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Identification of small-molecule urea derivatives as PTPC modulators targeting the c subunit of F_1/F_o-ATP synthase

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ABSTRACT

Maintaining a high percentage of living and functional cells in those pathologies in which excessive cell death occurs, such as neurodegenerative disorders and cardiovascular diseases, is one of the most intriguing challenges in the field of biochemical research for drug discovery. Here, mitochondrial permeability transition-driven regulated cell death is the main mechanism of mitochondrial impairment and cell fate; this pathway is still lacking of satisfying pharmacological treatments to counteract its becoming; for this reason, it needs continuous and intense research to find new compounds as modulator of the permeability transition pore complex (PTPC) activity. In this study, we report the identification of small-molecule urea derivatives able to inhibit PTPC opening following calcium overload and selected for future use in cytoprotection.

All cells of our body, sooner or later die, either naturally following a precise physiological program or upon pathological and accidental events. From this observation, it follows that different cell death types exist and each of them can be distinguished by quantifiable biochemical parameters[1]. Regulated cell death (RCD) is a pathway shared by both human physiology and in case of failing of adaptive responses to dangerous external stimuli. In the last years, one of the most studied RCD is that one driven by the mitochondrial permeability transition (mPT), because (but not exclusively related to this) it still lacks satisfying pharmacological approaches and genetic treatments to counteract its progression[2]. mPT consists in the irreversible permeabilization of the inner mitochondrial membrane (IMM) to solutes with a molecular weight of up to 1500 Da (as reviewed in[3]). Inducers of mPT like calcium overload, reactive oxygen species (ROS) production, but also increased phosphates concentration and a reduction in the adenine nucleotide intracellular pool, trigger the opening of the mitochondrial permeability transition pore complex (PTPC), that is responsible for mPT. This promotes severe and prolonged mitochondrial perturbation and the impairment of essential functions, seriously compromising cell life[4,5]. Indeed, in these conditions, failure of the mitochondrial electron transport chain (ETC) and the related break of ATP production occur. PTPC is a proteinaceous complex composed by an undefined number of proteins allocated at the crosstalk of inner and outer mitochondrial membranes (OMM)[6]. It is reported that mPT-driven RCD develops in neurodegeneration[7] and ischemia–reperfusion diseases like infarction[8–10]. Here, the maintenance of a balanced mitochondrial homeostasis and the "right" number of living cells allow minimizing the inauspicious phenotype arising with disease. Thus, pharmacological targeting of its modulators or pore-forming proteins would be beneficial against these deleterious effects of PTPC opening. Unfortunately, PTPC is still an evolving entity with cyclophilin D (CypD) as the only genetically confirmed modulator. But, from 2012, increasing evidences ascribed to the C subunit of F_O ATP synthase (Csub) a key role as regulator of the PTPC[11–15]. In our previous paper we have confirmed its usefulness as cardioprotective strategy following ischemia/reperfusion injury (IRI) by the selective targeting of the protein with small-molecule

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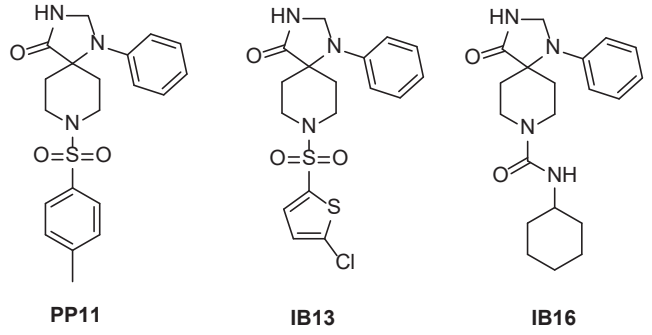


Figure 1. Structure of known PTPC inhibitors targeting the c subunit of F_1/F_o-ATP Synthase[16].

