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Nonsense codons suppression. An acute toxicity study of three optimized TRIDs in murine model, safety and tolerability evaluation

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ABSTRACT

Stop mutations cause 11% of the genetic diseases, due to the introduction of a premature termination codon (PTC) in the mRNA, followed by the production of a truncated protein. A promising therapeutic approach is the suppression therapy by Translational Readthrough Inducing Drugs (TRIDs), restoring the expression of the protein. Recently, three new TRIDs (NV848, NV914, NV930) have been proposed, and validated by several *in vitro* assays, for the rescue of the CFTR protein, involved in Cystic Fibrosis disease. In this work, an acute toxicological study for the three TRIDs was conducted *in vivo* on mice, according to the OECD No.420 guidelines. Animals were divided into groups and treated with a single dose of TRIDs molecules or Ataluren, an FDA-approved TRID molecule, as control. Mice were observed continuously for the first day post-drugs administration and the behavioral changes were recorded. On the 15th day, animals were sacrificed for histological examinations. The results showed that acute administration of 2000 mg/kg of NV914 and Ataluren and 300 mg/kg of NV848 or NV930, did not induce any mortality within 14 days. Moreover, histopathological analysis of treated mice showed no differences when compared to the experimental controls. In summary, our results suggest a good tolerability for the three molecules, and include NV848 and NV930 in a category 4 and NV914 in a category 5 of the Globally Harmonized System (GHS) of Classification and Labeling of Chemicals, classifying these compounds in a low-risk scale for health.

1. Introduction

Stop mutations are caused by a single nucleotide mutation that leads to the presence of a Premature Termination Codon (PTC) in the mRNA [1]. The main consequence of the presence of a nonsense codon in the mRNA is the premature termination of the translation process and the production of an incomplete and not functional protein [2].

Among the nonsense-associated diseases, there are included Cystic Fibrosis (CF), Duchenne Muscular Dystrophy (DMD), β-Thalassemia, Usher's Syndrome, Shwachman-Diamond Syndrome (SDS) [3,4], Choroideremia [5,6], PIDs [7] and some types of inherited cancer [8].

In the last decades, a new therapeutic strategy has been proposed to rescue the effects of stop mutations; this approach is based on the suppression therapy through the promotion of the mechanism known as readthrough. It consists in the insertion of a near-cognate tRNA at the premature stop codon which allowsthe synthesis of a full-length protein [9]. The molecules that show this particular activity were indicated as Translational Readthrough Inducing Drugs (TRIDs).

It is widely known that the first molecules showing this potential activity were aminoglycoside antibiotics that, by interfering with the ribosome machinery, lead to a translational misreading [10]. Unfortunately, due to their side effects of ototoxicity and nephrotoxicity, classical aminoglycosides have been declared unsafe for long-term use as TRIDs [11,12].

High-throughput screening (HTS) studies allowed to find a new compound, characterized by an oxadiazole core in its chemical structure, named Ataluren (or PTC124), that has been broadly tested and approved from FDA for the treatment of DMD due to nonsense mutations [13]. Unexpectedly, the same molecule failed to be approved for CF because of ineffective results of phase III clinical trial. Moreover, the

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molecule showed significantly higher rate of episodes of renal impairment and no improvement of respiratory function measures (FEV1) [14–16].

Considering the genetic variability of the stop mutations associated with specific genetic diseases and the fact that the genetic context, in terms of basis sequence surrounding the stop triplet, plays an important role in the readthrough effect, the research of wide spectrum active compounds is still ongoing [10,17–20]. Indeed, more recently, a new synthetic aminoglycoside, named ELX-02, has shown encouraging results *in vitro* and in phase I clinical trials [21]. This compound displayed a strong readthrough activity associated with safety and tolerability both *in vitro* and *in vivo* and is now in phase II trial for Cystic Fibrosis patients.

In this scenario, computational studies by our research group, considering Ataluren as a template, identified new 1,2,4 and 1,3,4-oxadiazole derivatives as promising hit-compounds [22–24]. Successively, a hit optimization strategy allowed us to discover three new optimized TRIDs. These compounds, named NV848, NV914 and NV930, were tested *in vitro* showing high readthrough activity in CF model systems [25,26].

Preclinical experimental steps are fundamental for drug development, before starting the clinical trial. In particular, preclinical toxicology studies are recommended to establish the toxicological features and safety profile of new drugs.

Our study was conducted according to the Organization for Economic Cooperation and Development (OECD), test guideline No. 420 (Acute Oral Toxicity-Fixed Dose Procedure) [27], a widely accepted standard guideline for acute toxicity testing. This guideline has been demonstrated to use fewer animals and cause them less suffering, whilst still providing reliable acute toxicity data compared to the traditional acute toxicity study method, which used death as an endpoint [28].

Thus, the purpose of this study is to assess the acute oral toxicity of the readthrough agents named NV848, NV914 and NV930, in mice, providing insights into the safety and tolerability of these new molecules in comparison to Ataluren.

2. Materials and methods

2.1. Synthesis of NV848, NV914 and NV930

NV848, **NV914** and **NV930** were synthesized as previously reported [26], purified by automated flash chromatography Biotage Isolera equipment, by using pre-packed flash columns and mixtures of petroleum ether (fraction boiling at 40–60 ∘C) and ethyl acetate as eluents. Analysis of all the samples performed by HR-MS with 6540 UHD Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a Dual AJS ESI source, allowed to assess their purity grade (>95%).

2.2. In silico ADME properties

The QikProp program (QikProp, version 4.4, Schrödinger, LLC, New York, NY, 2015) was used to obtain the ADME properties of our compounds, and Ataluren. It predicts both physically significant descriptors and pharmaceutically relevant features, such as principal descriptors and physicochemical properties. It also evaluated the drug-like acceptability of the compounds, based on Lipinski's rule of five, essential for rational drug design.

