Effect of Sulindac Sulfide on Metallohydrolases in the Human Colon Cancer Cell Line HT-29

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Abstract

Matrix metalloproteinase 7 (MMP7), a metallohydrolase involved in the development of several cancers, is downregulated in the Apc<sub>Minn/+</sub> colon cancer mouse model following sulindac treatment. To determine whether this effect is relevant to the human condition, HT-29 human colon cancer cells were treated with sulindac and its metabolites, and compared to results obtained from <i>in vivo</i> mouse studies. The expression of MMP7 was monitored. The results demonstrated that sulindac sulfide effectively downregulated both MMP7 expression and activity. Furthermore, activity-based proteomics demonstrated that sulindac sulfide dramatically decreased the activity of leukotriene A4 hydrolase in HT-29 cells as reflected by a decrease in the level of its product, leukotriene B4. This study demonstrates that the effect of sulindac treatment in a mouse model of colon cancer may be relevant to the human counterpart and highlights the effect of sulindac treatment on metallohydrolases.

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), such as sulindac, are chemo-preventive reagents towards colorectal cancers [1,2,3]. This effect is consistent with observations <i>in vitro</i> in human colon cancer cells [4], as well as <i>in vivo</i> in the Apc<sub>Minn/+</sub> mouse model, where a large decrease in tumor burden is observed [5]. The gastrointestinal complications that develop in NSAID users are well documented [6], and represent an obstacle in the use of these drugs as chemo-preventive agents. The pleiotropic effects of sulindac on colon cancer prevention are still unclear and hinder the development of more specific treatments with diminished side effects. Regarding this issue, it has been shown that sulindac treatment causes changes in extracellular matrix remodeling events, including downregulation of matrix metalloproteinase 7 (MMP7) in intestinal adenomas of Apc<sub>Minn/+</sub> mice [7].

MMP7 belongs to a group of metal ion-dependent proteases. According to the MEROPS database (www.merops.sanger.uk), matrix metalloproteinases (MMPs), consisting of 24 members, comprise the M10 subfamily of the MA family of the metallohydrolases clan M. MMPs have been strongly implicated in the development of many cancers. Deletion of MMP2 and MMP9 in mice have been shown to reduce pancreatic tumorigenesis by reducing angiogenesis [8]. Pertinent to this study, MMP7 deletion in Apc<sub>Minn/+</sub> mice has been shown to strongly reduce intestinal tumor burden [9]. These observations implicate MMP7 as a viable target for the development of novel treatment regimes.

The current study determined the effect of sulindac treatment on MMP7 in the human colon cancer cell line, HT-29, and utilized a global approach to detect altered activities of other metallohydrolases. The results of these investigations are described herein.

Materials and Methods

Cell culture

HT-29 cells (American Type Culture Collection, Manassas, VA) were maintained in McCoy’s 5A medium (Gibco, Grand Island, NY) with 10% fetal bovine serum at 37°C in an atmosphere of 95% air/5% CO2. Sulindac, sulindac sulfide, and sulindac sulfone were purchased from Sigma Aldrich (St. Louis, MO). These drugs were diluted in dimethyl sulfoxide (DMSO). DMSO was added to the media at a final concentration of 1% and cells were cultured in 6-well plates. Once cells were 80% confluent, they were treated with various concentrations of sulindac and its metabolites. After 24 hr, the cells were trypsinized and viable cells counted using the trypan blue method.

Gene expression

HT-29 cells, at the time of harvest, were trypsinized, centrifuged, and the cell pellet used to extract RNA following the Qiagen protocol. Concentration and quality of the samples were assessed using the spectrophotometer obtained from the NanoDrop-1000 (Thermo Scientific, Wilmington, DE). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described [7]. Primers and probes designed for human MMP7, MMP25, Trypsin1, and RPL-19 are shown in Table 1.
Activity-based proteomics for the identification of activated metallohydrolases

Proteomes were adjusted to a concentration of 1 mg/ml in PBS. A cocktail of alkyne probes targeting the active site of metallohydrolases was a generous gift of Benjamin F Cravatt (The Scripps Research Institute, La Jolla, CA). Labeling was performed as previously described [10]. Briefly, probes were added to a final concentration of 1 uM and incubated under UV light for 1 hr. Rhodamine azide was then bound to the probes using click chemistry [11]. Samples were electrophoresed on a 10% polyacrylamide gel and scanned using either a Hitachi FMBio IIe flatbed scanner (MiraBio, Alameda, CA) or the KODAK In-Vivo Multispectral System FX (Carestream Health, Rochester, NY).

