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Therapeutic applications of NSAIDS in cancer: special emphasis on tolfenamic acid

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1. ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are primarily used for the treatment of acute or chronic conditions with pain and inflammation. Evidence from a wide range of sources suggested that chronic administration of NSAIDs reduced the risk of cancer incidences. Both the epidemiological and animal studies showed an inverse association between the incidence of various cancers and the use of aspirin or other NSAIDs. The chemopreventive and therapeutic interventions of NSAIDs in cancer are obvious; however, the instigation of drug and treatment period depends on the study objective. Typically, prevention involves initiating the medication before the appearance of clinical symptoms and lasts long-term; while treatment could be short-term and contingent to the response of patient to the medication. Recent studies from our laboratories provided substantial evidence on the anti-cancer activity of tolfenamic acid, a NSAID for the potential applications in pancreatic, esophageal and lung cancers. In this review, we provide a summary on the potential benefits of NSAIDs in a variety of human cancers with more emphasis on tolfenamic acid.

2. NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)

2.1. NSAIDs and their medical application

Non-steroidal anti-inflammatory drugs (NSAIDs) have been extensively used for the treatment of diverse conditions associated with pain and inflammation (1-3). Acetylsalicylic acid (or aspirin) is the most popular NSAID with multiple therapeutic applications. Aspirin was initially developed in the late 1800s for the treatment of arthritis and pain relief. Currently, this is one of the most widely used over-the-counter drugs globally. Aspirin and a few other NSAIDs are known for their protective effects against cardiovascular and other diseases (4-7). Figure 1 illustrates the diverse structures of NSAIDs, which include salicylates (ortho-hydroxy benzoic acids), structurally diverse arylacetic acid and aryl proponic acid derivatives, ortho-carboxyl diaryl amines (fenamates), pyrazoles and oxicams.

The anti-inflammatory and analgesic properties of NSAIDs have been linked to their activities as inhibitors of cyclooxygenases (COX) that convert the fatty acid

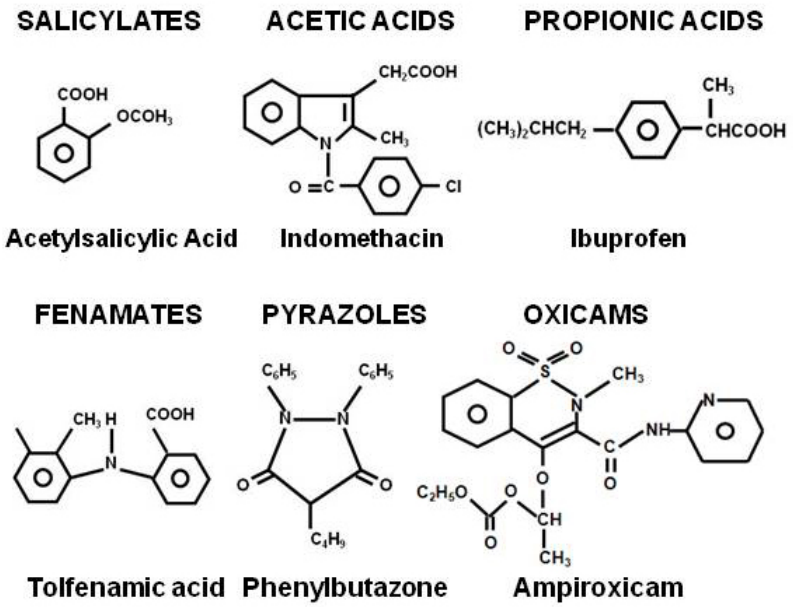

Figure 1. Structural classes of NSAIDs. Chemical structures of important classes of NSAIDs, salicylates, acetic acids, propionic acids, fenamtes, pyrazoles and oxicams are shown. Tolfenamic acid belongs to the class, Fenamates.

