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Choline Transporters in Human Lung Adenocarcinoma: Expression and Functional Implications

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Abstract Choline is an essential nutrient for cell survival and proliferation, however, the expression and function of choline transporters have not been well identified in cancer. In this study, we detected the mRNA and protein expression of organic cation transporter OCT3, carnitine/cation transporters OCTN1 and OCTN2, and choline transporter-like protein CTL1 in human lung adenocarcinoma cell lines A549, H1299 and SPC-A-1. Their expression pattern was further confirmed in 25 human primary adenocarcinoma tissues. The choline uptake in these cell lines was significantly blocked by CTL1 inhibitor, but only partially inhibited by OCT or OCTN inhibitors. The efficacy of these inhibitors on cell proliferation is closely correlated with their abilities to block choline transport. Under the native expression of these transporters, the total choline uptake was notably blocked by specific PI3K/AKT inhibitors. These results describe the expression of choline transporters and their relevant function in cell proliferation of human lung adenocarcinoma, thus providing a potential "choline-starvation" strategy of cancer interference through targeting choline transporters, especially CTL1.

Key words choline transporter; lung adenocarcinoma; proliferation; CTL1

Choline is a precursor for the synthesis of a component of membrane phospholipids, the neurotransmitter acetylcholine and other metabolites [1–3]. As cells have only a limited capacity to synthesize choline, the major source of choline is principally from the extracellular choline pool through uptake by the choline transport system [4,5]. According to the affinity for choline, the transport system has been categorized into three subgroups: (1) polyspecific organic cation transporters (OCTs) and the carnitine/cation transporters (OCTNs), both with low affinity for choline; (2) intermediate-affinity choline transporter-like proteins (CTLs); and (3) high-affinity choline transporters (CHTs) [5]. Till now, three OCT members (OCT1–3) and two OCTN members (OCTN1 and OCTN2) have been identified and broadly detected in human tissues. It is believed that these transporters non-specifically transport choline for phospholipid synthesis. The highly specific choline transporter CHT1 is principally responsible for supplying choline for acetylcholine synthesis in cholinergic neurons, although it also supplies choline for the production of the membrane phosphatidylcholine in some non-neuronal tissues [5]. Recently, a distinct member of choline transporters unrelated to the members of OCT or CHT subgroup, named choline transporter-like protein 1 (CTL1), has been characterized in neuronal and some non-neuronal human tissues [5,6].

Irregular choline transport and metabolism have been involved in growth retardation, apoptosis, renal or liver dysfunction and atypical phospholipid metabolism [4], and also been implicated in a wide array of neurological disorders such as Alzheimer's and Parkinson's disease [7–9]. Based on higher choline content in some malignant tissues (e.g. prostate, breast and lung cancer) than their adjacent non-cancerous counterparts, radiolabelled choline has been used as an imaging probe for the diagnosis of these cancers [10,11]. Therefore, the expression pattern and characteristics of choline transporters are of central importance to understand the choline metabolism that underlies membrane integrity, cell signaling and cell growth

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in these diseases.

In the present study, we examined the expression status of these transporters both in human pulmonary adenocarcinoma tissues and relevant cancer cell lines and the correlation between choline uptake and cell growth.