Identification of activity-based labeled proteins

Real-size gel scan prints were used as a template to extract the desired bands and scanned afterwards to assure correct slicing of the gel. Each gel plug was reduced with 10 mM dithiothreitol in ammonium bicarbonate and alkylated with 53 mM iodoacetamide. Samples were digested with 0.1 uC trypsin (Promega, Madison, WI) in 100 uC of 10 mM ammonium bicarbonate and incubated overnight at 37°C. Tryptic peptides were extracted from gel plugs with 50% acetonitrile/49.9% H2O/0.1% trifluoroacetic acid followed by 100% acetonitrile and finally with 0.1% trifluoroacetic acid.

Tryptic peptides were chromatographed on a 100 uC × 10 cm C18 column (particle size: 5.m) and eluted using a linear gradient of 3 to 45% acetonitrile in H2O over 70 min at room temperature, at a flow rate of 500 nl/min. The eluent was electro-sprayed into the linear quadrupole ion trap (LTQ) mass spectrometer (Thermo-Fisher Scientific, Waltham, MA). Sequest and X!Tandem algorithms were used to search the database utilizing the international protein index (IPI) mouse database.

Leukotriene B4 enzyme immunoassay

At 80% confluence, HT-29 cells were incubated with either sulindac sulfide or DMSO for 24 hr. The secreted proteome was obtained and concentrated by vacuum centrifugation. Proteomes were adjusted to 5 mg/ml and LTB4 quantified with an enzymatic immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Results

The reduced metabolite of sulindac, sulindac sulfide, selectively increases cell death of HT-29 cells

HT-29 cells were treated with sulindac, sulindac sulfide, and sulindac sulfone at concentrations ranging from 10 to 1000 uM (Figure 1). Starting at 250 uM, sulindac sulfide significantly increased cell death, reaching 100% cell death at 500 uM. In contrast, sulindac and sulindac sulfone failed to significantly increase cell death, even when the drugs where added at mM concentrations.

Sulindac sulfide reduces MMP7 at the mRNA, protein, and activity levels

It was reported that two days of sulindac treatment is sufficient to downregulate MMP7 in tumors of ApoE−/− mice [7]. In order to determine which metabolite effectively diminished MMP7, HT-29 cells were treated with sulindac and its metabolites at 100 uM, a concentration not toxic to the cells as demonstrated in figure 1, for 24 hr. The expression profile of MMP7 was compared to the results previously obtained in mouse studies (Figure 2A). Only the

Table 1. Primers and probes.

| Gene   | sequence (5'→3') | Tm (°C) | GC (%) |
|--------|------------------|---------|--------|
| MMP7  | Forward GGAATTCGAGCTAGGATTAACCT | 61      | 46     |
|        | Reverse GGAGATGCCTCCCATACCCAAGAA | 61      | 45     |
| Probe  | CCTGATGTCGCAACATTCAAGCTG | 68      | 50     |
| RPL-19 | Forward GCCAGTTCATGGAACACA | 58      | 50     |
|        | Reverse GGTCAGCCAGAAGCTTCTTG | 59      | 60     |
| Probe  | CCAACAGCTGAAGCAGAACAGGCCC | 70      | 60     |
| Trypsin | Forward CCCCCCTTTGATGATGAC | 59      | 40     |
|        | Reverse GATGTCATTGTCAGAGTCCTC | 59      | 40     |
| MMP25 | Forward TCGAGCTATGCGCTTGATG | 60      | 50     |
|        | Reverse AGGGCCCTCAATAAGGAG | 59      | 50     |

1All genes are human.

