Molecular Cancer Therapeutics

Preclinical Characterization of RSM-932A, a Novel Anticancer Drug Targeting the Human Choline Kinase Alpha, an Enzyme Involved in Increased Lipid Metabolism of Cancer Cells

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Abstract

Choline kinase α (CHKA; here designated as ChoKα) is the first enzyme in the CDP-choline pathway, implicated in phospholipids metabolism. It is overexpressed in several human tumors such as breast, lung, bladder, colorectal, prostate, ovary, and liver. The overexpression of ChoKα has oncogenic potential and synergizes with other known oncogenes. It has been proposed as a novel cancer drug target with a distinct mechanism of action. We have generated a set of ChoKα inhibitors with potent *in vitro* antiproliferative and *in vivo* antitumoral activity against human xenografts in mice, showing high efficacy with low toxicity profiles. Among these inhibitors, RSM-932A has been chosen for further clinical development due to its potent antiproliferative activity *in vitro* against a large variety of tumor-derived cell lines, a potent *in vivo* anticancer activity, and lack of toxicity at the effective doses. Here, we provide the preclinical evidence to support the use of RSM-932A as a good candidate to be tested in clinical trials as the "first in humans" drug targeting ChoKα. *Mol Cancer Ther; 14(1); 31–39. ©2014 AACR.*

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Introduction

Choline and related lipid compounds are vital metabolites to maintain cellular homeostasis and to support proliferation of both normal and tumor cells. It is required for the synthesis of phosphatidylcholine (PC), the major phospholipid in eukaryotic membranes, and other phospholipids which are relevant to maintain cell membrane integrity and signal transduction both in normal conditions and disease, including cancer (reviewed in refs. 1–3).

Generation of phosphocholine (PCho) by choline kinase α (ChoKα) is an essential event for growth factor signaling. There are data supporting the role of PCho in malignant transformation as *RAS*-transformed cell lines show increased ChoK activity and higher PCho levels than their normal counterparts (4). Furthermore, increased activity of ChoKα is observed in a large series of human tumors and tumor-derived cell lines including breast, lung, colorectal, prostate, bladder, ovary, liver, and osteosarcomas (5–11), and its activity has been found to be a critical requirement for the proliferation of primary human mammary epithelial cells and breast tumor progression (6, 7, 12). All of this evidence, as well as NMR studies of choline and related compounds in both normal and tumor cells, concludes that abnormal choline metabolism is a metabolic hallmark associated with tumor onset and progression, and further supports the proposal for using ChoKα as a novel therapeutic target and a biomarker for cancer prognosis and treatment (1–3).

Several different molecules were initially used as choline kinase antagonists (1). A successful series of symmetrical bis-quinolinium compounds based on the hemicolinium-3 (HC-3) molecule have been synthesized as inhibitors of ChoKα with promising activity as antiproliferative compounds against tumor-derived cells and as antitumoral drugs in xenografts models (13–20). MN58b, a first-generation ChoKα inhibitor, shows a potent *in vitro* antiproliferative activity and *in vivo* antitumoral activity (1, 13, 14). It is highly specific because it demonstrates a differential effect in normal cells versus tumor cells. In primary cells, blocking *de novo* PCho synthesis by MN58b promotes a reversible cell-cycle arrest at G_0–G_1 phase. In contrast, ChoK inhibition in tumor cells makes cells unable to arrest in G_0–G_1 as marked by a deficient pRb dephosphorylation (21, 22). In addition, MN58b has provided strong evidence that it is specific to ChoKα, with no effect in a variety of oncogene-activated signaling pathways (21–23). Furthermore, MN58b showed a much higher specificity against ChoKα1 (IC_{50} = 5 μmol/L) than against ChoKβ (IC_{50} = 107.5 μmol/L), this is 21.5 times more potent against ChoKα1 than ChoKβ isoform (24). All these results have been validated by ChoKα-specific RNA-interfering (siRNA) approaches (7, 25–27) and additional small molecules directed toward ChoKα (28–29).

Once the proof-of-principle that ChoKα is a validated target in oncology has been set up, a very intensive activity has been developed for the identification of novel ChoKα inhibitors with

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potential use as antitumoral drugs (15–20, 28–31) and also as antiparasitic compounds (32, 33).

In humans, there are two genes codifying for ChoK activity, *CHKA* and *CHKB*, and three different enzymes, ChoKα1, ChoKα2, and ChoKβ (reviewed in refs. 1, 2). Although ChoKα1 and ChoKα2 are almost identical and are generated by differential splicing from the *CHKA* gene, ChoKβ, which shares 60% of overall homology, is generated from *CHKB*. Thus, ChoKβ is a different gene product, and although is able to convert choline into PCho in cell extracts, its main role in living cells seems to be the phosphorylation of ethanolamine to phosphoethanolamine (24). Thus, probably due to this differential biochemical behavior, although ChoKα has been involved in the tumorigenic process, a similar role for ChoKβ in carcinogenesis has not been found (24). Recent evidence further supports the relevance of ChoKα as a bonafide anticancer drug target (34). This makes necessary the identification of specific ChoKα inhibitors as anticancer drugs.

