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Key Words
Small molecule • Tolfenamic acid • Sp1 • Survivin • Toxicity

Abstract
Background/Aims: The small molecule, Tolfenamic acid (TA) has shown anti-cancer activity in pre-clinical models and is currently in Phase I clinical trials at MD Anderson Cancer Center Orlando. Since specificity and toxicity are major concerns for investigational agents, we tested the effect of TA on specific targets, and assessed the cellular and organismal toxicity representing preclinical studies in cancer. Methods: Panc1, L3.6pl, and MiaPaCa-2 (pancreatic cancer), \textsuperscript{h}TERT-HPNE(normal), and differentiated/un-differentiated SH-SYSY (neuroblastoma) cells were treated with increasing concentrations of TA. Cell viability and effect on specific molecular targets, Sp1 and survivin were determined. Athymic nude mice were treated with vehicle or TA (50mg/kg, 3times/week for 6 weeks) and alterations in the growth pattern, hematocrit, and histopathology of gut, liver, and stomach were monitored. Results: TA treatment decreased cell proliferation and inhibited the expression of Sp1 and survivin in cancer cells while only subtle response was observed in normal \textsuperscript{h}TERT-HPNE and differentiated SH-SYSY cells. Mice studies revealed no effect on body weight and hematocrit. Furthermore, TA regimen did not cause signs of internal-bleeding or damage to vital tissues in mice. Conclusion: These results demonstrate that TA selectively inhibits malignant cell growth acting on specific targets and its chronic treatment did not cause apparent toxicity in nude mice.

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### Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly prescribed drugs for the relief of pain and inflammation. The primary mechanism of action of NSAIDs is through inhibition of cyclooxygenase (COX) enzymes, and the subsequent inhibition of prostaglandin synthesis at the site of inflammation [1]. Arachidonic acid is converted to prostaglandins by two COX enzymes; COX-1 which is ubiquitously expressed, and COX-2 which is regulated by various stimuli associated with inflammation [2]. First generation NSAIDs were primarily COX-1 inhibitors and unfortunately have been associated with severe upper gastrointestinal (GI) toxicity. This phenomenon led to the development of COX-2 selective NSAIDs (coxibs) that cause less GI damage relative to the nonselective NSAIDs. However, use of certain coxibs is also under scrutiny as their long-term use has been associated with severe cardiovascular toxicity [3-5]. These concerns facilitated the recommendation for the withdrawal of Vioxx (Rofecoxib) and Bextra (Valdecoxib) from the US market [6-8]. Even though the risks associated with various NSAIDs are continuously being monitored, their multiple applications in clinical settings has led to studies that are focused on selecting less hazardous NSAIDs and optimizing low doses. Understanding precise molecular mechanisms associated with NSAID toxicity and developing less toxic NSAIDs is also in progress [9, 10].

NSAIDs also have been extensively evaluated for their application in cancer prevention and therapy. The anti-cancer activity of NSAIDs is well studied using pre-clinical models. For example, laboratory studies showed that NSAIDs and COX-2 inhibitors such as aspirin, indomethacin, sulindac and celecoxib suppressed tumor growth in animal models for multiple cancers including bladder, esophageal, lung, breast and pancreatic [11-16]. The interest in NSAIDs has been heightened by several studies that have suggested a chemopreventive response to long term use. Some of the strongest evidence comes from epidemiological studies which have shown the association of prolonged use of NSAIDs with a decrease in the incidence of certain malignancies [17-21]. There is growing evidence indicating an association between the use of NSAIDs and the decrease in cancer incidence and/or disease progression in breast, colorectal, lung and prostate cancers [22, 23].

