**A dual acting compound releasing nitric oxide (NO) and ibuprofen, NCX 320, shows significant therapeutic effects in a mouse model of muscular dystrophy**

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**Abstract**

A resolutive therapy for muscular dystrophies, a heterogeneous group of genetic diseases leading to muscular degeneration and in the severe forms to death, is still lacking. Since inflammation and defects in nitric oxide generation are recognized key pathogenic events in muscular dystrophy, we have analysed the effects of a derivative of ibuprofen, NCX 320, belonging to the class of cyclooxygenase inhibiting nitric oxide donor (CINOD), in the α-sarcoglycan null mouse, a severe mouse model of dystrophy. NCX 320 was administered daily in the diet for 8 months starting 1 month from weaning. Muscle functional recovery was evaluated by free wheel and treadmill tests at 8 months. Serum creatine kinase activity, as well as the number of diaphragm inflammatory infiltrates and necrotic fibres, was measured as indexes of skeletal muscle damage. Muscle regeneration was evaluated in diaphragm and tibialis anterior muscles, measuring the numbers of centronucleated fibres and of myogenic precursor cells. NCX 320 mitigated muscle damage, reducing significantly serum creatine kinase activity, the number of necrotic fibres and inflammatory infiltrates. Moreover, NCX 320 stimulated muscle regeneration increasing significantly the number of myogenic precursor cells and regenerating fibres. All these effects concurred in inducing a significant improvement of muscle function, as assessed by both free wheel and treadmill tests. These results describe the properties of a new compound incorporating nitric oxide donation together with anti-inflammator properties, showing that it is effective in slowing muscle dystrophy progression long term. Of importance, this new compound deserves specific attention for its potential in the therapy of muscular dystrophy given that ibuprofen is well tolerated in paediatric patients and with a profile of safety that makes it suitable for chronic treatment such as the one required in muscular dystrophies.

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**1. Introduction**

Nitric oxide (NO) is involved in many mechanisms responsible for preserving muscle function [1] including both pre-synaptic and post-synaptic neuromuscular transmission, energy supply and mitochondrial biogenesis [1–4]. In addition, NO regulates repair of the damaged muscle via specific actions on satellite cells, mononuclear progenitor cells found in mature muscle and located between the basal lamina and sarcolemma which are normally quiescent, but they can be activated in response to muscle injury [5–8]. Alteration in the amount and localisation of NO synthesis has been implicated in the pathophysiology of skeletal muscle dystrophies. These genetic degenerative diseases of the muscle are due to defects in muscle proteins, leading to structure alteration and continuous damage of fibres during contraction [9]. Duchenne muscular dystrophy (DMD) is the most severe form, with a paediatric onset and often leading to rapid paralysis and a premature death, usually by respiratory and/or cardiac failure before 20th year of age [9]. In DMD, as well as in the mouse models of muscular dystrophies, mdx or α-sarcoglycan (SG) null mouse, the splice...
variant of neuronal nitric oxide synthase (nNOS\textsubscript{\text{\textalpha}}) is absent from the sarcolemma, and relocated to the cytosol, with total muscle NOS activity being thus reduced [10]. Overexpression of nNOS in the mdx mouse was shown to yield substantial recovery of muscle structure [11].

Histological analyses of dystrophic skeletal muscle in human and in mice show that skeletal muscle myofibres are progressively substituted by connective and adipose tissue [9]. This continuous fibre damage is counteracted, at least initially, by sustained proliferation and activation of satellite cells leading to regeneration of fibres; in later phases depletion of the pool of myogenic precursors, due to the repeated cycles of activation and proliferation, leads to significant decrease of the muscle repair capacity [12,13]. Enhanced fibres destruction is also due to the extensive inflammatory response occurring within the muscle, which contributes significantly to progression of muscular dystrophies [14]. Indeed, DNA microarray and biochemical data show that inflammatory mediators/effectors dominate the expression profile of muscles from the mdx mouse model of dystrophy [15,16].

A variety of pharmacological and genetic approaches, aimed at regulating NO supply to the muscle, have been used to ameliorate the disease progression in both the \(\alpha\)-SG-null and the mdx mouse models [6,11,17–23].

