In Vivo Antitumor Activity of Choline Kinase Inhibitors: A Novel Target for Anticancer Drug Discovery

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ABSTRACT

Transformation by some oncoproteins is associated with increased activity of choline kinase (ChoK), resulting in elevated constitutive levels of phosphorylcholine, a proposed second messenger required for DNA synthesis induced by growth factors. Here we describe the characterization of ChoK inhibitors with antiproliferative properties against human tumor-derived cell lines. The new molecules were tolerated in mice at doses that showed in vivo antitumor activity against human tumor xenografts derived from HT-29 and A431 cell lines implanted s.c. in nude mice. This first generation of inhibitors provides in vivo evidence that blockade of phosphorylcholine production is a valid strategy for the development of new anti-cancer agents, opening a new avenue for the development of antitumor drugs with a novel mechanism of action.

INTRODUCTION

Malignant cells are characterized by a number of alterations in signal transduction pathways controlling proliferation, differentiation, and apoptosis (1–4). The identification of these aberrant processes may allow the development of chemotherapeutic interventions to restore them or selectively destroy the transformed cells (5–7). However, besides the great progress made in our understanding of the molecular mechanisms involved in cell transformation, only a few of the molecules identified as critical events in the carcinogenic process can be used as novel targets for intelligent drug design.

Cancer is a major threat to humans in industrialized countries, with about a one-third to one-quarter of the population dying of this disease (8). Cancer treatment has evolved greatly in the last decades when improved therapies have managed to increase survival rates to >50% of diagnosed patients (9). Besides a much better knowledge of the natural history of many types of cancer and the establishment of different novel strategies based on it, still the most efficient ways for treatment are those described as “classical” methods, such as surgery, radiotherapy, and chemotherapy. There is a general belief that development of new chemotherapeutic agents is probably the most reliable way to improve our success against cancer, and intelligent drug design is a key factor for this end (5, 10). However, most of the very few drugs developed against novel targets thus far identified are still under investigation for their potential use in the clinic, and the results are uncertain. Thus, the identification of novel strategies and novel targets for anticancer drug discovery is needed.

Several molecules derived as a consequence of membrane phospholipid breakdown are well established as second messengers in mitogenic signal transduction pathways (11, 12). Apart from the well-known phosphatidylinositol-derived metabolites DAG and inositol trisphosphate, PC hydrolysis has more recently been demonstrated to play an important role in mitogenesis (13–18). PC is the most abundant membrane phospholipid in euakaryotic cells. Therefore, it can sustain a prolonged liberation of second messengers, without drastic changes in membrane phospholipid content (19). These long-lasting signals are thought to be important in the acquisition of the transformed phenotype (11, 12). Upon mitogenic stimulation by growth factors or oncogenic transformation, phospholipase D-driven PC hydrolysis is activated, and both PA and Cho are generated (17, 18, 20–22). PA can be further hydrolyzed or deacetylated to form DAG or LPA, respectively; both of them have mitogenic activity (23–25). More recently, our group has demonstrated that the generation of PCho from Cho by the enzyme ChoK is an essential event for growth factors such as platelet-derived growth factor or fibroblast growth factor to exert its mitogenic action in mouse fibroblasts (26–28). These growth factors were unable to stimulate DNA synthesis if choline kinase was inhibited by HC-3 or HC-3 analogues. This observation is supported by the fact that the exogenous addition of PCho to the culture medium of mouse fibroblasts induces the G1–S transition in these cells (26).

Evidence for a role of PCho in malignant transformation comes from studies performed in several types of human tumors using nuclear magnetic resonance spectroscopic studies. This technique has allowed the identification of high levels of phosphomonoesters (PCho and/or phosphorylethanolamine) in tumors, relative to the normal tissue in which they were originated (29–33). Moreover, a common characteristic in several transformed cell lines derived from human brain tumors is the elevated PCho levels relative to glycerophosphorylcholine. This spectroscopic parameter was able to distinguish malignant from normal cell lines, irrespective of their proliferation rate (34). Additional data supporting the role of PCho in malignant transformation is the fact that ras-transformed cell lines show increased ChoK activity and higher PCho levels than their normal counterparts (35–37). This observation, reported previously in stably transfected cell lines, was confirmed recently in a transient expression system (38). It rules out the possibility that the increased ChoK activity is the result of an increased PC biosynthesis in response to the elevated phospholipid breakdown in these cells. Oncogenic versions of the Ras protein are found frequently in human malignancies with a rate of one-third of all cancers and up to 90% in some tumors such as adenocarcinoma of the pancreas (39, 40). We have observed that other oncoproteins, such as src,raf, and mos, can also increase the endogenous ChoK activity when expressed in mouse fibroblasts (28).

