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Down-regulation of 20-HETE Synthesis and Signaling Inhibits Renal Adenocarcinoma Cell Proliferation and Tumor Growth

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Abstract

Background—We examined the ability of inhibitors of the synthesis or actions of 20-HETE, metabolite of arachidonic acid, to inhibit proliferation of human renal carcinoma cell lines.

Materials and Methods—786-O and 769-P cells were exposed to either 10 μ M HET0016 (selective inhibitor of 20-HETE synthesis), 10 μ M WIT002 (20-HETE antagonist), or vehicle. Subsequently, we assessed the effect of WIT002 on tumor growth *in vivo* using an ectopic mouse model of clear-cell renal carcinoma.

Results—Addition of HET0016 and WIT002 inhibited the proliferation of 786-O and 769-P human renal cell carcinoma lines. HET0016 and WIT002 had little effect on the proliferation of primary cultures of normal human proximal tubule epithelial cells. WIT002 (10 mg/Kg, s.c.) administered daily to athymic nude mice implanted subcutaneously with 786-O cells reduced the growth of the tumors by 84 % compared to vehicle ($p < 0.001$).

Conclusion—20-HETE is required for proliferation of human renal epithelial cancers.

Keywords

Renal Adenocarcinoma; CYP4A; 20-hydroxyeicosatetraenoic acid; eicosanoids; xenograft model

Renal cell carcinoma comprises 90-95% of all neoplasms of the kidney (12) and is the sixth leading cause of cancer deaths. Renal cell carcinomas are highly proliferative and metastatic and very resistant to conventional treatment (chemotherapy, hormonal therapy and radiotherapy). Renal clear cell carcinomas grow rapidly and are associated with very poor patient survival (16). Thus, there is a tremendous need for the development of new treatment strategies. Recent studies have implicated a potential role for cytochrome P450 metabolites of arachidonic acid in the pathogenesis of several forms of cancer. Cytochrome P450 enzymes of the 4A and 4F families (CYP4A and CYP4F) catalyze omega-hydroxylation of arachidonic acid (AA) to form 20-hydroxyeicosatetraenoic acid (20-HETE) (8). 20-HETE stimulates mitogenic and angiogenic responses both *in vitro* and *in vivo* (9). Inhibitors of the synthesis of 20-HETE have been reported to block the angiogenic response of a variety of growth factors including VEGF, FGF, EGF *in vivo* and the proliferation U235 and 9L gliosarcoma cells *in vitro* and the growth of these tumors *in vivo* (4-6). Since renal epithelial cells normally express high levels of enzymes of the CYP4A and CYP4F families and avidly produce 20-HETE when incubated with arachidonic acid it is likely that renal cell carcinomas retain the ability to produce 20-HETE. Thus, in the present study we examined the effects of an inhibitor of the

synthesis of 20-HETE and a 20-HETE antagonist on the proliferation of renal adenocarcinoma cells *in vitro* and tumor growth *in vivo*.

Materials and Methods

Cell culture

Human renal cell adenocarcinoma lines 786-O and 769-P were obtained from ATCC and grown according to the supplier recommendations. Normal primary cultured human renal proximal tubule epithelial cells (RPTC) were obtained from Cambrex Bio Science and were cultivated according to manufacturer protocols using REGM Bullit Kit.