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active metabolite, sulindac sulfide, was capable of significantly reducing the expression of MMP7. A titration curve using different concentrations of sulindac sulfide revealed that even at 50 μM, MMP7 showed a small (14%), but statistically significant downregulation of expression (p = 0.044) (Figure 2B). At 100 μM, a decrease of 63% was observed (p = 0.000001). The difference between 100 μM and 200 μM was less dramatic (11%), but was still significantly different between these two concentrations (p = 0.017).

Western blotting of MMP7, as well as the active site of MMP7, was performed on cytosolic, membrane, and secreted fractions of HT-29 proteomes after 24 hr of sulindac sulfide treatment at 100 μM (Figure 2C). In both cases, MMP7 was only detected in the secreted fractions of the proteome. Sulindac sulfide treatment showed a clear reduction of MMP7 in the secreted fraction at a 100 μM concentration. Western blot analysis targeting the active site of MMP7 revealed a complete disappearance of detectable active MMP7 after sulindac sulfide treatment.

In order to determine if other MMPs, other classes of proteases, and the housekeeping gene, RPL-19, were affected by sulindac in HT-29 cells, RT-PCR (whole cell extracts) and Western blot (secreted, cytosolic, and fractions) analyses were performed. mRNA for MMP25, trypsin1, and RPL-19 were not altered after sulindac sulfide treatment (Figure 3A, B). Western blot analysis of the membrane fraction of these cells confirmed RT-PCR results for MMP25 (Figure 3C).
Activity-based protein profiling targeting metallohydrolases reveals a decrease in LTA4H activity

Proteomes extracted from HT-29 cells after 24 hr of treatment with DMSO or sulindac sulfide were labeled with a probe cocktail targeting the metallohydrolase superfamily and run on a 1D gel. A strong band between 60 and 65 kDa was observed in HT-29 cells treated with DMSO. Sulindac sulfide-treated cells showed a faint band of similar molecular weight. The bands were excised and analyzed by mass spectrometry. The samples were run by linear quadrupole MS/MS (Figure 4) and the results matched with the MEROPS database for metallohydrolases. There were two peptides (LTYTAEVSVPK and LVVDLTDIDPDVAYSSPYEK) detected in the 70 kDa band that matched leukotriene A4 hydrolase (LTA4H). The predicted molecular weight of LTA4H is 69 kDa. LTA4H has previously been shown to be a target for this metalloprotease probe cocktail (10).

In order to validate the reduction in LTA4H activity, an enzymatic immunoassay was used to measure leukotriene B4 (LTB4) levels after sulindac treatment (Figure 5). Sulindac sulfide-treated cells showed strongly reduced levels of LTB4 when compared to DMSO-treated cells.

Discussion

The present study demonstrated that sulindac sulfide is capable of reducing the RNA and protein levels of MMP7, which is consistent with what was observed in mouse studies [7]. Through activity-based protein profiling, it was demonstrated that sulindac sulfide dramatically reduces the activity of LTA4H. This result was validated by decreased levels of LTB4.

