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SHORT COMMUNICATION

Inhibition of ecto-ATPase activity by curcumin in hepatocellular carcinoma HepG2 cells

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Abstract Effects of curcumin, a major constituent of turmeric, on ecto-nucleotidases have not been clarified. Here, we investigated whether curcumin affects ecto-nucleotidase activities in human hepatocellular carcinoma HepG2 cells. In the cells, high levels of Mg^{2+}-dependent activity of ecto-nucleotidases were observed in the presence of 1 mM adenosine triphosphate (ATP). The activity was inhibited by ecto-ATPase inhibitors such as suramin, $ZnCl_2$ and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid. On the other hand, the activity was significantly decreased at alkaline pH (pH 9) and was not inhibited by levamisole, an inhibitor of alkaline phosphatase. In the presence of ATP, curcumin inhibited the activity in a concentration-dependent manner ($IC_{50} = 6.2$ μM). In contrast, curcumin had no effects on ecto-nucleotidase activity in the presence of ADP (1 mM) or AMP (1 mM). The K_m value for ATP hydrolysis of curcumin-sensitive ecto-ATPase was similar to the value of NTPDase2, an isoform of ecto-nucleoside triphosphate diphosphohydrolase. These results suggest that curcumin is a potent inhibitor of ecto-ATPase and may affect extracellular ATP-dependent responses.

Keywords Curcumin · Ecto-ATPase · NTPDase · Hepatocellular carcinoma · Extracellular ATP hydrolysis

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Introduction

Adenosine triphosphate (ATP), which is released from cytosol to the extracellular spaces, binds to P_2 receptors (P_2X and P_2Y receptors) and regulates important physiological responses in many biological processes [1, 2]. The concentration of extracellular nucleotides is precisely regulated by ecto-nucleotidases such as ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), alkaline phosphatases and ecto-5′-nucleotidases [3, 4]. ATP and ADP are hydrolyzed by E-NTPDases (E-NTPDase1–3), E-NPPs (NPP1–3) and alkaline phosphatases at the plasma membrane [3, 4]. However, AMP is hydrolyzed to adenosine by ecto-5′-nucleotidase [3, 4]. Extracellular adenosine is a ligand for the P_1 receptor and also regulates important physiological responses [2].

Extracellular ATP regulates liver functions and is hydrolyzed by ecto-ATPase located at the plasma membrane of liver cells [5, 6]. It has been reported that NTPDase2 is abundantly expressed in human hepatocellular carcinoma HepG2 cells, whereas no significant expression of NTPDase1 and 3 is observed by semiquantitative RT-PCR [7].

Curcumin (diferuloylmethane) is a natural compound present in turmeric, a rhizome of the Asian plant *Curcuma longa*, and has been used in traditional ayurvedic medicine. It has diverse biological activities, including anti-inflammatory, antioxidant, antiviral and anti-infectious effects [8]. Curcumin also has hepatoprotective effects; it prevents liver damages induced by ethanol, thioacetamide, iron overdose, cholestasis, low density lipoprotein (LDL) and carbon tetrachloride [9–11]. Recently, curcumin has been reported to inhibit the growth of several cancer cells of the colon, duodenum, esophagus, stomach, liver, breast, leukemia, oral cavity and prostate [12, 13].

So far, p-glycoprotein, multidrug resistance protein 1 and 2, glutathione, protein kinase C, α-1-acid glycoprotein, CD13/aminopeptidase N, lipoxygenase and several transcription factors have been identified as targets for curcumin [14]. However, effects of curcumin on the purinergic signaling system have not been clarified. In this study, we examined whether curcumin affects the activities of ecto-nucleotidases in HepG2 cells.

Materials and methods

Materials

Curcumin, bafilomycin A1, sodium orthovanadate (V), levamisole hydrochloride, suramin sodium and zinc chloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). ATP, ADP, AMP, ouabain and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of molecular biological grade or the highest grade of purity available.

Cell culture

HepG2 cells, a human hepatocellular carcinoma cell line (Riken Cell Bank, Tsukuba, Japan), were maintained in minimum essential Eagle medium (Sigma-Aldrich) supplemented with 100 μM non-essential amino acid solution (Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen) and 10% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA).

