Nitric oxide donating non steroidal anti-inflammatory drugs (NO-NSAIDs) for the treatment of cancer.

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Background and rationale

Treatment of pain, inflammation, and fever most frequently implies non-steroidal anti-inflammatory drugs (NSAIDs) administration. Beside their primary role in treatment of inflammation, evidence clearly shows a chemopreventive effect for aspirin and other non-steroidal anti-inflammatory drugs on colorectal and gastric cancer and probably other cancer types [Cuzick et al. 2009; Puntoni et al. 2008; Dube et al. 2007]. However, although selective cyclooxygenase-2 (COX-2) inhibitors are now given to patients at high risk of colorectal cancer, data on the risk-benefit profile for cancer prevention are insufficient and no definitive recommendations can be made regarding the lowest effective dose, the age at which to initiate therapy, the optimum treatment duration, and the subpopulations for which the benefits of chemoprevention outweigh the risks of adverse side-effects [Cuzick et al. 2009].

Studies on the mechanisms by which NSAIDs might inhibit carcinogenesis have not provided conclusive evidence for pathways or molecular targets that are clinically most relevant [Cuzick, et al. 2009] but several NSAIDs properties have been proposed to play important roles in cancerogenesis prevention: stimulation of apoptosis, cell growth suppression, inhibition of angiogenesis, and metastasis prevention [Chan et al. 2002; Chan et al. 1998]. Furthermore, overexpression of COX-2 has been reported in tumor cells and tissues [Scartozzi et al. 2004; Lim et al. 2000]. The inhibition of COX by NSAIDs was previously thought to be the unique explanation for their antitumor effect [Hanif et al. 1996], but more recently, other COX-independent mechanisms have been identified [Sun et al. 2009; Yin et al. 1998; Grilli et al. 1996]. It has been recently demonstrated that aspirin and several other NSAIDs could promote apoptosis through the inhibition of NF-Kb activity, activation of mitochondrial pathways by cytochrome c release and activation of caspase-9 and extrinsic pathways by activation of caspase-8, induction of oxidative stress and inhibition of proteasome functions [Jana 2008].

Unfortunately, serious undesirable effects limit the application of those drugs. The most common adverse NSAID therapy-related events are the development of ulcers and subsequent bleeding in the upper gastrointestinal tract and different renal side effects, such as acute renal failure, acute interstitial nephritis, worsening of chronic kidney disease, salt and water retention and hypertension, leading to increased cardiovascular risk [Wallace JL et al. 2008; House et al 2007; Rigas and Kashfi 2004]. Moreover, selective COX-2 inhibitors have a reduced risk of gastrointestinal bleeding, alongside with a sim-
ilar renal toxicity profile and most likely a worsened cardiovascular toxicity [Whelton 2002].

Chemical modifications of these drugs based on the covalent attachment of a nitric oxide (NO) releasing moiety –ONO\textsubscript{2}, often via a spacer molecule, has been proposed to overcome the most common NSAID-associated adverse events [Lanas 2008]. This approach was supported by an idea that NO shares similar properties with prostaglandins (PGs) as regards the capacity of PGs to influence local blood flow [Rigas and Kashfi 2004]. It is indeed hypothesized that the NO molecules bound to the drug through the spacer molecule might be delivered to the damaged site, thereby decreasing gastric and renal toxicities induced by diminished PG levels [Lanas 2008; Rigas and Kashfi 2004].

In a phase 2, double-blind, randomized, parallel group study in patients with osteoarthritis, the novel NO-NSAID 4-nitrooxybutyl (2S)-2-(6-methoxynaphthalen-2-yl)propanoate (Naproxcinod), a NO modified derivative of naproxen, appeared safer than COX-2 inhibitor positive control rofecoxib [Karlsson et al. 2009]. Up to date, naproxcinod completed the pivotal phase III studies needed for a New Drug Application, that has been submitted to the Food and Drug Administration in September 2009 and accepted for filing, seeking approval for the treatment of the signs and symptoms of osteoarthritis [NicOx Press Release 18 October 2009].