Materials and Methods

Materials

[Methyl-^{3}H]choline chloride ([^{3}H]choline, 2.99 Tbq/mM) was from Amersham Biosciences (Piscataway, USA). Hemicholinium-3 (HC-3), phenoxybenzamine (PbA), tetraethylammonium (TEA), norepinephrine (NEP), LY294002, API-2 and rapamycin were from Sigma-Aldrich (St. Louis, USA). Goat anti-OCT3, OCTN1 and OCTN2 polyantibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). Mouse anti-CTL1 polyantibody was from Abnova (Taiwan, China). Human lung adenocarcinoma cell lines H1299 and A549 and human glioma cell line U87MG were from American Type Culture Collection (Manassas, USA), and SPC-A-1 was from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). H1299, SPC-A-1 and U87MG cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Carlsbad, USA). A549 cells were cultured in F12K (Gibco BRL) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, USA), 100 u/ml penicillin, and 100 μg/ml streptomycin. Paraffin-embedded bronchoscopic biopsies of lung adenocarcinoma cancer tissues and their paired normal lung tissues were obtained from the First Affiliated Hospital of Zhejiang University Medical School, Hangzhou, China. Sections of 5 μm were processed for immunohistochemistry.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells with TRI reagent (Invitrogen, Carlsbad, USA). The cDNA was reversed transcribed from 1 μg of RNA using 0.25 ng of oligo(dT_{12-18}) (Promega, Madison, USA). Aliquots of cDNA (2 μl) were amplified by PCR using a Supermix kit (Toyobo, Tokyo, Japan). RT was carried out according to the manufacturer's instructions (Invitrogen). The primers and the lengths of amplified genes were: CHT1 (AF276871), sense, 5′-TGGCCTACACTGATGTCGTT-3′, antisense, 5′-TACCAGAGCCCATACACAGT-3′, 670 bp; OCT1 (U77086), sense, 5′-GACCACATCGCTCAAAAGAAT-3′, antisense, 5′-CCAACACCGCAAACAAAAT-3′, 601 bp; OCT2 (X98333), sense, 5′-TTGGTTGCTGGAGGTCTGGT-3′, antisense, 5′-TGGTTGAGTTGTATGGGCTTTGT-3′, 255 bp; OCT3 (AF078749), 5′-TTTCGGAGTTTCGCTCTGTT-3′, antisense, 5′-TCAGGCAAAAGCATCACAAG-3′, 156 bp; OCTN1 (AB007448), sense, 5′-GCTGCCACTGTTTGCTTA-3′, 5′-GGGTCTTATTTTTCTGTTTTTCA-3′, 971 bp; OCTN2 (AF057164), sense, 5′-GGGAAAGTGAAAGGTAATGAAGA-3′, antisense, 5′-AAGGGCAAATAACAAGGAGAA-3′, 360 bp; CTL1(AJ420812), sense, 5′-CATGTGGTGGTACCATGTGGTGGG-3′, antisense, 5′-CGAATAAGGCGATTTACTGATGCC-3′, 161 bp; β-actin (M28424), sense, 5′-TGCTATCCCTGTACGCCTCT-3′, antisense, 5′-CTAGAAGCATTTGCGGTGGA-3′, 713 bp. All the PCR products were analyzed with 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Immunoblotting

Cells were lysed with lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM KH_2PO_4, 10 mM sodium molybdate, 2 mM sodium orthovanadate, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 6 mM dithiothreitol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting.

Immunohistochemistry

Paraffin sections of human lung cancers and their matched normal lung tissues were processed for immunohistochemistry assay. Sections (5 μm) from phosphate-buffered saline (PBS) buffer-fixed tissue were deparaffinized and rehydrated through descending alcohol series and in PBS. Antigen was retrieved in citrate buffer (0.01 M, pH 6.0) in a microwave oven for 10 min at 9 ºC, and endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After blocking non-specific antigen binding sites with SuperBlocking solution (Pierce, Rockford, USA), polyclonal antibodies to OCT3, OCTN1, OCTN2 and CTL1 (1:25) were applied overnight at 4 ºC. After rinsing with PBS, sections were incubated with an EnVision system-horseradish peroxidase (Dako, Glostrup, Denmark). Peroxidase was visualized with 3,3′-diaminobenzidine-tetrachloride chromogen (Dako) and counterstained with Mayer's hematoxylin. Intensity of immunohistochemical staining was scored from – to +++ by two independent readers (–, no staining; +, weak staining; ++, moderate staining; +++, intense staining).

Cell proliferation assay

Bromodeoxyuridine (BrdU) incorporation assay was used to evaluate the effect of choline transporter inhibitors on cell proliferation. The experiments for each inhibitor were carried out three times with a minimum of triplicates per each time interval. Cells were plated at 1000 cells per well in 96-well plates. Inhibitors were added after cell plating. The final medium volume of each well was 200 μl. Every day, the medium was replaced with fresh medium containing the same concentration of inhibitors. The procedure of the BrdU incorporation assay was carried out as described in the manufacturer's introductions (Roche, Basel, Switzerland).

