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Effect of isouronium/guanidinium substitution on the efficacy of a series of novel anti-cancer agents†

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Considering our hypothesis that the guanidinium moiety in the protein kinase type III inhibitor **1** interacts with a phosphate of ATP within the hinge region, the nature of the interactions established between a model isouronium and the phosphate groups of ATP was computationally analysed indicating that an isouronium derivative of **1** will interact in a similar manner with ATP. Thus, a number of compounds were prepared to assess the effect of the guanidinium/isouronium substitution on cancer cell growth; additionally, the molecular shortening and conformational change induced by replacing the di-substituted guanidine-linker of **1** by an amide was explored. The effect of these compounds on cell viability was tested in human leukaemia, breast cancer and cervical cancer cell lines and the resulting IC₅₀ values were compared with those of the lead compound **1**. Replacement of the di-substituted guanidine-linker by an amide results in the loss of cytotoxicity; however, substitution of the mono-substituted guanidinium by an isouronium cation seems to be beneficial for cell growth inhibition. Additionally, the effect of these compounds on the MAPK/ERK pathway was studied by means of Western blotting and the results indicate that the isouronium derivative **2** decreases the levels of phosphorylated, and thus activated, ERK (pERK) both in leukaemia and breast cancer cells, whereas lead compound **1** only shows an effect on pERK levels in breast cancer cells. This confirms that both compounds could interfere with the MAPK/ERK pathway although other targets cannot be ruled out.

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Introduction

Protein kinases, important components of intracellular cascades, have emerged as one of the most central targets in drug discovery for anticancer therapies.¹ To date, the FDA has approved 31 small-molecule protein kinase inhibitors (PKIs), half of which have been approved in the past three years.² However, most of the known PKIs are competitive (type I), targeting the ATP site which is highly conserved amongst most of the kinases and this makes cross-reactivity a very common problem.³ Other PKIs comprise type II inhibitors, which bind to the inactive form of the kinase, and type III inhibitors, which bind exclusively in an allosteric pocket. Such allosteric modulators bind to sites that are less conserved across the kinome and are likely to be specific for a particular

family of kinases, not facing the limitations of ATP-competitive inhibition that challenge the translation of current generation PKIs to the clinic.^{4,5}

The Ras/Raf/MEK/ERK pathway (MAPK/ERK pathway) is a kinase cascade that transmits signals from cell surface receptors to promote cell proliferation, differentiation and survival. Although much understanding has been gained in the last few years about the complicated regulatory steps that govern this signalling pathway, much remains to be elucidated about the pathway and its components.⁶ Under physiological conditions, ERK signalling is controlled at multiple levels essential for maintaining regulated cell growth and homeostasis, but when hyper-activated, it can play a causative role in the development of different types of tumours.^{7,8} Raf and MEK kinases also play a central role in cancer and their therapeutic opportunities have been intensely studied, both in terms of single kinase inhibition and interference in protein-protein interaction.^{9–11} In particular, their constitutive activation in many tumours indicates the urgent need for new inhibitors with an alternative mechanism of action.^{12,13} Trametinib (GSK) is the only allosteric type III inhibitor (MEK inhibitor) of the MAPK/ERK pathway approved for B-Raf(V600E) melanoma,^{8,14} while Ras, Raf and ERK still

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remain untargated through a pure type III allosteric mechanism.

Previous studies in Rozas' group identified compound **1** (Fig. 1) as a PKI capable of inhibiting the MAPK/ERK pathway through a type-III allosteric mechanism providing a new lead for further refinement of kinase selectivity and potency.^{15,16} Earlier and on-going molecular-modelling studies carried out in our laboratory indicate that this compound forms hydrogen bonds (HBs) and electrostatic interactions with the phosphate chain of ATP through the mono-substituted guanidinium cation, thus positioning the lipophilic (4-Cl-3-CF₃)-Ph moiety (see compound **1** in Fig. 1) in a hydrophobic pocket of the enzyme.^{15,17}

Guanidine's high basicity (pK_{aH} in water is 13.6 (ref. 18)) indicates that at physiological pH, it will be protonated forming the guanidinium cation. Even though appropriate substitution of this cation decreases its basicity (e.g. pK_{aH} of phenylguanidine in water is 10.9 (ref. 19)) and this functional group is present in many current therapies such as zanamivir,²⁰ cimetidine²¹ or clonidine,²² guanidinium derivatives are sometimes discarded as useful drugs because of potential pharmacokinetic problems. Regardless of its basicity, the guanidinium cation has important features very useful for target binding such as forming strong HBs and ionic interactions.^{23–25} Interestingly, the related isouronium cation retains these two binding features and, additionally, is less basic (pK_{aH} = 9.8 (ref. 18)); hence, this cation is very promising as a functional substitute in guanidinium derivatives with therapeutic potential.²⁶

Over the past 20 years we have prepared a large number of symmetric and asymmetric di-aromatic isouronium derivatives that, in some cases, showed rather promising cytotoxic activity against diverse cancer cell lines.^{27,28} Given these encouraging results and the possibility for isouronium to overcome the pharmacokinetic problems associated with guanidinium, we decided to explore the effect of substituting the guanidinium cation hypothetically involved in HBs and electrostatic interactions with the ATP's phosphate chain of protein kinases, by an isouronium.