Limited therapeutic outcomes of existing drugs, diverse toxic side effects, along with acquired resistance to multiple drugs, are frequent drawbacks of current cancer therapies. Therefore, there is a clinical need for new and more precise compounds that can specifically inhibit novel selected targets, guaranteeing the continuous improvement in the search for new cancer-targeted drugs, with a higher therapeutic window and less toxicity. Here, we report the preclinical development of RSM-932A as the first-in-man ChoKα inhibitor to enter phase I clinical trials. Compound RSM-932A is a rationally designed targeted agent with a potential for combinatorial treatments in patients with cancer due to its novel mechanism of action.

Materials and Methods

Cell cultures and compound sensitivity assays

All cell lines used in this study were obtained from the ATCC; no authentication was done by the authors. Cells were grown in the recommended growth media by the provider under standard conditions at 37°C, 5% CO_2, and 98% humidity. Cells were obtained at the following dates: H1299 and H460 (May 2007), SW780 (August 2007), 769-P, SK-OV-3, OV-Car-3 and Mia. PaCa.2 (February 2008), SAOS-2 and TccSup (March 2008), HepG2, Hep3B2 and HCT-116 (April 2008), SW620 and HT-29 (June 2008), MDA-MB-231 (August 2008), MDA.MB.468 (September 2008), A431 (November 2008), SKMel-28 (January 2009), A-375 (May 2009), U-87 and MCF10-A (July 2009), H510 (October 2009), SkBr-3 (November 2009), T47D (February 2010), J82 (March 2010), HT-1376 (August 2010), DLD-1 (October 2010), and HeLa (November 2011).

For the antiproliferative activity assays, cells were seeded at 10,000 cells (except for SW780 that was seeded at 5,000 cells and H510 at 20,000 cells) per well into 96-well plates (BD, Falcon, Bioscience). Exponentially growing cells for 24 hours were exposed at scalar concentrations (quadruplicates for each concentration) of the compounds for 72 hours. The colorimetric assay MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is used to assess cell viability. The reaction correlates with absorbance at 595 nm in a VersaMax Microplate Reader (Molecular Devices). The IC_{50} values of each compound (50% inhibitory concentration of a substance) are quantified by plotting the log OD (optical density) versus log drug concentration.

Three additional parameters of the drug RSM-932A were assessed in tissue cultures cells: GI_{50} (concentration of the compound that causes 50% growth inhibition with respect to control cells, considering time 0), TGI (concentration of the compound at which the remaining cell number is equivalent to that of time 0), and LC_{50} (concentration of the compound at which the remaining cell number is 50% equivalent to that of time 0). Twenty-four hours after seeding the cells and before the exposure to the drug, a time zero point was read to calculate these parameters.

Synthesis of compounds

Compounds were synthesized as previously described (15–20, 35). The structure of all the compounds are shown as derivatives of the general structure depicted in Supplementary Fig. S1.

Mouse strains

Swiss immunocompetent mice (Harlan Sprague Dawley, Inc.) were used for MTD experiments. Athymic nu/nu mice (Harlan Sprague Dawley, Inc.), CD1 nude mice (Charles River), and BALB/c nude (Charles River) were used for tumor growth studies. All research protocols were approved by the National Centre of Biotechnology (CNB) Ethics Committee and followed the EU Directive 2010/63/EU for animal experiments.

Effective dose

The experiments to find the optimal dosis for the *in vivo* experiment were performed using 4–5 Swiss immunocompetent mice for each dose. Groups of mice were treated with each substance with different concentrations. All compounds were diluted at the same concentration as a stock solution (5 mmol/L) and successive dilutions were done in PBS (1×) for injections. The treatment consists in a daily intraperitoneal injection during 5 days, followed by a 9-day rest and a final cycle of 5-day treatment. During this time, mice were daily observed and any change in behavior, fur, weight, or mobility considered. All toxicity data were recorded, specifying the details of death cases during and at the end of the treatment. Survivor mice were sacrificed and all the organs were fixed for histopathological analysis (data not shown). The effective dose was defined as the highest concentration that induced no deaths after two 5-day treatment cycles. This dose was used for further toxicity and antitumoral activity experiments.

Tumor growth inhibition studies

Six-week-old nude mice were used. Mice were subcutaneously inoculated at lowers flanks with 1×10^6 of HT-29 (human colon adenocarcinoma) cells suspended in 100 μL of serum-free DMEM (Invitrogen) media mixed (1:1) with Matrigel (BD Biosciences). Measurable subcutaneous tumors (~0.15–0.2 cm^3) were randomized to the treatment and control group. Tumor growth was monitored three times per week. Tumors were measured in two dimensions with digital calipers and tumor volume was estimated using the formula of a rational ellipse $(D \times d^2)/2$, where D is the length of the long diameter and d is the short one. The body weight of mice was assessed once per week.

At the end of treatment, all animals from each group were sacrificed. All macroscopic abnormalities observed during the dissection were documented. Lungs were perfused with 4% formaldehyde and the rest of the organs post-fixed in the same

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fixative for at least 24 hours. After fixation, representative samples for each organ were obtained and embedded in paraffin. Sections of 6 μm of thickness of each block of paraffin were cut and stained by the routine hematoxylin-eosin (H&E) method. The histologic material was assessed by an independent experienced pathologist with ample experience in mice pathological analysis (CitoPath S.L.) under a Leica DM LS2 light microscope. Micrographs were taken with a Leica EC3 camera attached to the microscope. Liver sections from mice in the control group showed normal liver histology and described as (−). Liver sections from mice in the treated group showed either no alteration (−), slight changes referred to some binucleated hepatocytes and some with enlarged nuclei (+). Moderate changes include also cleared cytoplasm with a single vacuole (++), and acute changes showed all previously mentioned characteristic plus a diffuse mottling of the cytoplasm (+++).

