The protein kinase promiscuities in the cancer-preventive mechanisms of NSAIDs
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NSAIDs have been observed to have cancer-preventive properties, but the actual mechanism is elusive. We hypothesize that NSAIDs might have an effect through common pathways and targets of anticancer drugs by exploiting promiscuities of anticancer drug targets. Here, we have explored NSAIDs by their structural and pharmacophoric similarities with small anticancer molecules. In-silico analyses have shown a strong similarity between NSAIDs and protein kinase (PK) inhibitors. The calculated affinities of NSAIDs were found to be lower than the affinities of anticancer drugs, but higher than the affinities of compounds that are not specific to PKs. The competitive inhibition model suggests that PK might be inhibited by around 10%, which was confirmed by biochemical screening of some NSAIDs against PKs. NSAIDs did not affect all PKs universally, but had specificities for certain sets of PKs, which differed according to the NSAID. The study revealed potentially new features and mechanisms of NSAIDs that are useful in explaining their role in cancer prevention, which might lead to clinically significant breakthroughs in the future. European Journal of Cancer Prevention 25:77–84 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction
NSAIDs are among the most widely used drugs that are accessible without a prescription. The primary role of NSAIDs is to inhibit cyclooxygenase (COX) activity leading to the synthesis of prostaglandins that cause inflammation-associated symptoms, like pain or fever (Vane, 1971). There are two main COX isoforms distinguished: COX-1 and COX-2. COX-1 is constitutively produced in most tissues, whereas COX-2 is induced by various stimuli, including cytokines and hormones (Davies et al., 2002). There are more than few dozen NSAIDs, which include well-known compounds such as aspirin, ibuprofen, sulindac, celecoxib, and others that bind most COX isoforms, or are more specific to COX-2. The toxicity of NSAIDs is most frequently associated with cardiotoxicity (Patrono and Rocca, 2009) or gastrointestinal bleedings (Bjorkman, 1998).

In addition to their anti-inflammatory activity, there is evidence that NSAIDs reduce the risks of some types of cancer. Among the earliest studies published was a report from the 1970s that showed the inhibitory effect of aspirin and indomethacin on the osteolytic activity of Walker sarcoma in rats (Powles et al., 1973). In addition, in a year-long study, sulindac was shown to inhibit and eliminate colon polyp growth, confirming the results on indomethacin and piroxicam in animals (Waddell and Loughry 1989). A decrease in death from colon cancer was found among men and women who used aspirin 16 times or more per month (Thun et al., 1991). Another early study showed that high levels of piroxicam in the diet of rats resulted in fewer colon tumors compared with control animals (Reddy et al., 1987).

Clinical correlations have been made between NSAIDs and cancer in the human population. In 2009, an international group of researchers published a consensus statement supporting the benefits of aspirin and other NSAIDs as cancer therapeutics (Cuzick et al., 2009). In 2011, Rothwell and colleagues presented an in-depth study that described statistical (25 570 patients) correlations between long-term aspirin use and cancer, finding a lower risk of death among patients consuming aspirin, because of a decrease in gastrointestinal and solid tumors (Rothwell et al., 2010). It was shown that the beneficial effects of aspirin in esophageal, pancreatic, brain, lung, stomach, and prostate cancers manifested after 5 or more years of regular use (Rothwell et al., 2011).

Cancer is a complex and heterogeneous phenomenon, appearing locally at initial stages of development, with no trivial relationships with other biologically complex processes such as inflammation. The link between inflammation and cancer was established many years ago by Rudolf Virchow (Balkwill and Mantovani, 2001). In the 1990s, DuBois and colleagues reported upregulated COX-2 levels in individuals with colorectal cancer (Eberhart et al., 1994), making a closer connection between NSAID pathways, inflammation, and cancer. Later, COX-2 upregulation was reported in other cancer types, and COX-2-selective drugs were shown to have potential therapeutic value (Cha and DuBois, 2007). Furthermore, the cancer and inflammation domains overlap in biological pathways (Dubois et al., 1998).
Among the mechanisms not involving COX-2 (Hinz and Brune, 2002) is the proapoptotic effect of NSAIDs against cancer cells (Zerbini et al., 2006). The Wnt pathway has been shown to be critical in cancer development (Nusse et al., 1984), and NSAIDs have been shown to interfere with targets in this pathway (Dihlmann and von Knebel Doeberitz, 2005). In addition, the involvement of p53 and MAPK signaling pathways has also been reported (Ho et al., 2003). However, despite extensive studies by different research groups and decades of research and thorough clinical correlations, the mechanism of NSAID action is far from being clear. Moreover, the discovery of clear cancer-preventive mechanisms of NSAIDs requires more research (Hudis et al., 2012).