Independent studies on NO-donating NSAIDs, alternatively known as cyclooxygenase inhibiting nitric oxide donators (CINODs), have consistently demonstrated that these compounds bear up to several thousands-fold augmented antitumoral potentials both in vitro and in vivo when compared to the parental compounds [Rigas and Williams 2008; Kashfi and Rigas 2007; Huguenin \textit{et al.} 2005; Rigas and Kashfi 2004]. However, in oncology, the actions of NO are highly variable as it showed to exert both anti- and pro-neoplastic activity [Huerta, Int J Oncol 2008]. Reflecting the duality of NO function in cancer, both anti-NO and NO-based anticancer strategies appear effective in several preclinical models [Mocellin \textit{et al.} 2007].

Most likely, the final activity of NO in tumors is dependent on its working microenvironment, including the type of cell exposed to the compound, the redox state of the reaction, as well as the final intracellular concentration and the duration of intracellular exposure to nitric oxide [Huerta, Int J Oncol 2008].

Current interpretations of the data suggest a dose dependent relationship between NO concentration and tumor response, and it is generally accepted that at high concentra-
tions of NO may have an anti-neoplastic function by exposing cells to high levels of nitrosative stress whereas at low levels it can stimulate angiogenesis, cancer cell proliferation and metastatic potential [Chinje and Stratford 1997]. There is, however, no unifying mechanistic explanation for the biphasic role of nitric oxide in cancer. When released under appropriate conditions, NO possesses multiple antineoplastic properties both in vitro and in vivo including inhibition of cellular proliferation by cell cycle arrest induction [Kroncke et al. 1998], stimulation of autophagic cell death [Maksimovic-Ivanic et al. 2008] or apoptotic cell death through different mechanisms like p53 upregulation or activation, [Forrester et al. 1996], proteosomal degradation of anti-apoptotic mediators [Glockzin et al. 1999], induction of Smac release [Li et al. 2004] increase in mitochondrial permeability and consequent cytochrome c release [Boyd and Cadenas, 2002], regulation of angiogenesis by modulation of several kinases like PKC, ERK and AP-1 [Jones et al. 2004], protection against metastasis formation through enhancement of Raf-1 Kinase Inhibitor Protein expression [Bonavida et al. 2008] or regulation of matrix metalloproteinase levels [Phillips and Birnby, 2004].

In addition, NO has a well characterized chemo-, radio-and immuno- sensitizing potential [Bonavida et al. 2006], that has been attributed respectively to nitrosation of criticals thiols in DNA repair enzymes such as alkyltransferase [Laval et al. 1994], to a mimicy of the effects of oxygen on fixation of radiation-induced DNA damage [De Ridder et al. 2008] and to inhibition of the multifactorial transcription repressor Yin Yang 1 [Vega et al. 2005].

Moreover, classical nitric oxide donors have been shown beneficial effects also in humans since low dose glyceryl trinitrate treatment significantly delayed the PSA doubling time in prostate cancer patients after surgery and radiotherapy [Siemens et al. 2009]. Thereby, NO-NSAIDs represent an emerging class of compounds with chemopreventive, chemotherapeutic chemio-, radio- and immuno-sensitizing properties against a variety of cancers, demonstrated in preclinical models including cell culture systems and animal tumor models of different origin [Rigas and Williams 2008].

Their mechanism of action appears complex and involves the generation of reactive oxygen species [Sun Y et al. 2009], suppression of microsatellite instability in mismatch repair-deficient cells [McIlhatton et al. 2007] alongside the modulation of several signaling cascades including nuclear factor kappa B [Williams et al. 2008], Wnt [ Lu et al. 2009] and mitogen activated protein kinases [Hundley et al. 2006] that culminate in inhibited cell renewal and enhanced apoptosis [Rigas and Williams 2008; Rigas 2007].
Remarkably, these effects seemed to be COX-independent [Rigas and Williams 2008]. NO-ASA (NO-aspirin) is the best-studied compound belonging to this group, but several other NO-NSAIDs, including NO-sulindac, NO-ibuprofen, NO-indomethacin, NO-flurbiprofen, NO-naproxen have been recently synthesized [Sun et al. 2009; Rigas and Williams 2008].