Although the exact mechanisms of the anticancer activities of NSAIDs are unknown, experimental evidence suggests the involvement of both COX-dependent and independent pathways [24, 25]. Several COX-independent NSAIDs are currently in pre-clinical testing for the treatment or chemoprevention of specific cancers. Pre-clinical studies from our laboratory and others demonstrated the anti-cancer and chemopreventive response of tolfenamic acid (TA, 2-(4-chloro-3-methylanilino)benzoic acid) in a variety of human cancers. TA has been studied in pre-clinical models for adult (pancreatic [26, 27], lung [28], esophageal [14, 29], ovarian [30], prostate [31, 32], and colon [33, 34]) cancers and pediatric (leukemia [35], neuroblastoma [36], and medulloblastoma [37]) malignancies. Experimental results from our laboratory also showed a chemopreventive effect by this drug in an animal model of esophageal cancer [29, 38]. TA exhibits anticancer and chemopreventive activities primarily via inhibiting specificity protein (Sp) transcription factors. Cell culture studies revealed that by targeting specific Sp transcription factors (Sp1, Sp3 and Sp4), TA modulates the expression of key candidates such as c-PARP, caspases, cyclins, survivin, c-Met, and VEGF that are associated with apoptosis, cell cycle, angiogenesis and suppression of cellular growth. Previously we showed that TA acts as a radio-sensitization agent that increases tumor growth inhibition and decreases metastasis when used along with radiation [39]. TA is currently under study in a Phase I clinical trial at MD Anderson Cancer Center Orlando for upper gastro-intestinal cancers in combination with radiation therapy.

TA is a commonly prescribed agent for treating migraine headaches in Europe and Africa. Since TA has been in clinical use (though not in the US) for decades, the pharmacokinetics and toxicity data of this drug are available in both pre-clinical and clinical settings; however,
previous studies had a number of limitations, and they were not conducted in models that are routinely used for cancer treatment. In this investigation, we have tested the toxicity and tolerance of TA using precise laboratory models consistent with pre-clinical screening for cancer. Experiments performed in vitro with both normal and malignant cells examined the effect of TA on cell viability and the expression of specific molecular markers. Studies in athymic nude mice evaluated the effect of TA on animal health and growth, blood chemistry, and potential organ damage. These analyses confirmed the specificity of TA in targeting cancer cells, and showed that at effective anti-cancer doses, TA does not cause serious adverse effects. Further study of TA as an anti-cancer agent is warranted.

Materials and Methods

Cell lines and reagents

hTERT-HPNE, MiaPaCa-2, Panc1, and SH-SY5Y cells were purchased from ATCC (Manassas, VA). Pancreatic cancer cell line L3.6pl was a gift from Dr. Fidler (University of Texas MD Anderson Cancer Center, Houston, TX). MiaPaCa-2, Panc1, and L3.6pl cells were cultured in DMEM supplemented with 5% FBS and 1X Pen-Strep. hTERT-HPNE cells were maintained in media recommended by ATCC (DMEM+M3 Base media supplemented with puromycin, HGF, Dextrose, and 5% FBS). SH-SY5Y cells were cultured in EMEM (ATCC) supplemented with 5% FBS and 1X Pen-Strep. All cell lines were maintained in a humidified incubator at 37°C and 5% CO₂.

Tolfenamic acid, DMSO, and anti-actin antibody were obtained from Sigma (St. Louis, MO), CellTiter-Glo reagent was from Promega (Madison, WI), anti-Sp1 antibody was purchased from Santa Cruz (Santa Cruz, CA), and anti- Survivin was procured from R&D Systems (Minneapolis, MN). Hemoglobin ELISA kit was obtained from Abnova (Taipei City, Taiwan).

Cell viability assay

Cells were seeded in a 96-well plate and treated with vehicle (DMSO) or TA (25, 50 and 75 μM). Cell viability was measured using CellTiter-Glo kit following the instructions provided by the supplier. Briefly, 4000 cells were seeded in each well of a white-walled clear bottom 96-well plate and incubated for 24 h in a 37°C humidified incubator. Cells were then treated with vehicle (DMSO) or increasing doses of tolfenamic acid (0, 25, 50, 75 µM) in triplicate. Following incubation with the drug (24, 48, and 72 h), 100 µl of CellTiter-Glo reagent was added to each well and the plate was incubated in the dark for 15 minutes. Luminescence was measured using a 96-well plate reader (Optima, BMG LABTECH) and cell viability was calculated as the percentage of relative luminescence units (RLU) of the drug treated versus vehicle treated cells. Error bars represent standard error, which was calculated using statistical tools in MS Office Excel or GraphPad Prism (La Jolla, CA).

