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New NSAIDs-NO hybrid molecules with antiproliferative properties on human prostatic cancer cell lines

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ABSTRACT

The design of profen hybrids containing a NO donor moiety connected to an aliphatic spacer led to compounds with a similar cyclooxygenase inhibition compared to their parent profen and with significant antiproliferative activities on PC3 cells. However, inhibition of COX-2 pathway alone did not seem sufficient to inhibit cancer cell proliferation, and NO-release in a time-dependent manner strongly contributes to this activity.

In vivo, in vitro and epidemiological studies have all demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) appear to be effective in the chemoprevention and possible treatment of many cancers.[1] On the other hand, recently and initially in order to counteract the side effects of these drugs on the gastric mucosa, nitric oxide (NO) donating NSAIDs have been synthesized to take advantage of the multiple roles of this ubiquitous messenger. In addition to its similar properties to prostaglandins within the gastric mucosa, evidence is accumulating for the role of NO as a new oncopreventive agent and as a novel therapeutic to overcome cell resistance.[2] Numerous new chemical entities with enhanced anticancer properties, safety and efficacy versus NO-free control compounds were then developed, and the archetypical hybrid drug (in preclinical development) is nitric oxide-donating aspirin (NO-ASA).[3] However, even if NO-ASA seems, in cultured cancer cells, 1000-fold more potent than aspirin, the role of NO and NSAID moiety is more complex than that previously thought. Several mechanisms of action have been proposed including (or not) COX-2 activity,[4] and several signalling factors such as NF-κB, IFN-β, Hsp90, and p75 and latest hypothesis even evoke the active influence of the spacer used to connect NO-donating group and NSAID.[5]

Based on these considerations, which emphasize the need of additional essential data on mechanism of metabolisation and action of these new therapeutic derivatives and of each part of the molecule, we studied the effect of several new NO-NSAIDs on the growth of human prostate cancer cells, and the influence of NO release kinetics.

In an attempt to rationalize the link between cyclooxygenase inhibition, NO liberation and cell proliferation and to study the

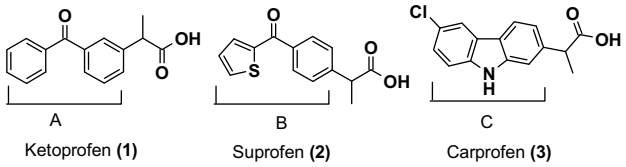
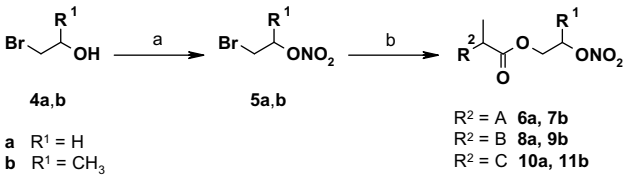


Figure 1. Structure of profens 1-3.



Scheme 1. Reagents and conditions: (a) 70% HNO_3, 95% H_2SO_4, 2 h, 0 °C; (b) 1—3, Cs_2CO_3, DMF, 12 h, 20 °C.

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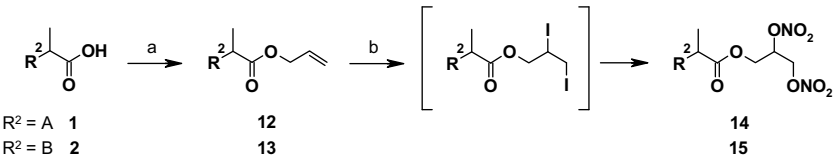


Scheme 2. Reagents and conditions: (a) Allyl bromide, Cs_2CO_3, DMF, 20 °C, 12 h; (b) $AgNO_3$, I_2, CH_3CN, 0 °C, then $AgNO_3$, reflux.

dissociation kinetics of such compounds, hybrid molecules were designed from representative compounds of these kinds of activity. Profens, that is, Ketoprofen **1**, Suprofen **2** (Fig. 1), were chosen because of their interesting inhibitory activity against cyclooxygenase and of their different selectivity versus the two isoforms COX-1/COX-2. Carprofen **3**, even if globally less effective, can be representative of a more selective COX-2 inhibition.

The NO-donor moiety was carried by a nitrooxy alkyl function brought by the ω–bromoalkanols **4a**,**b**: the nitric ester group was chosen for its kinetic profile of metabolization.[6] The aliphatic spacer was modulated in an attempt to modify the profen-release kinetics.

The NO-donating profens **6**–**11**, **14**, **15** were synthesized as described on Schemes 1 and 2.

The set of compounds **6**–**11** containing one nitrooxy function was obtained (Scheme 1) via nitration of the bromoalkanols **4a**,**b** using a 70% HNO_3 95% H_2SO_4 mixture, giving the corresponding nitrooxyalkyl bromides **5a**,**b** in quantitative yield. The nitrooxyalkyl esters **6**–**11** were prepared by condensation of the bromides **5a**,**b** with profens **1**–**3** in the presence of Cs_2CO_3.