The involvement of metalloproteases in colon cancer has previously been identified but the development of treatment strategies targeting these enzymes has been unsuccessful [12]. One problem is the low specificity of metalloprotease inhibitors that have made it to clinical trials. MMP7, however, has consistently been found to be relevant in colon cancer and its animal models [9,13]. The present study indicated that sulindac sulfide is able to reduce the expression of MMP7 in the human colon cancer cell line HT-29 which was also observed, in vivo, in ApcMin/+ mice [7]. In order to determine if other MMPs or other classes of proteases were also altered, MMP25, a membrane-associated MMP that is highly expressed in cancer cells [14], and trypsin1 were analysed and shown to be unaffected by sulindac sulfide treatment. Additionally, the housekeeping gene RPL-19 was also not affected by this NSAID. Nobiletin, another agent with anticancer properties, has also been shown to downregulate MMP7 [15] in HT-29 cells. Several mechanisms have been proposed by which MMP7 promotes tumor growth. Several of those studies link MMP7 with a disrupted Fas-mediated apoptotic response [16,17,18], and inflammation-related facilitation of tumor growth has been proposed to be caused by MMP7 cleavage of Fas ligand [19].
Zinc metallopeptidases consist of 12 members and belong to the family of metallohydrolases. These proteases have been implicated in several cancers including aminopeptidase N in colon cancer [20], cystinyl aminopeptidase in renal cancer [21], and LTA4H in lung and colon adenocarcinomas [22]. This is the first study linking sulindac to regulating LTA4H. The downregulation of activity of LTA4H, and decrease in tumor burden, after sulindac treatment supports studies in which it has been shown to be upregulated in colon cancer. Furthermore, [6]-gingerol, a natural component with antitumorigenic properties, has been found to suppress cancer growth by LTA4H inhibition [23]. The decrease in LTA4H activity was validated by the observation that lower LTB4 levels were found after sulindac treatment. LTB4 is a well known eicosanoid with chemotactic properties and has been shown to stimulate the proliferation, in vitro, of HT-29 colon cancer cells [24].

There has been no other report of the involvement of sulindac, or any other NSAID, on MMP7 or LTA4H expression. However, in preliminary studies, we have shown that sulindac is not the only NSAID capable of downregulating MMP7, i.e., aspirin (data not shown), which suggests that MMP7 is downregulated by a shared NSAID mechanism [7,25] and not a unique property of sulindac.

In summary, the present study identified two candidates, previously reported to be highly relevant in tumor development, that are altered after sulindac treatment. New treatment modalities selectively targeting MMP7 and LTA4H, individually or in combination, offer new therapeutic approaches that take advantage of the benefits of sulindac treatment, but potentially without the adverse side effects of this drug.

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Author Contributions
Conceived and designed the experiments: VAP FJC JT HGA. Performed the experiments: HGA JT. Analyzed the data: VAP FJC JT HGA. Contributed reagents/materials/analysis tools: HGA. Wrote the paper: VAP FJC JT HGA.

References
1. Hixson LJ, Earnest DL, Fennerty MB, Sampliner RE (1993) NSAMD effect on sporadic colon polyps. Am J Gastroenterol 88: 1652–1656.
2. Friend WG (1990) Sulindac suppression of colorectal polyps in Gardner’s syndrome. Am Fam Physician 41: 891–894.
3. Waddell WR, Loughby RW (1963) Sulindac for polypsis of the colon. J Surg Oncol 24: 83–87.
4. Hixson LJ, Alberts DS, Krutzsch M, Einspahr J, Brendel K, et al. (1994) Antiproliferative effect of nonsteroidal antiinflammatory drugs against human colon cancer cells. Cancer Epidemiol Biomarkers Prev 3: 433–438.
5. Boolbol SK, Dannenberg AJ, Chadburn A, Martucci C, Guo XJ, et al. (1996) Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. Cancer Res 56: 2536–2560.
6. Wolfe MM, Lichtenstein DR, Singh G (1999) Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. N Engl J Med 340: 1888–1899.
7. Guillen-Ahlers H, Buechler SA, Suckow MA, Castellino FJ, Ploplis VA (2008) Sulindac treatment alters collagen and matrixin expression in adenomas of ApcMin/+ mice. Carcinogenesis 29: 1421–1427.

Figure 4. Activity based proteomics and LTA4H in HT-29 cells. Metallohydrolase activity-based labeling of HT-29 cells secreted proteomes. MMP2 and MMP7 standards were added to cell proteomes (arrows). The band displaying a strong decrease (*) after sulindac sulfide treatment was extracted and analyzed by mass spectrometry. MS/MS spectra of LWDLTDIDPDVAASVereeDPEYK and LTYTAEVSVPK peptides corresponded to amino acids 366–386 and 155–165, respectively, of the LTA4H protein. Protein content across all samples was adjusted to 1 mg/ml and the equivalent of 15 μg of proteome was added per lane. MW denotes molecular weight; STDs, standards, Sd, sulindac sulfide-treated and D, DMSO-treated.