Expression of Sp1 and survivin

Expression of Sp1 and survivin in TA treated cells was determined by Western blot analysis. Cells were treated with TA (50 μM) for 48 h. Following incubation with TA, cells were washed once with PBS, harvested by scraping, and resuspended in Cell lysis buffer (Invitrogen, Carlsbad, CA). Cells were incubated for 30 min at 4°C with intermittent mixing followed by centrifugation at 14,000 g for 15 min at 4°C. Protein content in the supernatant was estimated using BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein(s) were separated on 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose membranes using iBlot Western Transfer System (Invitrogen, Carlsbad, CA). The membranes were blocked with 5% milk and incubated overnight at 4°C, with primary antibody (Sp1, survivin, or actin) followed by appropriate HRP-conjugated secondary antibody. Membranes were washed, and the signal was developed using Supersignal West Dura (Pierce).

Neuroblastoma cell differentiation studies

Anti-proliferative response of TA was evaluated using differentiated neuroblastoma (NB) cells and compared with un-differentiated NB cells. SH-SY5Y cells were seeded in a white-walled clear bottom 96-well plate (3000 cells/well) and incubated for 24 h in a humidified 37°C incubator with 5% CO₂. Cell
differentiation was induced by treating SH-SY5Y cells with 10 µM all-trans-retinoic acid (RA) for 3 days following established procedures [40, 41]. Cell count was performed in randomly selected wells and the mean value was calculated. Cells equal to this number were seeded in a new plate for control experiments. RA treated (differentiated) cells and freshly seeded cells were treated respectively with TA + RA or TA alone and cell viability was determined at 24, 48, and 72h post-TA treatment using CellTiter-Glo reagent.

Growth pattern in athymic nude mice

Nude mice were purchased from Charles River (Wilmington, MA) and maintained at the University of Central Florida College of Medicine (AAAS accredited) animal facility, Orlando FL. Animals were treated with vehicle (corn oil) or TA (50 mg/Kg) three times a week for 6 weeks through oral gavage. Mice were observed throughout the treatment period and the changes in body weight were recorded weekly. At the end of the treatment blood was drawn through cardiac puncture (under anesthesia) and used to measure hematocrit and plasma hemoglobin. Mice were euthanized and the organs of interest were examined under the microscope for overt pathological signs. Liver, intestine and stomach were harvested, cleaned and fixed in formalin.

Hematocrit

The hematocrit test was used to determine the percentage of packed red blood cells in whole blood. Blood obtained from cardiac puncture was placed in tubes containing EDTA. Blood was then drawn into heparinized microhematocrit capillary tubes. The tubes were placed in a microhematocrit centrifuge and spun for 8 to 10 minutes at high speed. Using a hematocrit reader (Critocaps) packed cell volume was determined as a percent of the total.

Hemoglobin ELISA

Hemoglobin levels in control and TA treated mice were determined by a double antibody sandwich ELISA kit (Abnova, Taipei City, Taiwan). Briefly, blood was collected into tubes containing heparin and carefully centrifuged to avoid hemolysis. Samples were diluted and 100 µl added to each well of a 96-well plate pre-coated with anti-hemoglobin antibody. The plate was incubated for an hour at room temperature followed by three washes with wash buffer. HRP-conjugated anti-hemoglobin antibody (100 µl) was added to each well and incubated for 30 minutes. Wells were washed three times and developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The hemoglobin amount in each sample was interpolated from a hemoglobin standard curve.

Histopathology assay

Mice tissues (gut, liver, and stomach) were collected at the termination of the experiment and fixed in formalin. Paraffin embedded sections (5 µM) were processed for H&E staining in the pathology laboratory of Sanford-Burnham Medical Research Institute (Orlando, FL). These stained sections were used for histopathological analysis to determine the changes between vehicle and TA treated mice.

