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**

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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).

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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 log*P* values were not available, they were supplemented with the Xlog*P* tool in Knime 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 log*P*. Structural and pharmacophore comparisons were accomplished using Align-it software (Silico IT, Wijnegem, Belgium; *http://silicos-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 log*P* 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 RSCB 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: 2GQG, 3CS9, 3HMI, 3V99, 4E26, 3C4C, 2I1M, 1M17, 2ITY, 1XKK, 1MQB, 3RCD, 3HNG, 1RJB, 2DQ7, 3VO3, 3G0E, 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, nepafenac, 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 salsalate. 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 replicas per compound.

Competitive inhibition

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

$$V = \frac{V_{\max}[S]}{K_M\left(1+\frac{[I]}{K_i}\right)+[S]}, \quad (1)$$

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 1×10^{-5} mol/l (Prowse and Lew, 2001; Knight and Shokat, 2005), respectively. K_i was calculated from docking energies.

$$K_i = \frac{1}{\exp(-\Delta G/RT)}, \quad (2)$$

where ΔG 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.

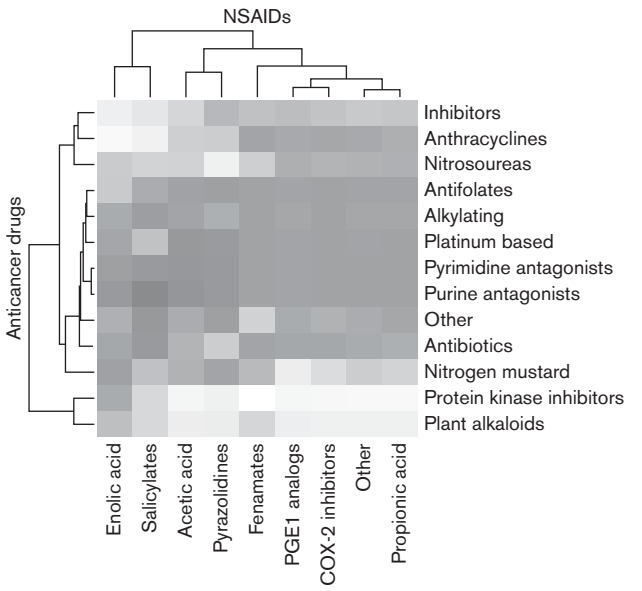
Results

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 that COX alone. Many NSAID families showed similarities to PK inhibitors, especially fenamate-based NSAIDs. Enolic acid derivatives and salicylates also showed similarities to anthracyclines and other inhibitors. No or very little similarity to chemotherapeutic compound families of antimetabolites 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 anthracyclines, 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 apolipoxygenase 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 nepafenac. On the basis of computed pharmacophoric and physicochemical similarities, no single NSAID shows similarity to all investigated PK inhibitors and anthracyclines. Most of the NSAIDs have narrowly defined similarities (Fig. 2); for example, diclofenac shows similarities to bexarotene, tretoin, everolimus, etodolac, masoprocol, imatinib, vindesin, and a few other compounds.