20-HETE and EETs measurements using LC/MS/MS

The renal cells were collected and homogenized in 1 ml of a 0.1 M KPO₄ buffer containing 5 mM MgCl₂ and 1 mM EDTA. The homogenate was incubated for 30 min at 37°C with a saturating concentration of arachidonic acid (40 µM) and 1 mM NADPH. The reaction was stopped by acidification with formic acid to pH 3.5 and extracted twice with 3 mls of ethyl acetate after the addition of 2 ng of an internal standard d⁶-20-HETE. The organic phase was dried under nitrogen. The metabolites of AA were separated by HPLC on a Betabasic C₁₈ column (150×2.1 mm, 3 µm; Thermo Hypersil-Keystone, Bellefonte, PA) at a flow rate of 0.2 mL/min using an isocratic elution with 51:9:40:0.01 mixture of acetonitrile: methanol:water:acetic acid for 30 minutes followed by a step gradient to 68:13:19:0.01 acetonitrile:methanol:water:acetic acid for 15 minutes. The effluent was ionized using negative ion electrospray and peaks eluting with a mass/charge ratio (m/z) of 319>245 (20-HETE), 319>301 (HETEs and EETs), 337>319 (DiHETEs), or 325>251 (internal standard) were monitored using an Applied Biosystems API 3000 LC/MS/MS. The ratio of ion abundance in the peaks of interest versus that seen in the internal standard were determined and compared with standard curves generated over a range from 0.2 to 10 ng for 20-HETE and from 1.0 to 10 ng for the other metabolites.

Real-time RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) following standard manufacturer's instruction. Reverse transcription of mRNA was carried out using iScript cDNA synthesis kit (BioRad Laboratories). cDNA was diluted 50-fold and a 2 microliter sample used in duplicate real-time PCR experiments using iQ SYBR Green (BioRad Laboratories) and Mx3000P real-time PCR system (Stratagene). Reaction cycling parameters were as follows: 1 cycle of 3 minutes at 95°C followed by 45 cycles of 10 seconds 95°C and 45 seconds at 58°C. LinRegPCR software was used to calculate PCR efficiencies (11) and geNORM software used to determine normalization genes (15). Primer sequences were: CYP4A11 – TGCTGATCAAGGCAGTTCAG (F) and GCTCCTGGTCCTGTTGG (R), CYP4A22 – GATCAAGGCAGCTCAG CTCT (F) and GTAGCTCCTGGTCGTGTTGG (R), CYP4F2 – AAGCACCCAGAATACCAGGA (F) and TCATGCACATGGTCAGGAAG (R), CYP4F3 – CTGTCCGACAGGAGGTACAAG (F) and CCTCAGGCTCTCCTTAATGC (R). Human housekeeping gene primer sets for *b2m* and *hprt1* were purchased from RealTimePrimers.com.

Ectopic mouse model of clear cell renal carcinoma

Experiments were carried out on 6-week immunodeficient athymic nude mice weighing 20-26g (Homozygous mice, strain code 194, Charles River Laboratories, Wilmington MA). Animals were acclimated for 1 week prior to injection with renal cell carcinoma. Immediately before each implantation, the cells were trypsinized, counted and resuspended in 10% serum containing RPMI media. The concentration of cells was adjusted to 40 millions/ml and 4

million cells/animal were injected subcutaneously. The cells were allowed to grow for 7-15 days until the size of the tumors reached approximately 0.1 cm³. The mice then received daily *s.c.* injections of WIT002 (10 mg/kg/day in 200 μ l) in an isotonic NaPO₄ buffer (pH 9.0) or vehicle (0.1 M NaPO₄ solution pH 9.0). The diameter of the tumor was measured on every 3-4 days for 2 weeks using precision calipers. Tumor volume was calculated using the formula: $\text{volume} = 4/3\pi r^3$ as previously described (1). At the end of the experiment the mice were euthanized with CO₂ and the tumors were excised to confirm the diameter measurements.

Results

Inhibition of 20-HETE synthesis decreases 786-O and 769-P cell proliferation

Recently, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo-2 ligand has received a lot of attention because the majority of tumor cell lines tested *in vitro* were sensitive to recombinant TRAIL, whereas it displayed minimal cytotoxicity towards normal cell lines (2;3). As demonstrated in Figure 1 we studied the effect of 20-HETE inhibitors on a TRAIL-sensitive (769-P) and TRAIL-insensitive (786-O) renal carcinoma cell lines. In these experiments the medium was changed to serum-free containing the mitogens EGF or Endothelin-1 (ET-1). Both EGF and ET-1 are known to play important role in the growth of renal cancer cells. We found that HET0016 [N-hydroxy-N'-(4-butyl-2 methylphenyl) formamidine], a selective inhibitor of the synthesis of 20-HETE reduced the proliferation of renal cell carcinoma 786-O and 769-P cells by 66% and 40%, respectively (Figure 1).

