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Synthesis and biological evaluation of thiazole derivatives on basic defects underlying cystic fibrosis.

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Abstract

Cystic fibrosis is a genetic disease caused by loss-of-function mutations in the cystic fibrosis transmembrane conductance regulator gene, encoding for CFTR protein. The most frequent mutation is the deletion of phenylalanine at position 508 (F508del), which leads to distinct defects in channel gating and cellular processing. In last years, several thiazole containing small molecules, endowed with dual F508del-CFTR modulator activity, proved to be able to target these defects. In search of new chemical entities able to restore CFTR function, we designed and synthesized a small series of sixteen thiazole derivatives. The designed compounds were studied as correctors and potentiators of F508del-CFTR. Although none of the molecules showed significant corrector activity, compounds **10** and **11** exhibited potentiator effects, thus allowing to determine some basic structural features which enable to obtain F508del-CFTR potentiator activity. *In silico* ADME studies showed that these derivatives obey Lipinski's rule of five and are expected to be orally bioavailable. Therefore, these molecules may represent a good starting point for the design of analogues endowed with improved CFTR potentiator activity and a good pharmacokinetic profile.

Keywords: cystic fibrosis; F508del-CFTR; ion channels; thiazole derivatives; structure-activity relationships.

Cystic fibrosis (CF) is a life-threatening genetic disease caused by loss-of-function mutations in the cystic fibrosis transmembrane conductance regulator (*cftr*) gene, encoding for CFTR protein.[1] CFTR functions is a cAMP-regulated chloride channel at the apical membrane of epithelia and the

disease affects the lungs, pancreas, liver, exocrine glands and other organs. More than 2000 mutations have been described in the *cftr* gene, however the most frequent is the deletion of phenylalanine at position 508 (F508del). F508del-CFTR displays several defects at the molecular level, including misfolding that results in premature degradation by the proteasome,[2] and if trafficked to the plasma membrane, reduced stability due to peripheral protein quality control mechanisms[3] and low open probability.[4] Thus, this kind of mutation leads to distinct defects in channel gating and cellular processing. Drug-like small molecules, known as 'CFTR modulators', can target these specific defects and restore, at least partially, CFTR function.[5] VX-809[6] or Lumacaftor™ (Vertex Pharmaceuticals Inc) was the first corrector drug to be approved by the FDA and is combined with the potentiator Ivacaftor™ (VX-770)[7] (Fig. 1) in the drug Orkambi™ (Vertex Pharmaceuticals Inc) to treat CF patients homozygous for the F508del mutation.[8] High-throughput screening of large chemical libraries and *in silico* approaches have led to the identification of several chemical entities able to act as mutant CFTR correctors and/or potentiators, among which thiazoles are particularly interesting (Fig. 1).[9-12]