Fig. 1



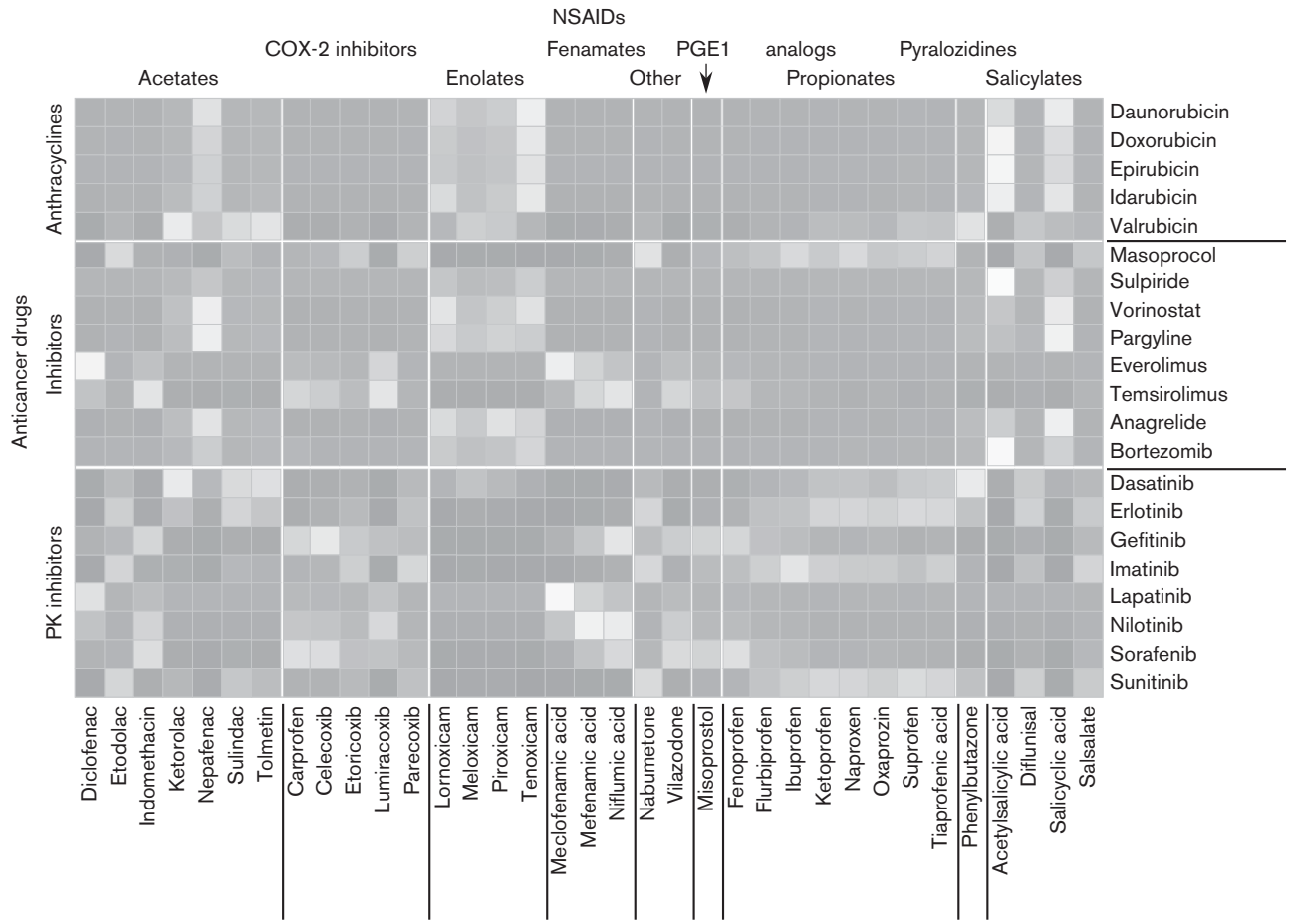
Heatmap of a similarity matrix with a corresponding dendrogram based on pharmacophoric alignments and physicochemical pairwise comparisons of different classes of anticancer drugs and NSAIDs. Brighter shades show greater similarities. COX, cyclooxygenase.

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, leading to a root mean square deviation of 0.37 Å. Meclofenamic acid docking into ABL1 reveals identical overlap with fragments of the crystal structure of nilotinib. It was interesting to explore docking of NSAIDs to DNA. Some

Fig. 2



Heatmap of a similarity matrix based on pharmacophoric and physicochemical ligand–ligand comparisons between anticancer drugs and NSAIDs. COX, cyclooxygenase; PK, protein kinase.