We have recently reported that combining NO donation with a non steroidal anti-inflammatory agent leads to a recovery of muscle function which is both significant and persistent [18,24]. Specifically, chronic administration of the NO-donating flurbiprofen HCT 1026, belonging to the CINOD (cyclooxygenase-inhibiting nitric oxide (NO)-donator) class [25], exerted significant therapeutic effects in two different mouse models of dystrophy [18]. HCT 1026 significantly slowed disease progression, maintaining the functional capacity of muscles by reducing necrosis and inflammation, and preserving the regenerative potential in both the \(\alpha\)-SG-null and the mdx mouse models. Since flurbiprofen is a potent anti-inflammatory agent associated with marked gastro-intestinal side effects and it is not approved for use in paediatric patients, we have focused our attention on a chemically related drug, ibuprofen, which is more widely used also in paediatric patients because of its profile of safety [26–29].

In this study we have analysed the long-term efficacy of a derivative of ibuprofen, namely NCX 320, which targets two mechanisms: NO donation and cyclooxygenase inhibition. We have defined its pharmacological profile in terms of kinetics of ibuprofen and NO release using biochemical and functional assays. We have then examined the therapeutic potential of NCX 320 in the \(\alpha\)-SG-null mice, which are characterised by a severe dystrophic phenotype with progressive damage and reduced regeneration capacity. NCX 320 induced persistent and significant reduction of both fibre damage and inflammation, thus preserving muscle integrity. Moreover, NCX 320 significantly increased the myoblast precursor number and differentiation capacity, maintaining the long-term regeneration capacity of muscle. The data with the prototype NCX 320 show that a dual-acting compound possesses a potential for treatment of muscular dystrophies.

2. Materials and methods

2.1. Materials

NCX 320 (Fig. 1), 4-(nitrooxy)butyl 2-(4-isobutylophenyl)propanoate, was synthesized at the NicOx Research Institute (Bresso, Milan, Italy). Ibuprofen was obtained from Albe- marle Corporation (Baton Rouge, LA, USA). Interferon-\(\gamma\) (IFN\(\gamma\)) was from Roche Molecular Biochemicals (Mannheim, Germany), Dulbecco’s modified Eagle’s medium (DMEM), foetal calf serum, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). PGE\(_2\) EIA-kit was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). The protease inhibitor cocktail was purchased from Roche (Basel, Switzerland). Elisa kits for cytokines determinations were purchased from R&D System (Minneapolis, MN, USA). Fluorescein isothiocyanate-conjugated CD34 antibody was purchased from AbD Serotec (Oxford, UK) and phycoerythrin-conjugated \(\alpha\)-7-integrin antibody from MBL (Woburn, MA, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. In vitro profile of NCX 320

2.2.1. Cyclooxygenase activity inhibition

To assess cyclooxygenase (COX)-inhibiting properties of the compound, COX-1 and COX-2 activities were studied using resting and LPS/IFN\(\gamma\)-stimulated RAW 264.7 cells respectively, as previously described [30]. The experiments were carried out in the presence of 1 \(\mu\)M of each enzyme and porcine liver esterases (1.7 units per sample) were added to facilitate the release of ibuprofen from NCX 320.

2.2.2. Vasodilating effects

NO-donating activity was studied in the vascular relaxation assay in rabbit aorta as previously described [31]. Briefly, the aortic rings were suspended in organ chambers containing Krebs solution and connected to a force displacement transducer (Grass FT03) for the measurement of isometric force. After equilibration, aortic segments were pre-contracted submaximally with methoxamine (3 \(\mu\)M) and, after obtaining a stable tone, a cumulative concentration–response curve of test drugs (0.01–100 \(\mu\)M) was established in the absence or presence of the soluble guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-\text{a}]quinoxalin-1-one (ODQ: 10 \(\mu\)M) to determine the vasodilating effects dependent on NO/cyclic guanosine monophosphate (cGMP). Data were expressed as percent of relaxation and plotted vs concentration.