arachidonic acid to various prostaglandins (1, 3). There are two major isoforms of COX and these include a constitutive form (COX-1) that plays an important role in cellular homeostasis and an inducible form (COX-2), which is primarily involved in inflammation (1-3). The development of compounds such as celecoxib, which preferentially inhibit COX-2 has resulted in a new and more specific subclass of NSAIDs (8). COX-2 inhibitors are highly effective as anti-inflammatory drugs and avoid the adverse gastrointestinal side effects of NSAIDs, which also inhibit COX-1 (9-11). However, other studies suggest that prolonged use of COX-2 inhibitors may also be problematic due to increased incidence of cardiovascular problems in some patients (12, 13). Although NSAIDs and more specific COX-2 inhibitors inhibit COX enzyme activity, there is extensive evidence that many responses induced by these compounds are COX-independent (14). For example, aspirin inhibits COX activity by irreversibly acetylating the enzyme; however, the doses of aspirin needed to treat chronic inflammatory diseases are much higher than that required for the inhibition of COX activity. Although the tissue-specific mechanisms associated with the therapeutic activity of individual NSAIDs have not been unequivocally determined, there is evidence that NSAIDs modulate several factors/pathways (14). These include altered expression or activity of various transcription factors, and cell cycle genes, heat shock proteins, apoptotic pathways and direct activation of nuclear receptors such as peroxisome proliferators-activated receptor γ (PPARγ) and PPARβ (15-19).

2.2. NSAIDs for cancer therapy

Cancer is essentially a critical pathology with complex mechanisms involved and hence both physicians and scientists are constantly working on discovering multi-model therapies and identifying vital targets. Recently, the use of small molecules for the therapeutic interventions in cancer is highly appreciated due to their use in targeted therapy and relatively lower side-effects. The NSAIDs are a class of small molecules that are extensively investigated for their application in cancer therapy. This class of molecules exhibit anti-tumor activities as assessed by using various human cancer cells and animal models for cancers. Laboratory animal and cell culture studies confirm the efficacy of NSAIDs for inhibiting growth of colon cancer and tumors derived from other tissues (20-22). NSAIDs inhibit cancer cell growth through modulation of cell cycle genes. Furthermore, in combination with these anti-proliferative properties, the NSAIDs also induce apoptosis and exhibit anti-angiogenic activities (23-25). Thus, the profile of NSAID-induced responses in cancer cells/tumors is highly desirable for an anti-cancer drug, and currently there has been focus on discovering anti-cancer NSAIDs as a new class of mechanism-based drugs for treating various cancers. The role of NSAIDs in both prevention and treatment of colon cancer has been extensively investigated (26, 27), and there is evidence from epidemiology studies that NSAIDs, such as aspirin and some COX-2 inhibitors, decreased the incidence and/or mortality of colon cancer (28-30). It is also apparent that the anti-cancer activities of NSAIDs and COX-2 inhibitors can be both COX-2-dependent and -independent (14, 26, 27, 31). The NSAIDs/COX-2 inhibitors modulate several pathways in cancer cell-lines that lead to inhibition of growth, apoptosis and angiogenesis, and COX-2 inhibitors are being investigated for colon cancer prevention and chemotherapy (1, 3, 26, 27, 31). The NSAIDs exhibit anti-tumor activities in models for several cancers; however, the prolonged use of NSAIDs is also associated with decrease in the incidence of some human cancers (chemoprevention). Several laboratory and *in vivo* studies showed that NSAIDs/COX-2 inhibitors such as aspirin, indomethacin, sulindac and

celecoxib suppress carcinogen-induced or xenograft/orthotopic models of lung, mast cell, fibrosarcoma, esophageal, bladder, pancreatic and mammary cancers (32-38). Although the mechanisms of these anti-tumorigenic effects induced by NSAIDs are not completely understood, there is strong evidence that NSAIDs inhibit cancer cell growth through modulation of cell cycle genes. Apart from anti-proliferative properties, NSAIDs also induce apoptosis and exhibit anti-angiogenic activities in several models of cancer (26, 27, 31). Thus, the profile of NSAID-induced responses in cancer cells/tumors is highly desirable for an anti-cancer drug, and several studies have focused on the development of NSAIDs (including COX-2 inhibitors) as a new class of mechanism-based drugs for treating various cancers including pancreatic, prostate, lung and ovarian cancers.