Preparation of the membrane fraction of HepG2 cells

HepG2 cells were resuspended and homogenized in a solution containing 0.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.4), and the homogenates were centrifuged at $500\times g$ for 10 min at 4°C. Then, the supernatants were centrifuged at $100{,}000\times g$ for 90 min at 4°C, and membrane fractions were prepared by resuspending the pellets in a solution containing 250 mM sucrose and 5 mM Tris-HCl (pH 7.4).

Measurement of ecto-nucleotidase activities

Ecto-nucleotidase activities of membrane fractions of HepG2 cells (30 μg of protein) were measured in solutions containing 3 mM $MgSO_4$, 5 mM NaN_3, 100 nM bafilomycin A1, 10 μM sodium orthovanadate (V) and nucleotide (1 mM ATP, 1 mM ADP or 1 mM AMP). These solutions were buffered with 10 mM Mes-Tris (for pH 5–6), 10 mM Hepes-Tris (for pH 7.4) or 10 mM Taps-Tris (for pH 8–9). After incubation for 30 min at 37°C, the reaction was terminated by addition of ice-cold stop solution containing 12% perchloric acid and 3.6% ammonium molybdate. Then, the released inorganic phosphate was quantified as previously described [15]. Kinetic constants (apparent K_m and V_{max} values) for curcumin (30 μM)-sensitive ecto-nucleotidase activity were calculated by Lineweaver-Burk plots.

Measurement of Na^+,K^+-ATPase activity

In the presence and absence of 100 μM ouabain, Na^+,K^+-ATPase activity of membrane fractions of HepG2 cells (30 μg of protein) was measured in a solution containing 10 mM Hepes-Tris (pH 7.4), 120 mM NaCl, 15 mM KCl, 3 mM $MgSO_4$ and 1 mM ATP as previously described [16]. Na^+,K^+-ATPase activity was calculated as the difference between the activities in the presence and absence of ouabain.

Statistical analysis

Data are shown as mean ± SE. Comparison between the two groups was made by using Student's *t* test. Statistically significant differences were assumed at $p < 0.05$. The IC_{50} value of data shown in Fig. 3a was calculated using the KaleidaGraph program, version 4.00 (Synergy Software, Reading, PA, USA).

Results

Mg^{2+}-activated nucleotidase activity was measured in the solutions supplemented with 1 mM ATP, 10 μM sodium orthovanadate (V), a P-type ATPase inhibitor, 100 nM bafilomycin A1, a V-type ATPase inhibitor and 5 mM NaN_3, an F-type ATPase inhibitor. The major portion of nucleotidase activity was due to the EDTA (10 mM)-sensitive Mg^{2+}-activated ecto-nucleotidase activity (Fig. 1a). The minor portion was due to the ouabain (100 μM)-sensitive Na^+,K^+-ATPase activity in the presence of 120 mM Na^+ and 15 mM K^+ (Fig. 1b). It is noted that the ecto-nucleotidase activity (calculated as the difference between the activities in the presence and absence of EDTA) was approximately three times greater than Na^+,K^+-ATPase activity (calculated as the difference between the activities in the presence and absence of ouabain) (Fig. 1c).

To check whether alkaline phosphatase, which has maximal activity at pH 9–10 [17], contaminated the present ecto-nucleotidase activity, the activity was measured at various pHs. The ecto-nucleotidase activity at pH 9 was significantly smaller than that at pH 7.4 (Fig. 1d), suggesting no significant contribution of alkaline