It has been questioned whether the anticancer effect of the NO-SAIDs is directly dependent on the NO release or it may be, at least for the NO-ASA, dependent to the spacer molecule exerting its own pharmacological effects [Rigas and Williams 2008; Kashfi and Rigas 2007; Hulsman et al. 2007]. Currently, other NO-donating anti-inflammatory drugs with the NO-donating group covalently attached to the parental compound that possesses strong anticancer activity have been synthesized by our group of research [Maksimovic-Ivanic et al. 2009; Maksimovic-Ivanic et al. 2008]. Alternatively, it has been hypothesized that the effects of NO-NSAIDs may depend on multiple mechanisms somehow arising from a simple NO-release, that can be rather achieved with a classical NO donor. From this point of view, NO-release is not required but contributes to the anticancer effect [Rigas and Williams 2008].

Given the paradoxical effects of NO against cancer, long term therapy with NO-NSAIDs may actually promote cancer growth by releasing NO. However, critical analysis of the results of the Framingham Heart and Offspring Study for evaluating the effects of nitro-vasodilators on the risk of colorectal cancer show that there was no increase in colorectal cancer over a sufficiently long period of observation, suggesting that unlikely chronic therapy with NO donors may lead to cancer [Muscat et al. 2005].

Up to date, only one phase I clinical trial with NO-ASA for the prevention of colon cancer has been started but was unfortunately recently terminated prematurely due to concerns regarding the potential genotoxicity of one putative metabolite, not directly correlated with the –ONO₂ group [NicOx Press Release 18 June 2007].
Aim of study.

Nitric oxide donating non steroidal anti-inflammatory drugs (NO-NSAIDs) represent an emerging class of compounds with chemopreventive, chemotherapeutic chemio-, radio- and immunosensitizing properties against a variety of cancers, demonstrated in pre-clinical models including cell culture systems and animal tumor models of different origin. These compounds consist of a conventional NSAID to which an NO-releasing moiety $\text{ONOO}^-$ has been covalently attached.

The aim of this study will be to evaluate the anticancer potential of the novel NO-NSAID Naproxcinod, since it is the only NO-NSAID that, differently from other compounds belonging to the same class like Aspirin-NO, has so far demonstrated a clear safe profile in humans and has not been extensively studied yet as a potential novel anticancer therapeutic.

The final objective of the study will be to provide solid basis for appropriately designing phase II clinical studies based on Naproxcinod administration.

Materials and Methods

Reagents and cells

Cancer cell lines (see Table 1 for a complete list) were available at the Department of Bio-Medical Sciences, University of Catania or purchased from ATCC, LGC Standards srl, Milan, Italy. The cells were grown in the appropriate culture media as indicated by the protocols of American Type Cell Collection (ATCC). All culture media, supplements, antibiotics and fetal bovine serum were purchased from Life Technologies Italia. Cells were routinely maintained at 37°C in a humidified atmosphere with 5% CO$_2$. Cells were collected with 0.25% trypsin-1 mM EDTA solution in PBS, and seeded at density of $1 \times 10^4$ well in 96-well plates unless otherwise indicated.

Naproxcinod (Figure 1), Naproxen and Cisplatin were obtained from Sigma-Aldrich (Milan, Italy). MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide, Thiazole Blue) was purchased from Merck Chemicals Ltd. (Nottingham, UK).
**Animals**

Six- to 8-week-old male BALB/c mice, 4 to 5-week-old BALB/c male and female athymic nude mice, and male Wistar rats 8-weeks old were purchased from Harlan Laboratories (Udine, Italy).

The mice were kept under standard laboratory conditions (non specific pathogen free) with free access to food and water. The animals used in the experiments were protected in accordance with Directive 86/609/EEC. The animal studies were carried out in accordance to local guidelines and will be approved by the local Institutional Animal Care and Use Committee (IACUC).

**In vitro studies**

**Isolation of colonocytes primary cells**

Distal colon isolated from male Wistar rats was cut in 4 cm pieces and incubated for 5 minutes at 37°C in a 5% trypsin / 2% EDTA solution. Colon fractions were then transferred in a Petri dishes with complete medium to block trypsin activity and cells were detached by the mucosa with a scraper. The collected cellular suspension was centrifuged, washed, counted and suspended in freezing medium (RPMI, 10%FCS, 10% DMSO) at the concentration of 2x10^6 cell/mL.
Isolation of human PBMCs

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated from fresh buffy coats of healthy volunteers. The buffy coats were diluted with phosphate-buffered saline (PBS) supplemented with 2.5 mM EDTA and layered onto Ficoll-Hypaque gradients (Gibco, Invitrogen, Milan, Italy). After 30 min of centrifugation at 400g at room temperature, mononuclear cells were collected, washed twice with PBS and incubated in tissue culture multi-well plates.