Inhibition of 20-HETE signaling decreases 786-O and 769-P cell proliferation

We also examined the effects of the 20-HETE antagonist 20-6,15 HEDE on the proliferation of the TRAIL-sensitive (769-P) and TRAIL-resistant (786-O) renal carcinoma cells (Figure 2 A, B, C, D). 20-6,15 HEDE completely inhibited the proliferation of both cell lines whether proliferation was stimulated by ET-1 or EGF.

HET0016 and WIT002 are not cytostatic to normal proximal tubule epithelial cells

Experiments were also performed using early passages of human primary renal proximal tubule epithelial cells (RPTC) to access the direct cytotoxic effects of HET0016 and 20-6,15 HEDE. As illustrated in Figure 2E, both HET0016 and WIT002 had little effect on the proliferation of normal human renal proximal tubular cells.

Renal adenocarcinoma cells express Cyp4F2 and Cyp4F3 mRNAs

We examined renal adenocarcinoma cells 786-O and 769-P for the presence of mRNAs encoding 4 human Cyp450 isoforms: CYP4A11, CYP4A22, CYP4F2 and CYP4F3. The relative mRNA expression levels of CYP4F2 was about 3 times higher in 786-O cells when compared to 769-P cells, as detected by real-time RT-PCR. The levels of CYP4F3 mRNA did not differ significantly in 786-O and 769-P cells, whereas CYP4A11 and CYP4A22 mRNAs were not detected.

Effects of HET0016 on the metabolism of arachidonic acid in renal carcinoma cells

The results of these experiments are presented in Figure 3. LC/MS/MS analysis revealed the presence of 15-, 12-, 5-HETEs and 14,15-, 11,12-, 8,9-EETs, but not 20-HETE in renal adenocarcinoma cells 786-O (Figure 3A). Incubation of the cells for 30 minutes with AA increased the levels of 20-HETE and DiHETEs, whereas the levels of EETs, 15-, 12- and 5-HETEs were not altered (Figure 3B). Addition of HET0016 (10 μ M) selectively reduced the formation of 20-HETE in 786-O cells incubated with AA (Figure 3C).

WIT002 suppresses renal adenocarcinoma cell proliferation *in vivo*

The effect of the 20-HETE antagonist, WIT002 on the growth of 786-O clear cell renal carcinoma was assessed in ectopic mouse model of renal tumor (Figure 4). The growth of tumors was significantly suppressed by WIT002 administered daily to athymic nude mice implanted subcutaneously with cells 786-O. Tumor growth was inhibited by $84\% \pm 12\%$, where 84 is average and 12 is SEM. It is of note that in these experiments WIT002 treatment started only after the tumor was seeded for 7-14 days and was relatively large 0.1 cm. Thus, WIT002 was effective at arresting the growth of a fairly advanced tumor.

Discussion

Preceding studies have indicated that enzymes of the CYP4A and CYP4F families are expressed in normal human renal tissue and it avidly produces 20-HETE (13). However, no one has studied whether renal clear cell carcinomas retain the ability to produce 20-HETE. In the current study we found that renal adenocarcinoma cells expressed CYP4F isoforms and produce 20-HETE. Moreover, inhibition of 20-HETE synthesis by HET0016 and an administration of an analog of 20-HETE that interferes with its acting did suppress proliferation of cultured renal carcinoma cells *in vitro*. It was previously reported that 5-Lipoxygenase inhibitors attenuate growth of human RCC (7) whereas COX-2 inhibitors have only slight anti-proliferative effects against RCC (17). Our data is the first indication that another class of enzymes, which utilizes arachidonic acid, the CYP4F family of proteins, is playing important role in regulation of renal carcinoma growth.