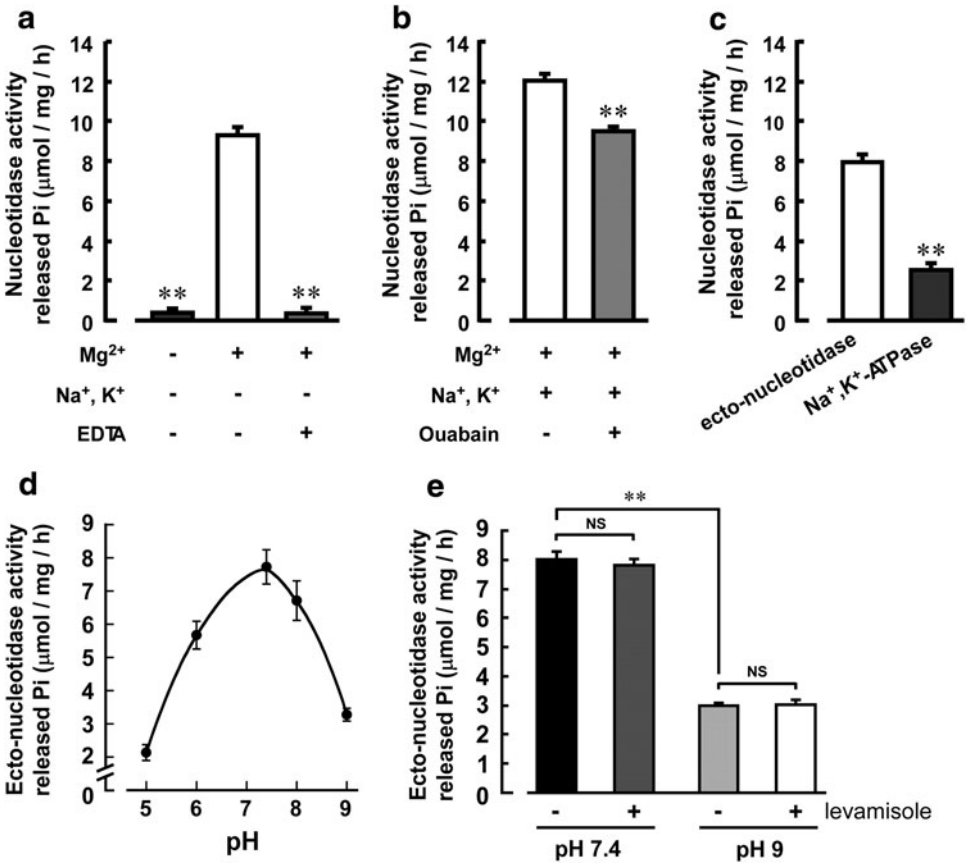

Fig. 1 Mg^{2+}-activated ecto-nucleotidase activity in the presence of ATP (1 mM). **a** Measurement of nucleotidase activity in the presence of 3 mM $MgSO_4$ (Mg^{2+}) and absence of both NaCl and KCl (Na^+, K^+) in HepG2 cells. EDTA (10 mM) strongly inhibited the Mg^{2+}-activated ATPase activity. $n = 4$. **b** Measurement of nucleotidase activity in the presence of 3 mM $MgSO_4$, 120 mM NaCl and 15 mM KCl in HepG2 cells. Inhibition by ouabain (100 μM) of the (Mg^{2+}, Na^+, K^+)-activated ATPase activity was slight but significant. $n = 7$. **c** Ecto-nucleotidase activity was calculated as the difference between the activities in the presence and absence of 10 mM EDTA. Na^+,K^+-ATPase activity was calculated as the difference between the activities in the presence and absence of 100 μM ouabain. Ecto-nucleotidase activity was about three times greater than Na^+,K^+-ATPase activity in HepG2 cells. **d** Ecto-nucleotidase activity was measured at pH 5–9. $n = 4$. **e** Ecto-nucleotidase activity was measured at pH 7.4 and pH 9 in the presence (+) and absence (−) of 100 μM of levamisole, an inhibitor of alkaline phosphatase. **Significantly different ($p < 0.01$). *NS* not significant ($p > 0.05$). $n = 4$

phosphatase to ecto-nucleotidase activity. In addition, ecto-nucleotidase activity was not inhibited by 100 μM levamisole [18], an inhibitor of alkaline phosphatase, at both pH 7.4 and 9 (Fig. 1e). It has been reported that NPPs are activated at alkaline pH as is the case with alkaline phosphatase [3, 19]. Therefore, NPPs may not contribute to this activity.

Suramin, DIDS and $ZnCl_2$ have been reported to inhibit the ecto-ATPase activity of NTPDases, including NTPDase1–3, in tissues of several species [3, 20–22]. In the presence of 1 mM ATP, the ecto-nucleotidase activity in HepG2 cells was significantly inhibited by 1 mM suramin, 500 μM DIDS and 500 μM $ZnCl_2$ (Fig. 2), suggesting that most of the ecto-nucleotidase activity may be derived from NTPDases.