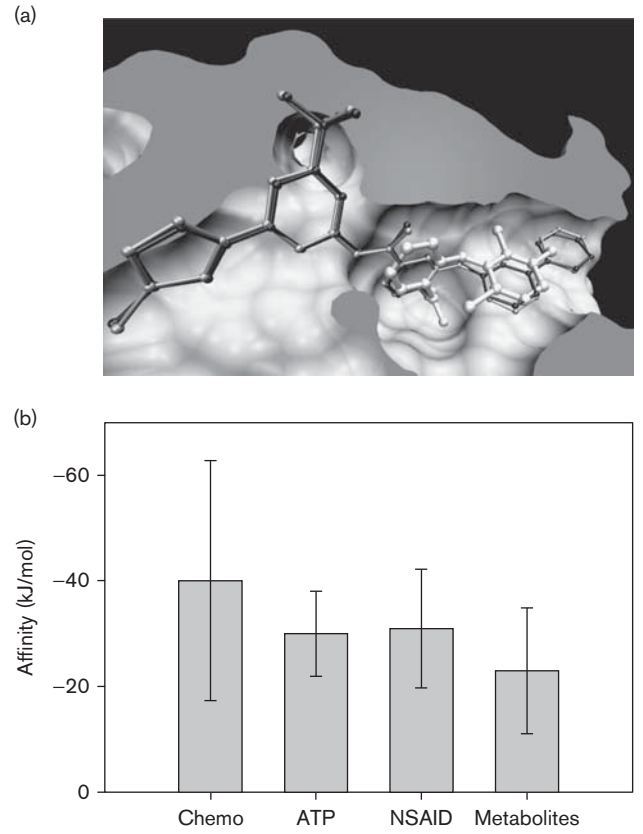
experimental data suggest that aspirin could intercalate DNA (Neault *et al.*, 1996). The comparison of individual ligands revealed similarities between aspirin and other anthracyclines that intercalate DNA as well. The docking of doxorubicin to DNA showed a perfect match to the crystal structure, and 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

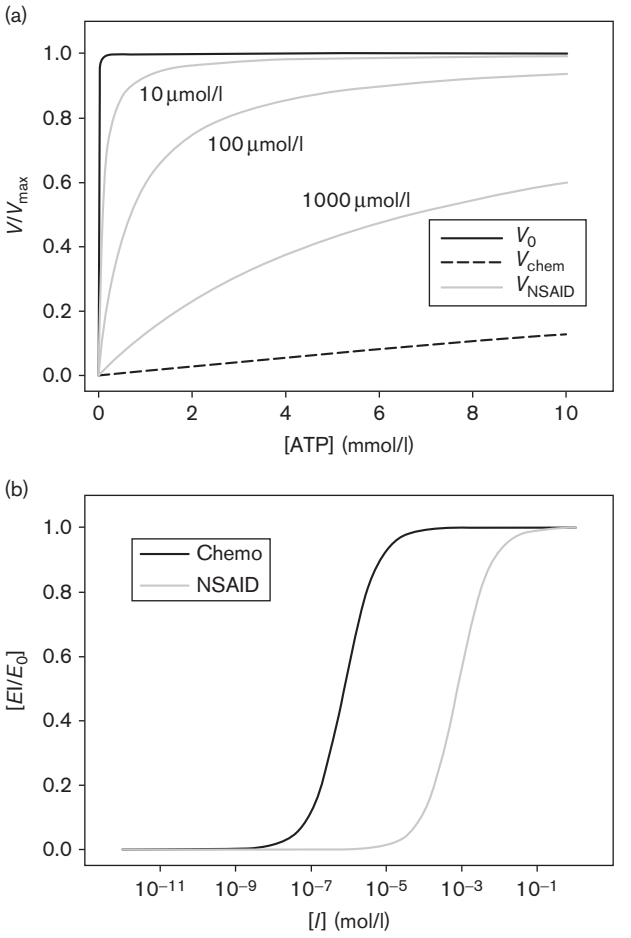
We have applied the competitive inhibition model to evaluate to what level NSAIDs could inhibit PK targets assuming the physiological ATP concentration and how the inhibitory strength of NSAIDs could compare to that of anticancer drugs. On the basis of the average calculated affinity from the docking results, we calculated $K_i = 1.5 \times 10^{-9}$ and 1.5×10^{-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_{max} values that fluctuate around 10^{-4} mol/l (Ross-Lee *et al.*, 1983; Türck *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 [*S*]. The calculated fraction of PKs occupied by NSAIDs and anticancer drugs is

Fig. 3



The virtual screening results of NSAIDs against PKs. (a) Docked nilotinib (gray structure) perfectly overlaps with the crystal structure of nilotinib (black structure), with a root mean square deviation (RMSD) of 0.37 Å. Docked meclofenamic acid (white structure) overlaps with nilotinib fragments in the ABL1 pocket. (b) Comparison of average calculated affinities of chemotherapeutic compounds, ATP, NSAIDs, and metabolites. Dockings were performed in the selected targets of anticancer drugs. Chemo, chemotherapeutic; PK, protein kinase.