2.3. Animals

Animals of both sexes were utilized in this study to examine potential gender discrepancies in response to the selected molecules.

15 male and 15 female adult mice C57BL/6 (6–8 weeks old, weight 18 ± 4 g) were purchased from ENVIGO Srl (San Pietro al Natisone UD, Italy) and housed in temperature-controlled rooms with 22–25 °C, 50–60% humidity and on a 12 h light/darkness cycle. Standard pellet chow and water were available *ad libitum* until the start of the protocol. After their arrival, they were acclimatized for one week before experimentation.

The experimental protocol was conducted in conformity with the Italian D.Lgs 26/2014, following the criteria determined by Organization for Economic and Co-operation Development (OECD) established in 1992 and in accordance to the ARRIVE guidelines. With respect to OECD guideline No. 420, animals were given a single acute dose of the test substance and toxic effects were observed for 14 days. The long recovery period of 14 days was intended for the observation of any potential 'delayed' acute toxicity to the single-dose administration. All animal procedures and care were approved by the Animal Care and Use Ethics Committee of the University of Palermo and by the Italian Ministry of Health (Authorization n.1235/2020) and performed by national and EU Directive 2010/63/EU for the handling and use of experimental animals.

2.4. Administration protocol and groups constitution

Firstly, according to OECD No.420 for the acute single fixed dose procedure, we proceeded with a pilot study, consisting of the administration by oral gavage of the highest dose of 2000 mg/Kg to 1 animal, to determine the dosage for the main study. Then, based on the outcomes (A, B or C) indicated in annex 3 of the guidelines, we proceeded with the same or a lower dosage of 300 mg/Kg.

15 male and 15 female mice were randomly assigned into 6 independent groups receiving by oral gavage the following treatments: (I) (N = 3 per group) treated with 250 μl of water; (II) (N = 3 per group) treated with 250 μl of a solution of 40% DMSO (dimethyl sulfoxide) and 60% olive oil; (III) (3 mice of each sex; N = 6 per group) treated with 250 μl of Ataluren (PTC124) (2000 mg/Kg); (IV) (3 mice of each sex; N = 6 per group) treated with 250 μl of NV848 (300 mg/Kg); (V) (3 mice of each sex; N = 6 per group) treated with 250 μl of NV914 (2000 mg/Kg); (VI) (3 mice of each sex; N = 6 per group) treated with 250 μl of NV930 (300 mg/Kg).

The sample size was determined by G Power analysis [29].

Before receiving treatments, all animals were fasted for 3–4 h and weighed.

All the solutions were prepared on the same day of the experiment. **NV848** was dissolved in distilled water and sonicated at 13% power *in continuum* for 2 min using Branson Digital Sonifier (Marshall Scientific, Hampton, NH, USA). **Ataluren (PTC124), NV914** and **NV930** were prepared in-stock solution of DMSO and further diluted in olive oil (~75%). The maximal percentage of DMSO in the final solution was 25% for **NV914** and **NV930** and about 40% for **PTC124**. No side effects were observed in the animals treated with the higher percentage of DMSO (control group II).

The signs of toxicity described in Tables 1–4 (Supplementary materials) included changes in the skin, fur, hypoactivity, hyperactivity, abdominal pain, itching and scratching, tremors, convulsions, changes in eyes, mucus membranes, salivation, lethargy, diarrhea, coma and/or mortality and were signed in accordance with the designed veterinarians and Ethical Committee of the University of Palermo. All the animals were observed periodically during the first 24 h (0.5, 1, 2, 3, 4, 24 h), then once daily for 14 days. During this period, not only possible toxicity signs were considered, but also body weights, food and water intakes were registered daily.

2.5. Histological analyses

Post-treatment, animals were weighed daily. On day 15, all animals were euthanized using 2% isoflurane anesthesia followed by cervical dislocation, by undergoing gross necropsy. The internal organs were observed.

Besides, all tissue samples were fixed in 10% v/v buffered formalin

for 24 h. Successively, they were washed with abundant water and, finally, preserved in 70% ethanol before proceeding with paraffin fixation. Four-micrometer-thick tissue sections were deparaffinized and rehydrated. Slides were stained with hematoxylin and eosin. All the sections were analysed under Zeiss Axio Scope A1 optical microscope (Zeiss, Germany) and microphotographs were collected using an Axiocam 503 Color digital camera with the ZEN2 imaging software (Zeiss Germany).

All organs dissected from mice, encompassing heart, lung, small and large intestine, liver, pancreas, brain, ovary, testis, kidney, lymph node, spleen and bone marrow, were evaluated on sections routinely stained with hematoxylin and eosin (H&E) for histopathological analysis.

A semi-quantitative scoring system was applied which included many variables for each organ. Interstitial fibrosis, cardiomyocyte changes (nuclear atypia, disarrangement, tapering, and cytoplasmic vacuolation) were considered for heart; interstitial inflammatory infiltration and Interstitial cellularity increase for lung; villi atrophy, crypts hyperplasia, regenerative/displastic alterations, inflammatory infiltration, cryptitis for small intestine; cryptitis, chorion inflammatory infiltration and suppression of mucosecretory activity, regenerative/displastic alterations and atrophy for large intestine; periportal and centrolobular inflammatory infiltrate, lobular architectural disarray, sclerosis and necrosis for liver; loss of acinar tissue, duct changes loss of acinar cells, periductal inflammatory infiltrate, fibrosis and preservation of islets for pancreas; cortical necrosis, intracerebral hemorrhage, neuronal degeneration edema, and inflammatory responses for brain; maturation defects, fibrosis, necrosis and inflammatory infiltrate for ovary; maturation defects, apoptosis figures, Sertoli cells alterations and necrosis for testis; tubular damages, glomerular alterations, vascular congestion, glomerulosclerosis, interstitial fibrosis and arteriolar hyalinosis, for kidney; follicular hyperplasia, progressive transformation of germinal centers, interfollicular expansions, variable degrees of fibrosis, increased histiocytes for lymph node; white pulp effacement, red pulp hyperplasia, myeloid/erytroid precursor increase, megakaryocyte hyperplasia and clustering for spleen;erythroid colonies expansion, myeloid cells expansion (segmented), myeloid cells expansion (immature), megakaryocyte clustering, megakaryocyte pleiomorphism and erythro/hemophagocytosis for bone marrow (Tables 5–17 in the Supplementary results).