Figure 5. Enzymatic immunoassay for LTB4. Secreted proteomes of DMSO-treated and sulindac sulfide-treated cells were analyzed for LTB4 levels using an enzymatic immunoassay. doi:10.1371/journal.pone.0025725.g005
8. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, et al. (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2: 737–744.

9. Wilson CL, Heppner KJ, Labosky PA, Hogan BL, Matrisian LM (1997) Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Natl Acad Sci U S A 94: 1402–1407.

10. Sieber SA, Niessen S, Hoover HS, Cravatt BF (2006) Proteomic profiling of metalloprotease activities with cocktails of active-site probes. Nat Chem Biol 2: 274–280.

11. Speres AE, Cravatt BF (2006) Profiling enzyme activities in vivo using click chemistry methods. Chem Biol 11: 535–546.

12. Mannello F (2006) Natural bio-drugs as matrix metalloproteinase inhibitors: new perspectives on the horizon? Recent Pat Anticancer Drug Discov 1: 91–103.

13. McDonnell S, Navre M, Coffey RJ, Jr., Matrisian LM (1991) Expression and localization of the matrix metalloproteinase pump-1 (MMP-7) in human gastric and colon carcinomas. Mol Carcinog 4: 527–533.

14. Sun Q, Weber CR, Sohaef A, Bernardo MM, Toth M, et al. (2007) MMP25 (MT6-MMP) is highly expressed in human colon cancer, promotes tumor growth, and exhibits unique biochemical properties. J Biol Chem 282: 21986–22000.

15. Kawabata K, Murakami A, Ohigashi H (2005) Nobiletin, a citrus flavonoid, down-regulates matrix metalloproteinase-7 (matrilysin) expression in HT-29 human colorectal cancer cells. Gastroenterology 122: 576–586.

16. Almendro V, Ameller E, Garcia-Reicio S, Collazo O, Casas I, et al. (2009) The role of MMP7 and its cross-talk with the FAS/FASTL system during the acquisition of chemoresistance to oxaliplat. PLoS ONE 4: e4728.

17. Powell WC, Fingleton B, Wilson CL, Boothby M, Matrisian LM (1999) The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Current Biology 9: 1441–1447.

18. Wang WS, Chen PM, Wang HS, Liang WY, Su Y (2006) Matrix metalloproteinase-7 increases resistance to Fas-mediated apoptosis and is a poor prognostic factor of patients with colorectal carcinoma. Carcinogenesis 27: 1113–1120.

19. Guillaum-Alders H, Suckow MA, Castellino FJ, Ploplis VA (2010) Fas/CD95 deficiency in Apo(Min/+); mice increases intestinal tumor burden. PLoS ONE 5: e9070.

20. Hashida H, Takabayashi A, Kanai M, Adachi M, Kondo K, et al. (2002) Aminopeptidase N is involved in cell motility and angiogenesis: its clinical significance in human colon cancer. Gastroenterology 122: 576–586.

21. Larrinaga G, López JL, Calvo L, Gil J, et al. (2007) Cystinyl aminopeptidase activity is decreased in renal cell carcinomas. Regulatory Peptides 144: 56–61.

22. Chen X, Wang S, Wu N, Yang C (2004) Leukotriene A4 hydrolase as a target for cancer prevention and therapy. Curr Cancer Drug Targets 4: 267–283.

23. Jeong CH, Bode AM, Pughese A, Cho YY, Kim HG, et al. (2009) 6-Gingerol suppresses colon cancer growth by targeting leukotriene A4 hydrolase. Cancer Res 69: 5584–5591.

24. Bertuozzo C, Hanif R, Kashfi K, Staiano-Coico L, Shiff SJ, et al. (1996) The effect of leukotrienes B and selected HETEs on the proliferation of colon cancer cells. Biochim Biophys Acta 1300: 240–246.

25. Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS (2005) Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. Science 310: 1504–1510.