For intraperitoneal drug administration, the compounds were dissolved every week of treatment at 5 mmol/L and kept protected from light at 4 °C, and successive dilutions were done fresh every day of treatment in sterile PBS. For the intravenous route of administration, RSM-932A was dissolved at 50 mmol/L (100% DMSO, Baker) and successive dilutions were done fresh every day of treatment in sterile 5% dextrose water (Viaflo).

Enzymatic activity assays

Recombinant bacterial extracts, in which it has been introduced the expression of human ChoKα or ChoKβ, were used. The enzymatic reaction in 50 μL total volume contained a constant volume of bacterial extract in the presence of 100 mmol/L Tris-HCl, pH 8.0, 100 mmol/L $MgCl_2$, and 10 mmol/L ATP, 200 μmol/L choline and methyl-[^{14}C]-choline, and different concentrations of the drug. The reaction was incubated 30 minutes at 37°C and stopped on ice. The samples were solved by thin layer chromatography (TLC 60A Silica Gel Whatman). The radioactivity corresponding to phosphocholine was quantified with the Cyclone Plus Scanner. Concentration of each compound that produces 50% inhibition was calculated and expressed in μmol/L (IC_{50}).

IHC

Tumors from xenografts grown in athymic mice were dissected and fixed in 4% formaldehyde for at least 24 hours and paraffin embedded and stained by routine H&E methods. IHC was performed on additional sections with the following antibodies; anti-cytokeratin 20, anti-cytokeratin clone MNF116 (ProgenBiotechnik GMBH), anti-carcinoembryonic antigen (Novocastra), and Ki-67 (DAKO) following the manufacturer's instructions. Negative antibody control was done replacing the primary antibody with the antibody diluents. The slides were examined at CitoPath S.L. by a consultant pathologist under a Leica DM LS2 light microscope. Micrographs were taken with a Leica EC3 camera attached to the microscope.

Statistical analysis

Comparisons in tumor volumes between the untreated and treated groups were done using the nonparametric Mann–Whitney test. Two side *P* values less than 0.05 were considered statistically significant. All calculations were performed using SPSS software, version 19.0 (SPSS Inc).

Results

Inhibition of specific enzymatic activity of ChoKα and antiproliferative effect in human tumor-derived cells

A battery of designed ChoKα inhibitors previously described (35) was tested for its ability to efficiently blocking the conversion of choline into PCho by the human ChoK-driven enzymatic reaction. To differentiate between the inhibitory activity against ChoKα and ChoKβ, the two human isoforms of choline kinase were expressed in an *Escherichia coli* bacterial strain that lacks choline kinase activity, thus the observed enzymatic activity is due exclusively to the recombinant-expressed isoform of the human choline kinase. As shown in Table 1, all compounds tested are more specific for ChoKα than for ChoKβ by 6 to 400-fold.

Subsequently we tested the *in vitro* ability of these compounds to inhibit cell proliferation using the human colon adenocarcinoma HT-29 cell line as model. Table 2 shows that although the sensitivity varies for some compounds, most of them have good antiproliferative IC_{50} values maintaining their main features: selective inhibition of the enzymatic activity of ChoKα and potent antiproliferative activity.

MTD in mice of ChoKα inhibitors

HC-3 is a potent inhibitor of the choline transporter with drastic toxic effects on the cholinergic terminals (36). In the first generation of derivatives, poor tolerated compounds were obtained that, however, were sufficient to display antitumoral activity. Among them, MN58b shows an efficient antitumoral effect in several systems but has a limited therapeutic window (13, 14). The MTD was determined for later experiments in athymic mice. To that end, 4 to 5 mice were tested with increasing concentrations of each compound for 5 consecutive days followed by 9 days rest and a final cycle of a 5-day treatment. Table 2 shows animal survival at the experimental endpoint for the different concentrations tested. All external signs of toxicity (weight loss, behavioral changes, and fur appearance) as well as lethality were recorded and evaluated to choose a safe dose for subsequent experiments whose objective is to find the final active and nontoxic dosage. A safe dose was established for all

Table 1. Relative inhibitory potencies for selected inhibitors against the human recombinant ChoKα and ChoKβ enzymes

	Enzymatic activity IC_{50} (μmol/L)		
	Chokα	Chokβ	Fold
MN58b	1.4 ± 0.17	>50	>36
RSM-932A	1 ± 0.12	33	33
RSM-820C	6 ± 0.68	40	6.7
RSM-964A	5 ± 0.6	>50	>10
RSM-828B	7 ± 0.59	>50	>7.1
ACG-604B	5 ± 0.17	>50	>10
RSM-936A	5.5 ± 0.32	44.6	8.1
ACG-548B	0.12 ± 0.02	48.9	408
ACG-560B	2 ± 0.17	>50	>25
RSM-824B	6 ± 0.24	>50	8.3
ACG-416B	0.13 ± 0.03	>50	>385

NOTE: Recombinant bacterial extracts expressing either human ChoKα or ChoKβ were tested as described in Materials and Methods in the presence of different concentrations of each drug. Concentration of each compound that produces 50% inhibition is indicated in μmol/L (IC_{50}). Fold inhibition relative to each enzyme is also indicated and describes the relative specificity of each compound towards ChoKα.