All the variables were scored according to the degree of severity (0, absent; 1, mild; 2, moderate; 3 severe) and extent (1, focal; 2, multifocal; 3, diffuse). Slides were analyzed under Zeiss Axio Scope A1 optical microscope (Zeiss, Germany) and microphotographs were collected using an Axiocam 503 Color digital camera with the ZEN2 imaging software (Zeiss Germany).

3. Statistics

All data relative to body weight variation are expressed as mean ± SEM. A two-way repeated-measures analysis of variance was used to determine differences between the groups in the acute toxicity study. Tukey's post-hoc test was conducted for pairwise comparisons when there was a significant overall difference between groups. All statistical analyses were performed using Prism ver. 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). *p*-values of < 0.0001 were considered significant.

4. Results

4.1. Evaluation of the acute toxicity after NV848, NV914 and NV930 administration and macroscopic observation of specific behavioral parameters

The results of the pilot study showed that no signs of mortality were observed in mice after acute administration of 2000 mg/Kg Body Weight (BW) of Ataluren and NV914. This dose was then used for acute oral toxicity evaluation in the main study. Instead, NV848 and NV930 induced mortality at 2000 mg/kg, so, following annex 3 of OECD 420 guidelines, we proceeded with a lower dosage of 300 mg/Kg BW. This dosage was well tolerated for both NV848 and NV930 molecules.

Even if the selected doses did not result lethal, 100% of mice presented moderate signs of toxicity after molecules administration, but all side effects were temporary and disappeared within 4 h post drug administration.

In the group of mice treated with NV848 at a dose of 300 mg/Kg, 3 out of 6 mice presented hypo-activity, tremors, convulsion, and abdominal pain, while only 2 out of 6 mice presented hyperactivity (Tables 1, 5–17; Supplementary results). All these behavioral changes disappeared within 4 h. According to the OECD criteria, the occurrence of toxicity signs in more than one animal and/or one death classified the product in the Globally Harmonized System (GHS) category 4, indicating a low risk for health [27,30].

In the group of mice treated with 2000 mg/Kg of NV914, 5 of 6 treated subjects displayed hypoactivity, and 4 of 6 mice presented hyperactivity and signs related to abdominal pain. Furthermore, 3 of 6 mice presented tremors and convulsions. Moreover, compared to the first tested molecule, we noticed the presence of itching and scratching in 2 of 6 animals (Tables 2, 5–17; Supplementary results).

No toxicity signs were observed after 3 h. Thus, according to the OECD criteria, the occurrence of toxicity signs in more than one animal and/or one death classified the product in the GHS category 5, indicating, also in this case, a low warning for health [27,30].

In the group of mice treated with NV930 at 300 mg/Kg, 5 of 6 mice presented hypoactivity and 1 of 6 mice presented hyperactivity, 3 of 6 abdominal pain and 5 of 6 mice exhibited tremors and convulsions. Also, as already observed in NV914 treated mice, NV930 caused itching and scratching in 4 of 6 animals. Once more, NV930 induced toxicity signs that disappeared within 3 h post-treatment (Tables 3, 5–17; Supplementary results).

As for the NV848 molecule, NV930 can be classified in the GHS category 4, indicating a low warning for health [27,30].

For the positive control group constituted by mice treated with 2000 mg/Kg of Ataluren, 3 of 6 mice presented hypoactivity and 1 of 6 presented hyperactivity and tremors and convulsion, while 4 of 6 displayed abdominal pain. Moreover, 2 of 6 cases showed itching and scratching, exactly like NV914 treated mice (Tables 4, 5–17; Supplementary results). For Ataluren toxicity signs were also observed 30 min post-treatment and within 3 h.

In general, the three TRID tested molecules showed similar toxicity effects to the control (Ataluren/PTC124).

Finally, all the treated mice were monitored until 14 days after the ingestion and did not show any behavioral changes compared to the animals treated with vehicles (water or DMSO/olive oil).

4.2. Evaluation of the body weight in mice treated with NV848, NV914, and NV930 acute dose

The body weight of each animal was recorded before starting drug administration, on the first day of experimentation, then daily for the following 14 days after drug administration, just before euthanasia. The effect of different treatments on the body weight growth pattern during the 14 days is shown in Fig. 1-A and -B, for female and male mice, respectively. Body weight of both treated and control groups arose progressively, and treated animals did not show any significant difference in body growth patterns. No significant difference was observed in the mean values of the body weights of male and female mice treated with ***NV848, NV914 and NV930 or Ataluren*** when compared to their respective controls. However, a transitory increase in the mean values of the body weights of mice treated with 300 mg/kg NV930 was observed in females during the third day ($p < 0.0001$), whereas in males during the third and the ninth day, when compared to the mean body weights of the mice of the control group. In addition, a significant decrease was registered in subjects treated with 2000 mg/Kg of Ataluren, specifically