Fig. 4



The catalytic kinetics of PK targets interpreted by competitive inhibition. (a) The reduced reaction rate without inhibition (V_0) and with inhibitors (V_{chem}, anticancer drugs; V_{NSAID}, NSAIDs) as a function of the physiological ATP concentrations. The numbers denote the NSAID C_{max} concentration used to calculate V/V_{max}. (b) The target fraction inactivated by chemotherapeutics and NSAIDs on the basis of their average calculated affinities as a function of their concentrations. Chemo, chemotherapeutic; PK, protein kinase.

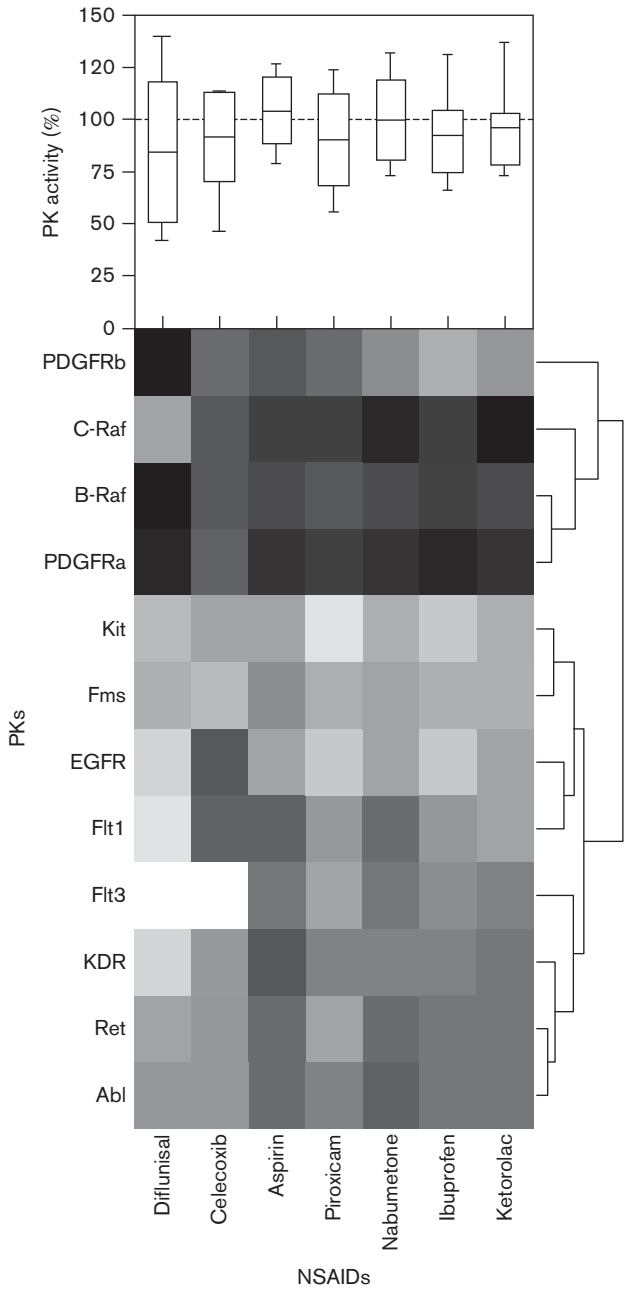
shown in Fig. 4b. Considering the physiological concentrations, 10% of PKs should be bound with NSAIDs, whereas up to 90–95% of PKs could be bound with the corresponding anticancer drugs. However, mutated PKs found in cancers usually have increased activity, with a 10-fold or even a 100-fold increase 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 ~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

Fig. 5



Activity of PKs with some NSAIDs at a 100 μmol/l concentration in the biochemical screening. Top: averages (the horizontal line inside boxes) with SD (box vertical size) and min/max values (whiskers). Bottom: activity heatmap (brighter shades indicate higher inhibition). PK, protein kinase.