All of these results justified the consideration of ChoK as a novel target for the development of new anticancer drugs. Generation of new ChoK inhibitors based on the structure of HC-3 allowed us to establish a good correlation between improvement in the inhibitory activity against ChoK and the acquisition of antimitogenic potency in vitro (28). Thus, a higher efficiency in the reduction of intracellular PCho levels, achieved by different substances, had a similar effect on mitogenesis, ruling out a possible nonspecific effect on cell proliferation by high doses of HC-3. Furthermore, we did not find interference of these compounds with other elements of the mitogenic signal transduction pathways. Finally, these new compounds had antiproliferative properties against oncogene-transformed cell lines in the low.
micromolar range. Here we report the in vivo antiproliferative activity of ChoK inhibitors against human tumor-derived cell lines.

MATERIALS AND METHODS

Cell Cultures and Reagents. Human tumor-derived cell lines used in this study were maintained under standard conditions of temperature (37°C), humidity (95%), and carbon dioxide (5%). Culture medium for the HT-29 cell line (ATCC HTB 38, colon adenocarcinoma) was DMEM supplemented with 15% FBS (Life Technologies, Inc., Grand Island, NY). Cell lines HeLa (ATCC CCL 2, cervix carcinoma) and A431 (ATCC CRL 1555, epidermoid carcinoma) were maintained in DMEM supplemented with 10% FBS. Cell lines MCF-7 (ATCC HTB 22, breast adenocarcinoma), U937 (ATCC CRL 1593, histiocytic lymphoma), and K562 (ATCC CCL 243, chronic myelogenous leukemia) were maintained in RPMI supplemented with 10% FBS. Commercially available reagents were [methyl-14C]choline chloride from ICN pharmaceuticals, Inc. (Costa Mesa, CA) and Crystal Violet from Merck (Darmstadt, Germany).

Analysis of PCho Production in Cells (in Vitro ChoK Assay). HT-29 cells (10^5/well) were seeded on 24-well plates (Falcon; Becton Dickinson, Franklin Lakes, NJ), and the rest of the cell lines analyzed were seeded on six-well plates (typically, 1.5 × 10^5/well). Cells were incubated for 3 days and then rinsed in TD buffer (137 mM NaCl, 5 mM KCl, and 20 mM Tris, pH 7.4). One h before labeling with 0.5 mCi/ml methyl[14C]choline chloride, cells were incubated in DMEM supplemented with different concentrations of ChoK inhibitors and maintained for 14 additional h in the presence of radioactivity and inhibitors. Then cells were rinsed in ice-cold TD and fixed with 16% ice-cold trichloroacetic acid. Trichloroacetic acid-soluble material containing Cho and PC was washed three times with four volumes of diethylether, dried under vacuum, and resuspended in water. Samples were resolved by TLC on 60 A Silica gel plates (Whatman, Clifton, NJ), using as liquid phase 0.9% NaCl/methanol/ammonium hydroxide (50:70:5, v/v/v). Radioactivity corresponding to PCho was automatically quantified by an electronic radiography system (Instantimagerr; Packard, Meriden, CT). The concentrations at which 50% inhibition of PCho production was reached (IC50) were calculated using a semilogarithmic plotting of the PCho versus concentration of the inhibitors.

Cell Proliferation Assays. HT-29 cells were seeded on 24-well plates (35 × 10^3 cells/well) and incubated for 24 h. Then, cells were treated with different concentrations of ChoK inhibitors in the usual culture medium. Three days later, wells were aspirated, fresh medium and treatment were added, and cells were maintained for 3 additional days. Alternatively, different cell lines were seeded on 96-well plates, treated, and incubated for only 3 additional days in the specific culture medium before processing. Quantification of the cells remaining in each well was carried out using the Crystal Violet method (41), with some modifications. Briefly, cells were washed with TD buffer and fixed with 1% glutaraldehyde for 15 min. After washing again with TD, cell nuclei were stained with 0.1% Crystal Violet for at least 30 min and washed 3 times with distilled water. Adsorbed dye was resuspended in 10% acetic acid, and absorbance at 595 nm was determined in a spectrophotometer. For non-adherent cell lines (U937 and K562), washings were preceded and followed by centrifugation of the plates at 10^5 rpm for 10 min.