Hence, we present in this article, first, the computational study of the potential interactions between a simple arylisouronium system and the phosphates in the ATP molecule. Although kinase active site structure limitations need to

be considered for a deep understanding of the mode of action of our inhibitors, the aim of this first part of the study is to obtain a theoretical comparative description of all possible interacting modes between ATP and isouronium/guanidinium. Next, the preparation of isouronium **2** (Fig. 1) is presented; in this derivative of **1** the (4-Cl-3-CF₃)-Ph lipophilic moiety was retained because its importance in keeping potent kinase inhibition.¹⁶ In addition, the synthesis of compounds **3** and **4** (Fig. 1) is reported to explore optimal length and conformation in these molecules by changing the di-substituted guanidinium in compounds **1** and **2** by an amide functionality.

This change orients the lipophilic moiety to a different position providing information on the structural requirements for improved kinase affinity.

Finally, we present the study of the effect of these compounds on cancerous cell growth, which was assessed on human leukaemia (HL-60), breast cancer (MCF-7) and cervical cancer (HeLa) cell lines by means of cell viability experiments. Additionally, the effect of these compounds on the MAPK/ERK kinase pathway, which was explored by Western blot experiments in HL-60 and MCF-7 cells, is also presented.

Results and discussion

Computational studies

We have previously computationally studied the interactions between some arylguanidinium systems and the different phosphate groups of ATP to understand their possible mode of action in kinase inhibition; as a result, a theoretical model was proposed.¹⁷ Now, we have carried out a similar study with 4-phenoxyphenyl-*O*-isouronium (simplified model of the molecules proposed in this work, see Fig. S1, ESI†) interacting with the different ATP phosphates.

In our previous study,¹⁷ the most stable interaction formed between ATP and 4-phenoxyphenyl-*N*-guanidinium, was the one simultaneously established between two H atoms of the guanidinium moiety and two O atoms of the phosphate. Thus, with the aim of comparing isouronium and guanidinium contacts, only those interactions involving H2 and H3 of the isouronium (see Fig. S1†) and two O atoms of a phosphate were considered.

Calculated interaction energies (E_i , kJ mol⁻¹) between ATP and 4-phenoxyphenyl-*O*-isouronium are presented in Table S1 (ESI†); those with the 4-phenoxyphenyl-*N*-guanidinium cation (in brackets in Table S1†) have been included for comparison purposes. The E_i values calculated for the 4-phenoxyphenyl-*O*-isouronium...ATP complexes are larger than those previously reported by our group for 4-phenoxyphenyl-*N*-guanidinium (Table S1†).¹⁷ However, an exception to this trend is found in complexes that form multiple simultaneous interactions with different phosphates (Fig. S2, ESI†). Since the larger the number of interactions, the larger the E_i values, it is clear from Table S1† that these complexes are more stable than similar dimers with interactions at only one of the ATP phosphates.

Fig. 1 Structure of compounds **1**, **2**, **3** and **4**, highlighting the comparison of the two cationic moieties (guanidinium in the green box and isouronium in the blue box). The guanidine and amide linkers are indicated by the red boxes.

All the interactions established within the 4-phenyloxyphenyl-*O*-isouronium-ATP complexes were characterized by analysing their electron density topology using the atoms in molecules (AIM) theory.²⁹ Thus, the electron density characteristics found in the bond critical points (BCPs) between the interacting moieties (ρ_{BCP} and $\nabla^2\rho_{\text{BCP}}$, a.u.), were calculated and are presented in Tables S2 and S3 (ESI†). The ρ_{BCP} values and positive Laplacians, indicate that all the interactions established within the complexes studied are of a non-covalent nature and can be classified as HBs. All the AIM molecular graphs are presented in Fig. S2 (ESI†).

In summary, the theoretical studies performed clearly indicate that the proposed isouronium derivatives can form similar interactions with the ATP phosphates as those proposed for the guanidinium lead compound **1** potentially exhibiting similar biological activity.

Synthetic procedures

Based on the positive results from the computational studies, we proceeded to the preparation of compounds **2** to **4** (Fig. 1). Synthesis of compound **2** required the preliminary preparation of a conveniently functionalised phenol (Scheme 1); thus, the phenol moiety was introduced by the initial protection of iodophenol **5** with pyridinium *p*-toluenesulfonate (PPTS) and 3,4-dihydro-2*H*-pyran in dry CH_2Cl_2 to yield compound **6**.³⁰ PPTS is the most common catalyst for the tetrahydropyranylation of alcohols, and seems to be superior to other catalysts such as hydrochloric acid, phosphoryl chloride, and boron trifluoride etherate.³¹ Further-

more, since the THP protecting group was stable under the conditions of the next reaction, compound **6** was used in the subsequent Ullman coupling with commercially available 3-aminophenol **7**, CuI and picolinic acid in DMSO at 80 °C to obtain compound **8** (Scheme 1).³²

Following the procedure already described by our group,¹⁶ Boc-protected thiourea **9** was reacted with the commercially available aniline **10** to yield the corresponding amidylating agent **11** (Scheme 1). Coupling **8** with the synthesized thiourea **11**, in presence of HgCl_2 and triethylamine, afforded **12** in good yield (58%).