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 log*P* values of 2–6 (Hopkins *et al.*, 2006), which are highly similar to NSAIDs: molecular weight = 289.5 ± 64.2 Da and log*P* = 3.3 ± 0.9.

The ligand-family-based comparison of NSAIDs with anticancer drugs using log*P* 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.

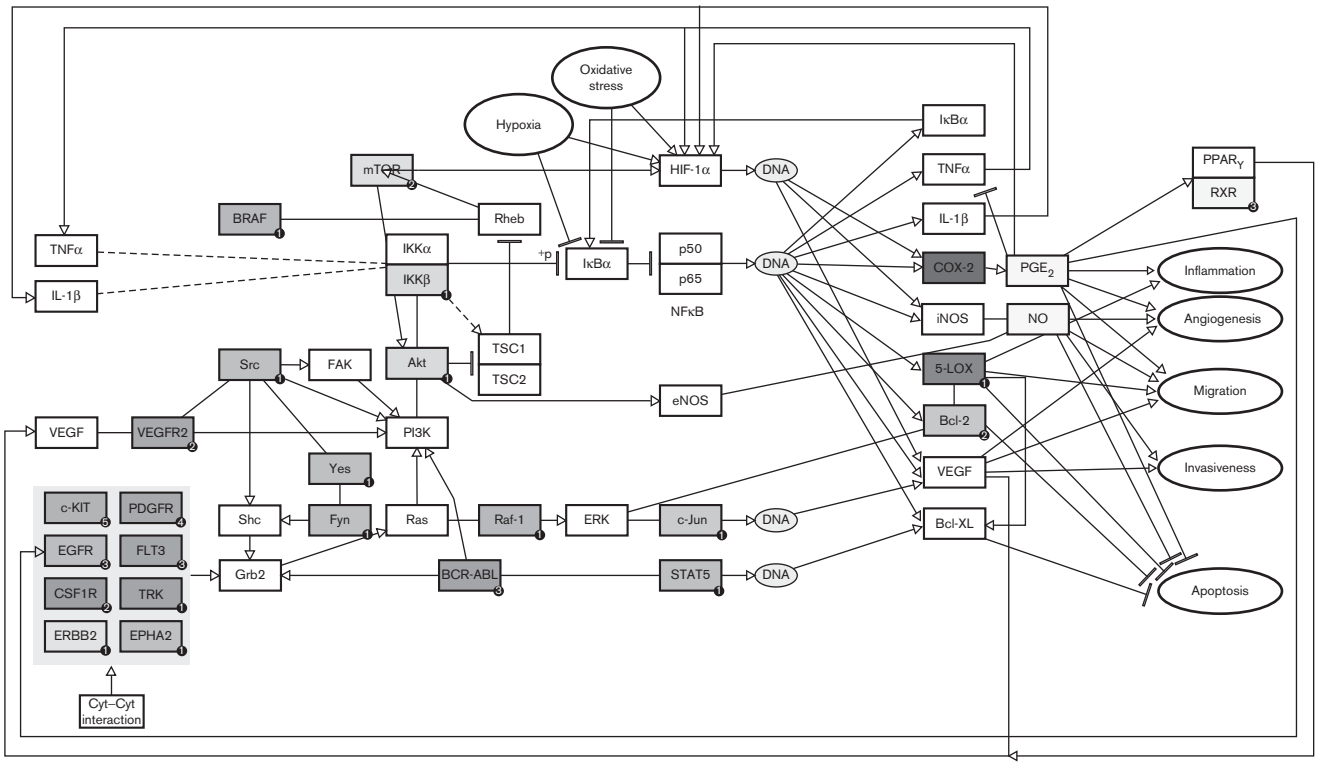
The investigated targets against NSAIDs and their immediate partners have been consolidated into pathways, as shown in Fig. 6. The pathways integrate COX-1/2, its related counterpart 5-LOX, and the recently identified IKKβ. We have included DNA, as previous studies have shown that aspirin can reside 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

Fig. 6



Network with common targets for anticancer drugs, color-coded according to the cumulative affinity (grayscale background: darker shades, higher affinity) for NSAIDs.

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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