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Table 2. Properties of ChoKα inhibitors

Compound	IC_{50} (μmol/L)	LD_{50} μmol/kg (mg/kg)	Effective dose (mg/kg)	% Inhibition at the end of treatment	% Inhibition 15 days after treatment
RSM-820C	2.38 ± 0.67	41 (39.9)	11.2	68	73
RSM-932A	1.15 ± 0.14	12 (10.9)	7.5	69	70
RSM-964A	1.75 ± 0.21	29.5 (23)	9.4	65	73
RSM-828B	1.01 ± 0.36	19.6 (20)	12.2	64	66
ACG-548B	2.08 ± 0.26	>32 (>25)	9.3	57	37
ACG-560B	4.25 ± 1.06	33 (23.2)	8.5	53	43
ACG-604B	2.05 ± 0.07	26.5 (22.4)	10.2	55	43
RSM-936A	0.98 ± 0.38	26.5 (24.5)	11.1	55	57
RSM-824B	1.48 ± 0.45	13 (12.5)	11.5	46	56
ACG-416B	1.94 ± 0.36	5.9 (4.5)	1.5	5	26

NOTE: *In vitro* antiproliferative activity against HT29 cells (IC_{50}) values is represented for each compound, as well as the dose that induced 50% deaths in mice (LD_{50}), the effective dose used in the *in vivo* experiments, and the *in vivo* antitumoral activity at the effective dose for each compound in immunocompetent mice. *In vivo* activity is reported as percentage inhibition of tumor growth compared with control, untreated mice, inoculated with tumor cells under identical conditions and treated with vehicle alone.

compounds as shown in Table 2 and used for further experiments as the effective dose.

Tumor growth inhibition by ChoKα inhibitors

On the basis of these toxicity results, compounds were subjected to growth inhibition studies at the specified effective dose. The human colon cancer HT-29 cell line was chosen for tumor growth inhibition studies due to its relative well-differentiated and aggressive phenotype. Mice bearing HT-29-derived tumors were treated intraperitoneally with a daily dose for 5 days, followed by 9 days rest and a second cycle of five consecutive daily treatments. Under these conditions, all the analyzed compounds displayed significant tumor growth inhibitory activity when compared with the tumor growth of the control mice treated with vehicle with RSM-820C, RSM-964A, RSM-932A, and RSM-828B as the most active compounds (Table 2; Fig. 1A). Next, all drugs were tested following a more restrictive treatment schedule consisting on one single injection per week for 4 weeks. Figure 1B and C shows the differences found between the two compounds with the best activity under the more restrictive conditions (RSM-932A and RSM-820C).

Data of external toxicity were recorded, and at the end of the treatment, all mice were sacrificed and all organs fixed for histopathology analysis. Table 3 illustrates the antitumor efficacy for each drug as well as the toxicity found in liver, although all main organs were analyzed. No significant organ damage was observed apart from hepatotoxicity that was found in all treatments except for the less aggressive treatment with RSM-932A that showed no liver toxicity while retaining a potent antitumoral activity (77%). Table 3 summarizes toxicity effects for all compounds along with their antitumoral activities. The histologic changes found in liver in all cases but one (RSM-824B) were only moderately different to normal tissue. Compound ACG-416B was not evaluated because of its low effect on tumor growth inhibition. On the basis of all these results, RSM-932A was chosen for further preclinical studies because of its performance with both treatment schedules and its low toxicity.

RSM-932A activity in tumor xenografts

The tumor growth inhibition of RSM-932A under different conditions of doses (7.5 mg/kg, 6 mg/kg, 5 mg/kg, 3 mg/kg, 1 mg/kg, 0.3 mg/kg), administration routes (intraperitoneal or intravenous), treatment schedule (5 consecutive days, 3 days per week, 2 days per week, 1 day per week), strain of mice (athymic nu/nu, CD1 nude, BALB/c nude), and human tumor xenografts (colon adenocarcinoma HT29, non–small cell lung cancer (NSCLC) H-460, breast adenocarcinoma MDA-MB-231) is summarized in Table 4.

Two routes of administration were compared, intraperitoneal and intravenous RSM-932A was used at 3 mg/kg on a 3 days per week schedule during 4 weeks by either route (Fig. 1D), and the inhibition of tumor growth registered along the duration of the experiment, 25 days. Comparison between both routes of administration versus control mice ($n = 19$ tumors) rendered similar results for intraperitoneal injections (58.1% inhibition; $n = 20$ tumors; $P \leq 0.001$) and for intravenous administration (62.4% inhibition; $n = 19$ tumors; $P \leq 0.0002$), both statistically significant. Pharmacokinetics assays were performed in parallel to determine the clearance of the drug in the plasma of treated mice comparing intravenous versus intraperitoneal administration, showing comparable clearance in keeping with the observed antitumoral activity (data not shown).

To confirm that the drug was properly delivered to the tumors and exerted its inhibitory action under these experimental conditions, tumors from the intravenous treated mice were surgically removed at the end of the experiment, protein extracts obtained from treated and untreated tumors and ChoK enzymatic activity assayed under standard conditions. A 40% inhibition of the enzymatic activity was assessed in the intravenous treated tumors compared with the untreated tumors (Fig. 1E).