Evaluation of cell viability by MTT

Cells were seeded in 96-well plates, incubated for 24-72 hrs in the presence of different concentrations of Naproxcinod, Naproxen and Cisplatin and viability was estimated using MTT assay as previously described [Mosmann, J.P. (1983) J. Immunol. Methods 65, 55-63]. The viability of treated cells was shown as percentage of value obtained for untreated cultures that was arbitrarily set to 100%. The MTT assay involves the conversion of the water soluble MTT to an insoluble formazan. The formazan is then solubilized in 0.1 N HCl in isopropanol and the concentration determined by optical density measured at 570 nm.

In vivo studies

Prostate cancer xenograft models

Tumours were induced in female or male Balb/c athymic nude mice by subcutaneous injection of cultured PC3 (androgen-independent human prostate cancer) or LNCaP (androgen-dependent human prostate cancer). Cells were dispersed by trypsin, washed (twice) in serum-free medium RPMI-1640 (10 min centrifugation, 200 x g), resuspended at the concentration of 2.5 x 10^7 cells/ml in the same medium and injected (0.2 ml) s.c. in the right flank of each mouse using a 0.6 mm needle. Tumour growth was observed daily and measured with calipers (2 perpendicular diameters), and tumour volume was calculated using the formula 0.52 x a x b^2, where a is the longest and b is the shortest diameter.
Three independent experiments were performed and each group consisted of 7-8 mice. Treatment with Naproxen or Naproxcinod started when the tumors were already palpable with a range volume of 60-70 mm³. The mice were randomly assigned to each experimental group. Post randomization analysis revealed no significant differences in tumor volumes at the beginning of the treatment among the different groups. Naproxen or Naproxcinod were prepared immediately before treatment and they were administered orally (per os) at a dose of 40 mg/kg for 20 consecutive days. A group of mice was treated with the vehicle carboxymethylcellulose (CMC 1% in water for injection), and another group with cisplatin intraperitoneally (i.p.) at the dose of 1mg/kg twice a week as positive control. The animals were observed for further 16 days after the interruption of the treatment.

Colon cancer xenograft model

Balb/c mice were inoculated subcutaneously with 2x10⁵ cells CT26CL25 (murine colon adenocarcinoma) and have started to be palpable tumors 10-12 days after inoculation. The animals were treated orally with Naproxcinod and Naproxen at a dose of 40 mg/kg or vehicle. Mice were treated under a therapeutic regimen starting when the tumor began to be palpable and continued for 2 consecutive weeks. The animals were treated with cisplatin (i.p.) twice a week at a dose of 1 mg/kg as a positive control. Tumour growth was observed daily and measured with callipers (2 perpendicular diameters), and tumour volume was calculated using the formula 0.52 x a x b², where a is the longest and b is the shortest diameter.

Induction of Lung Metastasis

Tumors were induced in BALB/c mice by injection of cultured mouse colon cancer CT26.CL25 cells. The cells were detached by trypsin, washed (twice) in serum-free medium RPMI (10 min centrifugation, 200× g), resuspended at the concentration of 2 x 10⁵ cells/ml in the same medium and injected (0.2 ml) i.v. in the tail of each mouse. The animals were treated orally with Naproxcinod and Naproxen at a dose of 40 mg/kg or vehicle starting on day 3, when, from the literature, the micro-metastases are beginning to be present in the lung and continued for 9-12 days. The animals were treated with cisplatin (i.p.) twice a week at a dose of 3 mg/kg as a positive control.
On day 12-14 post tumor challenge, mouse lungs were removed and weighted on an analytical scale.

**Statistical analysis**

The results are presented as mean ± SD of triplicate observations from one representative of at least three experiments with similar results, unless indicated otherwise. Student’s t-test was used to determine statistical significance. Values of p < 0.05 were considered to be statistically significant.
Results

Toxicity data

In order to assess the toxic effects of Naproxcinod and the parental compound Naproxen on primary cells, rat colonocytes and human PBMCs were incubated for 24-72hrs in the presence of scalar concentrations of these drugs, and viability measured as cellular respiration using mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan. None of the compounds reached the LC50 at concentrations up to 200 $\mu$M (Figure 2). However, compared to Naproxen, Naproxcinod showed a safer profile with almost no toxic effects on both cell lines. Viability of rat colonocytes at 72 hrs incubation with 200 $\mu$M Naproxcinod and Naproxen was 94% and 59%, respectively (Figure 2A). Viability of human PBMCs at 72hrs incubation with 200 $\mu$M Naproxcinod and Naproxen was 90% and 63%, respectively (Figure 2B).