3. SPECIFICITY PROTEIN (Sp) TRANSCRIPTION FACTORS

3.1. Sp transcription factors and cancer

Transcription factors are now recognized as novel targets for the development of anti-cancer agents. Specificity protein (Sp) family of transcription factors that bind GC/GT-rich promoter elements regulate the expression of multiple genes associated with growth and development (39). There is growing evidence that some Sp proteins play critical role(s) in the growth and metastasis of many tumor types by regulating expression of cell cycle genes and vascular endothelial growth factor (VEGF). Sp1, Sp3 and Sp4 are the sequence-specific transcription factors that recognize and bind to GC rich sequences (39). Since large number of genes, including the genes that are associated with the tumor growth and metastasis contain GC-box in promoter region, Sp proteins play critical role in the transcriptional activation of these genes and hence provide potential targets for cancer therapy (40, 41). For example, a study from our laboratory identified tolfenamic acid (TA), a NSAID that induces the degradation of Sp1, Sp3 and Sp4, decreases the expression of VEGF and inhibits pancreatic cancer cell/tumor growth (42). Recently, we showed that Sp proteins (Sp1, Sp3 and Sp4) and c-Met are over-expressed in human lung cancer cells and tumors in nude mice, and TA decreases the lung cancer cell survival, represses Sp proteins, c-Met and other key candidates in its downstream signaling including Akt phosphorylation (32). The Sp proteins also regulate the expression of X-linked inhibitor of apoptosis (XIAP) and survivin and we showed that TA inhibits the expression of survivin in pancreatic cancer cells and tumors in mice through inducing the inhibition of Sp proteins (43).

3.2. Targeting Sp proteins with NSAIDs: tolfenamic acid, novel anti-cancer agent

To investigate the effects of NSAIDs structure and activity relationship on altering the expression of Sp proteins, different structural classes of NSAIDs were screened for their ability to decrease the levels of Sp1, Sp3, and Sp4 in human pancreatic cancer cells (42). NSAIDs were selected from important classes for the screening and this screening was done with biphenyl/biphenylamine carboxylic acids (tolfenamic acid, diclofenac and diflunisal), oxicams (ampiroxicam), acetic acid derivatives (acemetacin and tolmetin), and propionic acid derivatives (ibuprofen, naproxen, fenbufen, and ketoprofen). The compounds were tested in two different concentrations using Panc1 and L3.6pl cells. Interestingly, tolfenamic acid treatment resulted in maximum response showing a time-dependent decrease in the expression of Sp1, Sp3, and Sp4 proteins in Panc1 cells (~80% decrease in the levels of all three proteins at 50 μM treatment for 48 h), while treatment with other NSAID, ampiroxicam (used as a negative control) did not affect the expression of Sp proteins. Furthermore, tolfenamic acid inhibited VEGF mRNA and protein expression in pancreatic cancer cells and this inhibition was associated with the decreased Sp-dependent activation of the VEGF promoter. In a seminal study, these *in vitro* studies were further tested using orthotopic mouse model for pancreatic cancer. In the mouse model for pancreatic cancer and found that tolfenamic acid (50 mg/kg of body weight) significantly decreased tumor growth, liver metastasis, and the expression of Sp1, Sp3, Sp4 and VEGF (42). These results facilitated the identification of tolfenamic acid as a new anti-pancreatic cancer NSAID that activates degradation of transcription factors Sp1, Sp3, and Sp4; reduces VEGF expression; and decreases tumor growth and metastasis (42-44).

3.3. Tolfenamic acid enhances radiosensitization in pancreatic cancer cells and tumors

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is a bifunctional protein that has been implicated in the control of cell division and inhibition of apoptosis (45). Its expression is minimal in most normal adult tissues (46); however, it is widely expressed during fetal development (47). Survivin is found in most human carcinomas, including pancreatic cancer (45, 48-50) and is involved in the resistance of tumor cells to both chemotherapy and radiation**.** A major strategy for modulating survivin expression is to control its transcription process. Previous reports show that in some cancer cell lines, survivin expression is dependent, in part, on Sp proteins (51-54) and Sp1 and other Sp proteins are over-expressed in cancer cells and tumors including pancreatic tumors (55-59). Since we showed that tolfenamic acid inhibits pancreatic cancer cell and tumor growth through degradation of Sp1, Sp3 and Sp4 proteins (42), we also investigated whether tolfenamic acid inhibits survivin expression and sensitizes pancreatic cancer cells and tumors to radiation therapy. Our results showed that combination therapy (tolfenamic acid and radiation) is highly beneficial in inhibiting pancreatic cancer cells proliferation and tumor growth in orthotopic mouse model for pancreatic cancer. We found that tolfenamic acid inhibits survivin protein/mRNA expression in Panc1 and L3.6p1 cells and this was dependent on the down-regulation of Sp1, Sp3, and Sp4 proteins. Tolfenamic acid not only inhibited pancreatic cancer cell growth but also increased their sensitivity to radiation therapy. *In vivo* studies using mice orthotopic pancreatic cancer model showed that radiation moderately inhibited tumor growth; however, the inhibition was more robust when tolfenamic

