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Original Articles

Inhibition of mutant *Kras*^{G12D}-initiated murine pancreatic carcinoma growth by a dual c-Raf and soluble epoxide hydrolase inhibitor *t*-CUPM

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ABSTRACT

Mutant *Kras* and chronic pancreatitis are the most common pathological events involved in human pancreatic cancer. It has been demonstrated that c-Raf is responsible for transmitting signals from mutant *Ras* to its downstream signals including MEK–ERK and for initiating carcinogenesis. The soluble epoxide hydrolase (sEH), a pro-inflammatory enzyme, generally inactivates anti-inflammatory and anti-pain epoxyeicosatrienoic acids (EETs). Herein, we have synthesized a novel compound of *trans*-4-{4-[3-(4-chloro-3-trifluoromethyl-phenyl)-ureido]-cyclohexyloxy}-pyridine-2-carboxylic acid methylamide (*t*-CUPM) via modifying the central phenyl ring of sorafenib and confirmed its dual inhibition of sEH and c-Raf by recombinant kinase activity assay. Pharmacokinetic analysis revealed that oral dosing of *t*-CUPM resulted in higher blood levels than that of sorafenib throughout the complete time course (48 h). The effect of *t*-CUPM on the inhibition of mutant *Kras*^{G12D}-initiated murine pancreatic cancer cell growth was determined using the mouse pancreatic carcinoma cell model obtained from *LSL-Kras*^{G12D}*/Pdx1-Cre* mice and showed that *t*-CUPM significantly inhibited this murine pancreatic carcinoma cell growth both *in vitro* and in mice *in vivo*. Inhibition of mutant *Kras*-transmitted phosphorylations of cRAF/MEK/ERK was demonstrated in these pancreatic cancer cells using Western blot assay and immunohistochemical approach. Modulation of oxylipin profile, particularly increased EETs/DHET ratio by sEH inhibition, was observed in mice treated with *t*-CUPM. These results indicate that *t*-CUPM is a highly potential agent to treat pancreatic cancer via simultaneously targeting c-Raf and sEH.

Introduction

Over one-third of human tumors carry an activated mutant *Ras* oncogene, including human pancreatic carcinomas in which more than 90% have the *Kras* gene mutation [1,2]. However, efforts to directly inhibit *Ras* posttranslational modifications have thus far been unsuccessful. An alternative approach is the development of inhibitors targeting signaling pathways downstream of RAS, including the mutant Ras-activated Raf–MEK–ERK pathway. b-Raf selective inhibitors block proliferation of b-Raf mutant cells *in vitro* and *in vivo*. But, surprisingly, they are not only ineffective but also paradoxically cause MEK–ERK activation in *Ras* mutant cells [3–5]. Further studies indicate that activation of the MEK–ERK pathway in the context of mutant *Ras* requires c-Raf [6]. Using mutant *Kras*-driven lung tumorigenesis in mice, it has been demonstrated that signaling through c-Raf, MEK and ERK, but not b-Raf, are essential for tumor initiation by mutant *Kras*, and c-Raf is responsible for transmitting signals from mutant *Ras* to MEK–ERK [6]. Thus, targeting c-Raf would be crucial for suppressing mutant *Kras* activated signaling and tumorigenesis.

Chronic pancreatitis is a well-recognized risk factor for pancreatic cancer [7]. In the process of long-standing chronic inflammation, aberrant metabolites of arachidonic acid, particularly cyclooxygenase and lipoxygenase mediated metabolites, play a crucial role in promoting carcinogenesis [8]. The third pathway of arachidonic acid metabolism is cytochrome P450-mediated epoxygenated and hydroxynated products. Epoxygenated products such as epoxyeicosatrienoic acids (EETs) inhibit inflammation through decreasing cytokine-induced endothelial cell adhesion molecule (VCAM) and reducing NF-κB and Iκκ kinase activities [9]. The soluble epoxide hydrolase (sEH) catalyzes the conversion of epoxyeicosatrienoic acids (EETs) into the dihydroxyeicosatrienoic acids (DHETs) and inactivates the EETS anti-inflammatory activities [10]. sEH inhibitor results in stabilizing EETs and increasing levels

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of EET/DHET ratios and has shown a potent anti-inflammatory activity in various rodent inflammatory disease models, mainly via reducing the production of nitric oxide, pro-inflammatory lipid mediators as well as inflammatory cell infiltration [9,11,12].