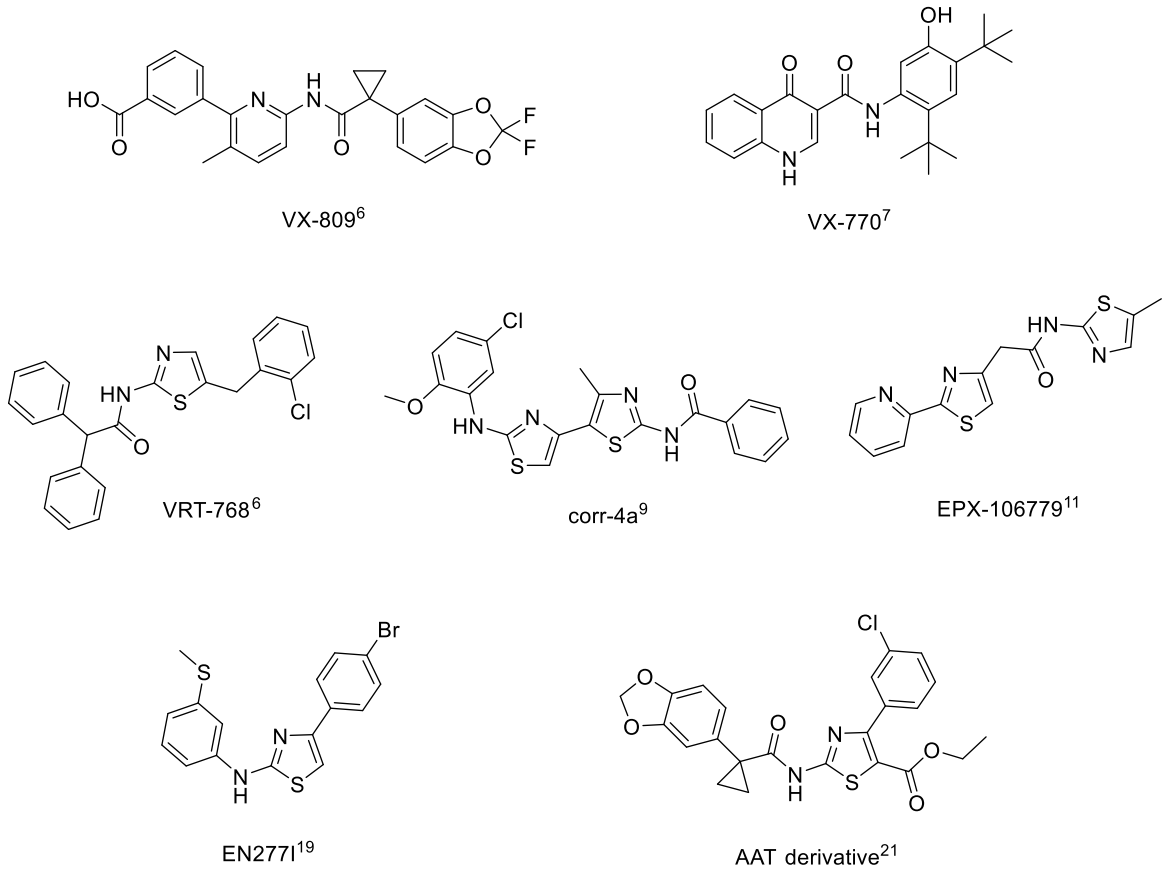


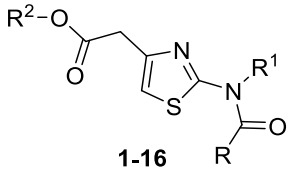
Fig. 1. CFTR modulators: VX-809, VX-770 and some thiazole derivatives.

Thiazole is a well-known five-membered heterocyclic compound. This scaffold is very versatile and widely used by the industry and pharmaceutical academic researchers in the design of drugs with

analgesic, anti-inflammatory, antimicrobial, antiviral, antioxidant, diuretic, anticonvulsant, neuroprotective and antitumor activity.[13-15] In 2005, Verkman and colleagues disclosed the first F508del corrector ever identified, corr-4a (Fig. 1).[9] Further optimization led to improved thiazole-containing correctors, including conformationally-locked bithiazoles, pyrazolylthiazoles and triazolo-bithiazoles.[9,16-18] VX-809 also was obtained after a lead optimization process, starting from a thiazole-containing lead compound, named VRT-768 (Fig. 1).[6] In addition, some aminoarylthiazole (AATs) derivatives exhibit dual F508del modulator ability, being correctors and/or potentiators of the channel (Fig. 1),[19-21] and thiazole based compounds proved also to act as multitarget agents useful in CF.[22,23] Therefore, thiazole can be considered an interesting framework for the design of small molecules addressed to overcome the underlying defects in the cellular processing and chloride channel function of CF. Indeed, the thiazole ring possesses a quite simple structure and electronic properties that allow to extend the synthesis to new molecules.[13]

On this basis, we designed a small series of thiazole derivatives (Fig. 2), inspired to well known CFTR modulators (Fig. 1), but endowed with a simplified structure, which was progressively decorated in order to get some basic SAR information. Specifically, the thiazole derivatives bear at position 2 a benzamido group, which is contained in known CFTR modulators[9] and an acetic residue at position 4. The benzamido group can be unsubstituted, or bear a halogen or methoxy groups, substituents present in various CFTR modulators previously described. Both the ethylacetate (**1**-**4**) and the acetic acid derivatives (**5**-**8**) were considered. Furthermore, the effect of the introduction of an ethyl group at the benzamide nitrogen was investigated (**8**-**16**). Finally, since pharmacokinetic properties have to be carefully evaluated in designing compounds potentially used in therapy, we considered also this important aspect and proceeded to obtain molecules consistent with the Lipinski's rule of five. All the designed compounds (**1**-**16**) were studied as correctors and potentiators of F508del-CFTR.