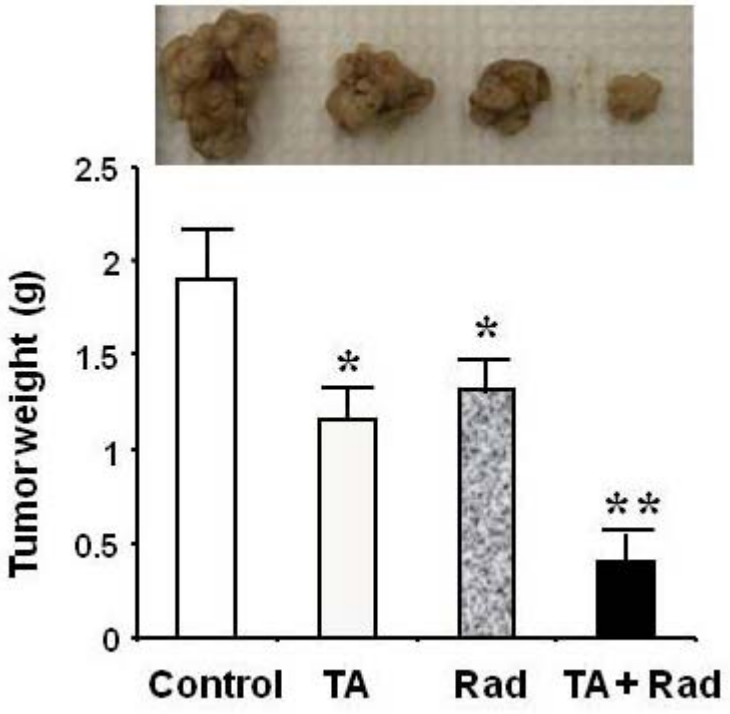

Figure 2. Tolfenamic acid enhances the response of pancreatic tumors to radiation therapy. Mice were injected with L3.6pl cells in the pancreas and treated with monotherapy [radiation (5Gy/week) or tolfenamic acid (50 mg/kg/every other day)] or combination therapy (radiation and tolfenamic acid) for four weeks. Results showed that tumor growth was significantly inhibited with either therapy and the response was more pronounced with the combination therapy, suggesting that tolfenamic acid sensitized the tumors and enhanced tumor response to radiation therapy. Con: Control; TA: tolfenomic acid; Rad: radiation.

acid was administered along with radiotherapy (Figure 2). Interestingly, TUNEL staining of mice tumors revealed that apoptosis was significantly higher when mice were co-treated with tolfenamic acid along with radiation. The results of this study clearly demonstrated that radiosensitizing effects of tolfenamic acid resulted due to modulations in Sp-dependent survivin expression.

The other possible mechanism for the enhanced pancreatic cells and tumors response to radiation could be attributed to the effect of tolfenamic acid on inhibiting tumor angiogenesis. The inhibition of tumor angiogenesis may occur through reduced levels of VEGF (60). It is evident from our previous studies that tolfenamic acid inhibits VEGF and VEGF receptor type 1 (VEGFR1) expression in pancreatic cancer cells and tumors (42, 61). It is plausible that the delay in tumor growth and enhancement in response to radiotherapy in part may happen through tolfenamic acid-induced inhibition of angiogenesis.

3.4. Tolfenamic acid for the treatment of lung cancer

The effect of tolfenamic acid on cell proliferation and cell viability in human lung cancer cells, A549 and CRL5803, was tested and we found that tolfenamic acid inhibited cell growth in a dose/time-dependent manner. We have also reported that tolfenamic acid significantly increased the number of apoptotic cells both in A549 and CRL5803 cells 48 h post-treatment in a dose-dependent manner. Selected pro-apoptotic markers, c-PARP and Bax showed increased expression in both A549 and CRL5803 cells. Such an up-regulation of the major executioners of mitochondrial apoptotic pathway, suggests the activation of intrinsic apoptotic pathways following treatment with tolfenamic acid leading the lung cancer cells to apoptosis.

