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Commentary

Is inhibition of cyclooxygenase required for the anti-tumorigenic effects of nonsteroidal, anti-inflammatory drugs (NSAIDs)? *In vitro* versus *in vivo* results and the relevance for the prevention and treatment of cancer

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Abstract

Active research is being conducted to unravel the cellular mechanisms mediating the anti-tumorigenic effects of nonsteroidal anti-inflammatory drugs (NSAIDs) and their association with cyclooxygenase (COX) inhibition. The majority of NSAIDs inhibit either COX-1, COX-2, or both and exert their anti-COX, anti-inflammatory, and anti-tumorigenic effects *in vivo* in a parallel dose-dependent manner. The effects are seen at NSAID blood plasma concentrations of 0.1–5 μM. Significantly, the same compounds tested at the same concentrations in incubations with cultured tumor cells *in vitro* similarly inhibit COX activities but are devoid of anti-proliferative activity. Yet, at much higher concentrations (100–20,000 μM), these same NSAIDs do exert anti-proliferative effects *in vitro* due to apparent non-specific toxic effects, as evidenced by disruption of ion transport and mitochondrial oxidation in some cells. A small group of NSAIDs (e.g. sulindac) do not inhibit COX enzymes significantly but can reduce the synthesis of prostanoids by alternate mechanisms. One such mechanism is inhibition of agonist-stimulated phospholipase-mediated release of arachidonic acid from phospholipids leading to depressed synthesis of prostanoids, especially prostaglandin E_2 (PGE_2). Another group of non-COX inhibitors are the *R*-isomers of NSAIDs, based on the structure of 2-arylpropionic acid. These compounds exert anti-proliferative effects *in vivo*, acting by an as yet undetermined mechanism. A possible caveat in these data is an *R* to *S* chiral transformation *in vivo* that would render the *R*-isomer effect as being due to the *S*-isomer generated *in vivo* from it. Demonstration of minimal or no *R* to *S* inversion under the experimental *in vivo* conditions employed is, therefore, a necessary control in these studies. The overall body of data supports the conclusion that, for COX-inhibiting NSAIDs, their anti-tumorigenic effect *in vivo* is due to, and depends upon, inhibition of tumor COX enzymes, primarily COX-2. The cellular effects seen when adding high concentrations of NSAIDs to tumor cells cultured *in vitro* and the mechanisms proposed to mediate these effects may not have substantial relevance to the mechanisms that mediate the effects of NSAIDs *in vivo*.

Keywords: Prostanoids; PGE_2; Cyclooxygenase; COX-1; COX-2; NSAIDs; Cancer

1. Introduction

There is a growing interest in unraveling the mechanisms mediating the anti-proliferative effects of NSAIDs and in determining if such effects are due to their COX-inhibiting activities or are independent of them. Evidence to date indicates that the majority of NSAIDs attenuate tumor growth *in vivo* by either induction of apoptosis or inhibition of angiogenesis or both. In studies with cultured cells *in vitro*, NSAIDs were shown to cause apoptosis in many cell types but only at high concentrations of >100 μM, whereas only submicromolar–micromolar concentrations are required to achieve 75–80% inhibition of COX-1 and COX-2 in most cell types. Are the high concentrations of NSAIDs needed to cause apoptosis *in vitro* relevant to the chemopreventive or chemotherapeutic activities of NSAIDs as seen *in vivo*? The aim of this commentary is to critically evaluate the existing data pertinent to this question and propose experiments that will yield answers to it.

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Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; CI, COX inhibitor; NCI, non-COX inhibitor; PGE_2 prostaglandin E_2.

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2. Mechanism-based classification of NSAIDs

NSAIDs are compounds with a nonsteroid-like structure that possess one or more of several anti-inflammatory properties such as analgesic, anti-pyretic, and edema-reducing effects. It is sometimes stated that all ''traditional'' NSAIDs inhibit both COX-1 and COX-2. This generalization is, however, not accurate since there are several compounds that are classified and act as NSAIDs yet do not inhibit either of the COX enzymes (sulindac sulfone and salicylic acid are two prime examples). It is therefore imperative that, when evaluating the cellular mechanisms mediating the anti-neoplastic effects of NSAIDs, we divide the NSAIDs, based on their known mechanism of action, into COX inhibitor (CI) and non-COX inhibitor (NCI) groups, with the new COX-2 selective compounds being part of the CI group.