[^{3}H]choline uptake

Cells were washed twice with Krebs-Ringer-HEPES buffer (130 mM NaCl, 1.3 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO4, 10 mM glucose, and 10 mM HEPES) at pH 7.4 and incubated at 37 ºC for 2 h, followed by an additional incubation in Krebs-Ringer-HEPES buffer containing 1 μM [^{3}H]choline for 10 min. Choline uptake was terminated by washing cells three times with ice-cold Krebs-Ringer-HEPES buffer. The level of accumulated [^{3}H] choline was determined by solubilizing cells in 1% sodium dodecyl sulfate and 0.2 N NaOH, and radioactivity was measured with a liquid scintillation counter (LS 6500; Beckman Coulter, Fullerton, USA). The sensitivity of [^{3}H]choline uptake to some agents was assessed by measuring [^{3}H]choline uptake in the presence of agents at the indicated concentrations. In these experiments, specific [^{3}H]choline uptake was presented as percent uptake.

Statistical analysis

Data were expressed as the mean±SEM. Statistical significance was assessed by Student's *t*-test. $P<0.05$ was considered to be statistically significant.

Results

Expression pattern of choline transporters in human pulmonary adenocarcinoma cell lines and primary cancer tissues

First of all, we examined the expression status of choline transporters including OCT1–3, OCTN1 and OCTN2, CHT1 and CTL1 in human lung adenocarcinoma cell lines A549, SPC-A-1 and H1299, as shown in **Fig. 1**. With the exception of CHT1 (U87MG as a positive control), all choline transporters tested in this experiment were expressed in the three cell lines at mRNA level, as revealed by RT-PCR [**Fig. 1(A)**]. The protein expression patterns of CTL1, OCT3, OCTN1 and OCTN2 in all four cell lines were validated by Western blot analysis [**Fig. 1(B)**]. We also validated that the band of CTL1 detected was approximately 72 kDa, the expected size. The expression patterns of CTL1, OCT3, OCTN1 and OCTN2 were further confirmed by tissue immunohistochemistry observation [**Fig. 1(C)**]. The results revealed that most of the positive staining cells were cancer cells from lung adenocarcinoma tissues. For semiquantitative determination of the patterns of transporter expression, we used 25 paired lung adenocarcinoma and non-cancerous lung tissues for immunohistochemistry studies. As illustrated in **Table 1**, CTL1, OCT3, OCTN1 and OCTN2 were generally expressed in both cancer and non-cancerous lung tissues. Among these transporters, a high level of expression (++/+++) of CTL1 was detected in 20 of 25 (80%) lung cancer tissues versus 11 of 25 (44%) in non-cancerous lung tissues ($P<0.05$).

Effect of transporter inhibitors on choline transport in human adenocarcinoma cells

To better understand the specific function of these

Table 1 Immunohistochemical detection of the protein expression of OCT3, CTL1, OCTN1 and OCTN2 in primary tumors and matched noncancerous lung tissues

Antigen	Primary lung adenocarcinoma (*n*)					Non-cancerous lung (*n*)				
	–	+	++	+++	Total (%)	–	+	++	+++	Total (%)
OCT3	5	7	9	4	13 (52)	6	7	8	2	10 (40)
CTL1	2	3	14	6	20 (80) *	5	9	8	3	11 (44)
OCTN1	6	13	4	2	6 (24)	6	12	6	1	7 (28)
OCTN2	10	12	2	1	3 (12)	14	10	1	0	1 (4)

–, no staining; +, weak staining; ++, moderate staining; +++, intense staining. *$P<0.05$.