The molecular mechanisms of 20-HETE signaling in the proliferation of epithelial cancer cells are unknown, Nevertheless, it has been shown that enforced overexpression of CYP4A and, correspondingly, generation of 20-HETE in human glioma cells *in vitro* is accompanied by increased signaling via ERK cascade and enhanced expression of cyclin D1/2 and VEGF (4). When epithelial cells, which participate in formation of renal cysts, were genetically modified to overproduce CYP4A12, it caused a four- to five-fold increase in cell proliferation compared with control cells, and this increase was completely abolished when 20-HETE synthesis was inhibited (10). Long term inhibition of 20-HETE signaling was accompanied by decrease of EGFR phosphorylation in mouse kidneys suggesting a possibility of coupling 20-HETE with transactivation of EGFR (10). Aberrant signaling of multiple members of the epidermal growth factor receptor (EGFR) family often causes uncontrolled proliferation of cancer cells. It is of note that transactivation of EGFR by prostaglandins, products of Cox-2 activity, was reported in a number of cancer cells (14). Whether 20-HETE, product of CYP450 enzymes, is capable of EGFR transactivation remains to be determined.

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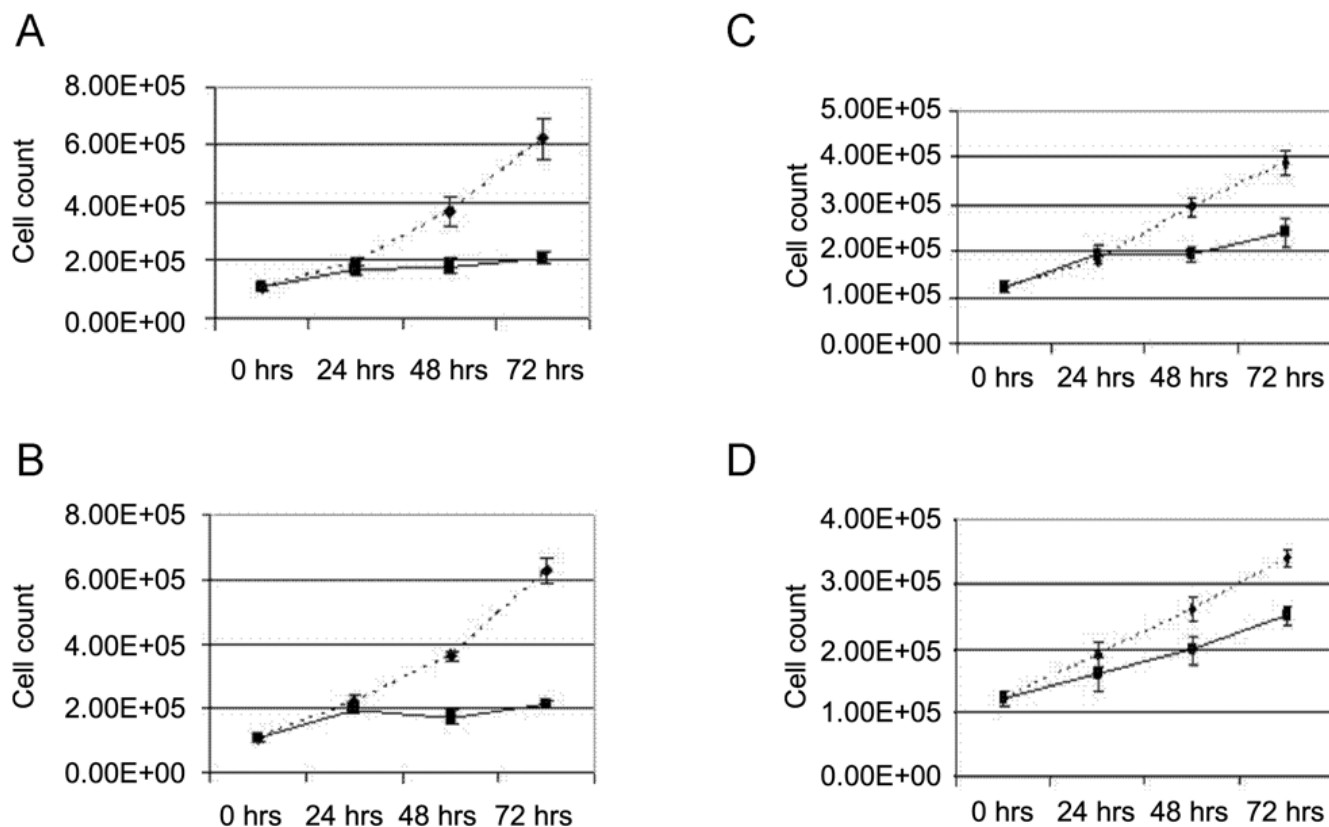