In the presence of 1 mM ATP, effects of curcumin on ecto-nucleotidase activity were examined. Curumin inhibited the activity in a concentration-dependent manner, and the IC_{50} value was 6.2 μM (Fig. 3a). On the other hand, curcumin (30 μM) had no significant effects on ecto-nucleotidase activities in the presence of ADP (1 mM; Fig. 3c) or AMP (1 mM; Fig. 3d), different from the case in the presence of ATP (1 mM; Fig. 3b). These results suggest that curcumin significantly inhibited the ATP-hydrolyzing activity, but not the ADP- or AMP-hydrolyzing activity in HepG2 cells.

To characterize the kinetics of ecto-ATPase inhibited by curcumin, curcumin (30 μM)-sensitive ecto-ATPase activity was measured at various concentrations of ATP (30 μM to 3 mM). The curcumin-sensitive ecto-ATPase activity increased in a concentration-dependent manner for ATP at up to 1 mM (Fig. 4a). Values of apparent K_m and V_{max} for ATP were 153.1 μM and 7.3 μmol Pi/mg/h calculated from Lineweaver-Burk plots (Fig. 4b).

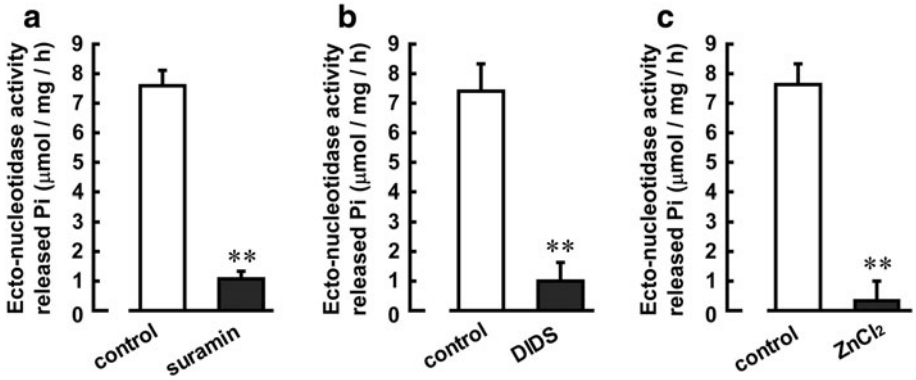

Fig. 2 Effects of ecto-ATPase inhibitors on ecto-nucleotidase activity in the presence of ATP. Ecto-nucleotidase activity was significantly inhibited by 1 mM suramin ($n = 4$), 500 μM DIDS ($n = 8$) and 500 μM $ZnCl_2$ ($n = 6$). **Significantly different ($p < 0.01$) versus the activity in the absence of inhibitors (control)