The OH free phenol derivative was then amidylated using standard conditions previously described by us²⁷ and others³⁴ using *N,N'*-bis-(*tert*-butoxycarbonyl)-*S*-methylisothiourea **14**, mercury(II) chloride and triethylamine to yield the corresponding Boc-protected isouronium **15** (Scheme 1). Compound **12** was then deprotected with montmorillonite KSF to obtain the corresponding phenol **13** (Scheme 1). Montmorillonite clays have been extensively used as efficient catalysts for a variety of organic reactions and they are a mild and efficient alternative procedure for the deprotection of THPEs.³³

The rationale behind the preparation of compounds **3** and **4** was that introduction of the amido functionality will not only shorten the total length of these potential PKIs, but also will position the lipophilic group in a different orientation due to the strict *trans* conformation of the CO-NH arrangement. Therefore, commercially available dianiline **16** was reacted with Boc-protected thiourea **9** in the presence of HgCl_2 and triethylamine to yield aniline **17**.¹⁶ Next, aniline **17**, was reacted with acid chloride **18** (previously prepared by reacting the corresponding benzoic acid and oxalyl chloride in the presence of catalytic DMF) to yield the Boc-protected guanidine **19** (Scheme 2).³⁵

Finally, for the preparation of the amide-linked phenol precursor of isouronium **4**, the THP-protected compound **8** was reacted with the acid chloride **18** to yield compound **20** which was deprotected using montmorillonite KSF, as previously described, to obtain compound **21**. This phenol was subsequently amidylated with *N,N'*-bis-(*tert*-butoxycarbonyl)-*S*-methylisothiourea **14** to produce compound **22** in a very good yield (97%) (Scheme 3).

Scheme 1 Reagents and conditions: (a) PPTS, 3,4-dihydro-2*H*-pyran, CH_2Cl_2 , rt, 2 h, 82%; (b) CuI, picolinic acid, K_3PO_4 , DMSO, 80 °C, 24 h, 86%; (c) NaH, TFAA, THF, 0 °C, 5 h, 61%; (d) HgCl_2 , Et_3N , CH_2Cl_2 , rt, overnight, 58%; (e) montmorillonite KSF, MeOH, 50 °C, 4 h, 65%; (f) HgCl_2 , Et_3N , CH_2Cl_2 , rt, overnight, 55%.

Scheme 2 Reagents and conditions: (a) HgCl_2 , Et_3N , CH_2Cl_2 , 25 °C, 12 h, 51%; (b) Et_3N , CH_2Cl_2 , overnight, 51%.

Scheme 3 Reagents and conditions: (a) Et_3N , CH_2Cl_2 , overnight, 43%; (b) montmorillonite KSF, MeOH, 50 °C, 4 h, 74%; (c) HgCl_2 , Et_3N , CH_2Cl_2 , 25 °C, 12 h, 97%.

Boc-deprotection of isourea 15 was carried out following standard conditions within our lab to yield the final hydrochloride salt 2; thus, 15 was treated with TFA, followed by chloride anion exchange, to afford the hydrochloride salt 2 in an excellent yield (95%). In the case of Boc-protected compounds 19 and 22, a different approach was considered. Although heat is often required to cleave an amide bond, as it is not very nucleophilic, it was thought that the amide link present in these compounds is particularly labile due to the electron withdrawing nature of the Ph–O–Ph oxygen and the electronegative halogen substituents present on the adjacent phenyl ring. These two elements lead to the electron donating capabilities of the NH in the amide bond to be somewhat diminished leaving the CO carbon atom more open to nucleophilic attack. Hence, use of HCl or TFA was not considered appropriate for such substrates and, hence, deprotection of 19 and 22 was carried out with the use of SnCl_4 in EtOAc (as described by Miel *et al.*³⁶) to obtain final hydrochloride salts 3 (47%) and 4 (51%).

Biological results and physicochemical properties

As mentioned, we had previously identified 4-chloro-3-trifluoromethylphenyl substituted 3,4'-bis-guanidinium phenoxyphenyl derivatives with potential as MAPK/ERK type III allosteric inhibitors. Compound 1 in particular was found

to be the best cell growth inhibitor for the human leukemia HL-60 cancer cell line with an IC_{50} of 9.72 μM .^{15,16} In order to assess the effect of the guanidinium/isourenium substitution as well as the effect of the conformational change, induced by an amide linker, on the growth inhibition properties of compound 1, the cytotoxicity of the newly synthesised compounds 2, 3 and 4 as well as that of the lead compound 1 was evaluated *in vitro* against the HL-60 (leukemia), MCF-7 (breast) and HeLa (cervical) cancer cell lines using the known type II kinase inhibitor sorafenib as a control (Table 1 and Fig. 2).

In these assays, compound 1 produced very similar results in the three cancer cell lines with IC_{50} values ranging from 9.48 to 9.84 μM . In contrast, compound 2 showed an improved effect on cell viability with low IC_{50} values of 5.49, 4.68 and 3.35 μM in the HL-60, MCF-7 and HeLa cell lines, respectively (Table 1 and Fig. 2). Moreover, the amide-linked guanidinium derivative 3, a shorter analogue of the lead 1, has a 2–3 fold decrease in activity compared to 1 and shows very similar cytotoxicity to the also short isourenium compound 4 (4–9 fold drop in activity). From these results we can deduce, firstly, that the amide linker present in compounds 3 and 4 probably orientates the lipophilic moiety in a different direction to that of the guanidinium link for compounds 1 or 2 resulting in the loss of activity. Secondly, the isourenium moiety seems to establish similar interactions to the mono-substituted guanidinium cation, resulting in related IC_{50} values.