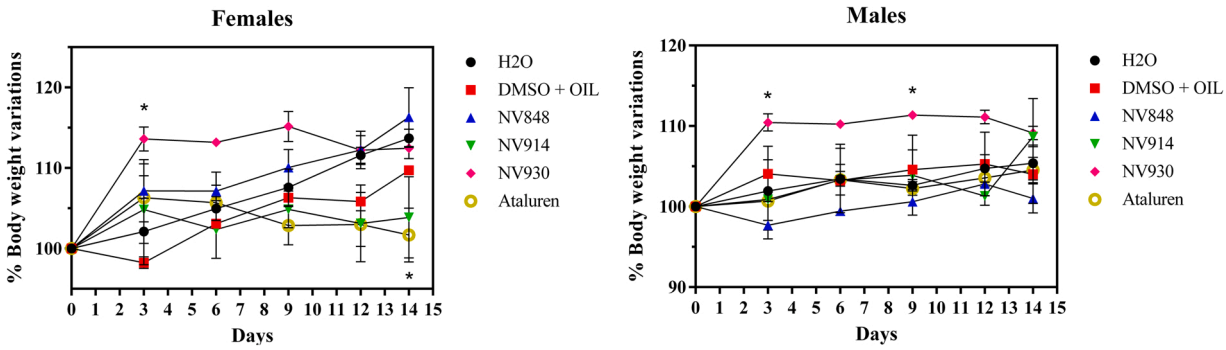

Fig. 1. Graphs showing the percentage of body weights variation in females (A) and males (**B**), during 14 days of observation post-treatment with the indicated molecules or vehicles (water or DMSO + olive oil). Body weight variations are calculated as percent difference relative to the original body weight. Data are mean ± SEM (n = 6) P<0.0001 *versus* the control group (water).

in females during the fourteenth day compared to negative control groups ($p < 0.0001$).

Moreover, no differences in daily food or water intakes were revealed among different groups or comparing males and females in the experimental groups.

4.3. Analysis of the histological features in mice treated with NV848, NV914, and NV930 revealed the absence of tissue or organ alterations 15 days post-acute dose administration

During the macroscopic examination, no treatment-related evident changes were noted on the organs analyzed.

In the histopathological investigations, heart, lung, small and large intestine, liver, pancreas, brain, ovary, testis, kidney, spleen and bone marrow were analyzed in control and treated animals.

No significant morphological alterations (score 0) were observed in the analyzed samples and no consistent differences among the different groups were noted, as shown in the panels of each tested molecule (NV848, NV914, and NV930) and control groups (PTC124, water, DMSO/olive oil) (Figs. 2–7, Tables 5–17 Supplementary results). Nevertheless, a slight tendency to maturation disturbance of neutrophil granulocytes (score 1) associated with a weak expansion of erythroid lineage was detected in two bone marrow samples (mice 10 and 11) belonging to the Ataluren (PTC124) treatment group and also in two samples (mice 19 and 20) belonging to the NV914 treatment group. In detail, the cytological features observed included neutrophilic

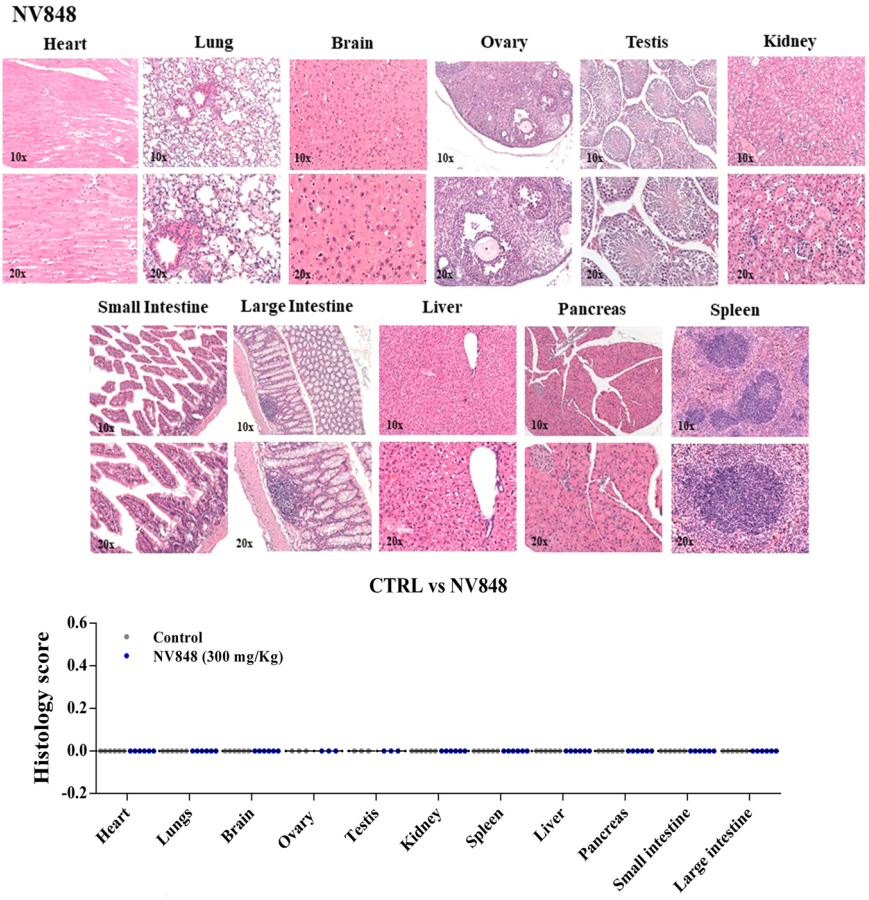

Fig. 2. Histological panel of the indicated organs in mice treated with NV848, 14 days post-acute dose administration (10X-20X). None of the analyzed organs showed morphological or structural alterations, as shown by the graph reporting the measured histology scores.