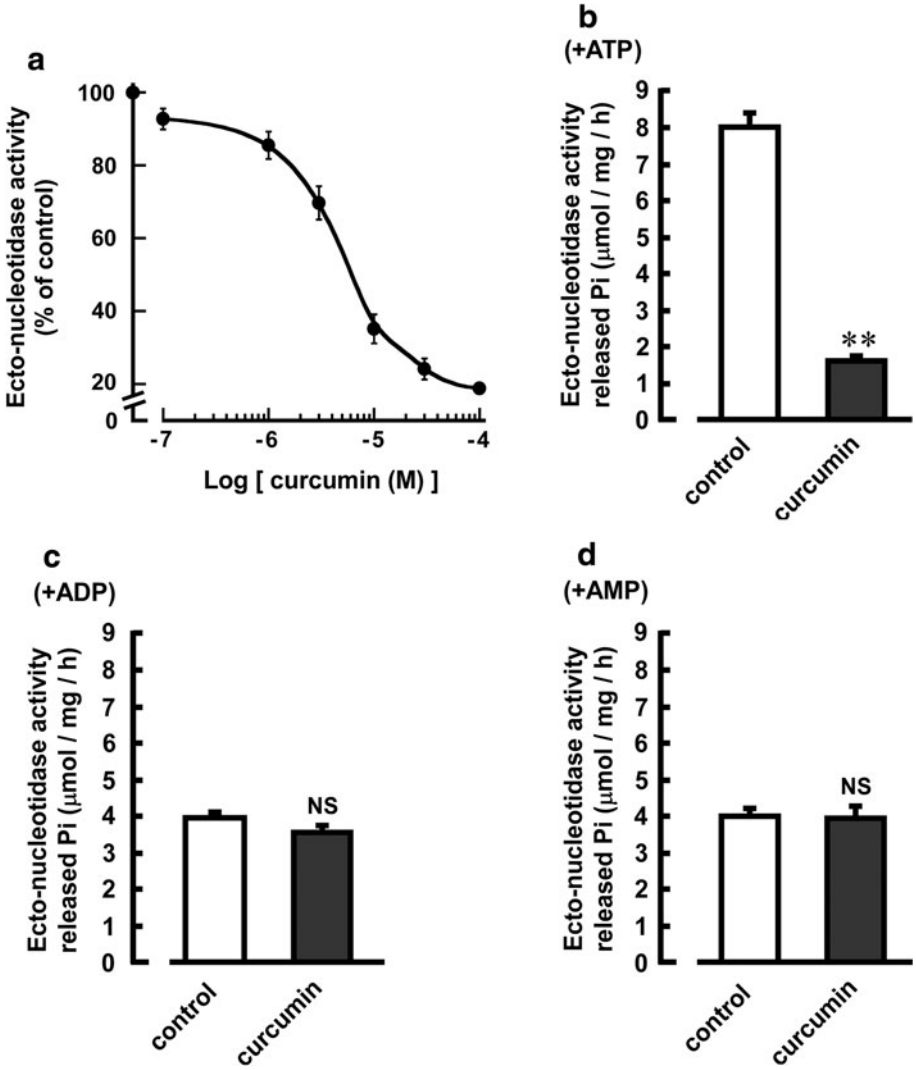

Fig. 3 **a** Concentration-dependent inhibition by curcumin of ecto-nucleotidase activity in the presence of 1 mM ATP. The activity in the absence of curcumin was taken as 100%. $n = 5$. Effects of curcumin (30 μM) on the ecto-nucleotidase activity in the presence of ATP (1 mM; **b**), ADP (1 mM; **c**) or AMP (1 mM; **d**) in HepG2 cells. **Significantly different ($p < 0.01$). *NS* not significant ($p > 0.05$). $n = 6$ (**b**), $n = 4$ (**c**, **d**)

Discussion

In this study, we found that HepG2 cells have a high Mg^{2+}-activated ecto-nucleotidase activity, which is approximately three times greater than the ouabain-sensitive Na^+,K^+-ATPase activity. Extracellular ATP and ADP are hydrolyzed by NTPDases, NPPs and alkaline phosphatases at the plasma membrane. The ecto-nucleotidase activity in HepG2 cells was decreased at alkaline pH (Fig. 1d, e). In the presence of ATP, the ecto-nucleotidase activity was inhibited by ecto-ATPase inhibitors such as suramin, DIDS and $ZnCl_2$, but not by levamisole, an inhibitor of alkaline phosphatase (Fig. 2). These results suggest that NTPDases (NTPDase1–3) but not NPPs and

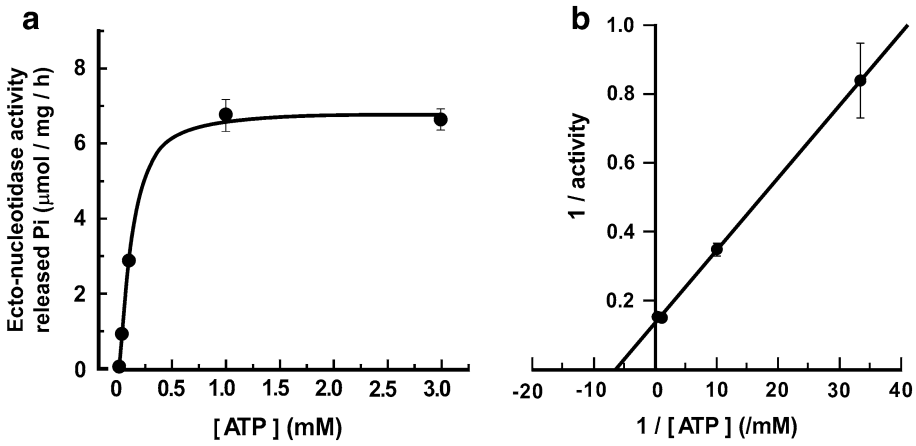

Fig. 4 Relationship between ATP concentration and the curcumin-sensitive ecto-ATPase activity. **a** Ecto-nucleotidase activities were measured in the presence and absence of 30 μM curcumin, and the curcumin-sensitive ecto-ATPase activity was estimated. $n = 4$. **b** Lineweaver-Burk plots of the curcumin-sensitive ecto-ATPase activity versus ATP concentration from 30 μM to 3 mM