3. Effects of NSAIDs on COX inhibition and cell cycle parameters: lessons from studies *in vitro*

Tumor growth rate is determined by the balance between the rate of cellular proliferation and the rate of cell death by apoptosis/necrosis. Many COX-inhibiting NSAIDs, including sulindac sulfide, indomethacin, piroxicam, naproxen, the ''profen''-type compounds, and aspirin, inhibit cell proliferation of colon cancer [1–8] and other tumor cells *in vitro* [9–11]. The cell quiescence induced by NSAIDs is due to reduction in the levels of cyclin-dependent kinases and increased D-type cyclins that participate in the transitions through the different phases of the cell division cycle [4–7]. The fact that inhibition of cell proliferation and/or stimulation of apoptosis/necrosis is seen at NSAID concentrations that are 10–250-fold higher than those needed to inhibit the COX enzymes has served for some investigators as suggestive evidence that the anti-proliferative effects of NSAIDs are unrelated to and independent of inhibition of COX activity [8,12–14]. Is this conclusion justified, and what is its relevance to neoplastic transformation and the growth of tumors in humans? The results reported for cell culture experiments describe effects that are obtained only when cells are treated with very high, often toxic concentrations of NSAIDs (e.g. piroxicam at 900 μM and sulindac sulfide at 200 μM [8]; indomethacin at 300 μM, sulindac sulfide at 200 μM [13]; aspirin at 1 mM, naproxen at 200 μM, piroxicam at 300 μM, indomethacin at 100–600 μM [2]; aspirin at 7 mM [15]; NS-398, indomethacin at 100 μM [16]). NSAIDs used under these conditions (both CI and NCI types) were shown to induce non-specific effects on cellular signalling including inhibition of several membrane-associated enzymes (e.g. phospholipase C, NADPH oxidase) as well as anion cell membrane transport and uncoupling of mitochondrial oxidative phosphorylation [12]. As the pro-apoptotic effects are unrelated to the inhibition of COX enzymes, they are seen in both cells that contain COX enzymes (e.g. HT-29 cells [1–5]) and cells that are devoid of them [8,13,14]. In agreement with this, the non-COX inhibiting sulindac sulfone given at 300 μM [13] or 600 μM [17] and salicylic acid given at the very high concentration of 20 mM [18–20] affect cellular proteins that inhibit cell proliferation *in vitro*. Furthermore, as the mechanisms of action mediating the effects seen at the high NSAID concentrations are indeed not dependent upon COX inhibition or reduction of cellular PGE_2 concentration, addition of exogenous PGE_2 would not be expected to reverse these NSAID effects, as indeed was observed [17].

A detailed, recent report demonstrated for Celebrex, a selective COX-2 inhibitor, a dose-dependent dissociation between COX inhibition and tumor reduction *in vivo*, on the one hand, and anti-proliferative effects seen *in vitro*, on the other [21]. The study showed that at drug concentrations of <10 μM (maximal concentration reached in blood plasma *in vivo*), the drug does not inhibit the growth of cultured tumor cells *in vitro*, yet it potently inhibits tumor growth *in vivo*. It further showed that two structurally related COX-2 inhibitors, Celebrex and SC-58125, possess equal inhibitory potencies for COX-2 activity and for tumor growth *in vivo* but have radically different potencies for inhibition of cell proliferation *in vitro*.

What then is the relevance of cell culture data to the effects observed under *in vivo* conditions? A direct answer to this question could be obtained if *in vivo* growing tumor cells could be subjected to the same concentrations as those employed in *in vitro* studies. This, however, is not practical since the blood/tissue levels needed to be reached are toxic to experimental animals and humans if given for the extended periods required to yield tumor growth arrest.