Anticancer effects of Naproxcinod

Determination of cancer cell sensitivity to Naproxcinod

We first evaluated the in vitro antitumoral effect of Naproxcinod on a panel of 20 cancer cell lines. To this aim, cells were incubated for 24-72 hrs with 7 log concentrations of Naproxcinod and of the parental compound Naproxen, and viability determined by MTT assay. Cell lines included were human or murine from prostate, colon, breast, SCLS, NSCLC, astrocytoma, melanoma, kidney and leukemia cancers (see Table 1 for a complete list). Sensitivity of each cell line was determined and results are shown in Table 2. Naproxcinod resulted to be effective in reducing cell growth/proliferation in both murine and human prostate cancer, colon cancer and astrocytoma cell lines (Table 2). On the contrary, breast, lung, melanoma, kidney and leukemia cell lines showed to be not sensitive to Naproxcinod, as the IC50 was higher than 200 $\mu$M.
Comparative anticancer effects of Naproxcinod

After determining the cancer cell lines that were sensitive to Naproxcinod, we evaluated the comparative effects between Naproxcinod, the parental compound Naproxen and the positive control drug Cisplatin (Figure 3). To this aim, cells were treated for 24 h with a previously determined range of concentrations and cell viability was estimated using MTT assay.

Parental compound Naproxen did not show significant anticancer effects in all of the cell lines tested, since the IC50 was not reached at concentrations up to 160 \( \mu \)M (Figure 3 A-F).

Cisplatin was effective in reducing cell growth/viability of all of the cell lines tested, showing an IC50 lower than 20 \( \mu \)M (Figure 3).

Naproxcinod and the positive control drug Cisplatin showed superimposable IC50 values in both androgen-dependent and androgen-independent prostate cancer cell lines PC3 and LnCap (Figure 3A-B).

Overall, Naproxcinod was less potent than Cisplatin in reducing cell growth in all of the cell lines tested, however no statistically significant differences was observed between the two drugs (Figure 3).

In vivo anticancer effects of Naproxcinod in prostate cancer

To confirm the data obtained in vitro, we tested the antitumoral effects of Naproxcinod in vivo in mouse models of cancer. We first evaluated the efficacy of Naproxcinod on tumor growth in nude mice xenografted with PC3 cells. Mice were treated with Naproxcinod, Naproxen, Cisplatin or vehicle for 21 consecutive days starting from about 21 days after xenograft. The tumor volumes of the mice treated with Naproxcinod were significantly reduced (p<0.05) already starting from 9 days after the beginning of the treatment (day 30) until the end of the observational period. The parental compound Naproxen started to show significant effects after 19 days of treatment but the effect was lost around 7 days after the interruption of the treatment (day 50) (Figure 4).

We further tested the anticancer effects of Naproxcinod in the LnCap xenograft mouse model. Mice were treated with test compounds for 21 consecutive days starting at day 22 after xenograft. Relative to vehicle-treated mice, the volumes of the tumors of the mice treated with Naproxcinod were significantly inhibited (p < 0.05) starting from 12 days after the beginning of the treatment (day 34) until the end of the treatment. The same was observed for the positive control drug Cisplatin. The parental compound Naproxen showed significant effects after 21 days of treatment until the end of the study (Figure 5).
In vivo anticancer effects of Naproxcinod in colon cancer

Based on the results from the in vitro studies, we determined the antitumoral effects of Naproxcinod in the colon cancer setting. To this aim we evaluated the efficacy of Naproxcinod on tumor growth in balb/c mice xenografted with CT26CL25 cells. Naproxcinod treatment significantly inhibited cell growth starting from day 6 post-inoculation. The inhibitory potential of novel modified drug was greater than the parental drug, which reached the statistical significance at day 28 post-inoculation (Figure 6). The effects of Cisplatin treatment was superimposable to that of the Naproxcinod (Figure 6).