Even though the difference in growth inhibitory activity between isourenium (2) and guanidinium (1) derivatives is small, it is still interesting to note that compound 2 shows slightly better results probably due to improved pharmacokinetic properties. Accordingly, we have calculated different relevant physicochemical parameters such as the clogP (lipophilicity), PSA (polar surface area), calculated pK_{aH} (basicity) or calculated solubility (in water) with the help of the Chemaxon's Marvin package³⁷ and the results are shown in Table 1.

The difference between isourenium and guanidinium is evident with the PSA, which is an indicator of HB formation and a commonly used metric for the optimisation of a drug's ability to permeate cells, since molecules with a polar surface area $>140 \text{ \AA}^2$ tend to be poor at permeating cell membranes.³⁸ In our case, compounds 3 and 4 have lower PSA values (100.2 and 97.4 $\text{\AA}^2$, respectively) than compounds 1 and 2 (119.0 and 116.2 $\text{\AA}^2$, respectively), but similar to

Table 1 Inhibition of cell viability, expressed as IC_{50} ($\mu\text{M} \pm \text{SEM}$) values, in HL-60, MCF-7 and HeLa cells and calculated properties (clogP ; PSA, $\text{\AA}^2$; pK_{aH} and aqueous solubility, mg mL^{-1})³⁷ obtained for the guanidiniums 1 (ref. 15) and 3, isoureniums 2 and 4 and sorafenib as a positive control. IC_{50} values are mean $\pm$ S.E.M. representatives of at least three independent experiments performed in triplicate

Comp	HL-60 IC_{50}	MCF-7 IC_{50}	HeLa IC_{50}	clogP	PSA	pK_{aH}	Solubility
1	9.72 $\pm$ 0.9 (ref. 15)	9.84 $\pm$ 1.8	9.48 $\pm$ 0.2	5.09	119.0	8.71, 7.02	0.0106
2	5.49 $\pm$ 0.10	4.68 $\pm$ 0.2	3.35 $\pm$ 0.3	5.46	116.2	8.34, 7.03	0.0102
3	28.16 $\pm$ 0.6	20.75 $\pm$ 2.3	11.28 $\pm$ 0.4	5.02	100.2	8.71	0.0092
4	35.60 $\pm$ 1.1	28.72 $\pm$ 1.4	83.57 $\pm$ 8.3	5.39	97.4	8.32	0.0091
Sorafenib	2.59 $\pm$ 0.40	3.07 $\pm$ 0.1	4.59 $\pm$ 0.5	4.34	92.4	2.03	0.0100

Fig. 2 Effect of compounds 1, 2, 3 and 4 on the viability of HL-60, MCF-7 and HeLa cells determined by the AlamarBlue® assay. Cells were seeded at a density of 4×10^4 (HL-60), 5×10^3 (MCF-7) and 5×10^3 (HeLa) cells per well on a 96-well plate and treated with vehicle alone [1% (v/v) EtOH or 0.1% (v/v) DMSO], or the compounds at 0.1–100 μM . Sorafenib was used as control. Cells were incubated for 72 h at 37 °C after which they were treated with AlamarBlue® and left in darkness in an incubator for 4–5 h. The resulting fluorescence was read using a plate reader from which percentage viability was calculated. The points on the three graphs represent mean values $\pm$ S.E.M. from at least three independent experiments performed in triplicate.

sorafenib (92.4 Å²). Moreover, isouroniums 2 and 4 have lower PSA values than their guanidinium analogues (1 and 2). This optimal profile for the isouroniums is countered by a slight gain in *clogP* compared to 1, 3 and sorafenib which are still far from the optimal value of 3. Correlation between *clogP* and PSA is presented in Fig. S4 (ESI[†]), where all compounds are found in a similar region.

Looking at the calculated basicity, compounds 1 and 2 present two *pK_{aH}* values due to the di-substituted guanidine linker. Despite the difference in basicity of the isolated cations (guanidinium is approximately 4 units more basic than isouronium), both sets of derivatives have very similar *pK_{aH}* values (~ 8.3 and ~ 8.7 , respectively) and will be protonated at physiological pH. Finally, the calculated aqueous solubility of these compounds is in the range of moderate (1, 2 and sorafenib) to low (3 and 4) and no particular advantage in this property can be seen for the use of one cation over another.

Summarising, taking into account the cell viability results and calculated physicochemical parameters, both isouronium and guanidinium cations show very similar profiles and should be considered as interchangeable in drug design.