2.3. In vivo efficacy of NCX 320 in \(\alpha\)-SG mice

2.3.1. Animals and drug treatment

\(\alpha\)-SG null mice were a kind gift of Dr. K Campbell (Iowa University, Iowa City, IA, USA). Animals were housed in the pathogen-free facility at the San Raffaele Scientific Institute (Milano–Italy), and treated in accordance with the European Community guidelines, and with the approval of the Institutional Ethical Committee. Mice (15 animals/group) were treated with 50 mg/kg/die of ibuprofen or 65 mg/kg/die of NCX 320 (equimolar to ibuprofen) administered daily into the diet (Mucedola, Milano, Italy) or with the same food...
2.3.2. Determination of ibuprofen, nitrates and nitrates in plasma

One-week following NCX 320 treatment, plasma was obtained from the heart of animals and centrifuged 13,000 × g at 4 °C for 5 min to remove cells. Plasma ibuprofen concentrations were assessed using previously published methods [32,33]. The method was linear from 2.5 to 250 μM, imprecision as well as inaccuracy was below 10%.

For quantitative NO measurements, prior to the derivatization procedure, plasma (100 μL) were spiked with the 15N-labeled nitrite and nitrate as internal standards to achieve final concentrations of 0.2 μM for [15N]nitrite and 2 μM for [15N]nitrate. Derivatization and quantification of nitrite and nitrate was performed as described [34].

2.3.3. Biochemical analyses and functional tests in dystrophic muscle

Serum CK levels were measured in blood samples obtained by tail vein withdrawal using an indirect commercially available colorimetric assay (Randox, UK) [17].

Functional muscle activity was measured using both the running wheel to assess free locomotor activity and the exhaustion treadmill to assess resistance to fatigue [17]. For the running wheel, three tests were performed on the same animal allowing 2 days in between each measure, while the exhaustion treadmill test was performed after an appropriate training period and three tests were performed on the same animal allowing 1 week in between each test.

2.3.4. Histological analysis

The animals were sacrificed by cervical dislocation and diaphragm muscle was dissected and immediately frozen in liquid N2 cooled isopentane. Haematoxylin–Eosin (H&E) or the Masson trichrome stainings were performed in serial muscle sections following manufacturers procedures (Bio Optica, Milan Italy). At least 10 random images for each muscle were taken at 10× magnification with a 100 μL TV microscope (Carl Zeiss Microlining Inc, Jena, Germany) and analysed using a digitized imaging system (Image 1.3B × National Institute of Health) for counting necrotic and centronucleated fibres and for evaluating infiltrated inflammatory cell number. Necrotic cells were identified by hyper eosinophilia, thinning, waviness and eventually presence of many nuclei [35].

2.3.5. Cytokine determination in tibialis anterior muscle

Tibialis anterior muscles were isolated from sacrificed animals and rapidly homogenized in 20 mM Tris–HCl, containing 137 mM NaCl, 5 mM ethylenediaminetetraacetic acid and a protease inhibitor cocktail, pH 8.0, and centrifuged at 3000 rpm for 5 min at 4 °C. After protein content determination, equal amounts of protein were analysed using commercially available kits according to manufacturer’s instructions.

2.3.6. Primary myoblast cells isolation and analysis

Primary myoblasts were isolated from quadriceps and gastrocnemius muscles as previously described [20]. Myoblasts were then counted and stained with a fluorescein isothiocyanate-conjugated CD34 antibody and a phycocerythrin-conjugated α7-integrin antibody according to manufacturer’s protocol. The number of double positive cells was measured by flow cytometry analysing 10,000 events for each sample (FACS caliber, Becton Dickinson, San Jose, CA, USA). Aliquots (1 × 105) of isolated cells were cultured using matrigel-coated dishes in DMEM supplemented with 10% foetal calf serum, 3% chick embryo, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamycin for 1 week. To induce myotube formation cells were then cultured for 24 h in differentiated medium (DMEM supplemented with 2% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin). Cells were lysed and analysed for differentiation proteins expression by sodium-dodecylsulfate electrophoresis as described [20].