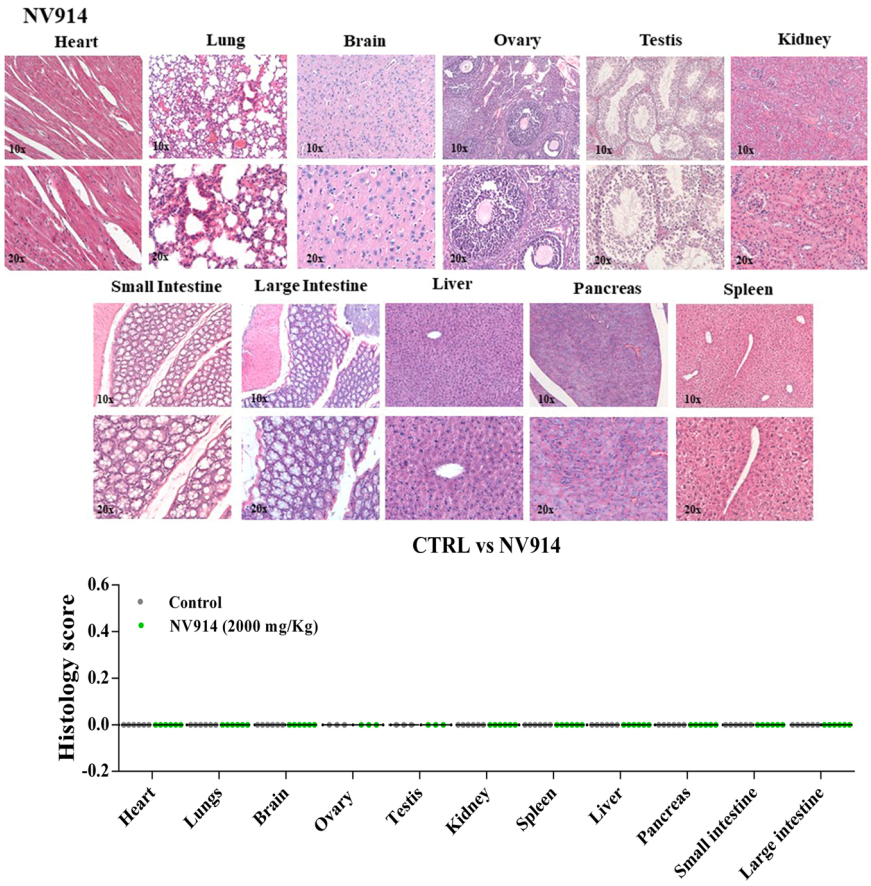

Fig. 3. Histological panel of the indicated organs in mice treated with NV914, 14 days post-acute dose administration (10X-20X). None of the analyzed organs showed morphological or structural alterations, as shown by the graph reporting the measured histology scores.

anisocytosis, nuclear hypo-segmentation and reduced cytoplasmic granularity (Fig. 8, Tables 5–17 Supplementary results).

4.4. NV848, NV914 and NV930 ADME properties

In the attempt to predict absorption, distribution, metabolism, and excretion (ADME) for the compounds, these were submitted to Qikprop calculations. Regarding ADME predictions the final result is expressed in terms of #stars, that is the number of property or descriptor values that fall outside the 95% range of similar values for known drugs. A large number of stars suggests that a molecule is less drug-like than molecules with few stars.

The properties and descriptors included in the determination of #stars are shown in the following Table 1, wherein all the calculated values for the compounds were compared with the calculated values for Ataluren.

Furthermore, the compounds respect Lipinski's rule of five, and they have a predicted human oral absorption.

5. Discussion

The three lead compounds, named NV848, NV914 and NV930 (Patent WO 2019/101709 A1, [25], identified by Ataluren optimization *in silico*, are characterized by an oxadiazole-based structure and showed translational readthrough properties *in vitro.* These molecules showed to promote CFTR rescue and functionality in different CF nonsense mutation cell systems [26].

The present toxicity investigation employed a fixed oral dose method as established by OECD test guidelines No. 420 for the preliminary acute oral toxicity evaluation, which allows classifying substances according to the Globally Harmonized System (GHS) for the classification of chemicals, providing information on the health hazards likely arising on acute exposure. In this study, the acute oral toxicity of TRIDs was investigated and compared with Ataluren.

Acute toxicity is a test performed mainly on rodents, usually promoted at the first steps of the development of a new substance, allowing for assessing the adverse effects that can occur in a short-time period after the administration of a single acute fixed dose of a compound [31].

In the present study, NV848 and NV930 showed mortality at 2000 mg/Kg and were safe at 300 mg/Kg; furthermore, even if moderate abnormal clinical signs were observed during the initial 3 h, for NV930, and 4 h, for NV848, following dosing at 300 mg/Kg, they did not produce any deleterious effect on body weight gain and organ structure as demonstrated by histological analyses.

Among various considered characteristics considered, body weight change is a good indicator of animal health. Indeed, fluctuations in this parameter are not only influenced by weight gain after food intake, but also by alteration of metabolism. Changes in body weight due to exposure to potentially toxic substances could indicate an effect of toxicity, so we monitored any possible significant decrease or increase in body weight from the initial value after substance administration. The increasing body weight of mice for all groups with no significant difference ($p < 0.0001$) at the end suggests that the molecules at the dosage tested are safe. Thus, it can be asserted that TRIDs did not affect energy metabolism of animals.

Only the group receiving NV930 treatment showed a transitory increase of body weight compared to control groups at day 3 and 9 after treatment.