We examined the concentration and time-dependent effects of tolfenamic acid on the expression of Sp1, Sp3, and Sp4 in A549 and CRL5803. Consistent with our findings on pancreatic cancer model (42, 43), tolfenamic acid decreased the expression of Sp1, Sp3, and Sp4 proteins in A549 and CRL5803 cells (32). In this investigation, we found that tolfenamic acid also targets another important candidate, c-Met and inhibits human lung cancer cells and tumor growth in orthotopic mouse model. The hepatocyte growth factor receptor c-Met is over-expressed in a variety of cancers including lung cancer, and it is clear that Sp1 and Sp3 mediate the expression of c-Met. Tolfenamic acid (50 μM; 48 h) decreased the expression of c-Met and its phosphorylated form (p-Met) in A540 and CRL5803 cells. *In vivo* studies showed TA treatment to be highly beneficial in inhibiting tumor formation/growth. Athymic nude mice bearing A549 cells in the lung were treated with vehicle or two doses (25 mg/kg/2days or 50 mg/kg/every other day) of tolfenamic acid for 4 weeks and the tumor tissues were analyzed for the tumor area. The results showed that tolfenamic acid significantly diminished the tumor growth and the response

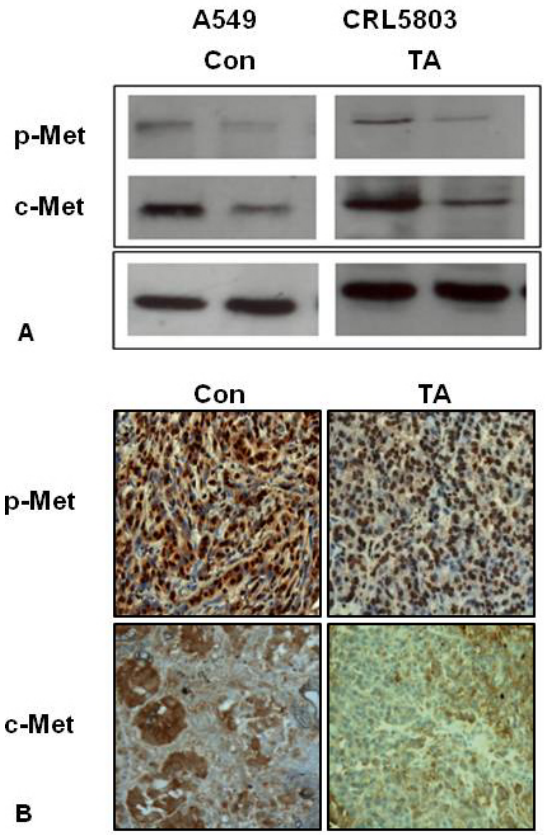

Figure 3. Tolfenamic acid decreases the expression of Met in lung cancer cells and tumors in orthotopic mouse model for lung cancer. (A) Lung cancer cells A549 and CRL5803 were treated with DMSO or 50 μM of TA for 48 h and whole cell lysates were prepared. The expression of c-Met and p-Met was evaluated through Western blot analysis and the results showed that tolfenamic acid diminished the expression of both c-Met and its phosphorylated form. (B) Mice were injected with A549 cells in lungs and treated with tolfenamic acid (50 mg/kg/every other day) for four weeks. Tumors were harvested and subjected to immunohistochemical analysis using specific antibodies for c-Met and p-Met. Consistent with the *in vitro* data (Figure 3A), tolfenamic acid decreased the expression of both c-Met and p-Met. Con: control; TA: tolfenomic acid.

was dose-dependent. Morphometric analysis of tumor area showed a significant dose-dependent decrease and this decrease was around 80% in the group that received 50 mg/kg/ tolfenamic acid every other day. Tumors harvested from these mice were tested for the expression of both c-Met and its phosphorylation (p-Met) and we found that the basal activity of both c-Met and p-Met is very high in control animals and the treatment with tolfenamic acid resulted in a massive decrease in their levels (Figure 3). These results further confirm the association of tolfenamic acid-induced inhibition of tumor growth and Sp-driven down-regulation of c-Met and its phosphorylation (32).