Next, the effect on the MAPK/ERK pathway of compounds 1 and 2 (the most cytotoxic agents for each family) and sorafenib was studied in HL-60 and MCF-7 cells by means of Western blotting (Fig. 3). From these experiments it can be reasoned that, after 16 hours in HL-60 cells, compound 2 has a similar effect to sorafenib in decreasing the levels of pERK, indicative of a decrease in activated ERK. However, lead com-

Fig. 3 Effect of compounds 1, 2 and sorafenib (as a control) on the level of expression of phosphorylated (activated) and total ERK. HL-60 and MCF-7 cells were treated with either vehicle (0.1% EtOH), sorafenib (10 μM), compound 1 (10 μM) or compound 2 (10 μM) for 16 hours. Cells were lysed and 20 μg protein was loaded, separated on 12% SDS-page gel and transferred to PVDF membrane. The membrane was then blocked and probed with anti-ERK and anti-pERK [1:1000] and anti-rabbit secondary antibodies. Anti-GAPDH [1:2500] was used as a loading control and probed with anti-mouse secondary. HL-60 and MCF-7 results are representative of 4 and 2 independent experiments respectively.

pound 1 does not show any change compared to control and vehicle. These results are in agreement with the *IC₅₀* values obtained (Table 1). Furthermore, after 16 hours in MCF-7 cells, the effect on decreasing pERK expression is again very similar between compound 2 and sorafenib, but also a weak effect is now observed for compound 1. Expression of total ERK remained unaffected by any of the three compounds.

In summary, we can conclude that compound 2 clearly affects the MAPK/ERK pathway both in HL-60 and MCF-7, which directly correlates with its potent inhibition of cancer cell growth. However, the actual interactions established with the target will only be identified if the crystal structure of the corresponding complex is resolved. Furthermore, regarding the mechanism of action of compound 1, even though targeting of the MAPK/ERK pathway cannot be completely ruled out, other mechanisms may be involved and, thus, further studies will be pursued.

Experimental

Computational details

The geometry of the monomers and the complexes were fully optimized at DFT $\omega\text{B97XD}/6\text{-}31\text{+G(d,p)}^{39}$ and then MP2/6-311+G(d,p) to obtain binding energies. All these calculations were carried out with Gaussian09 program.⁴⁰ Frequency calculations were performed at the same computational level to confirm that the resulting optimized structures are energetic minima. Interaction energies, *E_i*, (kJ mol⁻¹) were calculated as the difference between the total energy of the complex and the sum of the isolated monomers. The effect of water solvation was then accounted for using the SCFR-PCM approach implemented in the Gaussian09 package including dispersing, repulsing and cavitation energy terms of the solvent in the optimization. For the sake of clarity during the discussion, the atoms involved in each interaction have been labelled as indicated in Fig. S1[†]. The bonding characteristics were analysed by means of the atoms in molecules (AIM)

theory.²⁹ For this purpose we have located the most relevant bond critical points (BCP) and evaluated the electron density at each of them, by means of AIMall program.⁴¹ Electron density shift maps (EDS) have been obtained in order to provide an insight into the changes in the electron density of the monomers upon complexation. All the EDS maps have been obtained following the procedure previously reported.⁴²

Synthetic procedures

All commercially available chemicals were obtained from Sigma-Aldrich or Fluka and used without further purification. Deuterated solvents for NMR use were purchased from Apollo and Euriso-top. Chromatographic columns were run using silica gel 60 (230–400 mesh ASTM). Solvents for synthesis purposes were used at GPR grade. Analytical TLC was performed using Merck Kieselgel 60 F₂₅₄ silica gel plates or Polygram Alox N/UV254 aluminium oxide plates. Visualisation was by UV light (254 nm). NMR spectra were recorded on Agilent and Bruker Avance spectrometers, operating at 400.13 MHz and 600.1 MHz for ¹H NMR; 100.6 MHz and 150.9 MHz for ¹³C NMR. Shifts are referenced to the internal solvent signals. NMR data were processed using MestreNova software. HRMS spectra were measured on a Micromass LCT electrospray TOF instrument with a WATERS 2690 autosampler and methanol as carrier solvent. Melting points were determined using a Stuart Scientific Melting Point SMP1 apparatus and are uncorrected. Infrared spectra were recorded on a Perkin Elmer Spectrum One FTIR spectrometer equipped with a Universal ATR sampling accessory. HPLC purity analysis was carried out using a Varian ProStar system equipped with a Varian Prostar 335 diode array detector and a manual injector (20 µL). For purity assessment, UV detection was performed at 245 nm and peak purity was confirmed using a purity channel. The stationary phase consisted of an ACE 5 C18-AR column (150 × 4.6 mm), and the mobile phase used the following gradient system, eluting at 1 mL min⁻¹: aqueous formate buffer (30 mM, pH 3.0) for 10 min, linear ramp to 85% methanol buffered with the same system over 25 min, hold at 85% buffered methanol for 10 min. Minimum requirement for purity was set at 95%.