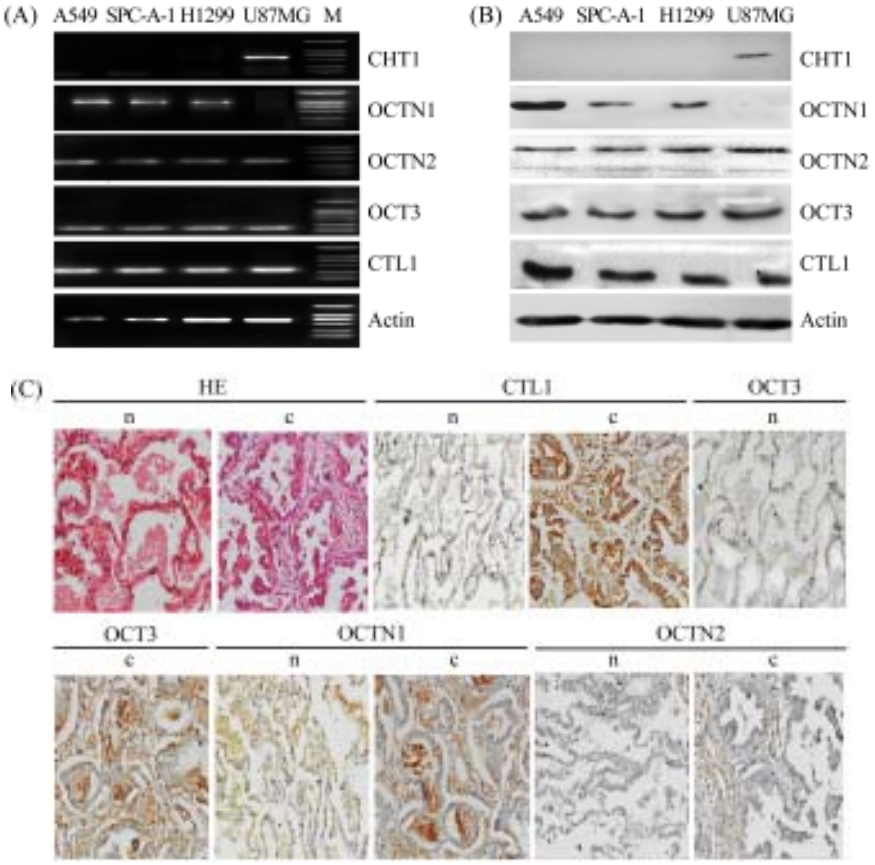

Fig 1 Expression of choline transporters OCT3, OCTN1, OCTN2 and CTL1 in A549, H1299 and SPC-A-1 human lung adenocarcinoma cells

(A) mRNA expression of OCT3, OCTN1, OCTN2 and CTL1 was detected by reverse transcription-polymerase chain reaction. (B) Protein expression of OCT3, OCTN1, OCTN2 and CTL1 was assayed by Western blot revealed with relevant antibodies. The detected band of CTL1 was approximately 72 kDa, the expected size. (C) The protein expression of OCT3, OCTN1, OCTN2 and CTL1 in human lung adenocarcinoma tissues (c) and their non-cancerous lung tissues (n). Five micrometer paraffin sections were prepared from human lung adenocarcinoma and the non-cancerous lung tissues, and were subsequently examined by immunohistochemistry assay with transporter-specific antibodies. HE, hematoxylin and eosin staining.

transporters expressed in lung cancer cells, we investigated the effects of selective inhibitors on the choline uptake in A549 and SPC-A-1 cells by assessing the ability of these inhibitors to block the uptake of [^{3}H]choline. In both A549 and SPC-A-1 cells, all these inhibitors showed significant inhibitory effects on [^{3}H]choline uptake. Their inhibitory rates on choline transport in A549 and SPC-A-1 cells are illustrated in **Fig. 2**: 76.35% and 81.81% for HC-3 (at 100 μM); 42.70% and 39.55 % for PbA (at 100 μM), 32.67% and 36.92 % for TEA (at 100 μM); and 24.95% and 23.37% for NEP (at 200 μM) in A549 and SPC-A-1, respectively.

Inhibitory effect of choline transporter inhibitors on cell proliferation

To explore the possible correlation between choline uptake and cell growth, the effect of transporter inhibitors on cell proliferation was examined by BrdU incorporation assays. As shown in **Fig. 3**, all the inhibitors could significantly suppress BrdU incorporation in both A549 and SPC-A-1 cells. The inhibitory potencies of these inhibitors on cell growth were as follows: HC-3>PbA/TEA>NEP.

Choline transport was suppressed by inhibitors of AKT pathway

To further explore the potential pathway for regulating choline transport, we examined the effect of specific inhibitors of signaling pathways on choline transport system in human lung adenocarcinoma cells. We found that LY294002 (PI3K inhibitor), API-2 (Akt inhibitor), and rapamycin (mTOR inhibitor) could block the choline uptake in these cancer cells to a significant extent (**Fig. 4**). These data strongly implied that the PI3K/AKT/mTOR pathway