The fact that NSAIDs may interfere with other pathways, can inhibit the development of tumors, and affect tumors of different phenotypes, as described above, lets us hypothesize that NSAIDs could interfere with fundamental pathways controlling cell growth and differentiation in a similar manner to anticancer drugs. Molecular promiscuities are common even for anticancer drugs that have more than a few targets, such as imatinib – the widely used protein kinase (PK) inhibitor (Broekman et al., 2011). Hence, here we investigate the similarities of NSAIDs to small-molecule anticancer drugs, seeking to understand more about NSAIDs. Using in-silico tools and in-vitro screening, we outline ligand-based and structure–physicochemical-based similarities between NSAIDs and anticancer drugs, leading to potential targets and pathways other than the COX pathway that NSAIDs could modulate.

Materials and methods

Databases

There were 35 NSAIDs and 81 chemotherapeutic substances analyzed in our study. Most of the structures of these drugs, together with accompanying data, were obtained from the DrugBank database. If experimental logP values were not available, they were supplemented with the XlogP tool in Kmine software (Berthold et al., 2008). Before further analysis, all of the structures were cleaned of salts and optimized using the OpenBabel MMFF94 method.

Ligand-based comparison

NSAIDs were compared with chemotherapeutic drugs and metabolites. Ligands were compared using a composite measure based on the following steric and physicochemical similarities: pharmacophoric alignment and logP. Structural and pharmacophore comparisons were accomplished using Align-it software (Silico IT, Wijnegem, Belgium; http://www.silico-it.com/software/software.html). In both of these tools, similarities between structures were measured in terms of the Tanimoto coefficient ($T_c$), which provides the similarity score in the unitary scale. To tackle physicochemical similarities, we used antilogarithmic logP values, which were also compared using $T_c$. The final score for each ligand–ligand pair was obtained by multiplying individual matrix scores.

Docking

Docking of the drugs into targets was performed using LeadIT software from BioSolveIT (LeadIT; http://www.biosolveit.de/LeadIT). The best docking poses were evaluated using the Hyde method to determine the contribution of hydration. Targets of the chemotherapeutics were identified according to the data in DrugBank and their corresponding structures were retrieved from the RCSB PDB database (Kouranov et al., 2006), giving preference to the structures with highest resolution. A docking study was performed in three steps. First, we docked the reference chemotherapeutic compounds and then evaluated the root mean square deviation by comparing with the crystallographic data to test the performance of the method. We then docked a negative control set of common metabolites to identify the activity of nontherapeutic compounds. We used randomly selected metabolites from the Human Metabolome Database (Wishart et al., 2007) as negative controls. This allowed us to set a scale on the results by comparing functionally divergent classes of compounds – drugs and metabolites. Common metabolites should not have any therapeutic role, but are present and can theoretically compete for binding to targets. Finally, we docked the selected NSAIDs into corresponding target structures. The following PDB structures were used: 2GQQ, 3CS9, 3HMI, 3V99, 4E26, 3C4C, 2H1M, 1M17, 2ITY, 1XKX, 1MQB, 3RCD, 3HNG, 1RJB, 2DQ7, 3VO3, 3GE0, 1NSG, 4AOJ, 3MJG, 3IQU, 2×2L, 2H8H, 3KMR, 2P1T, 4DM6, 1H9U, 1EXA, 2GL8, 3NT1, 1EQG, 1VTH, 2DES, 1D67. All docking poses were postprocessed with Hyde to evaluate the free energy of binding (Schneider et al., 2012).

Compounds

The following NSAIDs were studied: diclofenac, etodolac, indomethacin, ketorolac, naproxen, sulindac, tolmetin, carprofen, celecoxib, etoricoxib, lumiracoxib, parecoxib, lornoxicam, meloxicam, piroxicam, tenoxicam, meclofenamic acid, mefenamic acid, niflumic acid, nabumetone, vilazodone, misoprostol, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, naproxen, oxaprozin, suprofen, tiaprofenic acid, phenylbutazone, acetylsalicylic acid, diflunisal, salicyclic acid, and salicylate. Only small molecular weight chemotherapeutic compounds were selected in this study (~80). Forty-four metabolites were used as negative controls, having structures of amino acids, lipid acids, monosaccharides, and compounds: acetyl-CoA, fumaric acid, malonyl-CoA, oxalosuccinic acid, pyruvic acid, urea and water.