Toxicity Assays in Mice. Male Swiss mice (average, 40-g weight) were inoculated i.p. with different doses of the ChoK inhibitors dissolved in 0.25 ml of NaCl 0.9%. Control mice were inoculated with the same volume of vehicle alone. Mice were maintained under standard conditions, and their general status was monitored daily.

In Vivo Antitumoral Assays. Human tumor xenografts were established by s.c. injection of tumor-derived cell lines in athymic nu/nu nude mice. Mice were kept under standard laboratory conditions according to the guidelines of the Spanish Government. Cells were trypsinized and resuspended in DMEM just before inoculation (10^6 cells/0.1 ml). When tumors reached a volume of ~0.1 cm^3 (usually 3–6 days after inoculation), mice were randomized to a control and treated group (4–6 mice each). Treatments were performed i.p., as described in the above section. Different schedules were tested, as described in “Results.” Tumor size was measured at least weekly, and the volumes were calculated using the equation V = (D x d^2)/2, where V is tumor volume, D is longest diameter in mm, and d is shortest diameter in mm. Differences between control and treated mice were analyzed using the Student t test.

RESULTS

Antiproliferative Effects of ChoK Inhibitors on Human Colon Adenocarcinoma Cells HT-29. We have described previously the antimitogenic activity of HC-3, a molecule with ChoK inhibitory activity in the mM range (26, 27). More recently, we have described the enhanced antimitogenic action of newly generated ChoK inhibitors based on the chemical structure of HC-3, with potent activity in the μM range (28). These compounds were also shown to inhibit cellular proliferation of oncogene-transformed fibroblast cell lines, suggesting that this family of molecules can exert potent antiproliferative effects against human cancer cells. So as to address this possibility, we started screening HC-3 analogues on antiproliferative assays in vitro, using the human tumor-derived cell line HT-29. This cell line was established from a colon adenocarcinoma, one of the most frequent solid cancers in humans that are also mainly resistant to chemotherapy (42), making these cells appropriate for the search of new antitumor drugs. Recently, the function of the itinerant tyrosine kinase Src has been identified as an essential step for the tumorigenic activity of HT-29 cells (43). For all of these reasons and given the fact that ChoK inhibitors drastically impair proliferation of src-transformed murine fibroblasts (28), HT-29 cells seemed a good system to be used in the screening for ChoK inhibitors as new anticancer drugs.

As shown in Table 1, the compounds JCR89C, JCR1043B, JCR795B, JCR947A, JCR791B, and JCR987B, described previously as good ChoK inhibitors against purified ChoK and in murine fibroblasts (28), were also potent inhibitors of ChoK in HT-29 cells. All of these compounds inhibited proliferation of HT-29 cells with a 50% inhibitory concentration (IC50) below 4 μM. The assay was performed using monolayers of cells maintained under optimal conditions of growth (DMEM supplemented with 15% FBS) for 6 days in the presence or absence of different concentrations of each ChoK inhibitor, and the number of cells remaining at the end of the incubation period was quantified using the Crystal Violet staining method (41).

These results imply a dramatic increase in antiproliferative activity compared with the initial molecule (HC-3), which showed an IC50 of 2500 μM under the same assay conditions. As a negative control, an HT-3 analogue without enhanced inhibitory activity in vitro, such as FK21, was also tested. In keeping with previous results, FK21 did not inhibit proliferation of HT-29 cells, even at a concentration of 50 μM. Thus, all of the HC-3 analogues that were good ChoK inhibitors had potent antiproliferative activity with an IC50 value <4 μM, whereas the compound FK21 was inactive, even at 50 μM. Although both assays (proliferation and ChoK activity) have different methodologies and cannot be strictly compared in a quantitative manner, there is a good correlation between the two parameters tested. Thus, ChoK