To evaluate the anti-metastatic potential of parental and NO-modified Naproxen, we examined their effects in the lung metastasis model induced by i.v. injection of CT26CL25 cells. Cisplatin was effective in reducing the weight of the lung (Figure 7B) and mice exhibited a better clinical conditions, as observed from the significantly lower loss in body weight (Figure 7A). Treatment of mice with Naproxcinod resulted in a decrease in lung weight, although the statistical significance was not reached (Figure 7B). No protective effect was observed for the parental compound Naproxen (Figure 7A-B).

Discussions

Nitric oxide-releasing non-steroidal anti-inflammatory drugs (NO-NSAIDs) are a class of NSAID derivatives generated by adding a nitric oxide donating group to the parental NSAID. Several NO-NSAIDs have recently been developed, including NO-ASA (NO-aspirin), the first in class compound, NO-sulindac, NO-ibuprofen, NO-indomethacin, NO-flurbiprofen and NO-naproxen [Sun et al. 2009; Rigas and Williams 2008]. NSAIDs have been extensively investigated in clinical trials for their chemopreventive/chemotherapeutic effects in different types of tumors. A randomized trial conducted in 135 patients with advanced stage cancer (colorectal, liver, pancreatic, and gastric primary cancers) and an expected survival of more than 6 months, showed that the addition of indomethacin prolonged mean survival with 8.7 months compared to placebo-treated patients [Lundholm et al., 1994]. In a pilot study 12 patients, who had relapse of their prostate cancer received celecoxib. Five patients had a decline in their absolute PSA level, three patients had
stabilization of the level and of the remaining four patients, three had a marked decrease in their PSA doubling time [Lundholm et al. 1994]. Parallel to the anti-tumor activity of NSAIDs as single agents, interest has raised in the effects of a combined therapy of chemotherapy with NSAIDs. A retrospective study comparing capecitabine in combination with celecoxib compared to capecitabine alone in colorectal cancer patients showed an increase in median time to tumor progression (6 months versus 3 months, P = 0.002) and in the proportion of stable disease (62.5% versus 22.8%, P = 0.001) [Lin et al. 2002].

However, conventional NSAIDs are known for their gastrointestinal side effects and nephrotoxicity. About 15–30% of regular NSAID users have one or more ulcers when examined endoscopically, and 3–4.5% of NSAID users have clinically significant upper gastrointestinal events, including ulcers and ulcer complications [Laine et al. 2001; Wolfe et al. 1999]. Different renal side effects, such as acute renal failure, acute interstitial nephritis, worsening of chronic kidney disease, salt and water retention and hypertension, leading to increased cardiovascular risk have also been described [Wallace JL et al. 2008; House et al. 2007; Rigas and Kashfi 2004].

Chemical modifications of NSAIDs based on the covalent attachment of a nitric oxide (NO) releasing moiety, has been proposed to overcome the most common NSAID-associated side effects [Lanas 2008]. Beside their safer profiles in comparison to the parental drugs, several studies on NO-donating NSAIDs have also demonstrated that these compounds exert up to several thousands-fold increased anticancer effects both in vitro and in vivo [Rigas and Williams 2008; Kashfi and Rigas 2007; Huguenin et al. 2005; Rigas and Kashfi 2004]. NO can modify sulphydryl residues of proteins through S-nitrosylation. S-nitrosylation of proteins has been recently recognized as a critical cellular regulation mechanism [Lane et al., 2001]. NO can also modulate NF-kB activity by nitrosilation and oxidation of several different NF-kB proteins including IkB, kinaseB and p50 and p65 [Marshall et al., 2000; Reynaert et al., 2004]. In addition, S-nitrosylation can fine-tune cellular homeostasis by maintaining the balance between the induction and prevention of apoptosis [Son et al. 1995; Li et al. 1997].

Moreover, NO has a well characterized chemo-, radio-and immuno- sensitizing potential [Bonavida et al. 2006], likely by nitrosilation of thiols in DNA repair enzymes [Laval et al. 1994], a mimicry of the effects of oxygen on fixation of radiation-induced DNA damage [De Ridder et al. 2008] and inhibition of the multifactorial transcription repressor Yin Yang 1 [Vega et al. 2005].