4-{3-[3-(4-Chloro-3-trifluoromethylphenyl)guanidino]-phenoxy}phenylcarbamimidate dihydrochloride (2). A solution of the corresponding Boc-protected precursor 15 (100 mg, 0.13 mmol) in 10 mL of a 50% v/v solution of TFA and CH₂Cl₂ was stirred at room temperature. Once disappearance of starting material was observed by TLC (3 h), the excess of TFA and CH₂Cl₂ were removed under vacuum to generate the trifluoroacetate salt. The residue was dissolved in water (10 mL) and 500 mg of IRA400 Amberlite resin in its chloride-activated form was added, allowing the reaction to proceed overnight. The resin was then removed by filtration and the aqueous solution washed with CH₂Cl₂ (2 × 5 mL). Removal of water under vacuum yielded the final isouronium hydrochloride 2 as a white/yellow solid (67 mg, 95%). Absence of the trifluoroacetate salt was confirmed by ¹⁹F NMR. Mp: decom-

poses over 120 °C. δ_{H} (400 MHz, CD₃OD): 7.02 (dd, J = 8.2, 2.0 Hz, 1H, Ar), 7.09 (t, J = 2.1 Hz, 1H, Ar), 7.17 (dd, J = 8.0, 1.3 Hz, 1H, Ar), 7.20 (d, J = 9.0 Hz, 2H, Ar), 7.35 (d, J = 9.0 Hz, 2H, Ar), 7.47 (t, J = 8.1 Hz, 1H, Ar), 7.60 (dd, J = 8.6, 2.4 Hz, 1H, Ar), 7.70 (d, J = 8.6 Hz, 1H, Ar), 7.75 (d, J = 2.3 Hz, 1H, Ar). δ_{C} (100 MHz, CD₃OD): 116.5 (CH, Ar), 118.6 (CH, Ar), 121.1 (CH, Ar), 122.2 (2 CH, Ar), 123.8 (d, J = 271.0 Hz, qCF₃), 124.0 (2 CH, Ar), 125.3 (q, J = 4.3 Hz, CH, Ar), 130.5 (d, J = 31.8 Hz, qC), 130.8 (CH, Ar), 131.3 (qC), 132.5 (CH, Ar), 134.3 (CH, Ar), 136.1 (qC), 137.9 (qC), 146.8 (qC), 156.3 (qC), 157.5 (qC), 159.5 (qC), 163.3 (qC). δ_{F} (376 MHz, CD₃OD): -64.33 (s). ν_{max} (ATR)/cm⁻¹: 3183 (NH), 2963 (CH), 1574 (C=N), 1477, 1250 (CF₃), 1096, 1030 (C-O), 1012 (C-Cl), 785, 667. HRMS (m/z ES⁺) calcd for C₂₁H₁₇ClF₃N₅O₂ ([M + H]⁺): 464.1096, found 464.1101. HPLC: 98.4% (t_{R} 27.01 min).

4-[3'-(4-Chloro-3-trifluoromethylbenzamido)phenoxy]-phenyl guanidine hydrochloride (3). To a stirred solution of 19 (90 mg, 0.14 mmol) in EtOAc was added SnCl₄ (0.06 mL, 0.56 mmol). After 1 h of stirring at room temperature, the solvent and the excess of SnCl₄ were evaporated *in vacuum*. The product was purified by flash chromatography (silica gel, CHCl₃:MeOH, 100:0 → 95:5) and the purified fraction was then dissolved in MeOH and recrystallized from Et₂O to obtain 3 as a white solid (32 mg, 47%). Mp: decomposes over 120 °C. δ_{H} (400 MHz, CD₃OD): 6.86 (d, J = 7.8 Hz, 1H, Ar), 7.12 (d, J = 8.6 Hz, 2H, Ar), 7.30 (d, J = 8.7 Hz, 2H, Ar), 7.35–7.43 (m, 2H, Ar), 7.62 (s, 1H, Ar), 7.77 (d, J = 8.3 Hz, 1H, Ar), 8.16 (dd, J = 8.3, 1.5 Hz, 1H, Ar), 8.32 (s, 1H, Ar). δ_{C} (100 MHz, CD₃OD): 112.9 (CH, Ar), 116.3 (CH, Ar), 117.3 (CH, Ar), 121.0 (2 CH, Ar), 124.0 (d, J = 272.5 Hz, qCF₃), 128.2 (q, J = 5.5 Hz, CH, Ar), 128.9 (2 CH Ar), 129.4 (d, J = 31.6, qC), 130.9 (qC), 131.2 (CH, Ar), 133.1 (CH, Ar), 133.6 (CH, Ar), 135.3 (qC), 136.5 (qC), 141.2 (qC), 158.1 (qC), 158.3 (qC), 158.4 (qC), 166.0 (qC). δ_{F} (376 MHz, CD₃OD): -64.11 (s). ν_{max} (ATR)/cm⁻¹: 3413 (NH), 3343 (NH), 1602 (C=O), 1504 (C=N), 1450, 1412, 1261 (C-N), 1216 (C-O), 1134 (CF₃), 1035 (C-Cl), 924, 845. HRMS (m/z ES⁺) calcd for C₂₁H₁₆ClF₃N₄O₂ ([M + H]⁺): 449.0987, found 449.0988. HPLC: 98.9% (t_{R} 32.25 min).