Table 1: Antiproliferative and choline kinase inhibitory activity of HC-3 analogues on HT-29 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Choline kinase (HT-29) IC50 (μM)</th>
<th>Proliferation (HT-29) IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FK21</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>JCR89C</td>
<td>0.8</td>
<td>2</td>
</tr>
<tr>
<td>JCR1043B</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>JCR795B</td>
<td>3.5</td>
<td>2</td>
</tr>
<tr>
<td>JCR947A</td>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>JCR791B</td>
<td>2.5</td>
<td>4</td>
</tr>
<tr>
<td>JCR987B</td>
<td>0.6</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Choline kinase activity was analyzed on HT-29 cultured cells by measuring the conversion of labeled Cho into PCho in the presence of different concentrations of the HT-3 analogues. The antiproliferative activity was analyzed by measuring the number of cells remaining after 6 days of incubation with the same compounds, relative to control, not treated cells. IC50 refers to the concentration at which 50% inhibition of choline kinase or proliferation activities are reached.
inhibitors are valuable tools as antiproliferative drugs in human cancer cells.

Enhanced Antiproliferative Activity of Newly Generated ChoK Inhibitors on HT-29 Cells. The results compiled in Table 1 demonstrated antiproliferative activity of HC-3 derivatives on the HT-29 cell system. A more extensive screening of HC-3 analogues based on the structural information derived from those described in Table 1 was then carried out. The same parameters as those reported previously (28), inhibition of purified ChoK activity and inhibition of proliferation and ChoK activity in HT-29 cells, were analyzed in >150 new compounds. Those that gave best results as antiproliferative agents are listed in Table 2, where their action as inhibitors of ChoK is also reported. A group of more than 10 molecules with IC50 <2 μM was identified, and some others were at least as effective as the ones reported previously. All of these compounds can be considered as good ChoK inhibitors in HT-29 cells, with IC50 in the μM range. These results are interpreted as that in all of the HC-3 analogues analyzed (Tables 1 and 2, and results not shown), the ability of reducing the intracellular PCho content was associated with antiproliferative activity when measured in the same cellular system.

Antiproliferative Activity of New ChoK Inhibitors on Different Human Tumor-derived Cell Lines. The screening for new antiproliferative compounds using the HT-29 cell line allowed the identification of a large number of compounds that can be further selected according to other parameters. The next step was the analysis of the antiproliferative activity of some of them in a panel of human tumor-derived cell lines including HeLa (carcinoma of the cervix), A431 (epidermoid carcinoma), MCF-7 (breast carcinoma), U937 (histiocytic lymphoma), and K562 (chronic myeloid leukemia). The assay was performed using the same procedure described for Tables 1 and 2, except that the incubation period was 3 days instead of 6 days, avoiding the need of changing the medium for the nonadherent cells. IC50 refers to the concentration at which 50% inhibition of choline kinase or proliferation activities are reached.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Proliferation (HT-29)</th>
<th>Choline Kinase (HT-29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN288B</td>
<td>0.3</td>
<td>1.8</td>
</tr>
<tr>
<td>MN276B</td>
<td>0.3</td>
<td>3.7</td>
</tr>
<tr>
<td>MN352B</td>
<td>0.3</td>
<td>1.5</td>
</tr>
<tr>
<td>MN284B</td>
<td>0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>MN308B</td>
<td>0.4</td>
<td>2.5</td>
</tr>
<tr>
<td>MN356B</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>MN94B</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>MN336B</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>MN58B</td>
<td>0.5</td>
<td>4.2</td>
</tr>
<tr>
<td>MN280B</td>
<td>0.6</td>
<td>2.0</td>
</tr>
<tr>
<td>MN82B</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>MN90B</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MN304B</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>MN168B</td>
<td>1.5</td>
<td>10.5</td>
</tr>
<tr>
<td>MN332B</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>FK19</td>
<td>2.5</td>
<td>7.5</td>
</tr>
</tbody>
</table>

The cells were maintained in their respective optimal conditions of growth in the presence of different concentrations of the choline kinase inhibitors for a period of 3 days. Then, the number of cells was quantified and compared with the control, untreated cells. IC50 refers to the concentration necessary to reduce by 50% the number of cells at the end of the experiment.