Sorafenib is a multiple kinase inhibitor, especially for pan-Raf and vascular endothelial growth factor (VEGF) receptor kinase inhibitor, and has a dramatic effect in treating highly angiogenic malignancies [13]. Recently we have found that sorafenib possesses sEH inhibitory activity, which is due to structural similarity with sEH inhibitor *trans*/-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) [13]. To improve the inhibitory activity against sEH and c-Raf, we have modified the central phenyl ring of sorafenib based on the following: first, replacement of a phenyl ring with a cyclohexyl ring in *t*-AUCB increases sEH inhibitory activity [14], and second, the structure–activity relationship (SAR) study of sorafenib focuses on similar planar heterocyclic rings [15]. Such structural modification led us to first synthesize cyclohexyl derivatives (*trans*- and *cis*-) replacing sorafenib, and expect these compound/s to have high potent activities against c-Raf and sEH.

In the present study, we have first synthesize a novel *trans*-cyclohexyl derivative, called *trans-trans*-5-{4-[3-(4-chloro-3-trifluoromethyl-phenyl)-ureido]-cyclohexyloxy}-pyridine-2 carboxylic acid methylamide (*t*-CUPM). *t*-CUPM contains the relatively flexible cyclohexyl ring present in *t*-AUCB and has the left side of the molecule of a 4-chloro-3-(trifluoromethyl) phenyl ring and the right side of pyridine-2-carboxylic acid methyl amide of sorafenib. The effect of *t*-CUPM against b-Raf, c-Raf and sEH and on inhibiting mutant *Kras* activated Raf–MEK–ERK pathway was examined using sEH enzyme assay, recombinant kinase activity assay, and *in vitro* and *in vivo* mouse pancreatic ductal carcinoma cell model derived from *LSL-Kras^{G12D}/Pdx1-Cre* mice. Pharmacokinetic (PK) profiles of *t*-CUPM and its comparison with sorafenib and *t*-AUCB were analyzed in mice. Using unique mutant *Kras*-driven murine pancreatic carcinoma cells, the effects of *t*-CUPM on inhibiting pancreatic carcinoma cell growth and mutant *Kras*-activated signals and on modulation of oxylipin metabolic profile using LC–MS/MS approach were further analyzed *in vivo* in mice.

Materials and methods

t-*CUPM synthesis and recombinant kinase activity assay*

The detailed synthetic procedures and methods of *t*-CUPM were described in the supplementary data. *t*-AUCB was also synthesized with our previously published method [14]. Sorafenib (free base) was purchased from LC Laboratories (Worburn, MA).

sEH enzyme assay followed our previous published method [14]. Recombinant kinase activity assay of b-Raf and c-Raf was performed using the ADP-Glo™ Kinase Assay (Promega, Madison, WI) as described by the manufacturer. IC_{50} values were calculated by quantifying the end-point ADP production from each kinase reaction. Individual data sets were performed in duplicate and each IC_{50} was determined by three separate experiments. The data were fit to a saturation curve using KaleidaGraph graphing program (Synergy Software) to determine the inhibition at 50% activity (IC_{50}).

Analysis of the effect of t-*CUPM, sorafenib, and* t-*AUCB on mouse pancreatic carcinoma cell growth* in vitro *and* in vivo

The mouse pancreatic ductal carcinoma cell line (PK03) was established from our lab that was derived from the *LSL-Kras^{G12D}/Pdx1-Cre* mice [16]. *Pdx1-Cre* recombined or activated mutant *Kras^{G12D}* gene was confirmed with PCR assay using genomic DNA extracted from the cell line. The expression of cytokeratin-19, amylase, and E-cadherin was determined immunocytochemically.

The colony formation assay was performed to determine the effect of *t*-CUPM on inhibiting PK03 cell growth in a dose dependent manner. Giemsa staining was used to highlight the colony, the cell cluster for more than 50 cells was considered as a colony. PK03 cells were further treated with *t*-CUPM, sorafenib or *t*-AUCB at a final concentration of 3, 10 or 30 μM for 24 hours. The cells were harvested and the cell lysate was used for extracting protein and western blot assay.

For *in vivo* PK03 cell growth in C57 B6/J mice, PK03 cells (3×10^6 cells per 100 μL per mouse) were injected subcutaneously to two hind legs of 8- to 10-week-old mice. *t*-CUPM, sorafenib or *t*-AUCB was administered to mice in the drinking water in the dose of 10 mg/kg body weight (n = 5 mice/group). Animals were fed with AIN93M diet until the end of the experiment. Food and water consumption, and body weight were monitored weekly. Mice were housed under pathogen-free conditions in the facilities of Laboratory Animal Services, Northwestern University. All studies were conducted in compliance with the Northwestern University IACUC guidelines. Tumor development was monitored daily. Mice were sacrificed at the end of 35 days when tumor size reached 1 cm in diameter. Blood and plasma were collected via heart acupuncture after being euthanized by CO_2 and stored in a –80 °C freezer until analysis. Tumors were dissected and measured for size (length and width) and weight. Tumor volume was determined using the equation $V\ (mm^3) = L \times W^2/2$. Tumors were then fixed in 10% buffered formalin and processed for paraffin sections for histopathological and immunohistochemical analyses.