Moreover, Ataluren-treated mice, showed a decrease of 10% compared to control groups at the end of the observational period (14th

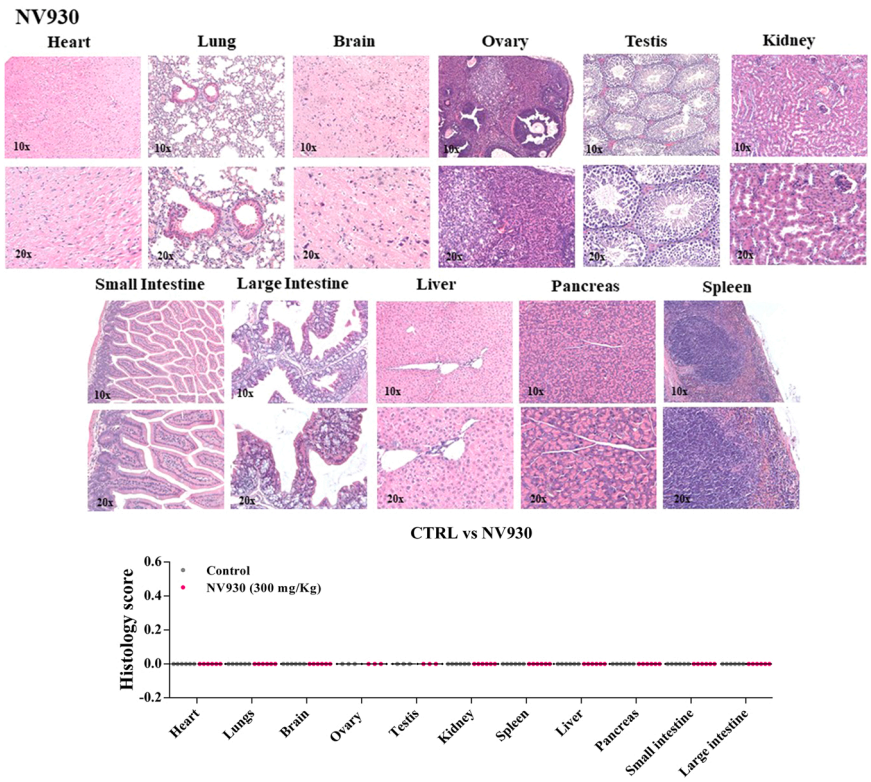

Fig. 4. Histological panel of the indicated organs in mice treated with NV930, 14 days post-acute dose administration (10X-20X). None of the analyzed organs showed morphological or structural alterations, as shown by the graph reporting the measured histology scores.

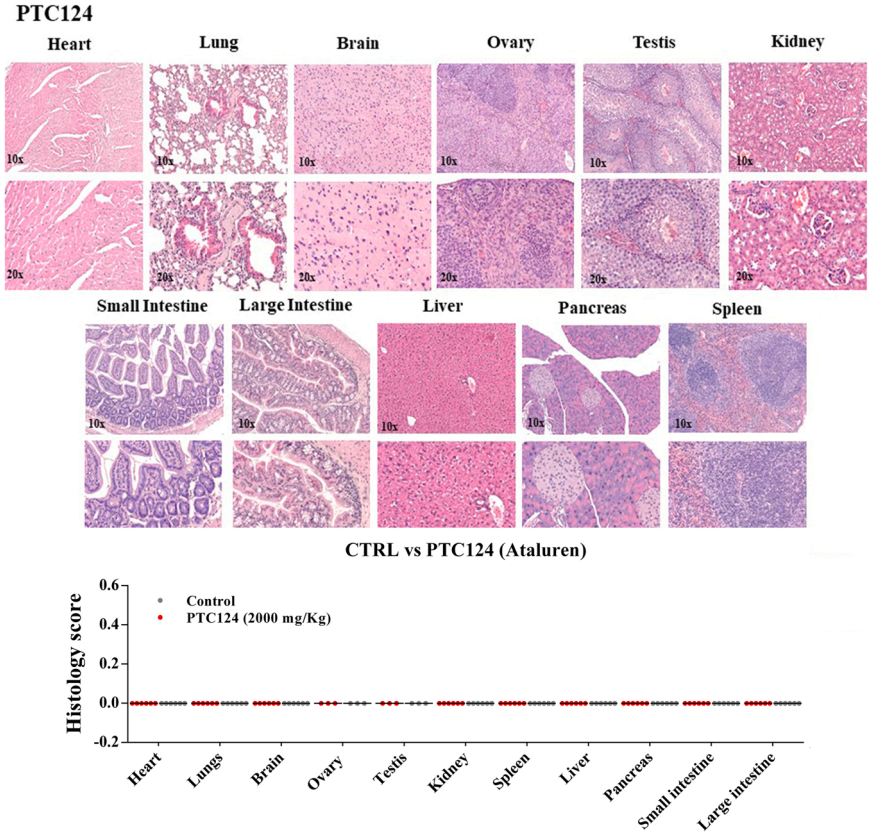

Fig. 5. Histological panel of the indicated organs in mice of the experimental positive control group treated with PTC124, 14 days post-acute dose administration (10X-20X). None of the analyzed organs showed morphological or structural alterations, as shown by the graph reporting the measured histology scores.

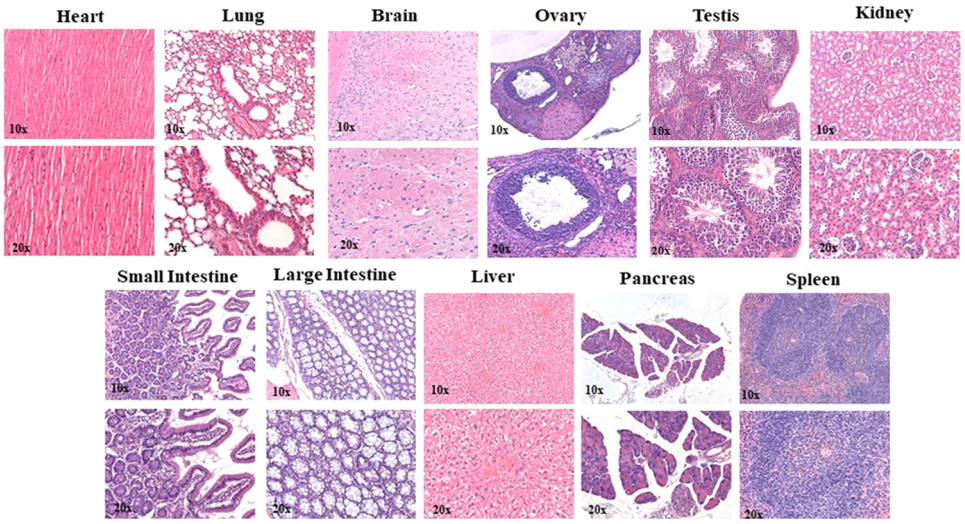

Fig. 6. Histological panel of the indicated organs in mice of the negative control group administered only with water, 14 days post-acute dose administration (10X-20X). None of the analyzed organs showed morphological or structural alterations.