2.4. Statistical analysis

Results are expressed as the mean ± SEM or median (interquartile range) according to their distribution based on results of the Kolmogorov–Smirnov normality test. For the parameters with a normal distribution, the Student’s t-test was used, whereas the Mann–Whitney test was used for parameters with non-normal distribution. A p-value <0.05 was considered as statistically significant.

3. Results

3.1. In vitro profile of NCX 320

3.1.1. NCX 320 inhibits COX-1 and COX-2 activities

To evaluate the anti-inflammatory effects of NCX 320, we investigated its COX inhibitory activity in resting (COX-1) or LPS/IFNγ-stimulated (COX-2) RAW 264.7 cells. NCX 320 inhibited in a concentration-dependent manner the COX-1 and COX-2 activities (IC50 of 8.1 ± 1.1 and 21.6 ± 1.2 μM respectively) similarly to ibuprofen, which displayed an IC50 of 11.7 ± 1.2 μM for COX-1 and 18.2 ± 1.5 μM for COX-2 (Fig. 2A). The experiments were carried out in the presence of porcine liver esterases (1.7 units per sample) to facilitate the release of ibuprofen from NCX 320.

3.1.2. NCX 320 induces NO-dependent vasodilation

We evaluated whether NCX 320 releases bioactive NO, investigating NO-mediated vasorelaxation on methoxamine-precontracted rabbit aortic rings. Cumulative concentration–response curves to NCX 320 evoked concentration-dependent relaxation with an EC50 of 20.9 ± 3.9 μM, achieving 85% relaxation at the highest concentration tested of 100 μM (Fig. 2B). These vasodilating effects depended on the activation of the NO/cGMP pathway, as they were fully antagonized by ODQ, a selective soluble guanylate cyclase (sGC) inhibitor (Emax: 22 ± 4%). Under the same conditions, ibuprofen (Emax: 35 ± 1%) was completely inactive up to the highest concentration tested (100 μM).

3.2. In vivo efficacy of NCX 320 in α-SG mice

3.2.1. NCX 320 releases ibuprofen and NO following oral administration in α-SG null mice

Following 1 week of treatment with NCX 320 incorporated into the diet (65 mg/kg/die), we assessed the steady state plasma concentrations of ibuprofen and of nitrates or nitrates, as product of NO metabolism. Plasma levels of ibuprofen were 22.0 ± 3.2 μg/mL, a value similar to that found after a paediatric oral dose of ibuprofen (150–300 mg/day) [36,37]. Nitrates and nitrates levels were significantly (p < 0.05) increased when compared to control animals (3.80 ± 0.10 μM and 39.7 ± 2.1 μM, respectively, for NCX...

3.2.2. Effect of NCX 320 on the amelioration of dystrophic muscle function and structure in α-SG null mice

To study the therapeutic effect of NCX 320 in muscular dystrophy, we relied on α-SG null mice, in which the progressive muscle degeneration with general impairment of locomotor function is severe and resembles the one observed in human muscular dystrophy [18]. We evaluated the muscle functional activity by analysing α-SG null mice performance in both the free wheel (voluntary activity capacity) and the treadmill (resistance to forced exercise) tests after 8 months of drug treatment. NCX 320-treated animals showed significant amelioration in both tests when compared to control α-SG null mice treated with the standard diet. Conversely, ibuprofen alone induced a modest increase of voluntary activity and was completely ineffective in ameliorating resistance to forced exercise (Fig. 3A and B).

To investigate the mechanism of this effect, we analysed skeletal muscle damage by evaluating serum CK levels, a hallmark of muscle damage, as well as fibre degeneration and inflammatory reactions at the level of the diaphragm that, as involuntary muscle, is one of the most damaged in skeletal muscle dystrophy.

NCX 320 treatment significantly reduced CK activity (by 48%, 40% and 41% at 2, 3 and 4 months of age respectively; p < 0.05), a significant effect greater than that observed with ibuprofen alone (Fig. 4A).