alkaline phosphatase may be responsible for the ecto-nucleotidase activity.

In HepG2 cells, NTPDase2 has been reported to be abundantly expressed, whereas no significant expression of NTPDase1 and 3 was observed [7]. NTPDase2 has been reported to be less sensitive to sodium azide than NTPDase1 and 3, in which 5 mM of sodium azide inhibited ATP-hydrolysis of NTPD1 and NTPDase3 by 40–50%, but NTPDase2 was not inhibited by 10 mM sodium azide [23–26]. In this study, all experiments for measurement of ecto-nucleotidase activity were performed in the presence of 5 mM sodium azide, the concentration of which significantly inhibits NTPDase1 and 3. Therefore, NTPDase1 and 3 may not contribute to the activity in the present experimental conditions.

It has been reported that the affinity of NTPDase2 for ATP is more than 20 times higher than that for ADP [27]. Curcumin selectively inhibited ATP-hydrolyzing activity, but not ADP- and AMP- hydrolyzing activities in the cells (Fig. 3). In addition, the K_m value for ATP of curcumin-sensitive ecto-ATPase activity was 157 μM (Fig. 4). This value was similar to that for ATP hydrolysis of NTPDase2, 210 μM [27] and 203 μM [28]. Taking this information together, we suggest that curcumin may inhibit the ATP hydrolysis of NTPDase2 in HepG2 cells.

So far, several compounds have been reported to be inhibitors of NTPDases. Suramin inhibits the ATP hydrolysis of human NTPDase2 with IC_{50} of 24 μM [29]. ARL67156 inhibits the ATP hydrolysis of human NTPDase2 with an IC_{50} value of 15 μM [30]. PSB-6426, a nucleotide mimetic derived from uridine-5′-carboxamide, inhibits the ATP hydrolysis of human NTPDase2 with an IC_{50} value of 42 μM [31]. NF279, a P_2 receptor antagonist, has been reported to be a most potent non-selective NTPDase inhibitor, and it inhibits the ATP hydrolysis of human NTPDase2 with an IC_{50} value of 4.2 μM [29]. In our study, curcumin had an IC_{50} of 6.2 μM for ecto-ATPase activity in HepG2 cells, indicating that curcumin is a potent inhibitor of ecto-ATPase as strong as NF279. To our knowledge, this is the first report to indicate the effect of curcumin on ecto-ATPase activity.

Inhibition of ecto-ATPase by curcumin may elevate the amount of extracellular ATP and decrease the amount of extracellular adenosine, and may result in the changes of purinergic signaling and cellular functions of cancer cells. It has been reported that extracellular ATP induces apoptotic cell death and inhibition of cancer cell growth through P_2X and P_2Y receptors in cancer cells [32, 33].

In in vivo and in vitro studies, anti-tumor effects of curcumin against a wide variety of cancers, including oral, breast, vulva, skin, liver, colorectal, bladder and cervical cancers, have been reported [12]. In HepG2 cells, it was reported that curcumin-induced apoptotic cell death with IC_{50} of 17.5 μM and 20 μM of curcumin reduced cell viability by ~80% [34]. Curcumin also inhibited the cell growth of hepatocellular carcinoma HA22T/VGH cells with IC_{50} of 17.4 μM [35]. These values for IC_{50} are similar to those of the inhibitory effects of curcumin on ecto-ATPase activity (Fig. 4).

In this study, we demonstrated that curcumin is a potent inhibitor of ecto-ATPase activity. It will be interesting to clarify the pathophysiological roles of curcumin-dependent inhibition of ecto-ATPase activity in hepatocellular carcinoma cells.

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Conflict of interest The authors declare no conflict of interest.

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