The synthetic route to the designed compounds is reported in Scheme 1. The ethyl ester of the 2-(2-aminothiazol-4-yl)acetic acid (**17**) was prepared according to literature[24] in order to protect the carboxylic group. Then, compound **17** was treated with the appropriate benzoyl chloride, in presence of DMAP and Et_3N, to obtain the corresponding benzamides (**1**-**4**), which in turn, treated with NaOH in EtOH, gave the acetic acid derivatives **5**-**8**. Compounds **9**-**12** were obtained by treating benzamides **1**-**4** with EtBr in presence of NaH, and treated with NaOH in EtOH to give the corresponding acids **13**-**16**. The structure of the new derivatives (**9**-**16**) was confirmed by ^{1}H NMR, ^{13}C NMR spectroscopy and high resolution mass spectrometry (HRMS). Detailed synthetic procedures and spectra of the new compounds are reported in the Supplementary data section.



1-16

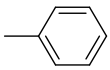
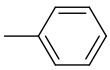
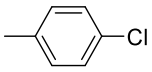
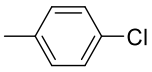
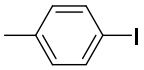
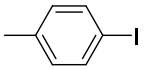
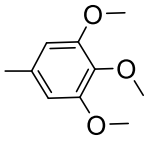
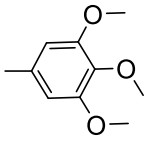
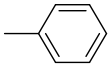
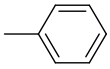
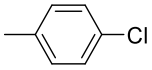
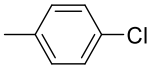
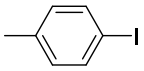
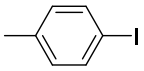
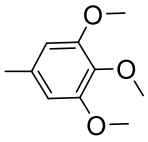
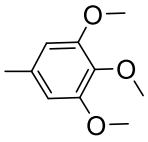
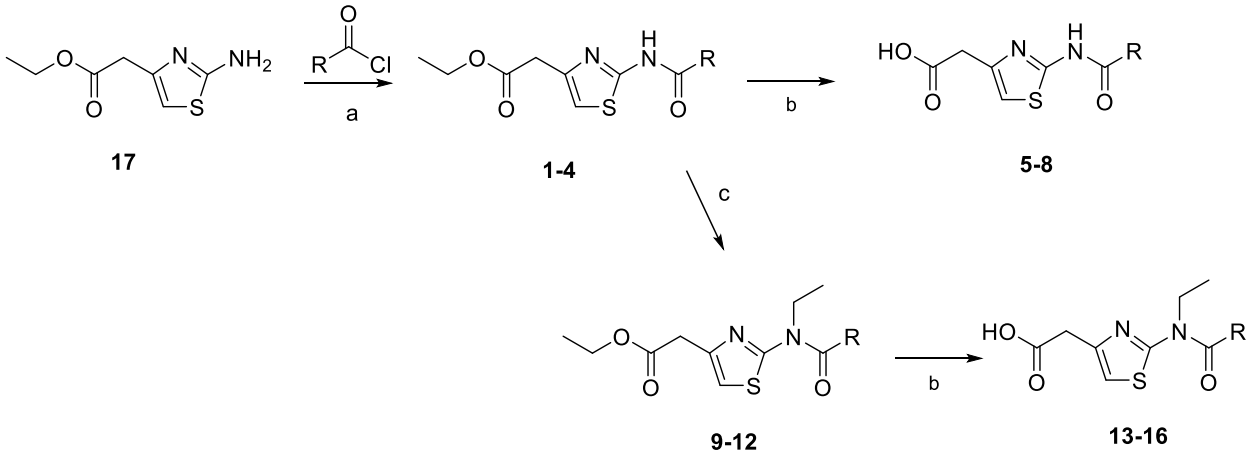
Comp.	R	R^1	R^2	Comp.	R	R^1	R^2
1		H		**9**			
2		H		**10**			
3		H		**11**			
4		H		**12**			
5		H	H	**13**			H
6		H	H	**14**			H
7		H	H	**15**			H
8		H	H	**16**			H

Fig. 2. Designed thiazole derivatives.

A bibliographic survey performed using Reaxys database (http://www.reaxys.com) showed that the synthesis of compounds **1**-**3** and **5**-**7** was already reported, although these compounds were studied as VEGFR and PI3K kinases (**1**, **2**, **5** and **6**)[25] or Xa factor (**3**, **7**) inhibitors.[26] Furthermore, the Reaxys database reports compounds **4** and **8** as commercially available, however we have described the synthesis and the experimental data of these compounds, since they are not reported in the literature.