Hence, the overall body of data discussed leads to the conclusion that the effects seen *in vitro* when using very high doses of NSAIDs (whether CI- or NCI-type NSAIDs), and the resulting cellular mechanisms proposed to mediate these effects, may have limited relevance to the effects seen and the cellular mechanisms that operate when treating *in vivo* with pharmacological, non-toxic doses of NSAIDs.

4. Effects of COX-inhibiting NSAIDs on tumor growth, tumor PGE_2 content, and cell cycle parameters: lessons from studies *in vivo*

Experimental and clinical data revealed that elevated cellular levels of PGE_2 are seen in cancer cells and in tumor biopsies and that treatment with COX-inhibiting NSAIDs can reduce tumor progression in rodents and tumor incidence in humans. The increased PGE_2 production in tumors was shown to result from greatly enhanced COX-2 expression and activity [22,23]. Furthermore, transfection with the *COX-2* gene increased COX-2 expression and led to decreased cellular apoptosis, increased cellular adhesion to extracellular matrix (ECM), and

elevated levels of Bcl-2 [24]. Genetic studies using APC knockout mice corroborated these findings, showing that both APC knockout animals (expressing very low COX-2) and control mice treated with NSAIDs had significantly reduced numbers of polyps [25]. The significance of COX-2 inhibition for the reduction of tumor growth in COX-2-containing cancer cells was clearly demonstrated by Sheng *et al.* [26] and Goldman *et al.* [27]. Both groups of investigators also showed that with tumors derived from cells having little or no COX-2 (HCT-116 cells), the COX-2 inhibitors were without effects. These findings and similar results of other studies [28] support the conclusion that, for COX-inhibiting NSAIDs, attenuation or inhibition of neoplastic transformation and tumor growth *in vivo* in COX-2-containing cells is, for the most part, dependent upon and linked to NSAID-inhibiting potency. In support of this conclusion is the recent report by Williams *et al.* [21], demonstrating a clear dissociation between Celebrex parallel anti-tumor and COX-2 inhibiting activities *in vivo* on the one hand and inhibition of cell proliferation *in vitro* on the other.

5. Effect of non-COX-inhibiting NSAIDs on tumor growth and tumor PGE_2: lessons from studies *in vitro* and *in vivo*

Among the traditional NSAIDs, two compounds stand out as being non-COX inhibitors: sulindac/sulindac sulfone and salicylic acid. With regard to the latter, an antiproliferative effect was seen only *in vitro* (cultured cells) and at very high concentrations (1–3 mM) that produce drug-related cell toxicity, resulting in increased DNA strand breaks and apoptosis [5]. As for sulindac, results from our recent studies on the growth of LL-carcinoma tumors in mice treated with the compound reveal that although the drug, as reported previously by numerous investigators (see [29]), does not inhibit tumor or host COX-1 or COX-2 significantly, it produces a substantial reduction of 60–70% in tumor PGE_2 content by potently inhibiting Ca^{2+} ionophore-stimulated release of arachidonate from cellular lipids and subsequent PGE_2 synthesis (Table 1). This observation indicates the need to further differentiate the NCIs into PGE_2-reducing and non-PGE_2 reducing compounds. Sulindac is an NCI but a potent reducer of tumor PGE_2 synthesis, with the resulting effect being similar to that of a weak COX-inhibiting NSAID. At the same time, the PGE_2-reducing effect exhibited by sulindac (Table 1) was not dose-dependent as was the inhibition of tumor growth (Raz, unpublished results), suggesting that sulindac possesses additional anti-tumorigenic effects that are unrelated to tumor PGE_2 content or modulation of COX activity.

Recent studies with enantiomeric pairs of certain ''profen''-type NSAIDs [30] provided results that support the notion that COX inhibition is not an obligatory mechanism for some NSAIDs. Comparison of the anti-tumorigenic activities of *R*-flurbiprofen (non-COX inhibiting) and *S*-flurbiprofen (COX inhibiting) in an experimental mouse tumor model found that the *R* isomer, at 10 mg/kg per day, significantly improved several tumor and biological endopoints (number of tumors, weight gain) as well as prolonged survival [31]. *R*-Flurbiprofen was also found to be effective in inhibiting the progression of prostate cancer in a mouse prostate cancer model [32]. These *in vivo* results strongly suggest involvement of a COX-independent mechanism in this process. This finding, however, should be tested for a possible *R* to *S* inversion *in vivo*, as such inversions were demonstrated to occur to varied extents in several species [33–35]. Overall, NSAIDs that do not block or otherwise reduce PGE_2 content *in vivo* appear to exert their effects by prostanoid-unrelated mechanisms.