The dinitrooxy-containing compounds **14**,**15** were prepared (Scheme 2) from profens **1**,**2**. Allyl esters **12**,**13** were prepared by nucleophilic substitution of allyl bromide by the carboxylic acid function of Ketoprofen **1** or Suprofen **2** (DMF, Cs_2CO_3). A subsequent vicinal iodination of the allyl function (products not isolated) followed by a two-step nitration of the primary and secondary halide carbons ($AgNO_3$, acetonitrile), according to a previously described procedure[7], led to dinitrooxy esters **14** and **15**. This route failed with the allyl ester of Carprofen **3** because of the instability of the intermediate iodonium ion. Every structure was confirmed by IR and NMR spectrums as well as LC/MS chromatograms, and purity of both profens and final products exceeded 98%.

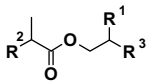
Assays for the inhibition of COX pathways were performed in human whole blood (HWB),[8] and Ketoprofen, Suprofen and Carprofen displayed inhibition values similar to those found in the literature. As expected, the modified profens have a very similar IC_{50} value (Table 1), and this can be explained by the esterase activity in human whole blood assays, enzymes able to cleave the ester bond by which the nitric oxide-donating moiety is linked to the parent cyclooxygenase inhibitor. Only the Carprofen derivatives present a significant difference: with a spacer and a NO donor, cyclooxygenase inhibition activity is cut in half. The release of this parent compound was verified by evaluating the amount of profen released via HPLC (Table 1). Results seem to indicate that the release was effective and almost complete after 22 h of incubation in human whole blood. In addition to the cleavage of the ester bond, biodegradation of the profen itself can and probably has occurred during the incubation time, hence the sub-100% release values are obtained.

The first series of assays on PC3 cell proliferation was done using the standard incubation time of 72 h with the compounds.[10] We first had to witness almost no activity at all from the profens on these very resistant cells. However, when linked to a NO-donating moiety, the derivatives showed some potency that can apparently be accounted only to the NO release, even though the difference between molecules having one and two nitric esters is not significant (Table 1).

Three molecules were then incubated for 24, 48 (data not shown) and 72 h in a proliferation kinetic assay. Moreover, molecules were chosen to evaluate if the inactive profen moiety had any influence on proliferation when linked to the exact same nitric oxide donor. Results are shown in Fig. 2. As expected, we observed that the effect of NO was much more visible after 24 h incubation; this is probably due to the metabolic activity.

EPR spectroscopy[11] was simultaneously performed to see if a link could be found between the amount of NO released in a given amount of time and the proliferation kinetics profile. The metabolization process of our molecules includes the cleaving of the ester bond by esterase, the release of ONO_2^- and the subsequent 3 electrons reduction to obtain NO. And since this radical half life is very

Table 1
COX pathways inhibition[a], profen release[b] in HWB assays and PC3 cells proliferation assays (percentage of inhibition at 100 μM or IC_{50})



Compound	R^1	R^2	R^3	Inhibition of COX-1 pathway IC_{50}, μM[a]	Inhibition of COX-2 pathway IC_{50}, μM[a]	Profen release ratio %, 10 μM	PC3
Ketoprofen	—	—	—	0,33	0,69	100	0
6	H	A	$-ONO_2$	0,45	0,77	73	26
7	CH_3	A	$-ONO_2$	0,60	0,73	70	26
14	$-ONO_2$	A	$-CH_2ONO_2$	0,58	0,74	79	21
Suprofen	—	—	—	0,56	2,75	100	0
8	H	B	$-ONO_2$	0,58	5,80	46	7
9	CH_3	B	$-ONO_2$	0,57	4,56	86	13
15	$-ONO_2$	B	$-CH_2ONO_2$	0,48	2,70	73	33
Carprofen	—	—	—	59	38	100	5
10	H	C	$-ONO_2$	110	65	88	IC_{50} = 48 μM
11	CH_3	C	$-ONO_2$	123	45	100	IC_{50} = 48 μM

[a] All IC_{50} values represent the mean of at least four experiments.
[b] Profen release ratio measured after an incubation time of 22 h, using the same blood pool as the COX inhibition experiments.