Scheme 1. Synthesis of the designed compounds (**1-16**). Reagents and Conditions: a) CH_2Cl_2, DMAP, Et_3N, room temperature; b) EtOH, NaOH 2N, room temperature; c) DMF, NaH, room temperature, then EtBr 90 °C.

The ability of thiazole derivatives **1-16** to correct the basic defects associated to F508del mutant was tested on immortalized CFBE41o- bronchial epithelial cells stably expressing F508del-CFTR and the halide-sensitive yellow fluorescent protein (HS-YFP). The compounds were first tested as correctors of the trafficking defect of mutant CFTR. To this aim, cells were incubated for 24 h with vehicle alone (DMSO), with test compounds at two concentrations (2 and 10 µM), or with corrector VX-809 (1 µM) as positive control. After incubation, the activity of F508del-CFTR in the plasma membrane was assayed by measuring the rate of HS-YFP quenching caused by iodide influx. Treatment with the thiazole derivatives did not cause any significant increase of the ion transport activity as compared to the one detected in cells treated with vehicle (Fig. 3).

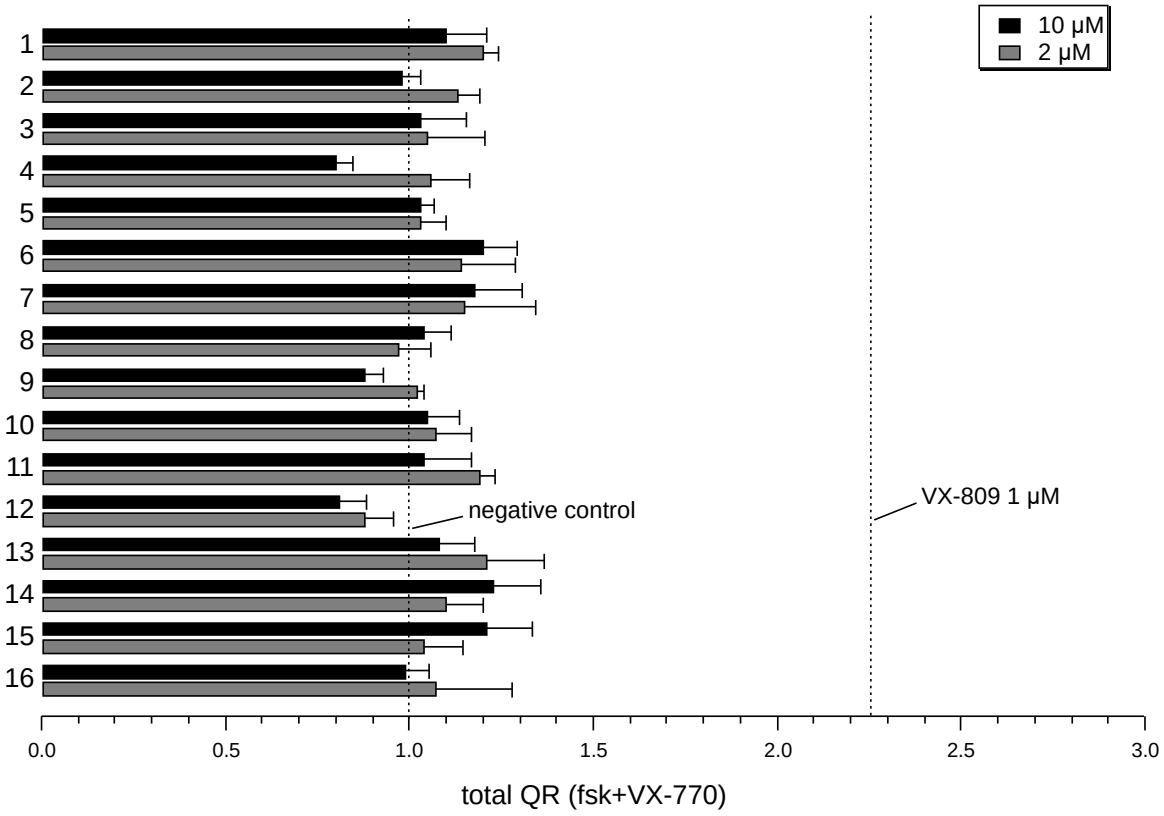


Fig. 3. Activity of novel thiazole derivatives as F508del-CFTR correctors. The bar graphs report F508del-CFTR activity in CFBE41o-cells after treatment with the indicated compounds (2 and 10 µM, 24 h). Activity was measured with the HS-YFP assay. The dotted lines indicate the level of activity in cells treated with vehicle alone (DMSO, negative control) and with the positive control, corrector VX-809 (1 µM).