Figure 1. HET0016 inhibits proliferation of 786-O and 769-P renal adenocarcinoma cells. Equal numbers of either 786-O (A, B) or 769-P (C, D) cells were plated and next day (0 hrs) transferred to serum-free medium containing either EGF (A, C) or ET-1 (B, D). Cells were exposed either to 10 μ M HET0016 (solid lane), or vehicle (dotted lane). Cell counting was performed at the day of transfer to serum free medium (0 hrs) and 24, 48 and 72 hours thereafter. Medium was changed to fresh containing mitogens and drugs every 24 hours. Data presented are characteristic experiment from at least two separate experiments, each performed in triplicate.

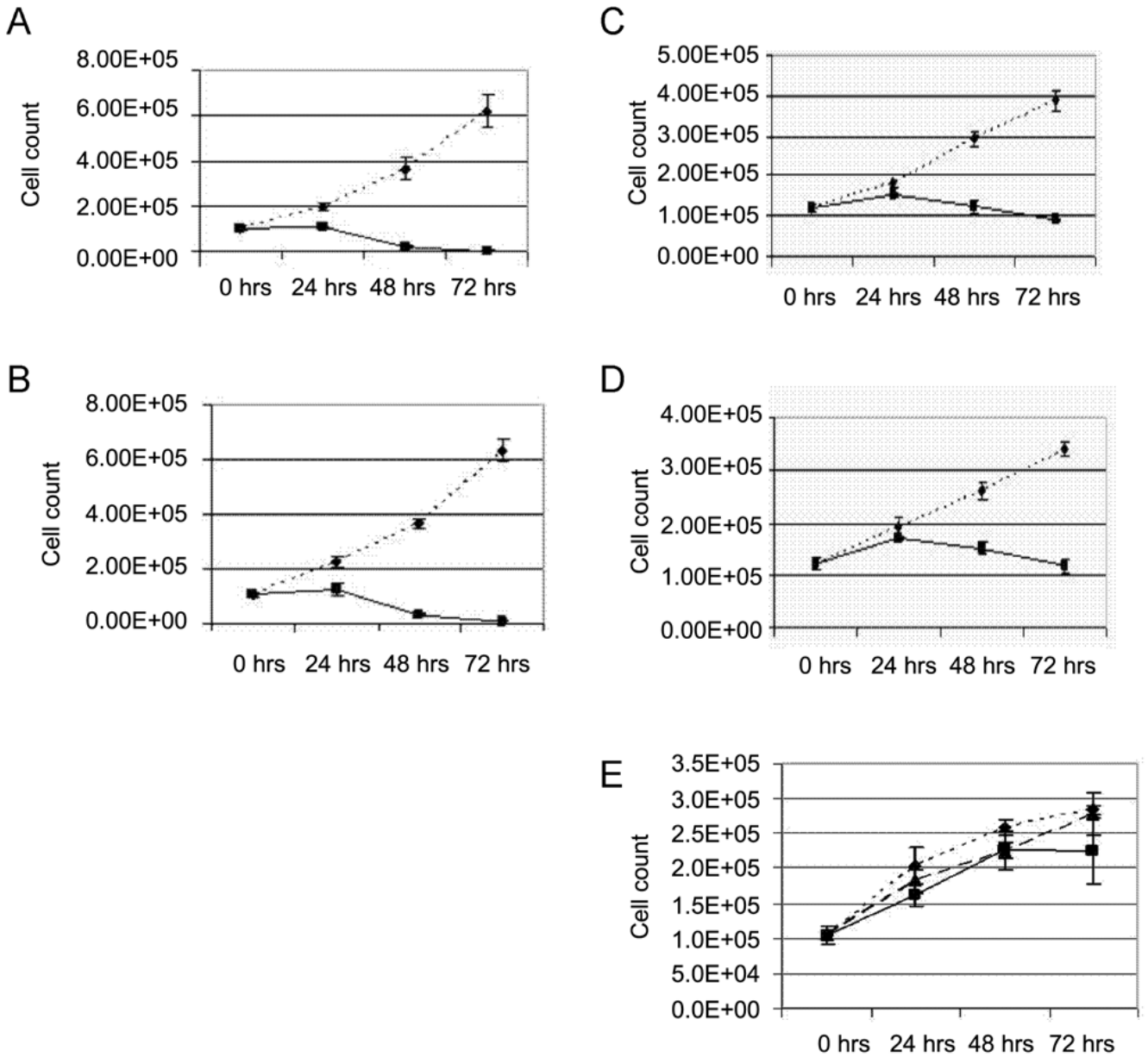


Figure 2. WIT002 inhibits proliferation of 786-O and 769-P renal adenocarcinoma cells (A-D), but HET0016 and WIT002 fail to inhibit proliferation of normal renal epithelial cells RPTC (E). Equal numbers of 786-O (A, B) or 769-P (C, D) cells were plated and next day (0 hrs) transferred to serum-free medium containing either EGF (A, C) or ET-1 (B, D). Cells were exposed either to 10 μ M WIT002 (solid lane), or vehicle (dotted lane). Cell counting was performed at the day of transfer to serum free medium (0 hrs) and 24, 48 and 72 hours thereafter. Medium was changed to fresh containing mitogens and drugs every 24 hours. Data presented are characteristic experiment from at least two separate experiments, each performed in triplicate. (E) Equal numbers of RPTC cells were plated and next day (0 hrs) transferred to medium containing either vehicle (diamonds, dotted lane), HET002 (squares, dotted lane) or WIT002 (triangles, solid lane). Concentrations of HET0016 and WIT002 were 10 μ M. Cell counting was performed at the day of transfer to serum free medium (0 hrs) and 24, 48 and 72

hours thereafter. Data presented are characteristic experiment from at least two separate experiments, each performed in triplicate.