Statistical analysis

All quantitative data were expressed as mean±SEM. Statistical comparisons between control and drug treated groups were performed using the Student’s t-test and a p value <0.05 was considered to be statistically significant.

Results

Tolfenamic acid selectively inhibits pancreatic cancer cell growth

We have tested the anti-proliferative effect of TA on hTERT-HPNE (Human Pancreatic Nestin Expressing) cells and the commonly used pancreatic cancer cells Panc1, L3.6pl and MiaPaCa-2. hTERT-HPNE are normal pancreatic duct cells immortalized by transduction with the hTERT gene. These cells have been used in various studies as a model for non-malignant pancreatic cells. Pancreatic cancer cells and hTERT-HPNE cells were treated with increasing concentrations of TA (0/25/50/75 µM) and cell viability was monitored for 3 days (72 h). As
presented in Figure 1, TA treatment resulted in a dose and time dependent decrease in cell viability of Panc1, L3.6pl, and MiaPaCa-2 cells, however, no significant effect was observed in hTERT-HPNE cells. For Panc1, 40.3% and 52.2% cell growth inhibition was observed at 72 h with 50 and 75 µM of TA respectively (Fig. 1b). Conversely, the growth inhibition of hTERT-HPNE cells was less than 10% with 75 µM of TA (Fig. 1a). Similar to Panc1 cells, other two malignant cell lines L3.6pl (Fig. 1c) and MiaPaCa-2 (Fig. 1d) also responded to TA treatment exhibiting significant growth inhibition (L3.6pl: 29.0% at 24 h and 64.9% at 72 h; MiaPaCa-2: 11.4% at 24 h and 43.6% at 72 h with 50 µM TA).

**TA treatment decreases expression of Sp1 and survivin**

Earlier studies demonstrated that TA acts via downregulation of Sp1 transcription factor and its downstream targets. Studies from our laboratory have also shown that TA decreases survivin expression in some malignant cells. We determined the changes in the expression of Sp1 and survivin in pancreatic cancer cells but not in normal cells. Normal pancreatic cells (hTERT-HPNE), and pancreatic cancer cells (L3.6pl and MiaPaCa-2) were treated with vehicle (DMSO) or 50 µM of TA. Cells were harvested at 48 h post-treatment and whole cell lysates were prepared and the expression of Sp1 and survivin was determined by Western blot analysis. Results were obtained from at least 3 different experiments. Representative gels are shown in the figure and β-actin was used as loading control. Con: Control (DMSO treated); TA: Tolfenamic acid 50 µM.
Sp1 and survivin in the pancreatic cancer cells (L3.6pl and MiaPaCa-2) and normal hTERT-HPNE cells following 48 h treatment with TA (50 µM). TA treatment did cause a decrease in the expression of both Sp1 and survivin in the cancer cells. No effect on Sp1 levels was observed in TA-treated hTERT-HPNE cells, and as expected, survivin was not detected in these cells (Fig. 2).
TA treatment has minimal effect on differentiated SH-SY5Y cells

The neuroblastoma cell line SH-SY5Y was grown in the presence of retinoic acid (RA) for 3 days and regularly observed microscopically. After the confirmation of cell differentiation, cells were counted and an equal number of differentiated and un-differentiated cells were plated for controlled experiments. Cells were treated with 25 or 50 μM of TA and cell viability was measured for 3 days. Interestingly, TA caused a significantly higher growth inhibition in un-differentiated SH-SY5Y cells relative to RA treated (differentiated) cells (Fig. 3a). The TA-induced growth inhibition was 9.13±0.28% (differentiated) and 27.29±1.77% (un-differentiated) at 48 h post-treatment; 14.01±2.78% (differentiated) and 35.93±4.39% (un-differentiated) at 72 h post-treatment. These results demonstrate that the anti-proliferative effect of TA was more than double in un-differentiated cancer cells than differentiated cells.