Up to date, only one phase I clinical trial with NO-ASA for the prevention of colon cancer has been
initiated but was unfortunately recently terminated prematurely due to concerns regarding the potential genotoxicity of one putative metabolite, correlated to the spacer [NicOx Press Release 18 June 2007].

In this study we have explored both in vitro and in vivo the chemopreventive/chemotherapeutic properties of the NO-NSAID, NO-naproxen. Naproxcinod is a cyclooxygenase (COX)-inhibiting nitric oxide (NO) donator, which has been designed to provide at least the same anti-inflammatory and analgesic efficacy as marketed NSAIDs with an improved safety profile, particularly with respect to gastrointestinal safety and blood pressure. This profile was intended to be achieved by designing a molecule with two active moieties both of which are released following rapid, systemic metabolic cleavage. The first moiety comprises the long established NSAID Naproxen to provide relief from the signs and symptoms of OA; the second is a novel NO-donating moiety the release of which is designed to counteract the detrimental effects of naproxen on BP and to some extent provide protection from the effects of Naproxen on the GI tract and other organs. NO possesses marked vascular smooth-muscle relaxant properties through activation of soluble guanylyl cyclase and consequent formation of cyclic guanosine monophosphate (cGMP) [EMA/657046/2011 © European Medicines Agency, 2011].

We show here that Naproxcinod is able to significantly inhibit cancer cell growth in vitro at doses comparable to those of conventional chemotherapeutic drugs, while exerting lower toxicity on primary cells. The magnitude of the effects exerted by Naproxcinod was, as expected, significantly higher than those of the parental compound naproxen. We found out that among all the cancer cell types tested, Naproxcinod is particular effective in both androgen-dependent and androgen-independent prostate cancer cells and colon cancer cells. Of note, was that the effects of Naproxcinod in these models was maintained also during the follow up period after the interruption of the treatment. We have further validated the results obtained in vitro in murine xenograft models. The data show a strong antitumoral effect of Naproxcinod in all of the models tested. However, Naproxcinod was not able to significantly decrease the metastatic potential of the CT26CL25 colon cancer cells to the lungs.

Overall, these data suggest the possible use of Naproxcinod as adjuvant therapy for the treatment and prevention of cancer. Tertiary prevention makes use of specific xenobiotics to prevent or delay the development of cancer. The epidemiological observation that NSAID administration is able to prevent colon cancers has driven the search for novel chemoprevention approaches against cancer. Indeed, two randomized trials with aspirin given at 300 mg, 500 mg, or 1200 mg daily showed a decrease in colon cancer incidence compared with placebo [Fiorucci et al. 2003]. A good
chemopreventive agent should be effective, devoid of significant side effects, inexpensive and convenient to administer. To this regards, Naproxcinod seems to meet the before mentioned characteristics.

In conclusion, these data provide strong support for the design of phase II trials based on the administration of Naproxcinod to cancer patients in association to conventional therapies or to prevent disease recurrence.
Tables and figures.

Table 1. Cancer cell lines screened for their sensitivity to Naproxcinod.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
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<tr>
<td>PC3</td>
<td>human prostate cancer</td>
</tr>
<tr>
<td>LnCap</td>
<td>human prostate cancer</td>
</tr>
<tr>
<td>CT26CL25</td>
<td>murine colon cancer</td>
</tr>
<tr>
<td>HCT116</td>
<td>human colon cancer</td>
</tr>
<tr>
<td>SW620</td>
<td>human colon cancer</td>
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<tr>
<td>HCC70</td>
<td>human breast cancer</td>
</tr>
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<td>MCF7</td>
<td>human breast cancer</td>
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Table 2. Naproxcinod IC50 values on cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3 prostate cancer</td>
<td>35 μM</td>
</tr>
<tr>
<td>LnCap prostate cancer</td>
<td>40 μM</td>
</tr>
<tr>
<td>CT26CL25 colon cancer</td>
<td>80 μM</td>
</tr>
<tr>
<td>HCT116 colon cancer</td>
<td>90 μM</td>
</tr>
<tr>
<td>SW620 colon cancer</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>HCC70 breast cancer</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>MCF7 breast cancer</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>TA3HA breast cancer</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>H69 SCLC</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>H1688 SCLC</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>H2126 NSCLC</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>H23 NSCLC</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>C6 astrocytoma</td>
<td>75 μM</td>
</tr>
<tr>
<td>A375 melanoma</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>COLO melanoma</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>MEWO melanoma</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>786-0 kidney cancer</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>CAKI-1 kidney cancer</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>HL60 leukemia</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>K562 leukemia</td>
<td>&gt;200 μM</td>
</tr>
</tbody>
</table>
Figure 1. Molecular structure of the NO-modified Naproxen, Naproxcinod.

