Tumour cells which migrated to the wound area were counted (**d**). Values significant in comparison to control (100%) at least with $*P < 0.05$ (paired *t* test)

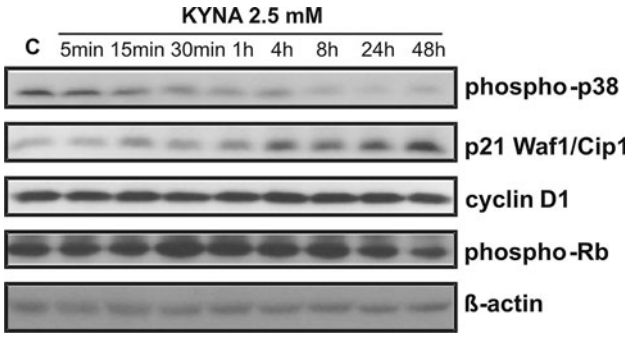


Fig. 4 Western blot analysis of protein expression or phosphorylation status of p38 MAPK and cell cycle-related proteins in Caki-2 cells after treatment with KYNA 2.5 mM for 5 min to 48 h (*C* control; not treated)

involvement of glutamate receptors in the antiproliferative action of KYNA can be suggested. To determine and clarify the molecular mechanism of the antiproliferative activity of KYNA, its effect on the intracellular signalling pathways in Caki-2 cells was analyzed.

Our study revealed that KYNA significantly decreased the phosphorylation of p38 MAPK. Intensified activity of all MAP kinases in various RCC cell lines was reported (Huang et al. 2008), whereas a decrease in MAPK activity inhibited the growth of renal tumours, as well as angiogenesis in vivo (Huang et al. 2008). p38 MAPK is involved in the control of cell cycle in G1/S and G2/M checkpoints through regulation of various genes coding cytokines, transcriptional factors and receptors (Zarubin and Han 2005). The activation of p38 MAPK can have a profound impact on cell fate. Depending on the nature of the signal and its cellular context, downstream effects include apoptosis, differentiation, growth and inflammatory responses (Holloway and Coulson 2006; Kyriakis and Avruch 2001). An increased level of p38 MAPK is associated with the overexpression of proinflammatory cytokines (IL-6, IL-1β, TNF-α) and COX-2 (Thornton and Rincon 2009). These results may suggest that KYNA-induced attenuation of TNF-α production by activated human mononuclear cells (Tiszlavicz et al. 2011) may be partially associated with the inhibition of p38 MAPK. Taking into consideration that the enhanced activity of MAPK in RCC is involved in angiogenesis and resistance to apoptosis-induced factors (Huang et al. 2008; Ambrose et al. 2006), KYNA may be

considered as an endogenous agent controlling tumour growth and/or as a chemopreventive agent.

We found that KYNA is also involved in the regulation of expression and phosphorylation status of some key cell cycle regulators. KYNA significantly enhanced the expression of p21 Waf1/Cip1, which is a CDK inhibitor regulating cell cycle, apoptosis and cell differentiation (Yang et al. 2001; Cheng et al. 2000). As an inhibitor of cell proliferation, it plays an important role in drug-induced tumour suppression, and disruption of its biological function may lead to carcinogenesis (Cheng et al. 2000). Although KYNA enhanced the expression of p21 Waf1/Cip1, no influence on the expression of cyclin D1 was observed. The basal level of cyclin D1 in Caki-2 cells is considerably high, which is in agreement with previous finding in human RCC (Hedberg et al. 2003). The role of cyclin D1 in renal cancer has not been unequivocally established. Although Aaltomaa et al. (1999) reported that cyclin D1 is not significantly related to the survival of patients with renal cell cancer, Hedberg et al. (2002) suggested that its low level is correlated with poor prognosis and lower survival rate.

Phosphorylation of Rb regulates cell proliferation by controlling progression through the restriction point within the G1 phase of the cell cycle. It is well known that enhanced phosphorylation of this protein may lead to disruption of the suppressor function and promote carcinogenesis. In fact, a high level of Rb phosphorylation in RCC was previously reported (Hedberg et al. 2004). Therefore, our finding that KYNA significantly inhibited the phosphorylation of Rb in Caki-2 cells pointed to its regulatory function in the control of the growth and proliferation of renal cells. Although alternations in the Rb gene and its protein product have been detected in numerous tumour malignancies, the role of Rb in pathogenesis and progression of RCC remains controversial. Vignoli and Martorana (1997) indicated that mutations of Rb in RCC can be associated with metastatic disease. However, other studies have revealed that molecular alternations of the Rb gene are infrequent in renal cancers, and Rb protein is present in the majority of primary and metastatic renal tumours (Presti et al. 1996). Moreover, further analysis of 14 RCC-derived cell lines confirmed that neither epigenetic changes nor loss of expression of Rb are present in these types of cells (Kawakami et al. 2003).

In this study, for the first time, we measured and compared the KYNA content in human normal renal tissue and RCC. Importantly, KYNA concentration in human renal tumours was significantly lower in comparison to healthy tissue. Both the antiproliferative action of KYNA demonstrated in in vitro experiments and the low level of KYNA in tumour tissue documented by chromatographic analysis support and strengthen the hypothesis of the plausible role of endogenous KYNA in renal carcinogenesis. Hypothetically, RCC cells exposed to low concentration of KYNA in kidney tumour tissue may escape from the antiproliferative action of this compound. However, another scenario should also be considered. It is worth mentioning that KYNA is eliminated by the kidneys and its concentration in urine is high ranging from 5 to 40 μM (Furlanetto et al. 1998). The concentration recorded in the renal tissue is ~10–100 times lower than in urine, but is ~5 times higher than in other organs, such as liver, lungs and spleen (Pawlak et al. 2003; Turski et al. 2009). The simple explanation of this phenomenon might be the presence of urine in non-perfused kidney tissue which is used to measure KYNA content. Similarly, a low content of KYNA in tumour tissue can be explained by reorganization of the tissue structure of kidney during carcinogenesis.

On the other hand, a previous study indicated that KYNA content in kidneys may be easily increased, which is crucial if KYNA is considered as a potential chemopreventive agent against RCC. Turski et al. (2009) reported that intragastrical administration of KYNA at the dose of 250 mg/kg resulted in an ~100-fold increase of its concentration in serum and kidneys in rats. Under this experimental condition, the highest content of KYNA recorded in serum was 3.4 nmol/ml (~0.003 mM) and in kidney 46.5 nmol/g wet weight (~0.05 mM) (Turski et al. 2009). Importantly, in our in vitro study, low micromolar KYNA concentrations, close to that found in the serum of rats intragastrically treated with KYNA (Turski et al. 2009), slightly but significantly decreased the proliferation of Caki-2 cells measured by means of MTT assay. Higher KYNA concentration, close to that found in the kidneys of rats intragastrically treated with KYNA (Turski et al. 2009), inhibited the proliferation of Caki-2 cells measured by means of MTT assay by more than 58%, and reduced DNA synthesis measured by means of BrdU assay by 24%. Importantly, in vivo studies revealed that administration of KYNA intravenously in the dose of 50 or 100 mg/kg/h in rats was well tolerated (Marciniak and Turski 2010). These findings further support the hypothesis of the potential therapeutic implications of KYNA in renal cancer. Accordingly, Mutsaers et al. (2011) very recently reported that KYNA inhibits transport by BCRP and MRP4 at clinically relevant concentrations.

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Conflict of interest The authors declare that they have no conflict of interest.

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