RSM-932A antiproliferative activity determined by *in vitro* screening

RSM-932A was tested against a wide panel of 27 human cancer cell lines and a nontumorigenic cell line as a control (Table 5) to define the spectrum of its antitumoral activity. Four standard parameters were calculated: IC_{50}, GI_{50}, TGI, and LC_{50} useful to determine whether it has cytotoxic or cytostatic activity. Results indicate that RSM-932A has a potent antiproliferative activity against most tumor-derived cell lines tested, including those derived from breast, lung, colon, bladder, liver, ovary, bone, cervix, kidney, pancreas, melanoma, and brain tumors, with IC_{50} in the low, single-digit micromolar range, consistent with a cytotoxic effect.

Discussion

Preclinical efficacy studies based on mice xenografts are the main strategy to select compounds that will be eventually

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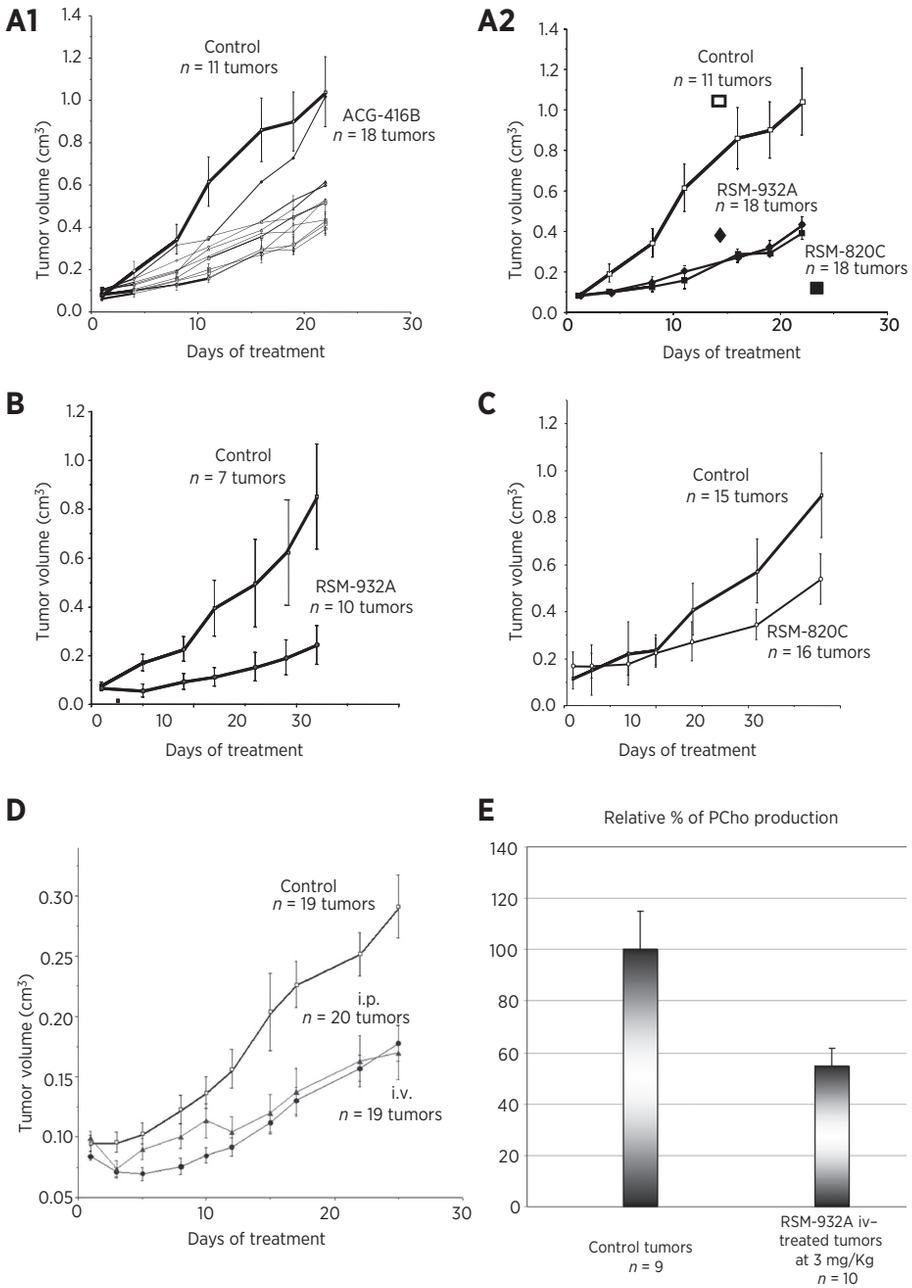

Figure 1.
Efficacy of selected ChoKα inhibitors against the human colon cancer cell line HT-29 xenograft. Athymic nu/nu mice were subcutaneously implanted in both flanks with 1×10^6 HT-29 cells. Tumors were allowed to form for 3 weeks and all drugs indicated in Table 2 were tested at the indicated effective concentrations. Mice were intraperitoneally treated with either vehicle (control) or with the drug, daily for 5 days, followed by a 9-day rest and then another 5 days of treatment. A1, all drugs showed antitumoral effects (46%–69% tumor growth inhibition) except for ACG-416B that showed no activity at the end of the experiment. A2, antitumoral activity in mice treated with the two most potent drugs, RSM-932A (7.5 mg/kg) or RSM-820C (11.2 mg/kg), are depicted. Mice were treated only once a week during 3 weeks with RSM-932A (B), or RSM-820C (C) to select for the most potent drug. The total number of tumors analyzed in each experiment is indicated. D, efficiency of RSM-932A in two different routes of administration. The antitumoral activity in balb/c nude mice was determined by measuring tumor volume in HT-29 cell line xenografts. Mice were treated with two routes of administration: i.p. and i.v. at 3 mg/kg. Both routes were administrated equally, three times per week for a total of 4 weeks. E, ChoK enzymatic activity in control and treated tumors with TCD-717. The enzymatic activity of ChoK was assessed in tumors extracts from intravenously treated mice ($n = 10$ tumors) with RSM-932A at 3 mg/kg, three times per week during 4 weeks. The 40% of activity inhibition validates the activity of the drug within the tumors.