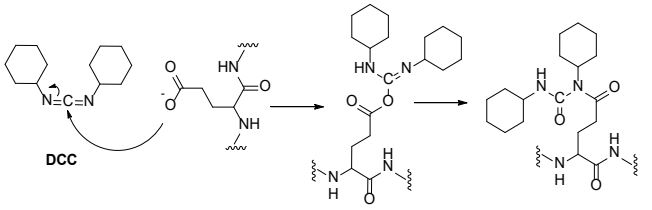


Figure 2. Interactions between DCC and Glu[59] of Csub.

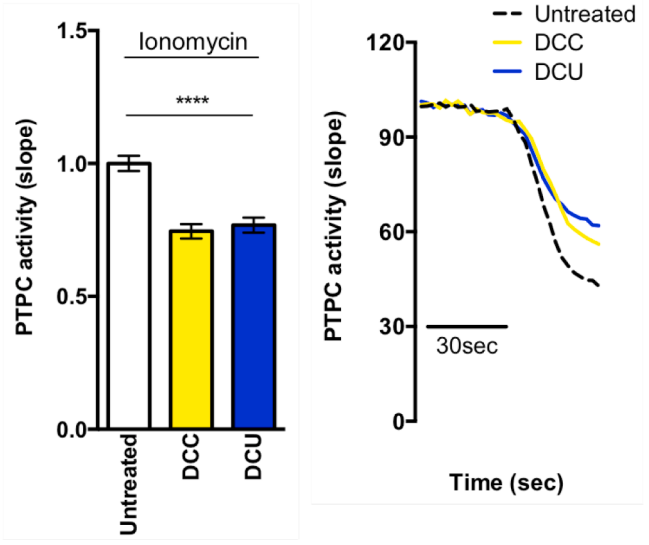


Figure 3. calcein-cobalt assay in AC16 cell line comparing PTPC inhibition between 15 μM DCC and 1 μM DCU.

inhibitors (see representative compounds in Figure 1)[16,17]. To the best of our knowledge, these triazaspiro[4.5]decane derivatives remain the only example of PTPC opening inhibitors with such mechanism of action reported thus far.

Given the difficulty for a functional compound to become a drug, intense efforts are needed in understanding chemical moieties that are involved in the modulation of the multiproteic components of PTPC. Also, a hyper-activation of this channel is useful in therapies, such as in cancers, where cells easily evade apoptosis.

In this manuscript we studied the chemistry of urea compounds for their ability to inhibit (but also to activate) PTPC opening as read out of putative mPT-driven RCD blockers.

Dicyclohexylcarbodiimide (DCC) has been considered as a starting point for the design of new PTPC inhibitors because of its known capability of binding Csub to Glu[59] (Figure 2)[18].

In particular, DCC induces an irreversible inhibition according to the formation of a dicyclohexyl-*N*-acylisourea (DCNU)-modified c-monomer. Despite this recognized mechanism we considered that DCC in aqueous solution is partially hydrated to the corresponding dicyclohexylurea (DCU) that was tested for its activity as PTPC opening inhibitor.

According to the calcein-cobalt assay[19] performed in human ventricular cardiomyocytes (AC16) and under conditions of mitochondrial calcium overload, DCU itself showed a higher activity by working at a 15-fold lower concentration (1 μM) than DCC (15 μM) to exert the same extend of PTPC inhibition (specifically 28% for DCU, Figure 3, Table 2). Of note, 15 μM DCC has been already tested by us[13] for PTPC inhibition and beneficial effects under IRI conditions. Even more important for the present project, DCU is not supposed to bind covalently the investigated target protein, thus, being devoid of the potential side effects typical of covalent ligands. In addition, covalent inhibitors of PTPC are not functional to our purpose because after the first block of its activity in IRI, cardiomyocytes will be able to start the ATP synthesis as in the physiological way. As described by Simersky and co-workers[20] the DCC interacts with the Glu[59] trough a covalent bond (Figure 2) with the cyclohexyl groups fitting into a hydrophobic pocket that was previously occupied by Phe[64] of the c -ring.