Biochemical screening

The biochemical screening services of selected NSAIDs against PKs were purchased from Millipore Co. (Billerica, Massachusetts, USA) at 10 mmol/l ATP and 100 μmol/l NSAID concentrations using two replicates per compound.
**Competitive inhibition**

The competitive inhibition model was applied to study the effect on NSAIDs:

\[
V = \frac{V_{\text{max}}[S]}{K_M + [I] + [S]},
\]

where \([S]\) is the concentration of ATP, \([I]\) is the concentration of the inhibitor (NSAID or chemotherapeutic drug), \(K_M\) is the Michaelis–Menten constant, \(K_i\) is the dissociation constant of the inhibitor, and \(V\) is the reaction velocity. Physiological average values were used for \([S]\) and \(K_M\), 5 mmol/l (Beis and Newsholme, 1975) and \(1 \times 10^{-5}\) mol/l (Prowse and Lew, 2001; Knight and Shokat, 2005), respectively. \(K_i\) was calculated from docking energies.

\[
K_i = \exp(-\Delta G/R T),
\]

where \(\Delta G\) is the inhibitor affinity or docking energy after analysis rescoring docking poses with Hyde software, \(R\) is the gas constant, and \(T\) is the absolute temperature.

**Results**

**Ligand comparison**

The anticancer and NSAID compounds were compared by consolidating them into related chemical families to explore the potential target promiscuities. The ligand-based comparison presented in Fig. 1 in the form of a heatmap helps understand whether NSAIDs can potentially be ligands for other targets rather than COX alone. Many NSAID families showed similarities to PK inhibitors, especially fenamate-based NSAIDs. Enolic acid derivatives and salicylates also showed similarities to antitumor agents and other inhibitors. No or very little similarity to chemotherapeutic compound families of antimitabolites or compounds involved in chemical modification, such as alkylating agents, was found (Fig. 1).

Using a family-based comparison, we have narrowed down on a drug–drug comparison focusing on PK inhibitors and antitumor drugs, because they have shown several similarities to anticancer drugs across many NSAID families. The analysis found aspirin to have high scores against a large number of chemotherapeutics (Fig. 2). This might be a result of the small molecular weight of aspirin, and an aspirin-like fragment could have been identified in corresponding anticancer molecules. Although salicylic acid is related to aspirin, it did not show a corresponding high similarity. NSAIDs have shown an average similarity to masoprocol, the anticancer agent whose main target is apolipoxigenase A (LOX) and is structurally and genetically related to COX-2. On the basis of the calculated highest score or the calculated highest average score against all anticancer drugs, nabumetone, aspirin, meclofenamic acid, mefenamic acid, etodolac, diflunisal, and niflumic acid are the NSAIDs that are most similar to PK inhibitors. Oxicam family compounds show the lowest similarity, together with napafenac. On the basis of computed pharmacophoric and physicochemical similarities, no single NSAID shows similarity to all investigated PK inhibitors and antitumor agents. Most of the NSAIDs have narrowly defined similarities (Fig. 2); for example, diclofenac shows similarities to bexarotene, tretoin, everolimus, ethodolac, masoprostol, imatinib, vindesin, and a few other compounds.

**Structural comparison**

Ligand–ligand comparisons suggested highest similarities, leading to specific targets associated with chemotherapeutics drugs. All targets were identified using DrugBank and available structures were used to identify docking studies. Each specific target was docked with ATP, the corresponding chemotherapeutic drug, the corresponding NSAID from the ligand–ligand comparison, and a set of common metabolites. The set of common metabolites were docked to each target also to establish nonspecific affinities toward targets, which helps judge the specificity of other docked compounds.

The docking results show examples of how ligand–ligand similarity is correlated with structural data. Figure 3a shows the crystal structure of nilotinib and the docked nilotinib in ABL1. The docked nilotinib almost perfectly overlies the crystal structure of nilotinib and the docked nilotinib in ABL1. The docking results show identical overlap with fragments of the crystal structure of nilotinib. It was interesting to explore docking of NSAIDs to DNA. Some
experimental data suggest that aspirin could intercalate DNA (Neault et al., 1996). The comparison of individual ligands revealed similarities between aspirin and other anthracyclines that intercalate DNA as well. The docking of doxorubicin to DNA showed a perfect match to the crystal structure, and the docking of aspirin led to the conformation in which the aromatic ring of aspirin intercalates DNA (Supplementary material). The examples of nilotinib/meclofenamic acid and doxorubicin/aspirin pairs illustrate the possible relations between physicochemical, pharmacophoric, and structural properties.