analyzed to determine pharmacologic safety for further clinical development in humans. The clinical results of agents used in routine cancer therapy demonstrate the importance of the efforts that should be made in preclinical research to reduce superfluous human studies and optimize clinical responses. Selective kinase inhibitors are considered a main division of cancer therapeutics. Therefore, development of new tailored kinase inhibitors into drugs is a priority in cancer research.

HC-3 is a known inhibitor of choline kinase (36). Its target is overexpressed in a variety of tumors that are the main cause of death worldwide (5–11). However, its extremely high toxicity makes it impossible to use it as a pharmacologic anticancer drug (1, 36). The development of HC-3 derivatives as a strategy to develop a potential novel anticancer treatment based on the inhibition of ChoK activity has been a successful strategy initiated in our group (1). Modifications on its structure have been introduced to eliminate the unwanted effects of HC-3 on cholinergic terminals and therefore reduce its high toxicity (13–20). MN58b was selected as the lead compound to further explore its suitability as anticancer drug (13, 14) and to unravel the mechanism of action and consequences of inhibitors of the ChoK enzymatic activity (21–23, 37). Thus, MN58b belongs to a first generation of HC-3 derivatives used as a prototype for the selective inhibition of ChoKα to study its effects in both normal and tumor cells (1). Its

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Table 3. Liver toxicity after treatment with ChoKα inhibitors in mice bearing tumors

		Antitumoral activity	Liver toxicity
RSM-932A	Large schedule	69%	++
	Short schedule	77%	–
RSM-820C	Large schedule	73%	++
	Short schedule	62%	+
RSM-964A	Large schedule	73%	+
RSM-828B	Large schedule	66%	++
ACG-604B	Large schedule	53%	++
RSM-936A	Large schedule	57%	++
ACG-548B	Large schedule	37%	++
ACG-560B	Large schedule	43%	++
RSM-824B	Large schedule	56%	+++
ACG-416B	Large schedule	25%	NA

NOTE: After the treatment with each compound, mice from each group were sacrificed, and organs dissected and fixed in formaldehyde, paraffin-embedded, and stained by routine H&E methods. IHC was performed as indicated in Materials and Methods and scored by an expert pathologist. Although all organs were analyzed, only liver toxicity is shown. A gradual estimation of reactive changes in tissue was established; acute changes correspond to those modifications observed were a diffuse mottling of the cytoplasm is present in almost all the hepatocytes and many nuclei are enlarged, being the maximum change observed with respect to normal tissue. Acute changes (+++); moderate changes (++); slight changes (+); no changes (−). The compound ACG-416 was not analyzed (NA) due to the low antitumoral activity observed. Antitumoral activity was analyzed for RSM-932A and RSM-820C under either a short schedule (1 i.p. injection per week) or a large schedule (daily i.p. injection during 5 days, followed by a 9 day-rest per cycle) as described in Materials and Methods.

effects have been shown to be selective for ChoKα (24) and while inhibiting cell proliferation in normal cells in a reversible manner, treatment of tumor cells induces a drastic metabolic change that triggers apoptosis (21).

ChoKα shares high homology with its isoform ChoKβ, and phoshorylates *in vitro* choline and ethanolamine into PCho and phosphoethanolamine, respectively. However, in whole cells, ChoKα has the ability to act efficiently as both choline kinase and ethanolamine kinase, whereas ChoKβ has shown only an ethanolamine kinase (24). Although the nature for this differential biochemical function is still not fully understood, it may be the basis whereas ChoKα is involved in tumorigenesis but a similar role for ChoKβ has not yet been found (24). This is the basis for the development of specific ChoKα inhibitors that should not affect ChoKβ because inhibition of both enzymes with same compound may have undesired side effects and nonspecific toxicity in nontumor cells.

Here, we have designed and characterized a set of a second-generation derivatives of the HC-3 structure, and obtained less toxic and better tolerated compounds at more effective doses as tumor growth inhibitors compared with MN58b. Compound RSM-932A was selected because it provided the best experimental results. It behaves *in vitro* as MN58b, promoting cell-cycle arrest in nontumorigenic cells, and apoptotic cell death in tumor cells (21, 23). Under i*n vivo* conditions, RSM-932A shows no detectable toxicity in mice at highly effective doses with 77% tumor growth inhibition. The results shown here provide a profile for RSM-932A that has made this compound candidate for further clinical studies in humans. Indeed RSM-932A (designated also as TCD-717) has completed a phase I clinical trial as the first ChoKα inhibitor to be tested in humans (38). This assay is a phase I dose-escalation study of TCD-717 in patients with advanced solid tumors. The objectives of this study are to evaluate the safety of the drug and to determine the MTD and appropriate dose for phase II studies. Secondary objective is to measure the efficacy of TCD-717. A substudy conducted in the MTD confirmation cohort only evaluates the potential correlation between the levels of tumor choline and tumor response to TCD-717, using magnetic resonance spectroscopy. Pharmacokinetics analysis has been performed on patients enrolled in the MTD confirmation cohort. Results of this trial will be released elsewhere.

