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Short communication

Exploring the readthrough of nonsense mutations by non-acidic Ataluren analogues selected by ligand-based virtual screening

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ABSTRACT

Ataluren, also known as PTC124, is a 5-(fluorophenyl)-1,2,4-oxadiazolyl-benzoic acid suggested to suppress nonsense mutations by readthrough of premature stop codons in the mRNA. Potential interaction of PTC124 with mRNA has been recently studied by molecular dynamics simulations highlighting the importance of H-bonding and stacking π–π interactions. A series of non-acidic analogues of PTC124 were selected from a large database via a ligand-based virtual screening approach. Eight of them were synthesized and tested for their readthrough activity using the Fluc reporter harboring the UGA premature stop codon. The most active compound was further tested for suppression of the UGA nonsense mutation in the bronchial epithelial IB3.1 cell line carrying the W1282X mutation in the CFTR gene.

1. Introduction

When a premature stop codon is present in the coding region of the mRNA, protein translation is interrupted thus producing truncated polypeptides. This situation is promptly detected by the nonsense mediated mRNA decay (NMD) pathway. The NMD is a surveillance mechanism that targets the cytoplasmic PTC-bearing transcript for rapid degradation [1]. Nonsense mutations are the cause of a significant percentage of most inherited diseases, including cystic fibrosis (CF), Duchenne muscular dystrophy (DMD), Usher's Syndrome, and a variety of other genetic disorders [2–4].

Due to the presence of premature stop codons in their CF *trans*-membrane regulator gene, approximately 10% of CF patients lack adequate levels of the CFTR protein, a chloride channel that is required for normal function of the lung, pancreas, liver, and other organs [5].

Gene therapy, that is potentially a method of choice to correct the mutated gene, is far from clinical routine. Alternative pharmacological approaches aim at modifying gene expression and have been applied to diseases caused by a premature stop codon. Indeed, the translational readthrough of a nonsense mutation might allow the synthesis of a full-length functional protein [6,7]. A well known category of drugs, the antibiotic aminoglycosides (e.g. gentamicin, tobramycin, paromomycin, amikacin, etc.) possess the ability to readthrough stop codons by disturbing the translation machinery and leading to the insertion of a near-cognate amino acid at a PTC [8–11]. However, aminoglycoside readthrough action lacks specificity thus resulting in readthrough of many correctly positioned stop codons, and originating toxic aggregates that after long-term treatments can cause nephrotoxicity and ototoxicity [3,12–14].

3-[5-(2-Fuorophenyl)- [1,2,4]oxadiazol-3-yl]-benzoic acid (also named PTC124 or Ataluren), was developed as a drug capable to promote selectively the readthrough of premature termination codons [6]. Recently, also the antiinflammatory drug amlexanox has been claimed to promote readthrough of nonsense mutations

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[15,16]. Overall, the general concept underlying therapeutic nonsense suppression is that a single drug, focused on a specific genetic defect, may be beneficial to different diseases whose common denominator is a nonsense mutation. PTC124 is not structurally similar to aminoglycosides and its activity was first assessed in HEK293 cells transfected with a luciferase gene (LUC190) harboring a premature stop codon at Thr190, replacing the normal ACA with UAA, UAG and UGA. Such ability was also assessed using MDX mice (mice with Duchenne Muscular Dystrophy caused by nonsense mutation) showing 20–25% recovery of full-length dystrophin [6].

In these past years PTC124 has been the subject of an intense debate about its mechanism of action, because some studies evidenced that it has an effective readthrough activity [4] while a few studies did not find enough evidence to prove the readthrough activity of PTC124 [17–19]. Our previous results on readthrough activity of PTC124 tested with a novel reporter vector harboring a premature stop codon (TGA) in the H2B-GFP fused gene (H2B-GFP-*opal*) and a molecular dynamics simulation on the hypothetical interaction between PTC124 and a mRNA fragment supported the hypothesis that PTC124 is able to promote the specific readthrough of internal UGA premature stop codons [20].

Recently, we also reported the synthesis of a set of variously fluorinated PTC124 analogues and their biological screening in the lower airway cell line (IB3.1) revealed three analogues showing comparable or higher activity than PTC124 as readthrough promoters [21].

However, despite recent progress on the topic, the precise biological site targeted by PTC124 is still unknown. This makes impossible to perform docking studies to suggest convenient modifications of the drug in order to improve its activity even against those stop codons for which PTC124 has shown a reduced effect.

In this context, based on biological data already available on some PTC124 analogues we decided to perform a Ligand Based Virtual Screening in order to identify promising PTC124-*like* candidates eventually providing optimized alternatives for readthrough drug development. Selected candidates were synthesized and tested for their readthrough activity of premature termination codons by using FLuc assay and IB3 cell lines.

2. Materials and methods

2.1. Ligand based virtual screening

Twenty PTC-124 analogs [22,23] were chosen to create the starting dataset for the modeling analysis. The dataset structures were processed with the LigPrep [24] software package in order to assign the appropriate protonation states at physiological pH(7.2 ± 0.2), employing the Ionizer option. Conformers were generated through Macro-Model torsional sampling using the OPLS_2005 force field as reported in a previous paper [25]. The pharmacophore modeling study was performed using the Phase software. Phase is a versatile product for pharmacophore perception, structural alignment, activity prediction, and 3D database creation and searching [26]. After the ligands preparation, the pharmacophore model was developed by using a set of pharmacophore features to generate sites for all of the compounds. A standard set of six pharmacophore features were used: hydrogen-bond acceptor (A), hydrogen-bond donor (D), hydrophobic group (H), negatively ionizable (N), positively ionizable (P), and aromatic ring (R); Hypotheses were generated by using a previously validated protocol [27,28]. Virtual high-throughput screening was performed on ZINC "drug-like" database [29], consisting of about $2^{*}10^{6}$ compounds and filtered according to the Lipinski's rule of Five [30].