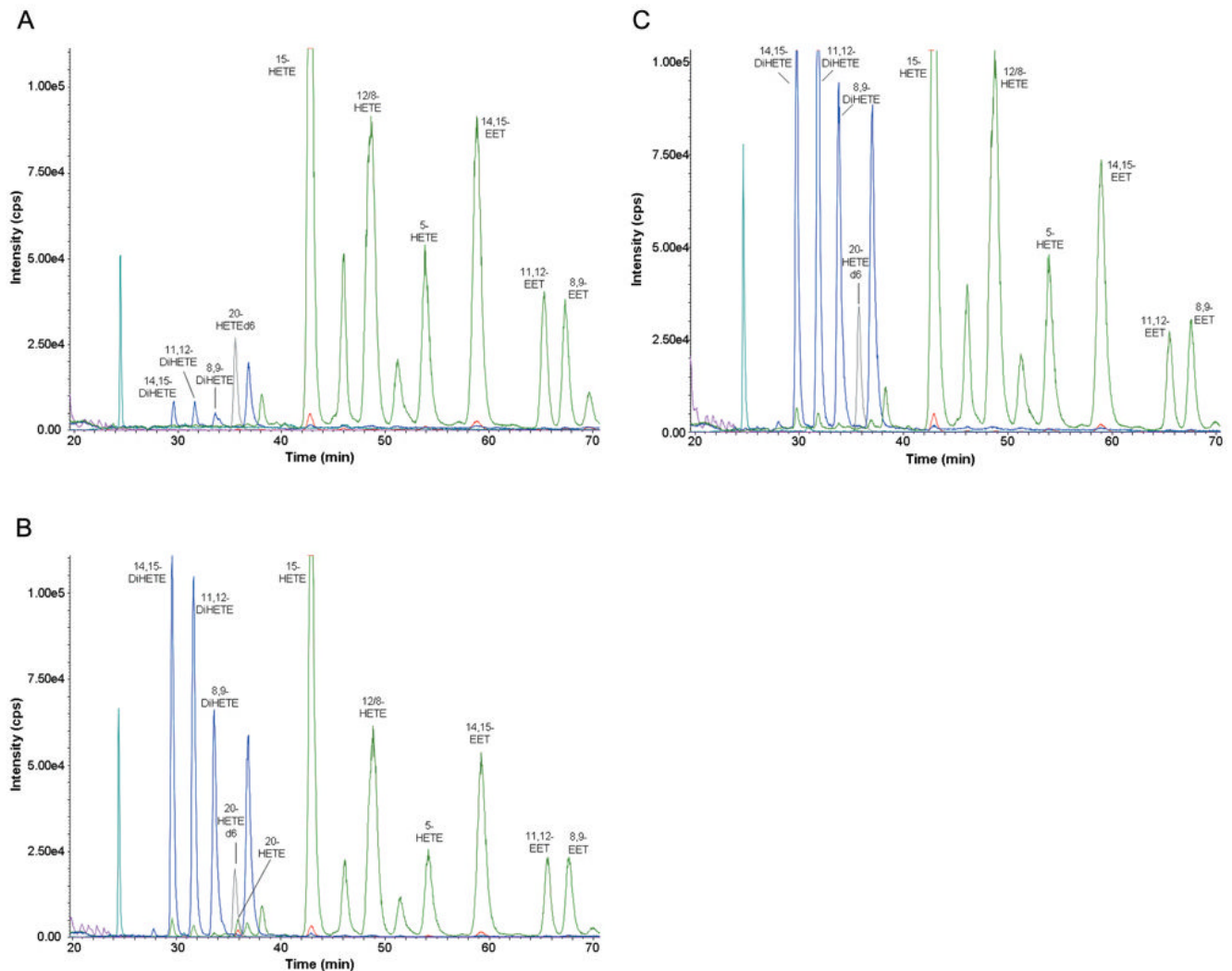


Figure 3. Liquid Chromatography/Mass Spectroscopy profiling of eicosanoids in 786-O renal adenocarcinoma cells. 786-O cells were plated, harvested, pelleted, and resuspended in serum-free medium and quickly frozen in liquid N₂. LC/MS analysis was performed as described in Materials and Methods. Profiling of eicosanoids was performed either without incubation with arachidonic acid (A), or after 30 min incubation with arachidonic acid in the absence (B) or presence (C) of HET0016.