Evaluation of H&E-stained sections revealed a significantly lower number of necrotic fibres (Fig. 4B and C) in diaphragm muscles from animals receiving NCX 320 for 8 months (2.9 ± 0.6 fibres/mm²) compared to control, α-SG null mice treated with the standard diet (10.7 ± 2.7 fibres/mm²). Moreover, NCX 320 decreased the accumulation of extracellular scar tissue as revealed by blue in the Masson trichrome staining (Fig. 4D). On the contrary, ibuprofen failed to significantly reduce necrotic fibres at 8 months of age in diaphragm muscles (Fig. 4B).

In addition, animals treated with NCX 320 showed a reduced inflammatory reaction compared to the control group as judged by a significant decrease by 36% of inflammatory infiltrates observed after H&E staining (Fig. 5A). NCX 320 inhibited the expression of the pro-inflammatory cytokines TGF-β, MIP-1α and MCP-1, measured in tibialis anterior muscle homogenates (Fig. 5B). These results were similar to those observed with ibuprofen (although it also reduced TNF-α activity), consistent with the NSAID activity of NCX 320.

3.2.3. NCX 320 induces preservation of muscle regeneration capacity

The ability of muscle myogenic precursor cells to proliferate and fuse to existing myofibres or to generate new myofibres is the key event responsible for muscle repair after damage. In DMD and α-SG null mice, this ability is significantly decreased because of the exhaustion of the myogenic precursor pool due to excessive repair [12].

We analysed sections of muscles isolated from mice after 8 months of treatment with NCX 320 or ibuprofen to measure number of centronucleated/regenerating fibres. Analysis of both diaphragm and tibialis anterior showed a significantly increased number of newly-formed fibres in the muscles of animals treated with NCX 320 with respect to control α-SG null mice while ibuprofen treatment was ineffective (Fig. 6A).

We also isolated the myogenic precursor cells from these muscles, analysed their number by flow cytometry based on co-expression of the markers CD34 and α7-integrin, and evaluated their ability to differentiate in vitro, a good proxy for their
Fig. 3. NCX 320 improves muscle function in α-SG null mice. (A) Free wheel running to test spontaneous movement and (B) treadmill test to measure resistance to fatigue were performed in α-SG null mice treated for 8 months with ibuprofen or NCX 320 incorporated into the diet. Animals receiving the same diet without any drug were used as control. Data are expressed as mean ± SEM (n = 10). *p < 0.05 vs control animals and #p < 0.05 vs ibuprofen-treated animals.

Fig. 4. NCX 320 reduces skeletal muscle damage in α-SG null mice. (A) CK serum levels analysed in mice from 2nd to 4th month of age; (B) number of necrotic fibres quantified in sections of diaphragm muscles after 8 months of treatment ibuprofen or NCX 320 incorporated into the diet. Animals receiving the same diet without any drug were used as control. Data are expressed as mean ± SEM (n = 10). *p < 0.05 vs control animals and #p < 0.05 vs ibuprofen-treated animals. (C) and (D) Representative images of one out of 10 reproducible experiments for H&E and the Masson trichrome staining respectively; scale bars = 100 mm.
regenerative potential [20]. Unlike ibuprofen, NCX 320 increased significantly the number of myogenic precursor cells (Fig. 6B) as well as their ability to express the differentiation markers myogenin and myosin heavy chain after 1 week of differentiation (Fig. 6C and D).

4. Discussion

The pharmacological approaches to human muscular dystrophies used in the clinic today are still based on glucocorticoids administration, usually prednisolone or deflazacort, administered according to various protocols, that delay the disease progression however only temporarily and in the presence of severe side effects [38]. Several alternative experimental pharmacological strategies are being proposed, including administration of calcium antagonists and antioxidant, protease inhibitors, compounds that correct dystrophin gene expression, modulate muscle growth, or stabilise the link between cytoskeleton and extracellular matrix. None of these therapies have so far yielded favourable outcomes in clinical trials [22].

Other approaches recently applied to dystrophy and aimed at reducing inflammatory reaction by monoclonal antibodies or genetic techniques seem to be promising, however still far from therapeutic application, because they are expensive and in some cases target only subsets of patients [39,40].