2.2. Chemistry

All solvents and reagents were obtained from commercial sources. All synthesized compounds were purified by chromatography and analyzed by IR, HRMS, and NMR. Purity of synthesized compounds was verified prior to biological tests by chromatographic analyses and NMR (see supplementary material) and in all the cases purity was higher than 95%. IR spectra have been registered (in Nujol) with a Shimadzu FTIR-8300 spectrophotometer; melting points have been determined on a Reichart-Thermovar hotstage Kofler and are uncorrected. NMR spectra have been registered on a Bruker AVANCE DMX 300 using $CDCl_3$ and DMSO as solvent. HRMS spectra were recorded by analyzing a 50 ppm solution of the compound in a 6540 UHD Accurate-Mass Q-TOF LC/MS (Agilent Technologies) equipped with a Dual AJS ESI source. GC-MS spectra have been registered by using either an Agilent 7890B/7000C GC-MS-TQ or a GC-MS Shimadzu QP-2010 Instrument. Flash chromatography was performed by using silica gel (Merck, 0.040–0.063 mm) and mixtures of ethyl acetate and petroleum ether (fraction boiling in the range of 40–60 °C) in various ratios. 3-Methyl-benzamidoxime [31], 2-picolin-amidoxime [32], isonicotin-amidoxime [33], nicotin-amidoxime [33], and benzamidoxime [34] were synthesized as reported. Generally, an acqueous solution of hydroxylamine was prepared by mixing $NH_2OH^{*}HCl$ (36 mmol) and NaOH (36 mmol) in water (20 mL). The hydroxylamine solution was then added to an alcoholic solution of the corresponding nitrile (30 mmol) dissolved in ethanol (100 mL) in a 250 mL round bottomed flask. The mixture was refluxed for 8 h. The solvent was then removed under vacuum and 100 mL water were added to the residue. The amidoxime was filtered as a white solid and re-crystallized from ethanol.

2.2.1. General procedure for the synthesis of 1,2,4-oxadiazoles

The synthesis of 1,2,4-oxadiazoles has been performed by the amidoxime route [35]. The appropriate amidoxime (0.3 g) was dissolved in 50 mL of toluene in a 250 mL round bottomed flask. Then, 1.2 eq. of the appropriate aroyl chloride and 1.2 eq. of pyridine were added and the reaction mixture was refluxed for 6–8 h monitoring the reaction by TLC until consumption of starting material. The solvent was removed under vacuum and water was added to the residue. Extraction with ethyl acetate and chromatographic separation on silica gel using mixtures of petroleum ether and ethyl acetate as eluent allowed to obtain the desired oxadiazole, further purified by crystallization.

3-(2′-pyridyl)-5-(3′-cyanophenyl)-1,2,4-oxadiazole (NV1859). (0.49 g; 89% Yield). White solid, m.p. 148–149 °C from petroleum ether (lit. [36].148–149 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 8.86 (dd, 1H, J_1 = 10.0 Hz, J_2 = 4.8 Hz), 8.60 (brs, 1H), 8.52 (1H, d, J = 8.0 Hz), 8.23 (1H, d, J = 8.0 Hz), 7.90 (2H, m), 7.73 (1H, t, J = 7.8 Hz), 7.49 (1H, dd, J_1 = 8.0 Hz, J_2 = 4.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 174.38, 169.01, 150.55, 145.93, 137.19, 135.88, 132.07, 131.75, 130.22, 125.82, 125.30, 123.36, 117.34, 113.88; HRMS for $C_{14}H_8N_4O$ found 249.0782 $[M+H]^+$ (Calcd. 249.0771).

3-(4′-pyridyl)-5-(3′-toluyl)-1,2,4-oxadiazole(NV1861). (0.32 g; 62% Yield). White solid, m.p. 109–110 °C from petroleum ether (lit. [37]. 111–112 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 8.78 (d, 2H, J = 5.5 Hz), 8.00–7.92 (m, 4H), 7.42 (d, 2H, J = 5.5 Hz), 2.45 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 177.40, 168.00, 151.09, 139.87, 135.38, 134.65, 129.80, 129.38, 126.06, 124.37, 122.09, 21.99; HRMS for $C_{14}H_{11}N_3O$ found 238.0989 $[M+H]^+$ (Calcd. 238.0975).

3-(3′-pyridyl)-5-(3′-toluyl)-1,2,4-oxadiazole (NV1879). (0.46 g; 88% Yield)White solid, m.p. 101–102 °C from petroleum ether. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 9.42 (d, 1H, J = 1.8 Hz), 8.78 (dd, 1H, J_1 = 5.0 Hz, J_2 = 1.8 Hz), 8.46 (dd, 1H, J_1 = 7.9 Hz, J_2 = 1.8 Hz), 8.05 (m, 2H), 7.47 (m, 3H), 2.49 (s, 3H); ^{13}C NMR