Chronic TA treatment has no effect on mouse growth, red blood cell count and hemoglobin levels

Athymic nude mice were treated with 50 mg/kg TA (3 times/wk) for 6 weeks. The growth pattern of treated and untreated mice was monitored throughout the study. TA did not cause any changes in body weight in any group after 6 weeks (Fig. 4a). At the beginning of the study the control group weighed 20.53±0.64 g and the TA-treated group weighed 20.04±0.43. At the end of the study (after 6 weeks) animals weighed 23.25±0.45 (control) and 22.61±0.67 (TA) with an average increase of 2.72 g and 2.57 g respectively. These results confirm that TA did not affect the growth pattern of athymic nude mice after treatment up to 6 weeks.

At the termination of the experiment (end of the 6th week), blood was drawn from control and TA-treated mice through cardiac puncture. These blood samples were used to measure red blood cell count (hematocrit) and plasma hemoglobin levels. Hematocrit
analysis showed that TA treatment had no effect on red blood cell count (Fig. 4b). We also measured the hemoglobin levels in the mice blood samples using a hemoglobin ELISA kit and found no difference between control and treated groups (Fig. 4c).

**TA treatment did not cause damage to the mouse gut, stomach, and liver**

Long term use of NSAIDs is often associated with upper gastrointestinal bleeding and ulceration. Stomach (gastric body together with forestomach), intestine tissues, and liver were harvested from mice in the 6-week TA-treatment experiment described above. Paraffin embedded tissue slices (slides) were prepared, stained with H&E and histopathological analysis was performed. Microscopic examination of collected tissues showed no difference in the cellular characteristics of control and TA-treated groups. Evaluation of gut (Fig. 5a), forestomach (Fig. 5b) and liver (Fig. 5c) revealed no sign of damage or ulceration in TA-treated mice.

**Discussion**

NSAIDs are the most commonly used drugs in clinical practice, generally prescribed for pain management. Long term use of specific NSAIDs has been associated with GI injury and cardiovascular problems. The anti-inflammatory effect of majority of NSAIDs is primarily linked to modulation of COX-1 and COX-2 which catalyze the conversion of arachidonic acid to prostaglandins [1]. Since prostaglandins show cytoprotective and antiulcerogenic properties in gastric mucosa [42], inhibition of cyclooxygenases decreases prostaglandins and results in severe toxicity to GI tract and other vital organs [1, 43].

A number of studies have demonstrated the antineoplastic and chemopreventive effects of NSAIDs; however the precise mechanisms remain ambiguous. Even though the involvement of COX-2 dependent mechanism is relatively well established in the anti-cancer activity of NSAIDs, there is increasing evidence for the association of both COX dependent and COX-independent pathways [24, 25, 44-47]. Pre-clinical studies from our laboratory and others have demonstrated that the anti-cancer activity of TA is mediated through COX-independent mechanism, and we have shown the involvement of the transcription factor Sp1, and an inhibitor of apoptosis protein, survivin.

In this study, we tested the effect of TA on cell viability in malignant (Panc1, L3.6pl, and MiaPaCa-2), and non-malignant (hTERT-HPNE) cells. We found that TA inhibits cancer cells but does not cause apparent toxicity in normal cells (Fig. 1). The anti-proliferative effect of TA on pancreatic cancer cells was in agreement with the published results [26, 27], showing a time/dose-dependent response. In order to correlate such selective cell toxicity, we investigated the effect of TA on the expression of Sp1 and survivin (Fig. 2). Cancer cell growth inhibition was accompanied with a decrease in the expression of both Sp1 and survivin in all three pancreatic cancer cell lines, which is also consistent with published pre-clinical work on pancreatic [26] and other cancers [30, 32, 35-37]. Notably, non-malignant hTERT-HPNE cells do not express survivin but do express Sp1, but TA treatment did not affect the expression of Sp1. These results support the idea that TA specifically inhibits cancer cells via disruption specific targets, including Sp1 and survivin, and that toxicity through these pathways is not discernible in non-malignant cells.