To directly monitor target engagement inside cells, we performed a cellular thermal shift assay (CETSA)[21,22] using a protocol based on ligand-induced thermal stabilization of the target protein, the c subunit of F_O-ATP synthase. We were able to detect DCU binding with the Csub between 53 °C and 65 °C. Despite the low sensitivity of the assay and an inability to perform any molecular docking assays, Figure 4 clearly shows that the Csub protein was thermally stabilized by DCU pretreatment (C subunit expression in Western Blot image and blue line of the linked quantification) and that this effect was absent in other mitochondrial proteins of the same complex, such as ATP5A (ATP5A expression in Western Blot image and brown lines of the linked quantification).

In light of this, DCU has been chosen as a promising hit compound suitable for structure–activity optimization. Except for very few examples (see compound IB16 in Figure 1), there are no previous evidences for Csub inhibition by urea derivatives, thus compounds **1**–**14** (Table 1) have been synthesized in order to obtain useful information about the relevance of different parts of these molecules for interactions within the protein. It is interesting to note that IB16 is an asymmetric cyclohexylurea derivative. In this work, punctual modifications have been introduced either to the urea moiety or to the N/N' substitutions. In addition, some of the most interesting modifications have been combined in a single compound. The general structure of these derivatives is depicted in Table 1.

Following this approach, five classes of DCU derivatives have been synthesized. Firstly, the intact urea moiety has been maintained and a number of symmetric (compounds **1**–**7**) or asymmetric (**8**–**9**) N/N' modifications were introduced by combination of different alkyl/cycloalkyl/(substituted)aryl/benzyl groups. Secondly, the urea function has been substituted with a thiourea group so to evaluate the effect of the removal of a potential hydrogen bond acceptor. Even in this case, symmetric (**10, 11**) and asymmetric (**12**) compounds have been prepared. Lastly, two carbamates derivatives were synthesized (**13**–**14**), in order to understand how a hydrogen bond acceptor instead of a donor

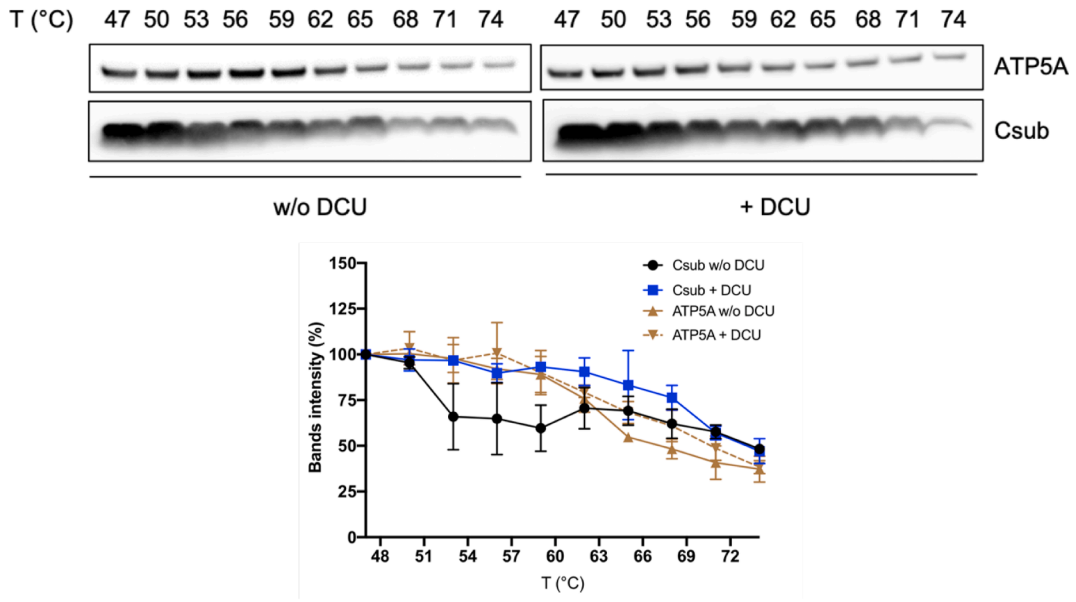
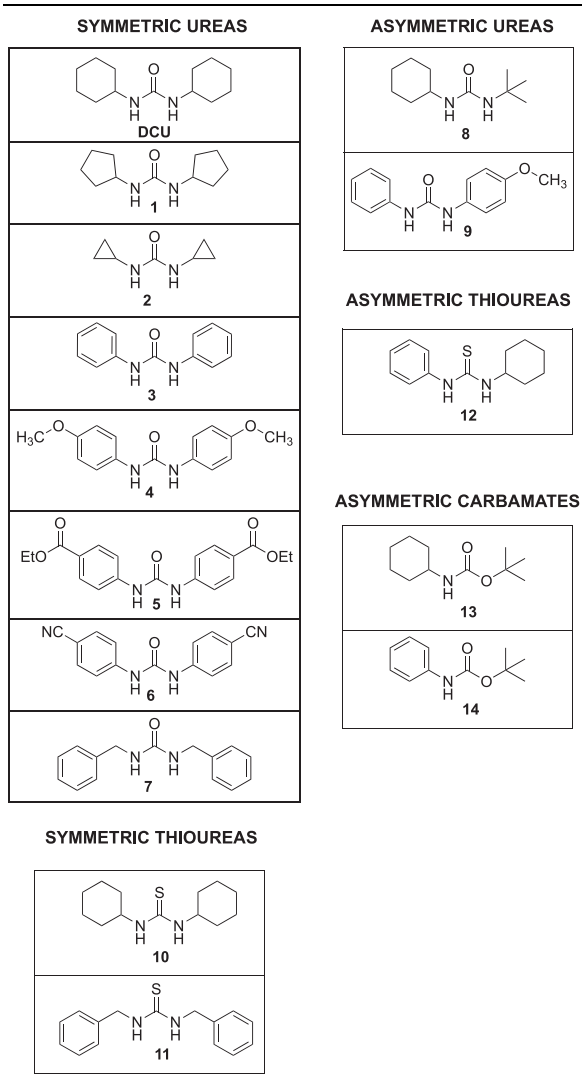