Cancer research must provide new molecular targets to expand current therapeutic strategies against cancer. Personalized therapies based on individual molecular profiles have proven more efficient, by reducing side effects and improving clinical outcomes (39). ChoKα expression levels have been reported as an independent prognostic biomarker for early-stage NSCLC (40) and for hepatocarcinomas (41). Thus, taking into consideration the status of ChoKα expression levels in the tumor may represent significant and valuable information to treat patients with this novel family of therapeutic tools.

Table 4. Antitumoral activity of RSM-932A in nude mice in different strains, models of xenografts, schedules, concentrations, and durations of treatments

Xenograft cell lines	Route of administration	Mouse strain	Concentration (mg/Kg)	Schedule of treatment	Weeks of treatment	Number of tumors analyzed	% Growth inhibition	Statistical significance
HT-29	IP	Athymic nu/nu	7.5	5-9-5[a]	3 wks	18	69%	0.001
HT-29	IP	Athymic nu/nu	7.5	1 d per wk	3 wks	10	77%	0.015
HT-29	IP	CD1 nude	6	5-9-5[a]	3 wks	8	68%	0.017
HT-29	IP	BALB/c nude	5	1 d per wk	4 wks	22	33%	no
HT-29	IP	BALB/c nude	5	2 d per wk	4 wks	14	55%	0.041
HT-29	IP	BALB/c nude	5	3 d per wk	4 wks	19	58%	0.001
HT-29	IV	BALB/c nude	3	2 d per wk	4 wks	20	45%	0.00001
HT-29	IV	BALB/c nude	1	2 d per wk	4 wks	20	41%	0.0005
HT-29	IV	BALB/c nude	0.3	2 d per wk	4 wks	20	33%	0.002
HT-29	IV	BALB/c nude	3	3 d per wk	4 wks	20	62%	0.0002
HT-29	IV	BALB/c nude	1	3 d per wk	4 wks	20	41%	0.0001
HT-29	IV	BALB/c nude	0.3	3 d per wk	4 wks	20	44%	0.0001
HT-29	IV	BALB/c nude	0.3	Daily	4 wks	20	40%	0.001
MDA-MB-231	IP	BALB/c nude	5	3 d per wk	3 wks	10	55%	0.042
H-460	IP	BALB/c nude	6	5-9-2[a]	3 wks	8	65%	0.047

NOTE: The xenograft model of human colon cancer cell line HT-29 was used using different genetic backgrounds of athymic mice, different concentrations of the drug, different schedules, and routes of administration. Number of tumors analyzed, the growth inhibition percentage taking as 100% the growth of the control tumors treated with vehicle, and the statistical significance are shown for each experiment. The human MDA-MB-231 and H-460 xenograft models are also shown.
[a]5-9-5 and 5-9-2 refer to daily treatment for 5 days, a 9-day rest, and a final 5 or 2 days of treatment.

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Table 5. Panel of human cancer cell lines tested with RSM-932A for 72 hours and determination of IC_{50}, GI_{50}, TGI, and LC_{50} parameters

		RSM-932A (72 h)							
Type of tumor	**Cell line**	**IC_{50}**	**±SD**	**GI_{50}**	**±SD**	**TGI**	**±SD**	**LC_{50}**	**±SD**
Breast	MDA-MB-231	1.3	0.5	1.2	0.2	2.0	0.1	2.7	1.2
	T47D	2,2	0,9	1,3	0,8	NA	NA	>6	NA
	MDA.MB.468	2.4	1.0	1.6	0.4	3.1	1.6	>6	0.5
	SkBr-3	3.1	0.6	1.0	0.6	1.6	0.5	13.2	6.7
Lung	H510	1.4	0.3	1.0	NA	1.1	0.5	3.9	1.8
	H1299	1.9	0.1	1.0	0.0	1.9	0.7	7.4	3.0
	H460	1.9	0.5	1.0	0.0	1.7	0.6	5.2	1.3
Colon	HT-29	1.7	0.4	1.3	0.2	5.6	6.6	11.0	5.4
	HCT-116	1.8	0.3	1.0	0.3	1.7	1.0	>6	NA
	DLD-1	2.1	0.7	1.6	0.5	3.3	0.9	6.3	2.8
	SW620	2.1	0.7	1.6	0.5	3.3	0.9	6.3	2.8
Bladder	HT-1376	1.5	0.3	1.0	0.0	5.7	1.2	>6.5	NA
	TccSup	1.6	0.2	1.1	0.5	1.5	0.0	>6.5	NA
	SW780	1.6	0.4	1.4	0.3	2.3	0.5	8.2	3.1
	J82	2.3	1.3	1.6	1.2	NA	NA	5.0	2.1
Epithelial carcinoma	A431	2.2	0.1	1.5	0.5	NA	NA	3.5	0.7
Liver	Hep3B2	2.4	0.5	1.2	0.1	2.7	1.4	3.2	0.8
	HepG2	6.4	2.2	1.3	0.3	8.4	1.3	>12	NA
Ovary	OV-Car-3	3.4	0.3	1.7	0.2	2.9	0.4	5.5	0.5
	SK-OV-3	6.4	2.2	1.3	0.3	8.4	1.3	>12	NA
Bone	SAOS-2	1.3	0.1	<1.6	NA	2.2	0.7	5.1	1.5
Cervix	HeLa	1.7	0.7	1.0	NA	1.5	0.0	5.7	1.2
Kidney	769-P	1.4	0.2	1.0	0.4	2.4	1.2	>3.5	0.7
Melanoma	SKMel-28	1.3	0.3	0.5	0.0	0.4	0.1	1.5	0.4
	A-375	6.2	1.9	2.6	1.4	5.6	1.1	7.7	1.4
Pancreas	Mia.PaCa.2	2.3	0.2	1.7	0.3	3.5	0.4	5.5	1.4
Astrocytoma, gliocystoma	U-87	1.9	0.5	1.4	0.2	5.0	1.2	8.9	4.3
Control	MCF10-A	7.1	0.5	3.4	0.8	7.2	1.3	14.7	1.3