Figure 3b illustrates the average affinity for each class of compounds. On the basis of the average docking energies, anticancer drugs have the highest affinity, whereas metabolites have the lowest affinity. This result serves as a validity test to show that NSAIDs might have a significant affinity for PKs. NSAIDs show intermediate affinity together with ATP. The data spread is large, but results average all different targets and well illustrate a general trend.

**Kinetics**

We have applied the competitive inhibition model to evaluate to what level NSAIDs could inhibit PK targets assuming the physiological ATP concentration and how the inhibitory strength of NSAIDs could compare to that of anticancer drugs. On the basis of the average calculated affinity from the docking results, we calculated $K_i = 1.5 \times 10^{-9}$ and $1.5 \times 10^{-6}$ for anticancer drugs and NSAIDs, respectively. Figure 4a shows the substantially reduced reaction rate of anticancer drugs at their concentration of $10^{-5}$ mol/l (Peng et al., 2004; Van Erp et al., 2009). The effect of NSAIDs was evaluated assuming NSAID concentrations at $C_{\text{max}}$ values that fluctuate around $10^{-4}$ mol/l (Ross-Lee et al., 1983; Türeck et al., 1996; Davies et al., 2000). The inhibition model shows that NSAID concentrations below $10^{-5}$ mol/l should have a very small effect on the reaction rate of PKs. On increasing the NSAID concentration, the reaction rate starts to decrease, leading to a 10–20% reduction in the PK reaction rate at 0–5 mmol/l [3]. The calculated fraction of PKs occupied by NSAIDs and anticancer drugs is
shown in Fig. 4b. Considering the physiological concentrations, 10% of PKs should be bound with NSAIDs, whereas up to 90–95% of PKs could be bound with the corresponding anticancer drugs. However, mutated PKs found in cancers usually have increased activity, with a 10-fold or even a 100-fold increase in $K_M$ (Brasher and Van Etten, 2000; Carey et al., 2006; Yun et al., 2008), and thus greater sensitivity to inhibition.

To evaluate whether NSAIDs could modulate PK activity, biochemical NSAID screening was performed for seven chosen NSAIDs (aspirin, celecoxib, diflunisal, ibuprofen, ketorolac, nabumetone, piroxicam) against ABL1, B-RAF, C-RAF, EGFR, FLT1, FLT3, FMS, FYN, KDR, KIT, RET, and SRC. Because anticancer drugs themselves show target promiscuities against PKs, the activities of PKs are presented as the average activity of PKs for each NSAID, as presented in Fig. 5. Inactivation by $\sim 10\%$ can be observed for the screened NSAIDs, which was predicted by the competitive inhibition model (Fig. 4). The exception is aspirin, which did not lead to a significant change in the average activity of PKs.

**Discussion**

There are many different studies suggesting different mechanisms of action of NSAIDs in cancer prevention. The fact that different NSAIDs can reduce the incidence of cancer across different phenotypes suggests that the mechanism possibly involves exploitation of universal biochemical pathways. PKs are universal and the key targets involved in the regulation of mostly all cellular processes. Small-molecule cancer therapeutics have been developed to bind PKs and suppress or arrest cell growth and proliferation. Our main aim was to explore whether it is possible that NSAIDs might exert similar, but much milder, effects to anticancer drugs, avoiding the well-known toxicities of...
anticancer drugs, but still suppressing tumor growth – that is, suppressing cellular proliferation and growth. For NSAIDs to cause suppression, they should show similarities to anticancer molecules and an affinity toward the targets of anticancer drugs. Promiscuities can certainly be a result of the physicochemical and structural properties of ligands. It has been shown that the highest promiscuity is observed for lower molecular weight compounds (∼300 Da), which have hydrophobic logP values of 2–6 (Hopkins et al., 2006), which are highly similar to NSAIDs: molecular weight = 289.5 ± 64.2 Da and logP = 3.3 ± 0.9.

The ligand-family-based comparison of NSAIDs with anticancer drugs using logP and pharmacophoric alignment differentiated functionally different families of compounds; for example, no NSAID family has shown similarities to alkylating agents, platinum-based compounds, or antibiotics (Fig. 1). In addition, no similarity was found between NSAID families and families of pyrimidine and purine antagonists (antimetabolites). However, the ligand-based comparison identified similarities between NSAIDs and anticancer compounds that function as ligands, such as PK inhibitors or anthracyclines.