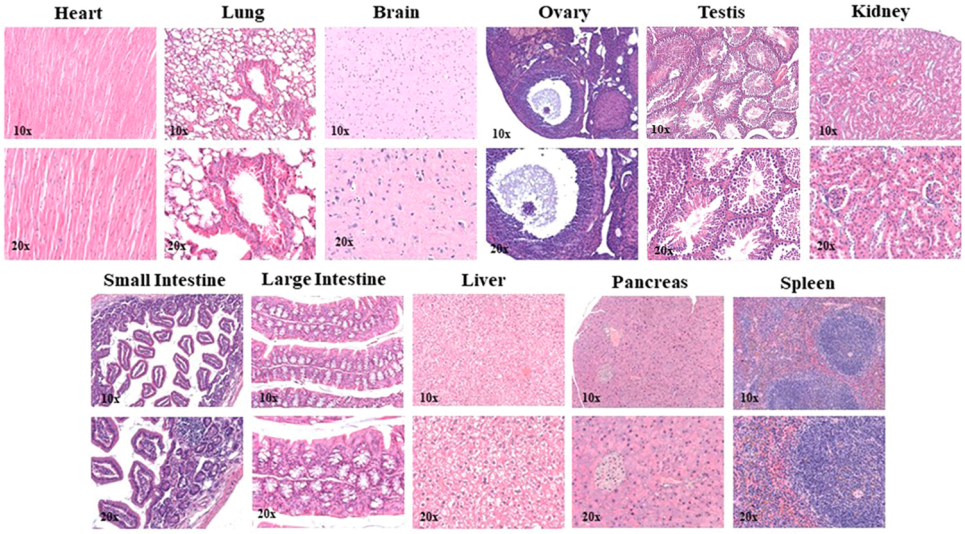

Fig. 7. Histological panel of the indicated organs in mice of the negative control group administered only with DMSO + olive oil, 14 days post-acute dose administration (10X-20X). None of the analyzed organs showed morphological or structural alterations.

day).

The difference in body weight observed in the groups even if transitory were not linked to differences in water and food intakes, that were similar among the groups and during all the observational periods, suggesting that the treatment did not affect the appetite or the feeling of thirst as well as the metabolism. Regarding the toxicity signs observed during the first 4 h after administration, we assessed similar side effects for all the three TRIDs with partial differences in the percentage of cases detected but not in timing nor the duration. Interestingly most of these side effects were similar in animals treated with Ataluren.

Side effects such as tremors or convulsions could be due to the activation of the central nervous system, suggesting the ability of tested molecules to pass the blood-brain barrier.

In addition, illness due to abdominal pain is a symptom induced in humans by Ataluren as well, as reported by Konstan et al. [16]. Furthermore, no morphological or pathological changes were observed in all vital organs, namely, the kidney, liver, heart, spleen, and lung, in the histopathological examination between the control and treated groups, thus indicating that all the treatments are nontoxic. In summary, all the molecules can be classified between categories 4 and 5 of GHS classification, indicating a low warning for health.

Moreover, *in silico* ADME properties calculation showed a high drug-like profile of these compounds respecting Lipinski's rule of five and ADME descriptors that fall inside the 95% range of similar values for known drugs.

Based on this evidence, we can assert that the three TRID molecules are eligible to be further studied in the next step of the investigation, specifically, exploring the biodistribution and the effectiveness in an in *in vivo* model characterized by a nonsense mutation.

6. Conclusions

In this study, our data allowed us to precisely establish the maximum tolerated dose in murine models for each TRIDs molecule tested precisely, in a range between 300 mg/Kg and 2000 mg/Kg for NV848 and NV930, and equal or more than 2000 mg/Kg for NV914.

Moreover, we compared our molecules to Ataluren and we found a similar profile among TRIDs and Ataluren in the side effects, symptoms

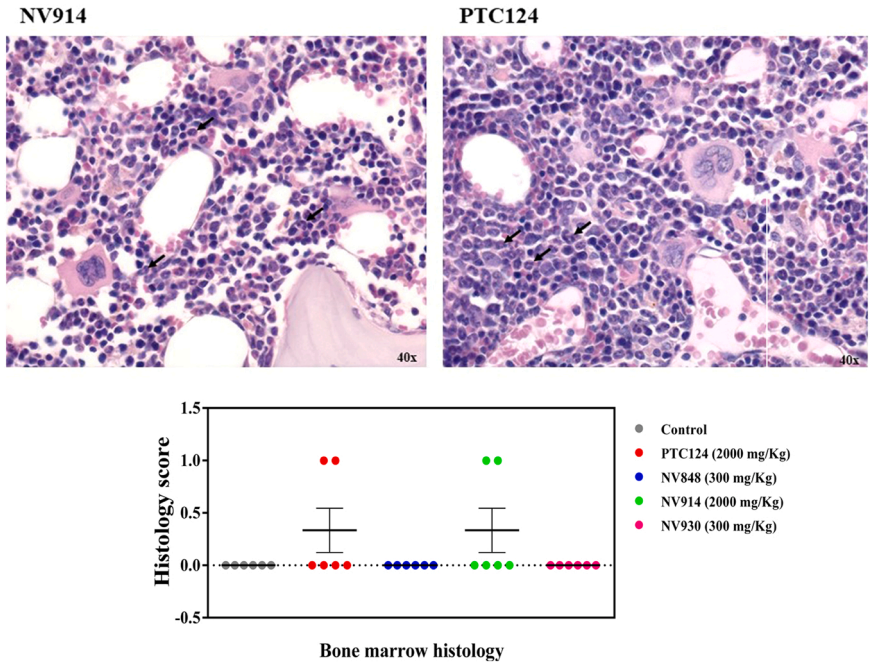

Fig. 8. Representative images showing bone marrow in NV914 and PTC124 samples (40X). Black arrows indicate the slight tendency of maturation disturbance associated to a weak expansion of erythroid lineage in two samples of NV914 and two samples of PTC124 2000 mg/Kg treated mice. The graph shows the relative measured scores.