Test compounds **1**-**16** were then evaluated for their ability to potentiate F508del-CFTR activity upon acute stimulation. Thus, CFBE41o-cells were incubated for 24 h at 32°C to promote correction of the trafficking defect of F508del-CFTR and its expression in the plasma membrane, and then cells were assayed following acute treatment with test compounds (2 and 20 µM) in the presence of forskolin (20 µM) to increase intracellular cAMP content. The ion transport activity detected under these conditions was compared to the one measured in cells treated with forskolin alone (negative control) or with forskolin plus the potentiator VX-770 (1 µM; as a positive control). Significant increase of the halide influx was observed when cells were acutely stimulated with thiazoles **10** and **11** (Fig. 4).

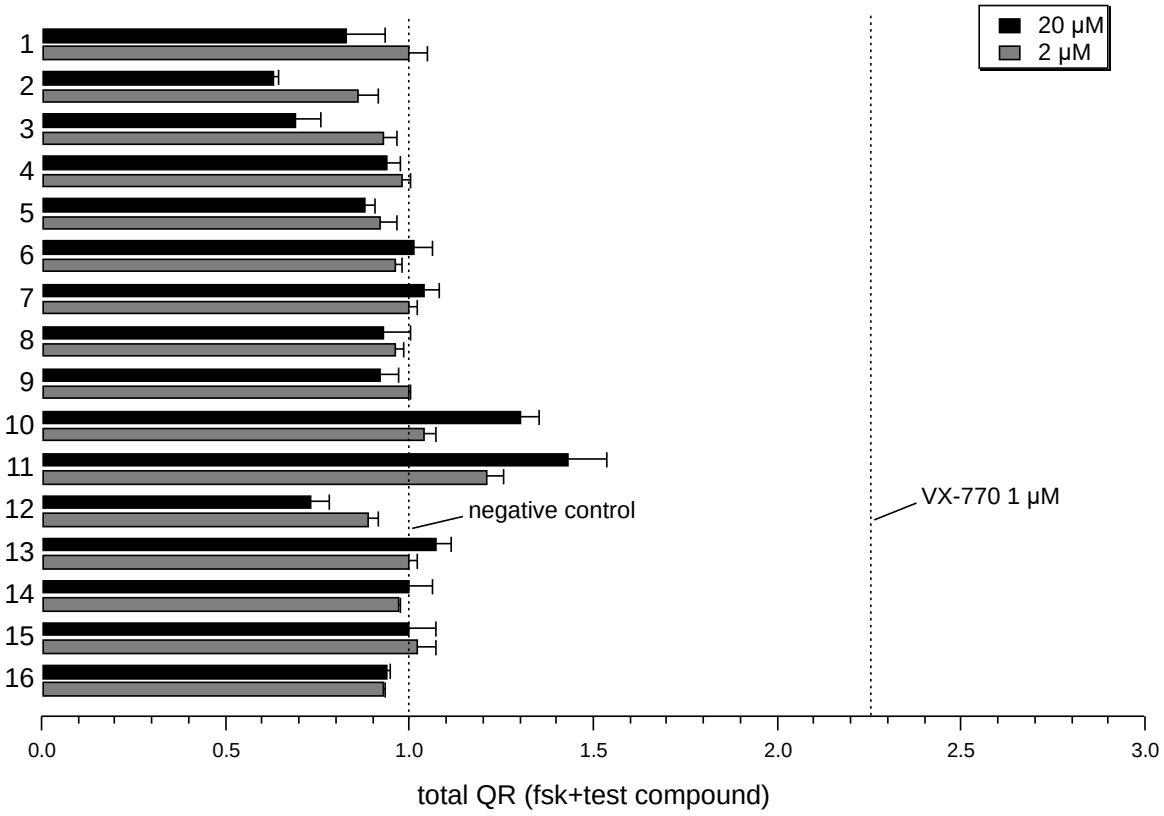


Fig. 4. Activity of novel thiazole derivatives as F508del-CFTR potentiators. The bar graphs report F508del-CFTR activity in CFBE41o-cells, incubated at 32°C for 24 h to rescue defective mutant CFTR maturation, after acute treatment with the indicated compounds (2 and 20 µM). Activity was measured with the HS-YFP assay. The dotted lines indicate the level of activity in cells treated with forskolin alone (DMSO, negative control) and with the positive control, potentiator VX-770 (1 µM).