Figure 4. Cellular thermal shift assay which supports the engagement of Csub by DCU.

Table 1
DCU and its derivatives.



one influences the activity.

The synthesis of DCU derivatives have been carried out following different straightforward approaches, through which the target molecules were easily produced, purified and almost all of the reactions led to high yields.

Symmetric ureas **1 – 7** have been prepared by the typical reaction between carbonyldiimidazole (CDI) and two equivalents of aromatic or aliphatic primary amines (see Scheme 1 and supporting information).

Since the reaction between CDI and primary amines rearranges into an isocyanate intermediate, this approach can conceivably be used for the synthesis of asymmetric ureas, by adding a different amine. However, because of its rather high reactivity, the isocyanate intermediate is not easy to isolate for the further reaction with amines. For this reason, asymmetric ureas, as well as thioureas (**8**–**12**) have been prepared by the direct reaction between aromatic or aliphatic primary amines and commercially available isocyanates or isothiocyanates.

Carbamates **13** and **14** were prepared by reacting either aryl- or cyclohexyl-amine with ditertbutyl-dicarbonate.

In producing urea derivatives, five classes of structural modifications have been introduced (Table 1), in order to investigate which parts of DCU are involved in the PTPC inhibition, to figure out which improvements can be undertaken for the biological activity optimization.

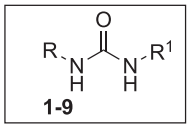
To test the biological activity of urea derivatives, AC16 has been chosen as cell model to study PTPC inhibition under mitochondrial calcium overload conditions induced by the ionophore ionomycin (see supporting information for details). The method used is a well-established approach[16] to evaluate if compounds may drive PTPC inhibition and thus potential beneficial effects against cell death.

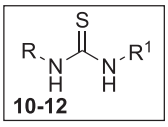
Among symmetric urea derivatives, we observed that aliphatic cyclic substituents with less than six carbon atoms demonstrated to have less inhibition potency if compared to DCU.

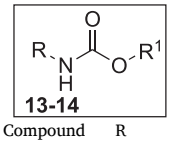
Moreover, in the case of N,*N*-diphenyl substitution, the presence of electron-withdrawing groups showed to be detrimental for the inhibitory activity contrarily to the electron-donor ones that, inducing the enolic or thioenolic form, lose the inhibition function in favor of an activation of PTPC.