4-{3-[4-Chloro-3-(trifluoromethyl)benzamido]phenoxy}-phenyl carbamimidate hydrochloride (4). To a stirred solution of 22 (47 mg, 0.07 mmol) in EtOAc was added SnCl₄ (0.033 mL, 0.28 mmol). After 1 h of stirring at room temperature, the solvent and the excess of SnCl₄ were evaporated *in vacuum*. The product was purified by flash chromatography (silica gel, CHCl₃:MeOH, 100:0 → 95:5) to afford the final hydrochloride salt 4 (17 mg, 51%) as a white/yellow gum. δ_{H} (400 MHz, CD₃OD): 6.84 (dd, J = 8.0, 1.8 Hz, 1H, Ar), 7.13 (d, J = 8.8 Hz, 2H, Ar), 7.30 (d, J = 8.9 Hz, 2H, Ar), 7.35 (t, J = 8.1 Hz, 1H, Ar), 7.44 (d, J = 8.0 Hz, 1H, Ar), 7.60 (s, 1H, Ar), 7.74 (d, J = 8.3 Hz, 1H, Ar), 8.17 (dd, J = 8.3, 1.6 Hz, 1H, Ar), 8.29 (d, J = 1.5 Hz, 1H, Ar), 8.66 (bs, NH). δ_{C} (150 MHz, CD₃OD): 113.0 (CH, Ar), 116.3 (CH, Ar), 117.5 (CH, Ar), 121.6 (2 CH, Ar), 122.2 (qC), 124.1 (d, J = 272.5 Hz, qCF₃), 123.8 (2 CH, Ar), 128.2 (q, J = 5.4 Hz, CH, Ar), 129.5 (d, J = 31.7 Hz, qC), 131.3 (CH, Ar), 133.1 (CH, Ar), 133.7 (CH, Ar), 135.4 (qC), 136.6 (qC), 141.3 (qC), 146.4 (qC), 158.2 (qC), 158.4 (qC), 166.1

(qC). δ_F (376 MHz, CD_3OD): -62.22 (s). ν_{max} 3329 (NH), 1695, 1648, 1602 (C=O), 1482 (C=N), 1249 (C-O), 1192 (CF_3), 1131, 1111 (C-Cl), 780, 660. HRMS (m/z ES⁺) calcd for $C_{21}H_{15}ClF_3N_3O_3$ ($[M + H]^+$): 450.0827, found 450.0835. HPLC: 97.69% (t_R 31.72 min).

Biochemical methods

Preparation of stock solution. Stock solutions (10 mM) of the compounds were prepared in sterile EtOH or DMSO and were then sterile filtered (0.2 μ M filters). Required concentration ranges (10–0.01 mM) of each drug were prepared in sterile EtOH or DMSO/ddH₂O and stored at -20 °C until required. Sorafenib was purchased from LC laboratories.

HL-60 cell line. The HL-60 (human caucasian promyelocytic leukemia) cell line was purchased from Sigma-Aldrich. They were maintained between 200 000 and 2 000 000 cells per mL in Roswell Park Memorial Institute (RPMI) 1640 medium with stable glutamate (GlutaMax I) supplemented with 10% (v/v) foetal bovine serum (FBS) and 50 μ g mL⁻¹ penicillin/streptomycin (pen-strep). The growth medium was stored in the fridge at 4 °C and heated to 37 °C prior to culture work. Cells were grown at 37 °C in a humidified environment maintained at 95% O₂ and 5% CO₂ and passaged at least twice weekly depending on their levels of confluency. When required for sub-culturing, cells were transferred to a sterile tube and centrifuged at 300 × *g* for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 15 mL of fresh medium. Cells were then counted and seeded at a density of 200 000 cells per mL (200 μ L per well or 4 × 10⁴ cells per well) in complete medium.

HeLa cell line. The HeLa (human epithelioid cervix carcinoma) cell line was purchased from ECACC. They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 50 μ g mL⁻¹ penicillin/streptomycin (pen-strep). The growth medium was stored in the fridge at 4 °C and heated to 37 °C prior to culture work. Cells were grown at 37 °C in a humidified environment maintained at 95% O₂ and 5% CO₂ and passaged at least twice weekly depending on their levels of confluency. When required for sub-culturing, cells were trypsinised and transferred to a sterile tube and centrifuged at 300 × *g* for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 15 mL of fresh medium. HeLa cells were then counted and seeded in 96-well plates at a density of 2.5 × 10⁴ cells per mL (200 μ L per well or 5 × 10³ cells per well) in complete medium.

MCF-7 cell line. The MCF-7 (human breast tumour) cell line was purchased from ATCC and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with stable glutamate (GlutaMax I) supplemented with 10% (v/v) foetal bovine serum (FBS), 50 μ g mL⁻¹ penicillin/streptomycin (pen-strep) and 2 mM L-glutamine. The growth medium was stored in the fridge at 4 °C and heated to 37 °C prior to culture work. Cells were grown at 37 °C in a humidified environment maintained at 95% O₂ and 5% CO₂ and passaged at

least twice weekly depending on their levels of confluency. When required for sub-culturing, cells were trypsinised and transferred to a sterile tube and centrifuged at 300 × *g* for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 15 mL of fresh medium. MCF-7 cells were then counted and seeded in 96-well plates at a density of 2.5 × 10⁴ cells per mL (200 μ L per well or 5 × 10³ cells per well) in complete medium.