In this study, we have characterised NCX 320, a member of CINODs, a class of compounds designed to provide the anti-inflammatory and analgesic efficacy of NSAIDs with additional beneficial effects due to NO donation.

In particular, we have found that NCX 320 retains the same anti-inflammatory effect of ibuprofen while releasing NO, and such NO donation endows it with significant and persistent therapeutic effects in the α-SG null mouse model of dystrophy, preserving muscle function, reducing muscle damage and maintaining muscle regeneration capacity. Indeed, those beneficial effects have not been observed with ibuprofen alone which was effective only in reducing inflammatory reaction.

The beneficial effects of NO on muscle function are well known and the mechanisms of its action well understood [3]. Approaches with NO-donors or L-arginine are indeed known to yield amelioration of the mdx dystrophic phenotype. However, these compounds have no therapeutic potential since they yield only transient effects and seldom have been shown to induce functional recovery [21,41–45].

A step forward in terms of the use of NO for the therapy of muscular dystrophy came with CINODs. The clear advantage of these compounds with respect to the classic NO donors is a sustained release of low concentrations of NO, and its combination with an anti-inflammatory activity [25]. Indeed, a recent study by our group showed that another CINOD, the NO-donating flurbiprofen HCT 1026, was effective in slowing muscle dystrophy and that, at least in animal models, it was superior to the glucocorticoid prednisolone [18]. However, flurbiprofen produces severe gastrointestinal damage which makes it unsuitable for long-term treatment, especially in paediatric patients [46]. Therefore, we have focused on NCX 320 because it is based on ibuprofen, whose profile of safety and tolerance is well established and consequently is accepted for paediatric use [47]. NCX 320 is indeed cleaved to its active metabolites ibuprofen and NO and shows the same efficacy of ibuprofen in COX-1 and COX-2 inhibition while donating bioactive NO, as demonstrated by its ability to induce NO/cGMP dependent vasodilation.

The results obtained in the muscular dystrophy model are significant. The administration of NCX 320 in the α-SG null model of dystrophy for 8 months reduced muscle damage through the multi-
NCX 320 increases muscle regeneration, myogenic precursor cells and regenerative potential. (A) Number of centronucleated-regenerating fibres quantified in sections of diaphragm and tibialis anterior muscles; (B) number of CD34+/α7 integrin-positive cells isolated from tibialis anterior muscles was measured by flow cytometry; (C) expression of the differentiation markers myogenin (Myog) and myosin heavy chain (MHC) in isolated myogenic precursor cells. All parameters were evaluated in mice treated for 8 months with ibuprofen or NCX 320 incorporated into the diet. Animals receiving the same diet without any drug were used as control. Data are expressed as mean ± SEM (n = 10). *p < 0.05 vs control animals and #p < 0.05 vs ibuprofen-treated animals. (D) Representative images of the immunoblotting experiments.

Fig. 6. NCX 320 increases muscle regeneration, myogenic precursor cells and regenerative potential. (A) Number of centronucleated-regenerating fibres quantified in sections of diaphragm and tibialis anterior muscles; (B) number of CD34+/α7 integrin-positive cells isolated from tibialis anterior muscles was measured by flow cytometry; (C) expression of the differentiation markers myogenin (Myog) and myosin heavy chain (MHC) in isolated myogenic precursor cells. All parameters were evaluated in mice treated for 8 months with ibuprofen or NCX 320 incorporated into the diet. Animals receiving the same diet without any drug were used as control. Data are expressed as mean ± SEM (n = 10). *p < 0.05 vs control animals and #p < 0.05 vs ibuprofen-treated animals. (D) Representative images of the immunoblotting experiments.

5. Conclusion

NCX 320 showed significant and persistent therapeutic effects preserving muscle function, reducing necrosis of fibres and inflammatory reaction and maintaining regeneration capacity of muscle. These data further confirm that NO donation together with anti-inflammatory activity improves pathological markers and locomotor function in a reference model. Given the well-established and wide use of ibuprofen, these findings indicate a path forward for the development of new potential effective agent for treatment of muscular dystrophy.

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