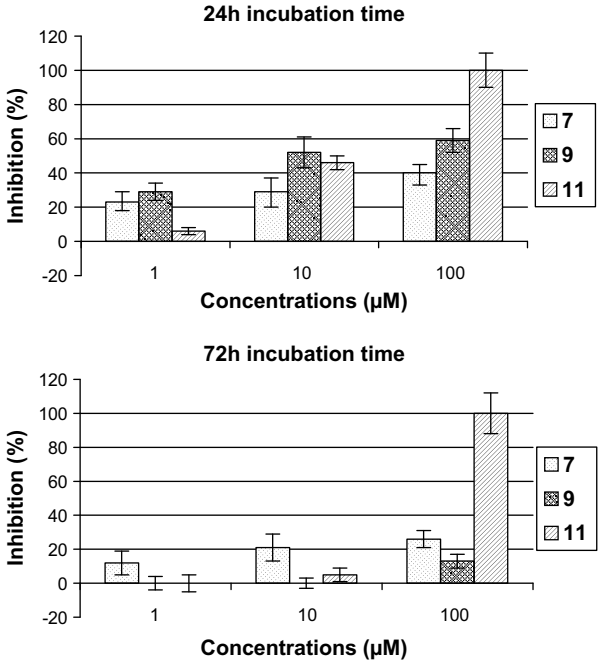


Figure 2. Effect of 7, 9 and 11 on PC3 cell proliferation after 24 and 72 h incubation.

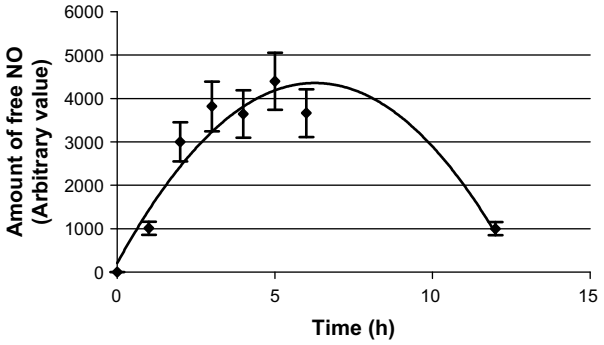


Figure 3. NO released from 7 evaluated by EPR spectroscopy.

short (a few seconds in HWB), it requires the use of iron (II) *N*,*N*-diethyldithiocarbamate, a radical scavenger.[12]

According to our measurement, NO release from the nitric ester in human whole blood is slow, and it shows a peak in concentration after approximately 6 h (Fig. 3). Afterwards, metabolic activity and chemical degradation of the NO-scavenger complex begin to be faster than the actual release.

These results first confirm that it is excluded to directly link inhibition of COX activity and antiproliferative properties. The radical NO was showed much more effective, but our data suggest that no general conclusion will ever be valid and that each part of hybrid molecules (NSAIDs, linkers, NO-donating group) and above all targeted tissues contribute to the effectiveness of such drugs.

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8. HPLC analysis of arachidonic acid metabolites in whole blood.[9] Fresh blood was collected in heparinized tubes from normal volunteers. Then, 1 mL aliquots were transferred to heparinized tubes preloaded with either 4 μL of vehicle (DMSO) or 4 μL of test compounds and incubated for 15 min at 37 °C (final compound concentrations used: 10^{-7}; 5.10^{-7}; 10^{-6}; 5.10^{-6}; 10^{-5}; 5.10^{-5}; 10^{-4} and 5.10^{-4} M as solubility allows). Then, 40 μM of calcium ionophore A 23187 alone was added, and incubation was carried out at 37 °C for 20 h to stimulate the COX-1 pathway. For the COX-2 pathway, samples stimulated with A 23187+LPS (500 μg) were incubated for 20 h. Eicosanoids were extracted from the samples into ethyl acetate. Samples were then evaporated to dryness under nitrogen, re-suspended in the mobile phase to precipitate proteins and then centrifuged. The supernatant was directly analyzed by HPLC using Hypersil ODS 3 μM columns (12.5 mm × 0.46 mm), a methanol/water/acetic acid (80:20:0.08) mobile phase at a flow rate of 1 mL/min and UV detection firstly at 270 nm and 12 min later at 245 nm.
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10. Cell culture and cell proliferation assays. Human prostate PC3 cancer cells were grown at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum, in a humidified incubator containing 5% CO_2. In the cell proliferation assay, cells were plated (1.8×10^4 cells/well) on 24-well plates. After 3 days, the cell medium was changed to serum free medium, and the cells were starved for 24 h for culture synchronization. Cells were then incubated in culture medium that contained various concentrations of test compounds (1; 10; 50 and 100 μM as solubility allows), each dissolved in less than 0.1% DMSO. After incubating for a given amount of time (usually 72 h), cell growth was estimated by the colorimetric MTT test.
11. EPR spectra were recorded using a Bruker ELEXYS E580 spectrometer operating at 9.5 GHz with a 100 kHz high frequency modulation and a modulation amplitude of 1 G. The sample solutions were examined in quartz cell inserted in standard thermoregulated cavity for the EPR spectra at liquid nitrogen temperature. The EPR signal of NO° trapped by iron (II) *N*,*N*-diethyldithiocarbamate consists in three lines with a nitrogen hyperfine splitting constant of 13 G. The concentration of NO° released was quantified by double integration of EPR signal using 2,2,6,6-tetramethyl-1-piperidinyloxy TEMPO as standard.
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