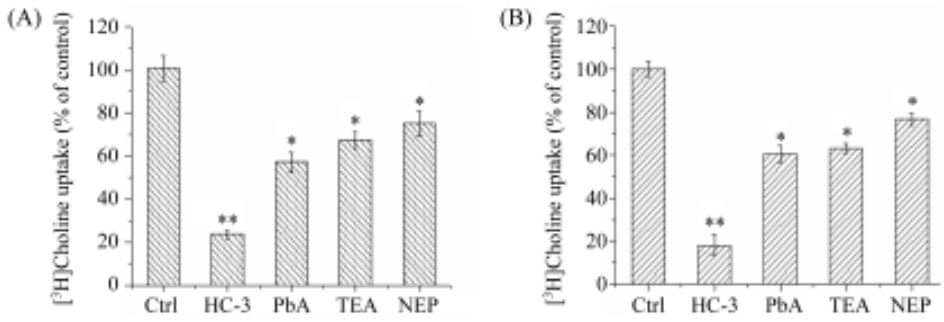

Fig. 2 Effect of transporter inhibitors hemicholinium-3 (HC-3), phenoxybenzamine (PbA), tetraethylammonium (TEA) and norepinephrine (NEP) on choline uptake of A549 (A) and SPC-A-1 (B) human lung adenocarcinoma cells

Concentrations of HC-3, PbA and TEA were 100 μM; NEP, 200 μM. The level of choline uptake in the cell population was assessed by measuring the radioactivity intensity of [^{3}H]choline in the absence or presence of these inhibitors. Data are representatives of independent triplicate experiments. **P*<0.05, ***P*<0.001.

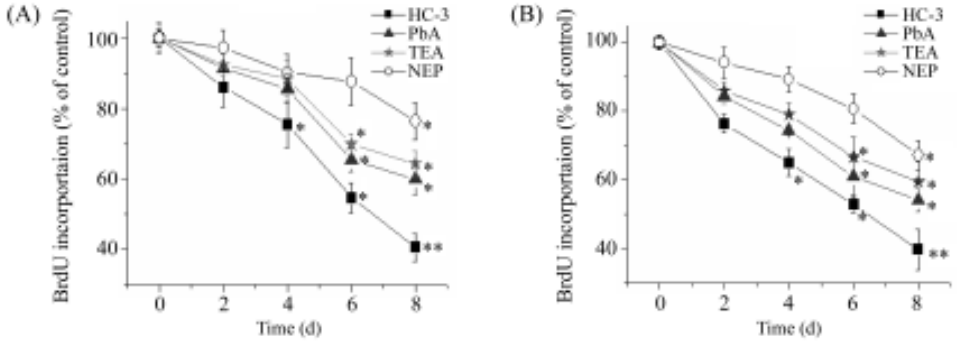

Fig. 3 Effect of hemicholinium-3 (HC-3), phenoxybenzamine (PbA), tetraethylammonium (TEA) and norepinephrine (NEP) on proliferation of A549 (A) and SPC-A-1 (B) human lung adenocarcinoma cells

The medium with various inhibitors was replaced each day with fresh medium containing the same concentration of inhibitors. Every other day, cells were measured by bromodeoxyuridine (BrdU) incorporation assay. HC-3, PbA and TEA were used at the final concentration of 100 μM, and NEP at 200 μM. Data are representatives of independent triplicate experiments. **P*< 0.05, ***P*<0.001.

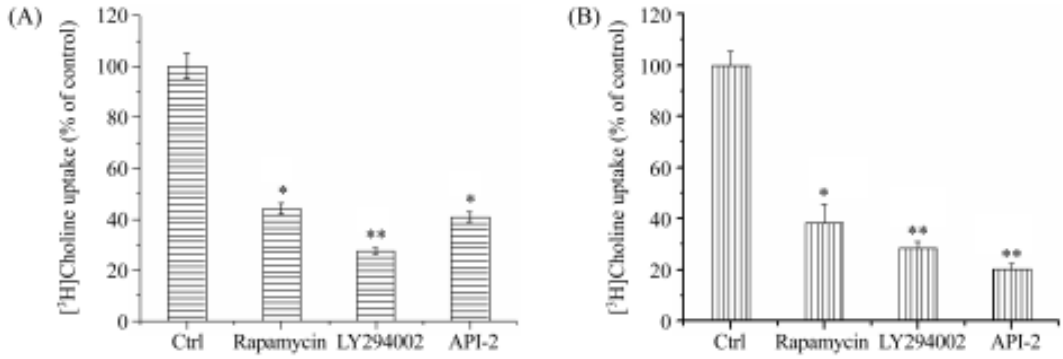

Fig. 4 Effects of LY294002 (PI3K inhibitor), API-2 (AKT inhibitor) and rapamycin (mTOR inhibitor) on [^{3}H]choline incorporation of A549 (A) and SPC-A-1 (B) human lung adenocarcinoma cell lines