Table 3

Antiproliferative activity of choline kinase inhibitors on different human tumor-derived cell lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>HT-29</th>
<th>HeLa</th>
<th>A431</th>
<th>MCF-7</th>
<th>U937</th>
<th>K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN58B</td>
<td>1.4</td>
<td>1.2</td>
<td>2.1</td>
<td>0.6</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>MN168B</td>
<td>2.7</td>
<td>1.5</td>
<td>1.5</td>
<td>2.6</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>MN82B</td>
<td>1.6</td>
<td>0.6</td>
<td>3.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>MN276B</td>
<td>0.6</td>
<td>0.3</td>
<td>0.6</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>JCR947A</td>
<td>5.6</td>
<td>3.3</td>
<td>6.0</td>
<td>1.1</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>JCR795B</td>
<td>16.5</td>
<td>5.5</td>
<td>6.2</td>
<td>2.2</td>
<td>2.1</td>
<td>4.5</td>
</tr>
<tr>
<td>JCR791B</td>
<td>22.5</td>
<td>5.8</td>
<td>25.0</td>
<td>7.1</td>
<td>3.9</td>
<td>24.7</td>
</tr>
<tr>
<td>FK19</td>
<td>25.0</td>
<td>5.6</td>
<td>4.5</td>
<td>25.0</td>
<td>6.0</td>
<td>30.0</td>
</tr>
</tbody>
</table>

The results are shown in Table 3. Similar results were found for the antiproliferative activity of all of the compounds tested for each of the six cell lines, although in general terms, hematopoietic cells were slightly more sensitive to the inhibitory effect by most of the compounds. We also investigated the effect of each compound on the intracellular PCho levels under optimal growth conditions for every cell line tested to directly correlate this effect and the inhibition of proliferation. Fig. 1 shows the results using one representative ChoK inhibitor, MN58B, at a concentration of 10 μM, acting on all of the transformed cell lines listed in Table 3. Consistent with previous data in the serum-free assays performed on HT-29 cells, MN58B caused a 75% reduction in PCho levels on this cell line, and even greater effects were observed in the rest of the cell lines tested. These results indicate that the antiproliferative activity of ChoK inhibitors can be extrapolated to tumor cell lines of human origin other than HT-29.

Toxicity of the New ChoK Inhibitors in Mice. Once a group of compounds with potent antiproliferative activity was identified using in vitro systems, the next step was to establish appropriate in vivo antitumoral assays. A prerequisite is to know the maximal tolerated dose of each compound in experimental animals. We first performed toxicity assays in mice using a similar schedule to that desired for the antitumoral assays, i.e., daily i.p. administration for 5 consecutive days. HC-3 has potent neurological toxicity, characterized by a respiratory paralysis due to the inhibition of Cho uptake and subsequent depletion of acetylcholine in neurons (44). The new HC-3 analogues had been designed to improve the inhibitory activity versus ChoK, but it could have caused a concomitant increase in its neurological tox-

Fig. 1. Inhibition of PCho production by MN58B on different human cell lines. The indicated cells were maintained in optimal growing conditions as described in “Materials and Methods” and labeled with 1 μCi/ml [3H]choline for 14 h. in the presence of 10 μM MN58B. Intracellular labeled PCho per mg of protein was then quantified and compared with the corresponding untreated, control cells for every cell line.
nicity. The results shown in Table 4 rule out this possibility and indicate that a dramatic reduction in HC-3 toxicity was actually obtained when ChoK is more efficiently inhibited. HC-3 caused the death of the injected mice in a single i.p. administration of 0.1 mg/kg (data not shown). Some of the new choline kinase inhibitors including MNS5B, MN280B, MN304B, MN168B, and MN82B were well tolerated at 5–10 mg/kg, injected for 5 consecutive days. Subsequent assays determined that the maximum tolerated dose for compounds MN304B and MN168B were 10 and 35 mg/kg, respectively. It was also shown that MN168B was not completely absorbed and accumulated in the peritoneum several days after the administration. Thus, some of the generated HC-3 analogues showed both increased ChoK inhibitory activity as well as antiproliferative activity without a parallel increase in toxicity to mice. These striking results allowed further analysis for antitumoral activity under in vivo conditions.

### In Vivo Antitumoral Activity of ChoK Inhibitors on Human Tumor Xenografts

Combination of data generated by the in vitro antiproliferative assays on human tumor-derived cell lines and the toxicity assays in mice allowed the identification of the first candidates to be tested as antitumoral agents in vivo. Two of the compounds, MNS5B and MN168B, were initially selected due to their high antiproliferative activity and relative low toxicity. Tumors were generated by s.c. injection of different human tumor-derived cell lines in the back of immunosuppressed mice (Swiss, nu/nu). The compounds were administered i.p. dissolved in sterile 0.9% NaCl. Control mice received an equivalent volume of vehicle alone, following an identical schedule. Treatment was initiated when the tumors became evident (mean of 0.1 cm³), and tumors were monitored at least weekly by measuring the major (D) and minor (d) diameters. Tumor volume was calculated using the equation \( V = (D \times d^2)/2 \).