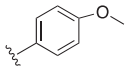
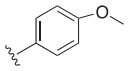
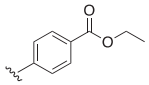
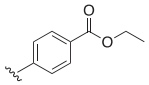
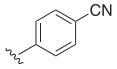
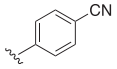
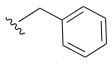
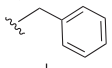
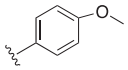
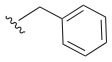
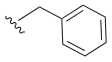
In particular, regarding the first statement of our supposed ratio, we observed that the inhibition activity of the compounds **1** and **2**, that have five and three ring carbon members respectively, decreases in a relevant way correlated with the steric hindrance (- 16,4% compound **1**, −3,9% compound **2**) (Table 2).

Table 2
Nomenclature, scheme and biological activity of urea-derivatives.







Compound	R	R'	PTPC activity (% at 1 μM)
DCU			−28%
1			−16,4%
2			−3,87%
3			−28,11%
4			+53,61%
5			−9,75%
6			−25,43%
7			+30,39%
8			−43,17%
9			−4,6%
10			+32,43%
11			+37,18%
12			+3%
13			+42,54%
14			+60,82%

Considering the binding site interaction of the known PTPC inhibitor Oligomycin with Glu[59] residue through a hydrogen bond with a molecule of water,[18] we speculated that the –NH group of the DCU is the one binding the oxygen of the water. The hydrogen of the water is able to interact with the carboxylate of Glu[59] residue through another H-bond. (Scheme 2).

This mechanism is evident in some derivatives namely compounds **3**, **5**, **6**, **8**, **9**; in particular, we noticed that compound **8** is more efficient than the others (see the dose–response curves of compound **8** and DCU in Figures S43 and S44, supporting information), not only because of these reasons but also due to the steric hindrance of the *tert*-butyl group: in fact, here we observed a synergistic combination that affects positively the inhibitory activity of the PTPC opening.

In the case for example of the compound **6**, we assumed that having an electron-withdrawing as substituent doesn't change much on the inhibition activity.

On the other side, to see the activator functionality we supposed that electron-donor groups as substituents promote the enolic or thioenolic form, subtracting the molecule of water from the interaction with Glu[59] (Scheme 2).

It is worth noting that compounds **4**, **7**, **10**, **11**, **12**, **13**, **14** showed a consistent behavior with the mechanism we support (Table 2). In fact, compounds with electron-donor substituents, such as methoxy group (compound **4**) or benzyl group (that as a protecting group can decrease the reactivity of the nitrogen: compounds **7** and **11**) as well as aliphatic substituents without any lone pair available (compounds **10**) favor the enolic (compound **7**) or the thioenolic form of the thiourea over the thioketonic form because of the weak stability of the latter.

Also, the compounds **13** and **14** were observed to be stronger activators of PTPC opening than the ones cited above. This could be due to the presence of a carbamate functionality which displays an -O- atom instead of a –NH- moiety. Here, the oxygen atom acts as H-bond acceptor with a molecule of water, which could prevent the water molecule from interacting through a hydrogen bond with Glu[59] residue (Scheme 2).