Cells were incubated in Krebs-Ringer-HEPES buffer with or without 50 μM LY294002, 5 μM API-2, or 10 μM rapamycin at 37 ºC for 2 h, then washed three times with buffer and treated with 1 mM [^{3}H]choline for 10 min. Data are representatives of independent triplicate experiments. **P*<0.05, ***P*<0.001.

might have a regulatory role in choline transport.

Discussion

The intracellular choline accumulation through choline transporters is the prerequisite for cancer cell proliferation [5,10,11]. The major choline transporters have been identified as OCT1–3 and OCTN1–3, CHT1, and CTL1, belonging to the OCT, CHT and CTL subgroups, respectively [12–19]. In human lung adenocarcinoma cells, we detected CTL1, OCT3, OCTN1 and OCTN2, but not CHT1, OCT1 or OCT2. By tissue immunohistochemistry array, we also found CTL1, OCT3, OCTN1 and OCTN2 expression in lung adenocarcinoma tissues and matched non-cancerous lung tissues. Thus, we concluded the presence of two subgroups for choline transport in human lung adenocarcinoma. Of further interest, CTL1 is more preferentially expressed in cancer tissues, as is OCT3, which is in line with the elevated level of choline in lung cancer and other cancer tissues in comparison with the matched non-cancerous tissues [10,11].

Based on the data of [^{3}H]choline uptake assay, with the addition of different transporter inhibitors, we found the order for inhibitory potency on choline transport was as follows: HC-3>PbA/TEA>NEP. As NEP is an inhibitor for the OCT subgroup, and PbA and TEA for the OCT and OCTN subgroups, it is not surprising to detect their relatively limited abilities in inhibiting choline transport by using individual inhibitors [12,16,18]. As HC-3 is a selective inhibitor for CHT1 and CTL1 [12,18,19], and CHT1 was not detected in lung adenocarcinoma cells, HC-3 highly potent inhibitory effect suggested that CTL1 might play a major role in choline transport in human lung adenocarcinoma cells. Based on the presence of partial choline inflow in HC-3 pretreated cells, we presume the presence of at least two transport pathways, one is HC-3-sensitive transport (CTL1) and the other is insensitive to HC-3 (OCT3, OCTN1 and OCTN2) as previously described [20, 21]. As choline is an essential molecule, various transport routes with multiple transporters might be beneficial to supply sufficient choline for cancer cell survival.

However, we found a close association between choline transport and cell growth. *In vitro*, these inhibitors on cell growth were well correlated with their capabilities of inhibiting choline transport in exactly the same order: HC-3>PbA/TEA>NEP. Therefore, targeting CTL1 might be an effective way to block choline transport and thus inhibit cell proliferation.

In the case of the respective ectopic expression of individual transporters, some signal pathways, such as Ca^{2+}-CaM and PKC signaling, are involved in choline transport [22–24]. Under the native expression of these transporters, it remains unclear which pathway is directly connected with the total choline uptake. An aberrant activation of the PI3K/AKT/mTOR pathway has been identified in a variety of human malignant cells, including lung cancer, and plays an important role in cancer proliferation [25–27]. Therefore, we hypothesize that the PI3K/AKT/mTOR pathway might be involved in choline transport regulation. We examined the effects of specific PI3K inhibitor LY294002, AKT inhibitor API-2 and mTOR inhibitor rapamycin on [^{3}H]choline uptake in A549 and SPC-A-1 cells. Our results showed that total choline uptake was blocked to a significant extent by specific LY294002 (50 μM), API-2 (5 μM) and rapamycin (10 μM) (**Fig. 4**). These results consistently suggested that the PI3K/AKT/mTOR pathway might participate in the regulation of choline transport in lung adenocarcinoma cells.

In conclusion, these results describe the expression profile of choline transporters and their relevant function in choline transport and cell proliferation of human lung adenocarcinoma. Our study might provide a potential "choline-starvation" strategy through interfering with choline transporters, especially CTL1, for human lung adenocarcinoma therapy.

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