The results obtained with the compound MNS5B on HT-29 tumor xenografts are shown in Fig. 2, using two different schedules. Average of the tumor volume in the course of the experiment is represented for control and treated mice. In the experiment represented in Fig. 2A, the treatment consisted of daily consecutive doses of 5 mg/kg for 5 days, separated by 1 entire week. In Fig. 2B, mice received daily doses of 3 mg/kg for 4 days, separated by 3 days. As is evident, both schedules caused an inhibition of ~70% in the tumor growth. The difference was shown to be statistically significant by the \( t \) test, with \( P < 0.05 \). When mice carrying tumors of <0.1 cm³ were treated with the same concentrations of MNS5B, an even more drastic reduction of >90% in tumor growth versus untreated mice was observed (data not shown). These results indicate that MNS5B has antitumor activity in vivo in nude mice injected with the human adenocarcinoma HT-29 cells.

Next, we investigated the effect of the compound MN168B on the same kind of xenografts using a schedule consisting of six daily doses of 35 mg/kg, separated by 1 week. As shown in Fig. 3, a reduction in tumoral growth was observed, similar to that obtained with MNS5B. However, when this compound was administered at 35 mg/kg, 3 days/week, <50% reduction was observed in the tumors as average (data not shown). A possible explanation for these results was poor absorption of the drug, because accumulation of the compound in the peritoneum forming a solid mass was evident in the course of the experiment.

Finally, we tested the antitumor activity of compound MNS5B on another human tumor xenograft, such as A431 cells, derived from a...
The search for new targets for the development of anticancer agents is a major topic in oncological investigation (51, 52). Recent advances in the knowledge of mechanisms controlling cell proliferation and transformation support the development of new strategies aimed at blocking the aberrant processes leading to malignant transformation (34). The importance of these findings in human malignancies is suggested by the identification of elevated PCho levels in several human tumor types relative to the normal surrounding tissues (29–33). Furthermore, we have recently reported the improvement of the previously known ChoK, HC-3, and demonstrated enhanced antimitogenic and antiproliferative activity of the new compounds in murine fibroblasts systems, which is more effective toward transformed cells (28). All of this information is the basis in considering the enzyme ChoK as a novel target for the development of new antiproliferative agents.

New chemotherapeutic agents based on the structure of phospholipids have been developed. Among them, hexadecyl phosphocholine (HePC) and 1-0-octadecyl-2-0-methyl-rac-glycero-3-phosphocholine (also known as ET-18-OCH₃ or edelfosine), are some of the best characterized members of this group of compounds (5, 13, 19, 63). Both have demonstrated antiproliferative activity in vitro and antitumor activity in vivo using animal models. Recent evidence suggests that at least some of these compounds may work by interference with the CTP:phosphocholine cytidylyltransferase (64), an enzyme critical for PC biosynthesis and next to ChoK in this pathway. Thus, the same explanation could be extrapolated to the ChoK inhibitors, because a similar effect on PC synthesis should be expected. However, we feel that is not the case because ChoK inhibitors do not affect the total PC levels due to the existence of alternative pathways for the generation of PC (data not shown; Refs. 5, 13, 19, 63, 26, and 28). Furthermore, a clear picture of the mechanism of action of these lipid-related compounds is far from being achieved, and in fact, it is controversial because other molecules have also been identified and postulated as have demonstrated that the activity of this enzyme is enhanced by oncogenes such as ras, src, mos, and raf (21, 28). This is in keeping with findings from other groups that have confirmed the activation of choline kinase by the ras and raf oncogenes (38), in addition to those indicating a specific elevation of PCho levels associated with malignant transformation (34). The importance of these findings in human malignancies is suggested by the identification of elevated PCho levels in several human tumor types relative to the normal surrounding tissues (29–33). Furthermore, we have recently reported the improvement of the previously known ChoK, HC-3, and demonstrated enhanced antimitogenic and antiproliferative activity of the new compounds in murine fibroblasts systems, which is more effective toward transformed cells (28). All of this information is the basis in considering the enzyme ChoK as a novel target for the development of new antiproliferative agents.