Recently we showed that TA inhibits neuroblastoma cells SH-SY5Y and LA 155n by targeting Sp1 and survivin [36]. We hypothesized that TA effects may be evident in undifferentiated and actively growing (malignant) cells, but may not be toxic to differentiated cells. To test this hypothesis, we evaluated the anti-proliferative response of TA on differentiated and un-differentiated SH-SY5Y cells. Several studies have shown that RA induces NB cell differentiation and RA is routinely used to differentiate SH-SY5Y cells [40, 41]. We grew these cells in the presence of RA for 3 days, and confirmed differentiation
by morphological changes. These cells along with a separate set (equal number) of undifferentiated SH-SY5Y cells were treated with TA and the anti-proliferative response was compared over the period of 3 days (Fig. 3a). As expected, TA caused minimal effect on differentiated NB cells when compared to un-differentiated SH-SY5Y cells. Recent studies from our group demonstrated that TA induces apoptotic pathways and disrupts cell cycle progression in cancer cells [30, 32, 35-37]. It is plausible that apoptotic and cell cycle processes/pathways are potentially inactive in differentiated cells, thereby reducing the effect of TA in RA treated (differentiated) SH-SY5Y cells. These results further support our hypothesis that TA can be toxic to malignant cells which are actively undergoing mitosis but is not harmful to differentiated cells or tissues. Thus, the collateral damage of TA may be minimal.

The major limitation for using NSAIDs long-term is their side-effects on the GI tract; however other side-effects such as hematological aberrations, hepato and nephro toxicities are also observed. In this study we have investigated the overt effect of TA with a maximum dose (50 mg/kg 3 times/wk) used in animal cancer models. In previous studies, we have given this dose of TA for 4 weeks in mice, however for this study we chose to continue the treatment for 6 weeks in order to assess for any pathological changes in the animals. We did not find any changes in behavior or growth of TA-treated animals (Fig. 4a). Furthermore, hematocrit and hemoglobin levels were unaltered in these animals (Fig. 4 b,c). Histopathological analysis also confirmed that TA did not cause any identifiable damage to the intestines, stomach (forestomach), or liver (Fig. 5). Prostaglandins ameliorate the balance in maintaining the secretions of gastric acid and mucus thereby facilitating the cytoprotection of gastric epithelium [48, 49]. It is believed that GI toxicity of NSAIDs is linked to alterations of prostaglandin synthesis potentially through perturbations in COX activities [48-50]. Since TA works independent of COX, this agent exhibits limited GI disturbances and toxicity.

Pharmacological analysis has shown that TA has limited toxicity when compared to other commonly used NSAIDs [51]. Even though our previous studies using rats showed limited toxicity of TA [38], this is the first study assessing the toxicity in detail, achieved through putting together the results of animal growth patterns, blood chemistry and organ histopathology. All these results showed limited cytotoxicity and GI effects. TA also has an excellent therapeutic index in humans [51, 52]. Relatively low GI toxicity could be an added advantage for TA when compared to other NSAIDs, apart from its specificity in targeting Sp transcription factors and survivin. Tumor cells express higher levels of survivin compared to normal cells and over-expression of survivin has been implicated in resistance of cancer cells to radiation and chemotherapy. The results of this investigation suggest that TA can act as a radiosensitizer due to its specific role on targeting Sp1 and survivin.

After efficacy, toxicity and side-effects are the key factors that impact the utility of therapeutic agents in clinical practice. Investigational agents often fail in clinical testing due to high toxicity and/or severe side effects. Our study shows that TA exhibits low toxicity and may serve as an ideal partner for use in combination therapy along with radiation or other chemotherapeutic drugs. Adding TA as a radio/chemo-sensitizing agent potentially facilitates the reduction of the required dose(s) of standard therapy, thereby minimizing the adverse side effects associated with aggressive therapy. Considering the characteristic potent therapeutic efficacy and low toxicity of TA, further study of this drug as an anticancer agent is warranted.

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Conflict of Interest

None declared.

References