Figure 2A. Toxic effects of Naproxcinod on rat colonocytes cells

Figure 2B. Toxic effects of Naproxcinod on human PBMCs

Figure 2. Naproxcinod is not toxic to human and murine primary cells. Rat primary colonocytes (A) and human PBMCs (B) were treated with a range of concentrations of Naproxcinod for 24 h, after which cell viability was determined by MTT assay. Data are presented as Mean ± SD from one representative of three independent experiments.
Figure 3. Comparative anticancer effects of Naproxcinod
Figure 3. Naproxcinod decreases the viability of cancer cells. Cells were treated with a range of concentrations of Naproxcinod, Naproxen, vehicle or positive control drug Cisplatin for 24 h, after which cell viability was determined by MTT assay. Data are presented as Mean ± SD from one representative of three independent experiments. (A) Effects of drug treatment on human prostate androgen-independent PC3 cells. (B) Effects of drug treatment on human prostate androgen-dependent LnCap cells. (C) Effects of drug treatment on murine colon cancer CT25CL26 cells. (D) Effects of drug treatment on human colon cancer HCT116 cells. (E) Effects of drug treatment on human colon cancer SW620 cells. (F) Effects of drug treatment on murine astrocytoma cancer C6 cells.
Figure 4. In vivo anticancer effects of Naproxcinod in prostate cancer (PC3).

A

PC-3 Androgen independent Human Prostate Adenocarcinoma in Nude Mice

5x10^6 PC3 cells injected s.c.

Treatment: day 21 to day 42 after tumour implantation

Readout: tumour volume in mm^3

B

PC3 Tumor growth

- Vehicle
- Naproxcinod 40 mg/kg
- Naproxen 40 mg/kg
- Cisplatin 1 mg/kg

Figure 4. Naproxcinod inhibits the growth of androgen-independent PC3 prostate cancer cells in nude mice. (A) Induction protocol and scheme treatment for PC3 xenograft induction. (B) Tumors were induced by subcutaneous implantation of 5 x 10^6 PC3 cells and mice treated with either Naproxcinod, Naproxen, vehicle or Cisplatin for 21 consecutive days starting when tumors were palpable. Tumor volumes were calculated twice times a week.
Figure 5. In vivo anticancer effects of Naproxcinod in prostate cancer (LnCap).

Figure 5. Naproxcinod inhibits the growth of androgen-dependent LnCap prostate cancer cells in nude mice. Tumors were induced by subcutaneous implantation of $5 \times 10^5$ LnCap cells and mice treated with either Naproxcinod, Naproxen, vehicle or Cisplatin for 21 consecutive days starting when tumors were palpable. Tumor volumes were calculated twice times a week.
Figure 6. Naproxcinod inhibits the growth of murine colon cancer CT25CL26 cells in balb/c mice. Tumors were induced by subcutaneous implantation of $5 \times 10^5$ CT25CL26 cells and mice treated with either Naproxcinod, Naproxen, vehicle or Cisplatin for 21 consecutive days starting when tumors were palpable. Tumor volumes were calculated twice times a week.
Figure 7. Effects of Naproxcinod in a metastatic model of colon cancer.

A. Body weight

B. Lung weight

* p<0.05 vs vehicle
Figure 7. Effects of Naproxcinod in a metastatic model of colon cancer in mice. Tumors were induced in BALB/c mice by injection of cultured mouse colon cancer CT26.CL25 cells i.v. in the tail of each mouse. On day 3, mice were treated with test compounds for two weeks. On day 12-14 post tumor challenge, mouse lungs were removed and weighted on an analytical scale. (A) Body weight variation of tumor-bearing mice upon treatment with Naproxcinod, Naproxen, vehicle or Cisplatin. (B) Weight of lungs isolated after two weeks of treatment. (C) Representative pictures of lungs isolated from tumor-bearing mice after two weeks of treatment.
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