NOTE: All cell lines were tested at the same density of population except for SW780 and H510. Twenty-four hours after seeding the cells and before the exposure to the drug, a time zero data is read to calculate the GI_{50}, TGI, and LC_{50} parameters. Seventy-two hours after the complete exposure to the drug in increasing concentrations, the rest of the experiment is read out. Not available (NA) is due to a high variability in the data obtained. The data shown are an average of at least three independent experiments.

Development of acquired resistance to standard therapies is a major concern nowadays in the management of patients with cancer. Combination of standard chemotherapy with new-targeted drugs represents a new strategy to efficiently and specifically target cancer cells to overcome resistance in some cases, reducing conventional schedules or dosage of the standard therapy (42). *In vivo* combination studies using ChoKα inhibitors with standard antineoplastic agents such as 5-fluorouracil (5-FU) have proven to be a promising strategy to increase efficacy in colorectal tumors (37) in keeping with previous treatments including ChoKα siRNA downregulation and 5-FU (43). Furthermore, resistance to ChoKα inhibitors is related to an increase of acid ceramidase (ASAH1) expression in lung primary tumors and derived cancer cell lines, making the combination of ChoKα and ASAH1 inhibitors a promising strategy to improve clinical outcome (44). Although these results are consistent with a relationship between interference of ChoKα enzymatic activity by specific inhibitors and their antiproliferative and antitumor activity, several convergent recent findings suggest that other possible mechanisms may be involved in the oncogenic activity of ChoKα. Thus, inhibition of ChoKα expression or its enzymatic activity is related to inhibition of mitogenic signals such as the PI3K/Akt and MAPK pathway (27, 45, 46), and ChoKα has been found to form a functional complex with EGFR (47). Furthermore, the interaction of different inhibitors with the ChoKα enzyme follows diverse mechanisms including both choline competitive and noncompetitive inhibition (30, 33) and as a consequence, may induce differential *in vivo* outcomes as recently proposed (48). In particular, we have recently demonstrated that RSM-932A induces the degradation of its target ChoKα (23, 33) allowing to test for its specific inhibitory action in treated tumors. Whether ChoKα has alternative roles in the regulation of cell proliferation other than its involvement in phospholipid metabolism is an intriguing question that merits further investigation. Recent interesting findings indicate that ChoK inhibitors induce drastic effects in the regulation of transcription of genes involved in cell-cycle regulation (49) as well as uridine metabolism (37) or endoplasmic reticulum stress and unfold protein regulation (23). These results, along with the very low toxicity profile of RSM-932A found in mice, will allow develop different formulations that may be effective as either oral or intravenous treatments in humans. Pharmacokinetic analysis of treated patients will help clarifying any potential secondary effects due to interaction with additional targets.

Unraveling the relationship between metabolism and deregulation of cell proliferation has allowed finding alternative tumor markers and molecular targets useful to address specific unmet needs in clinical oncology. Here, we demonstrate that RSM-932A (TCD-717) is a potent antiproliferative drug against a broad spectrum of human tumor-derived cell lines. Also, a potent antitumoral activity against xenografts of human tumors from colon, breast, and lung cancer cells has been demonstrated. Thus, RSM-932A has a broad-spectrum antitumoral activity and based on its profile against the large panel of tumor cells analyzed, most likely could be used in many tumor systems with similar efficacy. Different formulations have been tested with DMSO as the most effective solvent. However, due to its

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intrinsic properties, other solvents required to efficiently dissolve the drug for human assays needed to be developed. RSM-932A has already finished a phase I clinical trial as the first-in-human drug targeting ChoKα. The specific formulations developed will be described when reporting the clinical outcome of the phase I trial.

Disclosure of Potential Conflicts of Interest

J.C. Lacal has ownership interest (including patents) in TCD Pharma SL and CSIC patent (ES200400072; 2004, April 1). No potential conflicts of interest were disclosed by the other author.

Authors' Contributions

Conception and design: J.C. Lacal, J.M. Campos
Development of methodology: J.C. Lacal
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.C. Lacal
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.C. Lacal, J.M. Campos
Writing, review, and/or revision of the manuscript: J.C. Lacal, J.M. Campos
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.C. Lacal
Study supervision: J.C. Lacal, J.M. Campos
Other (design and synthesis of ChoK inhibitors): J.M. Campos

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