4. FUTURE DIRECTIONS

Sp Transcription factors play critical role(s) in cancer cells growth and their metastasis (15, 62, 63). There is clear evidence that Sp1 expression represents a negative prognostic factor for the survival in some cancer patients (55, 56, 64). Sp proteins also regulate the expression of survivin, a member of inhibitor of apoptosis gene family which is associated with resistance to chemotherapy and radiotherapy in several cancers. Hence the work from our laboratories focused on developing strategies to target these transcription factors for the treatment of various cancers. After obtaining promising results on pancreatic and lung cancer models, we are currently testing the application of tolfenamic acid for the treatment of prostate cancer and ovarian cancer. The on-going studies at our laboratories show that tolfenamic acid results in profound inhibitory effects on ovarian cancer cells proliferation, presumably through the degradation of Sp proteins. Sp proteins have high relevance in prostate cancer and we are also evaluating the anti-cancer activity of tolfenamic acid in prostate cancer model. Sp proteins regulate the expression of several genes, including prostate specific antigen (PSA) gene and c-Met, which are associated with prostate cancer and our preliminary studies demonstrate that tolfenamic acid inhibits prostate cancer cells proliferation and tumor growth in the mice bearing PC-3 cells in their prostates. Studies to test the effect of tolfenamic acid on critical genes associated with ovarian cancer and prostate cancer and the impact of this NSAID in enhancing these cancer cells and tumors responses to radiation therapy are currently underway. The strategies to use other Sp inhibitors (e.g., mithramycin A, Wp631, and curcumin) administered along

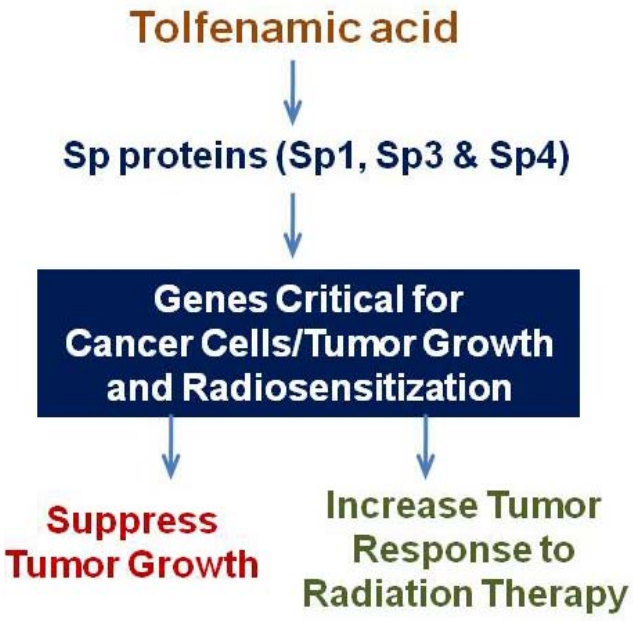

Figure 4. Tolfenamic acid, anti-cancer agent. Tolfenamic acid is emerging as anti-cancer agent due to promising data reported on pancreatic, lung and esophageal cancer models. The anti-cancer properties of this drug are primarily related to its actions on down-regulating the expression of Sp1, Sp3 and Sp4. Since Sp proteins mediate the expression of critical genes associated with tumor growth, metastasis and radiation resistance; down-regulation of these transcription factors instigate the suppression of tumor growth and also facilitate an increase in tumor response to radiation therapy.

with tolfenamic acid in order to achieve robust beneficial results for the treatment of various cancers thorough synergistic effects are also under investigation.

5. CONCLUSION

In conclusion, our experimental/pre-clinical data show that tolfenamic acid significantly suppresses cancer cells and tumor growth in various cancer models. Furthermore, tolfenamic acid enhances pancreatic cancer cells and tumor response to radiation therapy and these results are accompanied with decreased survivin expression due to tolfenamic acid treatment. However, tolfenamic acid-mediated induction of apoptosis and cell cycle redistribution may also enhance tumors/cells response to radiation therapy. Our results showed strong association of decreased survivin levels and radiosensistization in pancreatic cancer and suggest that tolfenamic acid has the potential to increase the radioresponse of unresectable or recurrent pancreatic cancer and current studies in our laboratory are focused on evaluating the mechanism of action of tolfenamic acid and its interactions with radiotherapy for developing therapeutic effectiveness. Tolfenamic acid has been in use for several years in Europe, Asia and Africa for treating migraine headaches. It is well tolerated by patients and its side effects are very limited and well documented. Currently, this drug is in Phase I clinical trials at M. D. Anderson Cancer Center Orlando, Orlando, FL to test on patients with upper GI cancer along with radiation therapy. Tolfenamic acid is emerging as a novel NSAID with potent inhibitory actions against Sp proteins to function as anti-cancer agents for the implications in the treatment of various cancers (Figure 4). Despite the advances in treatment options, resistance of tumors to current chemo regimen still constitutes a major concern. The advanced research in this area will serve as a model to fight against the resistance to chemo and radiation therapies. The studies on tolfenamic acid and other NSAIDs will provide the platform for the development of safe, cost-effective drugs, with minimum side-effects for the treatment of various malignancies.

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