Cell viability (Alamar Blue®). The 96 well plates were then treated with a 1:100 dilution of stock concentrations of drugs or EtOH (1% v/v)/DMSO (0.1% v/v) as vehicle control in triplicate. Three blank wells containing 200 μ L RPMI with no cells were also set-up as blank wells. After a 72 h incubation, 20 μ L of Alamar Blue was added to each well. The plates were incubated in darkness at 37 °C for 4–5 h. Using a molecular devices microplate reader, the fluorescence (*F*) was then read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Cell viability was then determined by subtracting the mean blank fluorescence (*F*_b) from the treated sample fluorescence (*F*_s) and expressing this as a percentage of the fluorescence of the blanked vehicle control (*F*_c). This is demonstrated in eqn (1). The results were then plotted as a nonlinear regression, sigmoidal dose-response curves on Prism, from which the IC₅₀ value for each drug was determined.

$$(F_s - F_b)/(F_c - F_b) \times 100 = \% \text{Cell Viability} \quad (1)$$

MAPK/Erk pathway analysis (Western blotting). MCF-7 and HL-60 cells were seeded in T25 flasks at a density of 50 × 10⁴ cells per mL for HL-60 and 25 × 10⁴ cells per mL for MCF-7. Cells were then treated with either vehicle [0.1% ethanol (v/v)], sorafenib (10 μ M), compound 1 (10 μ M) or compound 2 (10 μ M). Cells were harvested and washed with PBS. Cell pellets were re-suspended in ice-cold radioimmunoprecipitation assay buffer (RIPA buffer), supplemented with 1% (v/v) phosphatase inhibitor cocktail 2 and 3 (Sigma) and 10% (v/v) protease inhibitor (Roche). Lysis was carried out for 30 minutes on ice. Cellular lysates were clarified by centrifugation for 10 minutes at 14 000 × *g* at 4 °C. Protein concentration was determined and normalized using a BCA assay. Lysates were boiled with Laemmli sample buffer [Tris-HCl 50 mM (pH 6.7), glycerol 10% (w/v), sodium dodecyl sulphate 2% (w/v), bromophenol blue 0.02% (w/v)] containing DTT 50 μ M for 10 minutes at 90 °C. Equal protein concentrations of lysates were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF transfer membrane (EMD Millipore, Germany). The membranes were blocked in 5% non-fat milk and probed with primary antibodies for ERK, phospho-ERK (Cell Signalling, Danvers, MA) and GAPDH (Calbiochem) at 4 °C overnight and secondary antibodies the next day.

Conclusions

In this study, we have computationally characterised and analysed the nature of the interactions potentially established

upon complexation of 4-phenoxyphenyl-*O*-isouronium derivative with the ATP molecule and compared them with results previously obtained for a similar arylguanidinium. Considering our hypothesis that the guanidinium moiety of the previously identified lead compound **1** interacts with the γ -phosphate of ATP within the hinge region of an active protein kinase, our results now indicate that an isouronium analogue will be able to interact in a similar manner with the ATP molecule inhibiting kinase activity.

To evaluate the effect on antitumor activity of the guanidinium/isouronium substitution as well as the molecular shortening and conformational change, compounds **2**, **3** and **4** were prepared. Compound **3** is an amide-linked (shorter) version of the guanidine-linked lead compound **1** that will place the lipophilic 4-(Cl)-3-(CF₃)-Ph group in a different orientation, whereas compounds **2** and **4** are the corresponding isouronium analogues of **1** and **3**, respectively. Compounds **2**, **3** and **4** were successfully prepared through the amidylation of the corresponding aromatic amines or phenols by reaction with *N,N'*-bis-(*tert*-butoxycarbonyl)-*S*-methylisothiourea in the presence of mercury(II) chloride and triethylamine followed by Boc-deprotection to yield the corresponding hydrochloride salts in good to excellent yields.

The effect of all these compounds on cell viability was tested in human leukaemia (HL-60), breast cancer (MCF-7) and cervical cancer (HeLa) cell lines and the corresponding IC₅₀ values were compared with those of the lead compound **1**. It was observed that the replacement of the di-substituted guanidine by an amide results in lower activity probably due to the wrong orientation of the lipophilic moiety of the molecules. However, replacement of the mono-substituted guanidinium by an isouronium cation seems to be beneficial for this activity probably due to improved pharmacokinetic properties. Accordingly, a number of physicochemical parameters (PSA, *clogP*, *pK_{ah}* and aqueous solubility) were calculated and the values obtained were in agreement with the cytotoxicity observed.

Finally, the effect of compounds **1** and **2** on the Ras-Raf-MEK-ERK pathway was explored by means of Western blot experiments measuring the effect of these compounds on the expression of phosphorylated ERK (pERK) and total ERK both in HL-60 and MCF-7 cancer cells. These experiments showed that compound **2** clearly inhibits the expression of pERK in both cell lines, whereas compound **1** only affects the expression of pERK in MCF-7 cells. This indicates that the most likely mechanism of anticancer action of compound **2** involves targeting of the MAPK/ERK pathway; however, for compound **1**, even though this mechanism of action cannot be discarded for MCF-7 cells, other targets are likely for HL-60 cancer cells. Even though the exact mode of action and/or targeting of the compounds in the MAPK/ERK pathway cannot be accurately determined with these experiments, a clear indication of their interference with this pathway is obtained and further studies are being pursued.

Taking all these results together we can conclude that the isouronium functionality is a good alternative to

guanidinium and they can be interchanged for potential type III allosteric kinase inhibitors.

Conflicts of interest

The authors declare no competing interest.

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