Strong evidence links the Ras signaling pathways and the generation of phospholipid-derived second messengers (17, 18, 35, 60, 61). PC has been demonstrated to be a novel source of these molecules, including DAG, PA, and LPA (13–16, 62). In addition, we have proposed that PCho generated from the phosphorylation of Cho by the enzyme ChoK can also participate in mitogenic routes (26, 27) and

DISCUSSION

The discovery of human oncogenes and the identification of their role in the pathogenesis of cancer has focused the interest of many groups trying to interfere their dysregulated signal transduction pathways (5, 6, 45–47). Oncogenes are altered versions of normal proteins, the function of which is important in normal cells (1, 48, 49). However, the redundancy of signals controlling critical processes of cellular physiology may allow the blockade of altered pathways without deleterious effects in the whole organism (50).

The search for new targets for the development of anticancer agents is a major topic in oncological investigation (51, 52). Recent advances in the knowledge of mechanisms controlling cell proliferation and transformation support the development of new strategies aimed at blocking the aberrant processes leading to malignant transformation of cells. The newly designed antitumor agents are expected to be more specific and hence less toxic than the actual cytotoxic drugs used in conventional chemotherapy. Some of these strategies have shown promising results in animal models. For instance, the inhibitors of farnesyl-transferase enzyme, which inhibit the functions of the Ras protein (53–55) or specific tyrosine-kinase inhibitors (56–59), are able to elicit regression of certain tumors in mice.

Strong evidence links the Ras signaling pathways and the generation of phospholipid-derived second messengers (17, 18, 35, 60, 61). PC has been demonstrated to be a novel source of these molecules, including DAG, PA, and LPA (13–16, 62). In addition, we have proposed that PCho generated from the phosphorylation of Cho by the enzyme ChoK can also participate in mitogenic routes (26, 27) and

epidermoid carcinoma. Fig. 4 shows the antitumor effect of MN58B on A431 tumor xenografts in nude mice. The compound was administered at a dose of 3 mg/kg daily for 4 consecutive days, separated by 3 days. A reduction of 75% of the tumoral volume was achieved at the end of the treatment, with P < 0.05. All of these results demonstrate in vivo antitumoral activity of ChoK inhibitors against several human cancer cells injected into nude mice.
targets for their action. For instance, several studies have demonstrated inhibition of the agonist-induced phosphatidylinositol hydrolysis via phospholipase C, and the subsequent blockade of calcium release and DAG production, which abrogates PKC stimulation (65–68). Furthermore, it has been reported that hexadecyl phosphocholine (HePC) induces a rapid activation of phospholipase D activity, which is completely blocked by PKC inhibition due to down-regulation with phorbol esters or staurosporine treatment (69). Finally, other molecules involved in signal transduction and cell cycle regulation have been proposed as targets for these compounds (50, 64).

In the present work, we have used those reported previously and newly generated HC-3 analogues in an attempt to find new substances with potential antitumoral interest in humans. We first tested the antiproliferative activity of these compounds toward the human colon cancer cell line HT-29 and found potent inhibitory effects that correlated with the reduction of PCho levels. We then extended the screening to other human tumor-derived cell lines and demonstrated that the effect shown on HT-29 is common to the cell lines HeLa, A431, and MCF-7, representative of some of the most frequent and chemotherapy-resistant human solid cancers. We also observed antiproliferative effects toward the hematopoietic cell lines U937 and K562. These results opened the possibility of using an in vivo system to investigate the antitumoral activity of the ChoK inhibitors. It was particularly interesting to find that the enhanced antiproliferative activity of the new compounds was not accompanied by a potentiation of the neurochemical toxicity of HC-3. The dissociation of both effects is promising, because it allows further improvement in the ChoK inhibitory and antiproliferative activity of the molecules without a necessary increase in its adverse effects. We show in vivo data indicating antiproliferative effects of some of the ChoK inhibitors on human tumor xenografts from colon and epidermoid carcinomas. Our results also point out the importance of further improvements in the molecules to facilitate not only their efficiency but also their delivery to the tumoral tissue. Finally, a large-scale screening of new ChoK inhibitors can surely lead to the identification of better antitumoral agents based in this new concept. Here we provide in vivo evidence that ChoK is a novel target for the design of antitumor drugs. We believe this is an important contribution because it provides a novel mechanism for anticancer treatment, one of the most needed facts for the advance in chemotherapeutic clinical oncology.

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CHOLINE KINASE INHIBITORS AS ANTITUMOR DRUGS


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