The ligand–ligand comparison between families of PK inhibitors and anthracyclines, and NSAIDs revealed that similarity to NSAIDs is dependent on a specific ligand–ligand couple, even within the same family of compounds. The absence of universal similarity between NSAIDs and anticancer compounds suggests that different NSAIDs could interact differently with the targets of anticancer drugs in biochemical pathways. The biochemical screening results also show a large spread of effects of NSAIDs against the investigated PKs, confirming that NSAIDs could possess different specificities for different PKs. Mild activation of PKs was observed from the biochemical screening results, contrary to inhibition, which could be explained by other potential NSAID interactions with targets, such as possible allosteric modulation.

Structural analysis by in-silico screening has shown that NSAIDs can dock inside active centers of PKs. NSAIDs are slightly smaller compounds than chemotherapeutic molecules; therefore, they can be compared as fragments. This kind of behavior is illustrated in the case of meclofenamic acid in ABL1. However, this does not guarantee a strong affinity of NSAIDs toward a target. All docking poses were re-evaluated for free energy change, revealing that NSAIDs have a higher affinity toward chemotherapeutics compared with PKs, but a lower affinity toward common metabolites compared with PKs. The use of metabolites not associated with the activity of PKs helped show that calculated NSAID affinities are significant and might help NSAIDs lead in the competition for binding to PKs. The trend shows that the affinity of NSAIDs is numerically higher than that of metabolites by 10 kJ/mol. The evaluated average NSAID affinities from docking studies and experimental kinetic parameters have suggested that NSAIDs could inactivate PK activity by 10% on the basis of the competitive inhibition model. Biochemical screening has confirmed ~10% inactivation of PKs in the presence of NSAIDs. The limited inhibition of PKs supports the need for long-term administration of NSAIDs, typically 3–5 years, to observe statistically significant effects (Rothwell et al., 2011). In contrast, we can anticipate that stronger inhibition of PKs by NSAIDs would lead to stronger toxicities that are highly associated...
with anticancer drugs. However, the present toxicities of some NSAIDs could also be reviewed in the context of PKs. It is also possible that mutated PKs with higher activities would be more sensitive to NSAIDs.

The investigated targets against NSAIDs and their immediate partners have been consolidated into pathways, as shown in Fig. 6. The pathways integrate COX-1/2, its related counterpart 5-LOX, and the recently identified IKKβ. We have included DNA, as previous studies have shown that aspirin can reside in the minor groove of DNA and is capable of intercalation (Bathaie et al., 2010). On the basis of our analysis, individual NSAIDs show promiscuity and seem to have moderate affinities toward several targets. This should not come as a surprise, as PKs are closely related and retain similar structural patterns. The intended inhibitors of PKs, such as omatinib, dasatinib, gefitinib, erlotinib, and nilotinib, have also been reported to have considerable levels of promiscuity (Arora and Scholar, 2005; Vajpai et al., 2008; Zhang et al., 2009; An et al., 2010). Actually, it was reasoned that their efficacy is closely related to their ability to inhibit several related forms of kinases at the same time, as in the case of imatinib (Baselga, 2006).

Figure 6 shows that PK targets interact with DNA through biochemical pathways and affect common functions of cells and their microenvironment. Cellular features such as migration, inflammation, and angiogenesis are frequently associated with tumorigenesis, cancer, and metastasis. Whereas NSAIDs acting through COX targets affect those cellular features, those acting through PKs contribute a parallel and synergistic input toward the suppression of cellular functions aiding or facilitating cancer development. A variety of PKs have been implicated in the signaling cascades responsible for cancer development and growth (Krause and Van Etten, 2005). Therefore, NSAIDs can suppress cellular proliferation and growth though partially inhibited PKs, and indirectly – by acting on the microenvironment through inflammation and other processes by exploiting COX targets. One can also speculate that any mild suppression of cancer cell proliferation and growth can provide the immune system with more time to deal with cancerous cells.

**Conclusion**

Our in-silico and in-vitro results show that NSAIDs can act as mild PK inhibitors with low affinity and high promiscuity. Mild inhibition of PKs can suppress individual targets, but NSAID promiscuity might strengthen the effects by acting through several PKs due to low specificity for a target. Although no single NSAID possesses affinity toward all PKs in general, it is possible that different.
NSAIDs might exhibit differences on the basis of cancer phenotype, expression, or epigenetic profiles. The results offered a new perspective toward NSAIDs as a class of compounds, offering additional means of explaining their cancer-preventive features. Although this study presents a broad view on NSAIDs, further research focusing deeper on specific targets, cancer types, and biochemical heterogeneity of a patient population is needed to facilitate this research for translational purposes.

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Conflicts of interest
There are no conflicts of interest.

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