Table 1
COX-dependent and A23187-dependent synthesis of PGE_2 in *in vivo* grown tumor cells incubated *ex vivo* with sulindac

Treatment	COX activity *ex vivo* (ng/PGE_2 synthesized/mg protein)		
	−AA	+AA	Net change
Control	31 ± 3	72 ± 6	+41
Sulindac (50 μM)	28 ± 4	68 ± 5	+40
Treatment	**A23187-stimulated PGE_2 synthesis (ng PGE_2 synthesized/mg protein)**		
	−A23187	+A23187	Net change
Control	27 ± 5	52 ± 6	+25
Sulindac (50 μM)	27 ± 3	32 ± 4*	+5*

Mouse LL-carcinoma cells (2×10^5) were injected into the footpad of 10-week-old C57BL male mice. When the tumor reached approximately 0.2 ml volume (19–21 days post-injection), the mice were killed, the tumors were isolated, and the soft cell mass was resuspended in Dulbecco's Modified Eagle's Medium. Cells (2×10^5) in 1.5 mL were pipetted into 6-well plates and incubated with 50 μM sulindac or vehicle for 30 min at 37°/5% CO_2. Arachidonic acid (15 μM final concentration) or Ca^{2+}-ionophore A23187 (1 μM final concentration) or vehicle was then added, and the incubation was continued for 15 min, after which the medium and cells were isolated, the cells were pelleted by centrifugation, and the medium was assayed for PGE_2 by radioimmunoassay. Values are means ± SD of four wells per treatment group. (*) $P < 0.01$: significantly different from the appropriate control (*t*-test).

6. Conclusions

The group of therapeutic agents under the heading NSAIDs share anti-inflammatory, analgesic, and anti-pyretic properties and a nonsteroidal-type chemical structure. However, they do not all share a common mechanism of action that mediates their observed anti-inflammatory/anti-tumorigenic activities. For NSAIDs that act via inhibition of the COXs, especially COX-2, the anti-inflammatory and anti-tumorigenic effects are seen at blood plasma concentrations of 0.1–5 μM, concentrations that are reached by

consuming pharmacological, non-toxic doses of the drugs. When the same concentrations were tested in cultured tumor cells grown *in vitro*, they inhibited COX activities but had very little or no anti-proliferative effects (although they did show such effects *in vitro* when added at 10–250-fold higher concentrations).

The conclusions from these data are as follows.

(a) *In vivo*, COX-inhibiting NSAIDs attenuate tumor growth via a mechanism that requires COX inhibition. In agreement with this, the growth of tumors that do not express COX-2 is not inhibited significantly by COX-inhibiting NSAIDs.
(b) The anti-proliferative/pro-apoptotic effects of NSAIDs seen when the compounds are given at very high concentrations *in vitro* have little or no relevance to either the blood and tumor concentrations attained or the effects obtained *in vivo*.

In addition to COX-inhibiting NSAIDs, there are NSAIDs (typically sulindac) that, although not inhibiting COX, do reduce cellular/intercellular levels of PGE_2 by inhibiting phospholipase A_2-type activities, thereby reducing arachidonate availability for the COX enzymes. Sulindac and sulindac sulfone also appear to act via COX-independent and PGE_2-independent mechanisms to reduce tumor growth. Another type of unique NSAIDs are the *R*-isomers of an *S*/*R* racemic mixture NSAID (e.g. the ''profen'' family of NSAIDs). Here, the mechanism of action behind the anti-proliferative effect of the *R*-isomer is unknown, although a partial but significant inversion *in vivo* to the COX-inhibiting *S*-isomer is a possibility that should be evaluated when employing these drugs.

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