Table 1
MW, molecular weight; **IP**, PM3 calculated ionization potential (negative of HOMO energy); **EA**, PM3 calculated electron affinity (negative of LUMO energy); **SASA**, Total solvent accessible surface area (SASA) in square angstroms using a probe with a 1.4 radius; **FOSA**, Hydrophobic component of the SASA (saturated carbon and attached hydrogen); **FISA**, Hydrophilic component of the SASA (SASA on N, O, H on heteroatoms, carbonyl C); **PISA**, (carbon and attached hydrogen) component of the SASA; **WPSA,** Weakly polar component of the SASA (halogens, P, and S); **Volume**, Total solvent-accessible volume in cubic angstroms using a probe with a 1.4 radius; **#rotor**, number of rotatable bonds; **donorHB**, number of h-bond donor, **accptHB**, number of H-bond acceptor; **glob**, Globularity descriptor, (4πr2)/(SASA), where r is the radius of a sphere with a volume equal to the molecular volume; **QPpolrz,** Predicted polarizability in cubic angstroms, **QPlogPC16**, Predicted hexadecane/gas partition coefficient; **QPlogPoct**, Predicted octanol/gas partition coefficient; **QPlogPw**, Predicted water/gas partition coefficient; **QPlogPo/w**, Predicted octanol/water partition coefficient.; **QPlogKhsa**, Prediction of binding to human serum albumin; **QPlogBB**, Predicted brain/blood partition coefficient; **#metab**, Number of likely metabolic reactions. The following properties and descriptors are included in the determination of #stars: MW, dipole, IP, EA, SASA, FOSA, FISA, PISA, WPSA, PSA, volume, #rotor, donorHB, accptHB, glob, QPpolrz, QPlogPC16, QPlogPoct, QPlogPw, QPlogPo/w, logS, QPLogKhsa, QPlogBB, #metabol.

molecule	#stars (0–5)	MW (130–725)	Dipole (1.0–12.5)	IP (eV) (7.9–10.5)	EA (eV) (−0.9 to 1.7)	SASA (300–1000)	FOSA (0–750)	FISA (7.0–330)
Ataluren	1	284.246	1.915	9.725	1.341	520.123	0	154.63
NV 848	0	141.129	4.608	9.956	0.619	345.739	178.407	146.87
NV 914	3	445.176	4.123	10.476	2.313 **	591.639	0	101.555
NV930	1	218.212	2.767	9.953	1.238	478.415	140.814	115.047

molecule	PISA (0–450)	WPSA (0–175)	PSA (7–200)	Volume (500–2000)	#rotor (0–15)	donorHB (0–6)	accptHB (2–20)	Glob (0.75–0.95)
Ataluren	333.429	32.065	85.12	866.815	1	1	5	0.8453
NV848	20.461	0	80.17	518.729	1	1	5.5	0.90305
NV914	117.841	372.242 **	71.024	1002.895	2	1	5.5	0.81899
NV930	222.554	0	74.776	764.735	2	0	5	0.84534

molecule	QPpolrz (13–70)	QPlogPC16 (4–18)	QPlogPoct (8–35)	QPlogPw (4–45)	QPlogPo/w (−2.0 to 6.5)	QPlogKhsa (−1.5 to 1.5)	QPlogBB (−3.0 to 1.2)	#metab (1–8)
Ataluren	31.078	9.63	14.802	10.471	2.6	-0.174	-0.861	0 **
NV848	14.159	4.306	9.061	8.351	-0.567	-0.884	-0.697	1
NV914	33.823	6.818	17.188	9.207	4.127	0.192	0.347	0 **
NV930	25.292	7.491	10.819	7.651	1.733	-0.584	-0.63	0 **

and GHS classification.

Altogether, these results suggest that NV molecules can be good candidates for proceeding with further experimentation to assess their biodistribution, activity and efficacy *in vivo*.

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CRediT authorship contribution statement

The manuscript was written through the contributions of all authors. **Federica Corrao:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Maria Grazia Zizzo:** Methodology, Validation, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Marco Tutone:** Data curation, Writing – original draft. **Raffaella Melfi:** Formal analysis, Writing – review & editing. **Ignazio Fiduccia:** Writing

– review & editing. **Pietro Salvatore Carollo:** Writing – review & editing. **Aldo Di Leonardo:** Writing – review & editing. **Gaetano Caldara:** Methodology, Investigation. **Riccardo Perriera:** Writing – review & editing. **Andrea Pace:** Formal analysis. **Beatrice Belmonte:** Formal analysis, Investigation, Data curation. **Selene Sammataro:** Formal analysis, Data curation, Writing – review & editing. **Ivana Pibiri:** Conceptualization, Formal analysis, Resources, Data curation, Writing – original draft, Funding acquisition. **Laura Lentini:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

I.P., L.L., R.M., A.D.L., M.T. and A.P. are co-inventors of the patent WO2019101709 "Oxadiazole derivatives for the treatment of genetic diseases due to nonsense mutations". All other authors declare no conflict of interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113886.

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