Next, an in-depth analysis was performed to evaluate the potency and efficacy of the hits. To this aim, hits were tested as potentiators at multiple concentrations in the range 80 nM – 50 µM on CFBE41o-cells, after rescue of the maturation defect at 32°C for 24 h. Hits were found to be active in the low micromolar range, although the efficacy was lower than the positive control, potentiator VX-770 (Fig. 5).

The results obtained allow to get some information about the effects of the thiazole derivatives here described as correctors or potentiators of F508del-CFTR. Although several thiazole-containing correctors have been described so far,[9,16-18,19-23] when tested as correctors and compared with Lumacaftor™ (VX-809), none of the thiazole derivatives **1**-**16** showed significant effects, thus suggesting that the thiazole nucleus of these compounds is not adequately decorated to obtain a corrector activity. On the other hand, considering the potentiator activity, two compounds emerged (**10** and **11**) that, although less effective than the positive control Ivacaftor™ (VX-770), significantly

potentiated F508del-CFTR function. Interestingly, comparing the results obtained, it is possible to get some insights into the structural requirements necessary to achieve potentiator activity. In particular, the introduction of a halogen at para position of the phenyl group, led to an improvement of the activity. Indeed compound **9**, bearing an unsubstituted phenyl group, was not active, whereas compounds **10** and **11**, having as substituent chlorine and iodine, respectively, gave the best results as potentiators (Fig. 4). Furthermore, compounds **10** and **11** showed similar efficacy but slightly different potency (E_{max} = 1.8, EC_{50} = 1.8 μM for compound **10**; E_{max} = 1.9, EC_{50} = 3.2 μM for compound **11**), suggesting that both the halogen substituents contribute in enhancing the activity. Very important seems also the simultaneous presence of two ethyl groups, the one of the ester function and the other one at the benzamide nitrogen. Indeed, neither the ethyl esters **2** and **3**, which do not have the benzamide nitrogen ethylated, nor the acidic derivatives **6** and **7**, bearing an ethyl group only at the benzamide nitrogen, were active (Fig. 4).

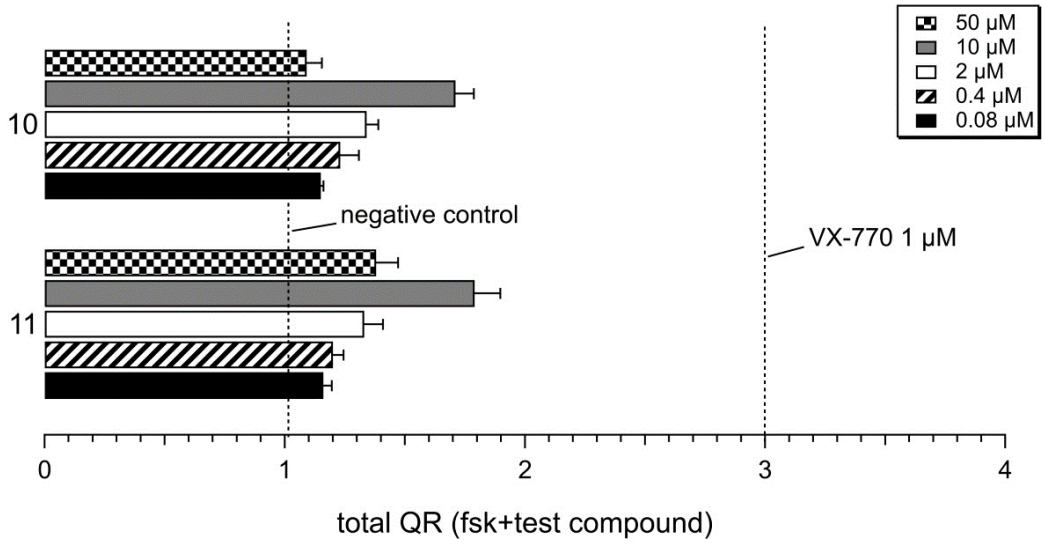


Fig. 5. Potentiator activity of novel thiazoles. The bar graphs report F508del-CFTR activity in CFBE41o-cells, incubated at 32°C for 24 h to rescue defective mutant CFTR maturation, after acute treatment with hits, at the indicated concentrations. Activity was measured with the HS-YFP assay. The dotted lines indicate the level of activity in cells treated with forskolin alone (DMSO, negative control) and with the positive control, potentiator VX-770 (1 μM).