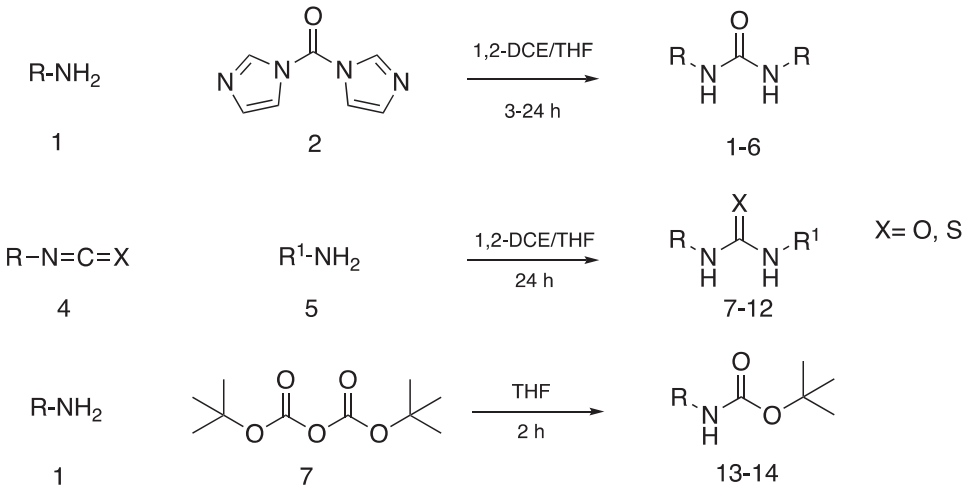
On the bottom part of the scheme, three different interactions between DCU-derivatives and the amino acid residue are shown:

a) Interaction between urea derivatives and Glu[59] through interposition of a water molecule which behaves as a "bridge-molecule" thanks to the capability of the urea –NH group and the carboxylate of glutamate to form H-bonds: this translates in an inhibitory potential;
b) In the case of thiourea derivatives, the more stable thioenolic form subtracts the water molecule from the interaction with the carboxylate. This could be translated in an activating behavior as observed.
c) In the case of carbamate derivatives, both the two oxygen atoms work as H-bond acceptors with water, which in turn is not able to prevent carboxylate of the glutamate from proceeding in the physiological way: also in this case, an activator potency is observed.

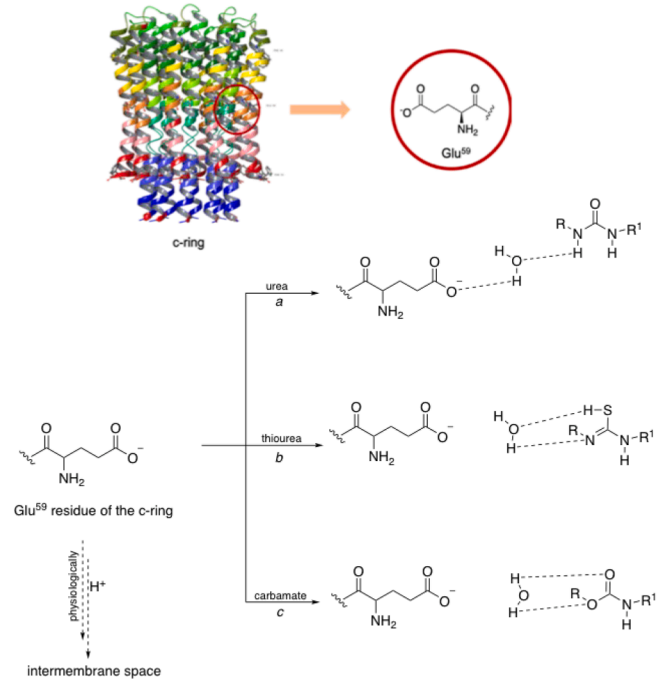
In conclusion in this work we have firstly demonstrated that part of the PTPC inhibition activity of DCC is due to its behavior as a prodrug, in fact the corresponding urea is a more potent inhibitor respect to DCC. Shading light to this mechanism we investigated the importance of steric hindrance and stereo-electronic effects on the ability of urea-based compounds to interact with the PTPC. Despite the chemical simplicity of the molecules in this study, the fine regulation of the electronic effects on the urea or thiourea carbonyl allowed us to hypothesize the different mechanism of action between inhibitors and activators of PTP multiproteic complex. Further studies will be necessary to confirm these hypotheses, also with the aid of molecular modeling or new experimental evidence with different classes of molecules.

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.



Scheme 1. reactions conditions.



Scheme 2. Supposed interaction mechanism of urea or thiourea compounds with Glu[59]. The c-ring crystal (PDB 4F4S,[17]) is shown on the top of the Scheme and some amino acid residues, involved in the proton translocation, are put in evidence. In particular, as mentioned above, residue Glu[59] (circled in red) plays a crucial role in the mechanism.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2022.128822.

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