Immunohistochemical staining was performed according to our routine protocol using an avidin–biotin–peroxidase method [16]. The primary antibodies include rabbit polyclonal anti-myeloperoxidase (MPO) (Abcam, Cambridge, MA, USA), mouse monoclonal anti-Ki-67 antibody (Calbiochem, Gibbstown, New Jersey, USA), and rabbit monoclonal anti-phospho-ERK1/2 (Cell Signaling Technology, Boston, MA, USA) antibody. The appropriate biotinylated secondary antibody and avidin–biotin–peroxidase complex (Vector Lab, Burlingame, CA, USA) were used for detecting antigen–primary antibody complex. A characteristic brown color was developed by incubation with 3,3-diaminobenzidine substrate chromogen system (Sigma-Aldrich, St. Louis, MO, USA). The negative control was established by replacement of primary antibody with normal serum, and an appropriate positive control was used for each primary antibody. Positive cells and staining intensity were quantified for all antibodies using an Olympus BX45 microscope and DP70 digital Camera. The full image of each pancreas was snapped under X2.5 and X20 objective lens and saved with >2560 resolution image. Using 'histogram analysis' in the Photoshop program, specific staining intensity and percentage of positive staining cell number in total cells counted were measured for at least 10 defined areas/images in each tumor and automatically expressed as mean value ± SD.

Protein extraction and western blot assay

Freshly harvested pancreases or cultured cell pellets were homogenized and lysed in ice-cold RIPA lysis buffer (Santa Cruz). The lysates were separated by centrifugation at 12,000 × *g* for 5 minutes at 4 °C; the supernatants were collected and aliquots were made. All protein concentrations were determined using the Bradford reagent (Thermo Scientific, IL, USA). An aliquot (30 μg protein/lane) of the tissue lysate was separated by 10% SDS-PAGE gel, and then the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The primary antibodies included antibodies against c-Raf, MEK, ERK and their phosphorylated form protein, and all of these antibodies were from Cell Signaling Technology (Boston, MA, USA). The membranes were further incubated with HRP-linked anti-rabbit IgG and HRP-linked anti-biotin antibodies (Cell Signaling Technology). The protein–antibody complexes were detected by using the chemiluminescent substrate according to the manufacturer's instructions and the emitted light captured on X-ray film. The intensity of each band was analyzed using 'histogram analysis' in the Photoshop program.

Pharmacokinetic (PK) study of t-*CUPM, sorafenib, and* t-*AUCB in mice*

Male Swiss Webster mice (10-week-old, 30–35 g) were used for PK studies. An equal amount of sorafenib, *t*-AUCB and *t*-CUPM was dissolved in oleic acid-rich triglyceride containing 10% PEG400 (v/v) to give a clear solution for cassette oral administration at a dose of 1 mg/kg body weight (N = 3). Blood (10 μL) was collected from the tail vein using a pipette tip rinsed with 7.5% EDTA(K3) at 0, 0.5, 1, 1.5, 2, 4, 6, 8, 24 and 48 h after administration of the inhibitors. Each blood sample was immediately transferred to a tube containing 50 μL of water containing 0.1% EDTA. After being mixed strongly on a Vortex for 1 min, all samples were stored at –80 °C until analysis.

The extraction of these inhibitors from blood was performed by a slight modification of a previous method [17]. The blood concentrations of compounds were determined using the HPLC–MS/MS method, which was validated to assure acceptable accuracy and precision (accuracy more than 95% with RSD less than 10%). Analytes were detected by negative mode electrospray ionizations tandem quadrupole trap mass spectrometry in multiple reaction-monitoring mode (MRM) on a Trap 4000 Mass Spectrometer (ABI, Milford, MA). The parameters of the MS condition were the same as presented previously [17]. The precursor and dominant product ions used to set up the transition monitored in the MRM mode were 463.1 and 194 for sorafenib, and 469.1 and 194 for *t*-CUPM, respectively.

Analysis of oxylipin metabolic profile using a liquid chromatography/tandem mass spectrometry (LC–MS/MS) method

Plasma specimens were spiked with 10 μL of 50 nM internal standard (d11-14, 15-DHET, d11-11(12)-EET, d4-PGE2, d4-LTB4, and d8-5-HETE) and were extracted by solid phase extraction using Oasis HLB cartridges (3 cc 60 mg, Waters, Milford, MA). LC–MS/MS analysis of oxylipins was performed using a modified method based on a previous publication [18]. An Agilent 1200 SL liquid chromatography series (Agilent Corporation, Palo Alto, CA) with an Agilent Eclipse Plus C_{18} 2.1 × 150 mm,