The evaluation of the pharmacokinetic properties of molecules designed to be eventually used in therapy is important in the early drug discovery phases. The theoretical absorption, distribution, metabolism and excretion (ADME) properties of compounds **1-16** were determined by *in silico* analysis, using the free online software SwissADME available at http://www.swissadme.ch/index.php.[27] The following physicochemical parameters, which according

to Lipinski's rule of five indicate crucial characteristics for oral bioavailability, were evaluated: molecular weight (MW), calculated logarithm of the octanol−water partition coefficient (LogP), number of H-bond donors (nHBD), number of H-bond acceptors (nHBA) and number of rotatable bonds (nRB), together with the topological polar surface area (TPSA). Lipinski's rule of five states that, to be orally active, a small molecule should have MW ≤ 500 Daltons, LogP ≤ 5, nHBD ≤ 5, nHBA ≤ 10 and nRB ≤ 10.[28,29]Absorption and membrane permeability are also influenced by TPSA, which should be lower than 140 $Å^2$ to have intestinal absorption. The results are reported in Table 1 together with the values obtained for the reference compound VX-770. All the thiazole derivatives obey the Lipinski's rule and have a TPSA lower than 140 $Å^2$, thus suggesting good gastrointestinal absorption.

Table 1. ADME *in silico* analysis (data obtained by using SwissADME software).

Comp	MW	LogP	nHBD	nHBA	nRB	TPSA ($Å^2$)	GI absorption	PAINS alert
1	290.34	2.36	1	4	7	96.53	High	0
2	324.78	2.88	1	4	7	96.53	High	0
3	416.23	3.02	1	4	7	96.53	High	0
4	380.42	2.38	1	7	10	124.22	High	0
5	262.28	1.61	2	4	5	107.53	High	0
6	296.73	2.16	2	4	5	107.53	High	0
7	388.18	2.29	2	4	5	107.53	High	0
8	352.36	1.53	2	7	8	135.22	High	0
9	318.39	2.86	0	4	8	87.74	High	0
10	352.84	3.45	0	4	8	87.74	High	0
11	444.29	3.59	0	4	8	87.74	High	0
12	408.47	2.87	0	7	11	115.43	High	0
13	290.34	2.19	1	4	6	98.74	High	0
14	324.78	2.71	1	4	6	98.74	High	0
15	416.23	2.81	1	4	6	98.74	High	0
16	380.42	2.18	1	7	11	126.43	High	0
VX-770	392.49	4.47	3	3	5	82.19	High	0

MW: molecular weight; LogP: values correspond to Consensus $LogP_{o/w}$; nHBD: number of H-bond donors; nHBA: number of H-bond acceptors; nRB: number of rotatable bonds; TPSA: topological polar surface area; GI: gastrointestinal; PAINS: Pan Assay INterference compoundS.

Finally, compounds **1**-**16** were not recognized by SwissADME software as PAINS (Pan Assay INterference compoundS), i.e. molecules containing substructures showing potent response in assays irrespective of the target. This evidence allows to exclude that the studied thiazole derivatives interact nonspecifically and yield false positive biological output.

In conclusion, although in the experimental model adopted the designed derivatives showed only poor activity as correctors, this preliminary study allowed to identify compounds **10** and **11**, which proved to have the structural requirements necessary to express some biological effects as potentiators, thus suggesting that the studied scaffold, adequately substituted, enables to obtain F508del-CFTR potentiator activity. Interestingly, *in silico* ADME analysis showed that these thiazole derivatives do not violate the Lipinski's rule of five, therefore they are expected to have good oral bioavailability. Moreover, SwissADME software did not recognize them as PAINS, thus excluding nonspecific effects. On this basis, compounds **10** and **11** could represent a valuable starting point for the design of analogues characterized by improved CFTR potentiator activity and endowed with promising pharmacokinetic properties.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Detailed synthetic procedures, ^{1}H NMR, ^{13}C NMR and HRMS spectra of the new compounds and description of biological assays are reported in the Supplementary data section.

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