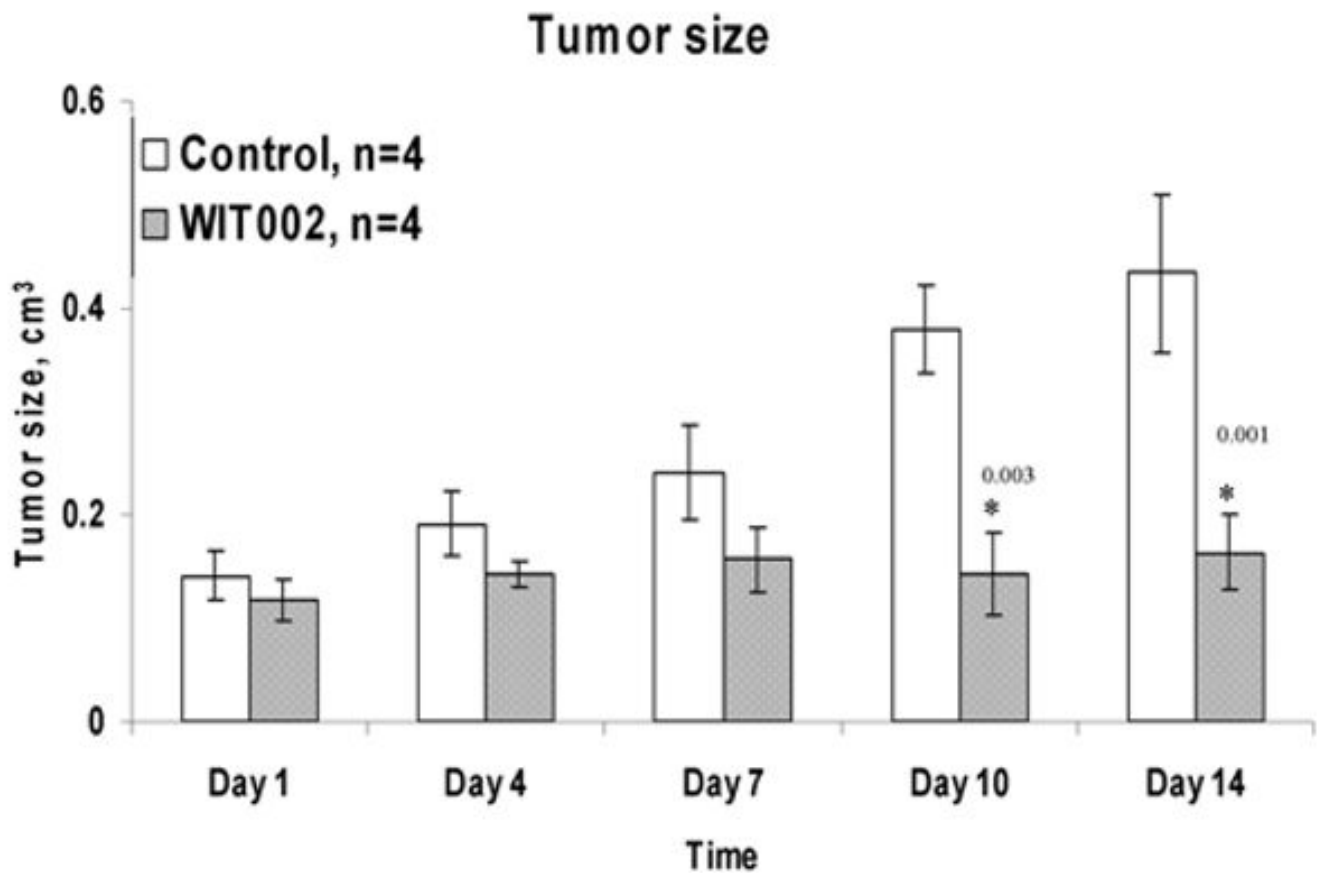


Figure 4.

WIT002 decreases tumor growth in ectopic mouse model of clear cell renal carcinoma. During the treatment period the mice had their tumor size measured at different time points by a precision hand measuring ruler (obtained from Scienceware) and tumor volume was calculated as described in Materials and Methods.