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Divergent response of homologous ATP sites to stereospecific ligand fluorination for selectivity enhancement†

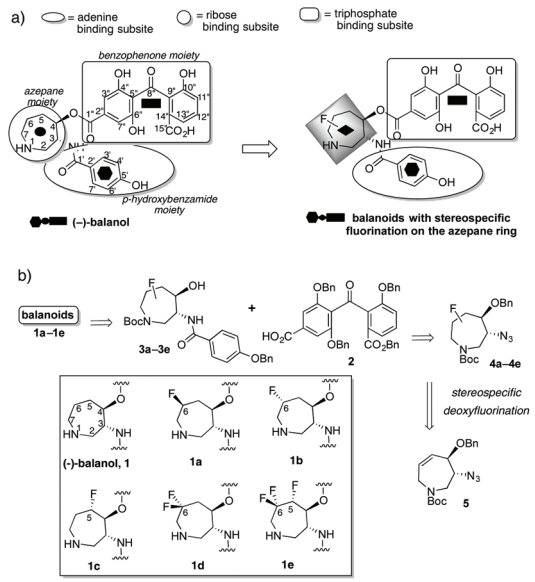
Alpesh Ramanlal Patel,‡ Ari Hardianto, Shoba Ranganathan and Fei Liu*

Acquiring a divergent response from homologous protein domains is essential for selective ligand–protein interactions. Stereospecific fluorination of (–)-balanol, an ATP mimic, uncovers a new source of selectivity from integrated chemical and conformational perturbation that differentiates homologous sites by the level of congruency in their response to local and remote fluorine effects.

Selectivity in ligand–receptor interactions is a general requirement in chemical and biological systems, and both chemical diversification and conformational regulation are important avenues for specificity optimisation.[1–4] However, selectivity in highly homologous receptors or protein domains remains a challenge. New insight into how conformational perturbation could be fused with chemical functionalisation to create compounded effects may open new avenues of selectivity optimisation. Here we investigate the potential of stereospecific ligand fluorination in instigating composite perturbation effects that challenge both the ligand and protein to adapt congruently. Fluorine is small with minimal steric interference yet a rich source of physical/chemical perturbation and stereoelectronic effects for shape control.[5,6] We employ the natural product balanol as a model system to demonstrate how new selectivity potential in highly homologous ATP sites of protein kinases can be unlocked by using fluorine through integrated chemical and conformational control in a protein-dependent manner. As protein kinases are validated drug targets but require high ATP site selectivity, this selectivity enhancement by perturbation congruency may introduce new design concepts in kinase drug development.

Balanol was originally discovered as a potent antagonist (IC_{50} 4–9 nM) for the ATP site of the protein kinase C (PKC) isozymes[7–9] and soon after for other related members, such as protein kinase A and B (PKA and PKB), belonging to the AGC superfamily of protein kinases.[10,11] Although a potent antagonist, balanol presents limited selectivity within the family.[12] The seminal PKA/balanol binary crystal structure[13] identified balanol as a type I ATP mimic that occupies the full ATP site, in which the *p*-hydroxybenzamide moiety of balanol occupies the adenine-binding subsite, the azepane moiety the ribose subsite, and the tetrasubstituted benzophenone moiety the triphosphate subsite (Scheme 1a). The aryl rings of the benzophenone moiety induce protein movement in the flexible glycine-rich loop, which allows the loop to interact extensively with the benzophenone moiety. Perturbation of benzophenone substitution frequently leads to severe loss of activity,[10,12,14,15] however the azepane ring is more amenable to derivatisation.[12]

We proceeded to constructing balanoids **1a–1e** with stereospecific C–F bonds on azepane. We have investigated exten-



Scheme 1 (a) General scheme of fluorinated balanoids as ATP site probes. (b) Retrosynthesis of fluorinated balanoids.

Department of Chemistry & Biomolecular Sciences, Macquarie University, Sydney, NSW 2109, Australia. E-mail: fei.liu@mq.edu.au

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‡Current address: Faculty of Pharmacy, The University of Sydney, Sydney, NSW 2109, Australia.

sively stereospecific fluorination by deoxyfluorination reactions on substituted azepanes,[16–19] and secured the synthesis and structural elucidation methods for preparing these stereospecifically fluorinated azepanes **4a–4e** from a common precursor **5**.[16] These fluorinated azepane fragments were converted to **3a–3e** after debenzylation and acylation, followed by esterification to furnish the final fluoro-balanoids **1a–1e** using reported protocols.[20,21] The natural product (–)-balanol (**1**) was also prepared for comparison.[21]

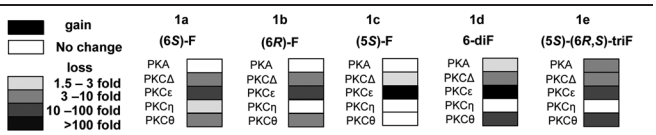
The initial hypothesis was that a minimal level of fluorination on balanol (*e.g.* monofluorinated **1a–1c**) should influence conservatively on the binding of this high affinity ligand. This would then permit more reliable structural modelling for fluorinated balanoids without drastic changes in the binding mode of the ligand. Multiply fluorinated balanoids **1d** (C6-(*R*,*S*) bisfluorination) and **1e** (C5-(*S*) and C6-(*R*,*S*) trifluorination) were prepared to examine the tolerance limit of these enzymes to fluorination. Given the central position of the azepane ring in the binding pocket, this balanol system offers a rare opportunity to examine how a protein could contend with shape-controlling fluorination that not only modifies interactions directly in the azepane-binding subsite but also indirectly in the other subsites by ligand conformation perturbation. Can new binding interactions in the azepane subsite arise from the stereospecific C–F bond in a protein-dependent fashion? Can the (sp^3)C–F bond potentially confer "remote control" in *p*-hydroxybenzamide or the benzophenone binding subsite, given the flexibility of both the ligand and the protein?

The binding affinities of balanol (**1**) and balanoids (**1a–1e**) to PKA and the PKC isozymes PKC*δ*, PKCε, PKCη, and PKCθ were then measured using the KINOME*scan*™ binding assay.[21,22] Isozymes PKC*δ*/ε/η/θ belong to the novel subclass of the PKC family that shares a very high level of sequence homology in their catalytic domain.[8] Without a crystal structure of balanol bound to any PKC isozyme available, the crystal structure of PKA/balanol[13] has served as a representative for the AGC superfamily. These proteins thus form a small but highly homologous panel for testing if this stereospecific fluorination approach could solicit divergent responses from these isozymes.

The observed K_d values of balanol **1** to the enzymes (4–25 nM, Table 1) generally are in good agreement with earlier, biological activity-based measurements for balanol,[7] except for PKCε that exhibited a higher binding affinity, a small variation that may be attributed to the biological activity measurements in earlier reports being performed with the substrate. However, as expected the overall specificity of balanol for these enzymes is limited (*i.e.* under 100 fold).[23]

In general, the binding profile of the monofluorinated balanoids **1a–1c** across the enzyme panel shows either no or mild affinity loss under 10 fold, except for PKCε that exhibited a substantial affinity reduction of 26–33 fold for **1a** and **1b** but a slight gain for **1c**. Single fluorine substitution at the C5-(*S*) position (**1c**) improves the affinity to 0.4 nM and also the relative selectivity ratio of the parent balanol **1** for PKCε, showing that fluorine perturbation at the C5-(*S*) position is productive

Table 1 Binding affinities of **1** and **1a–1e** against selected PKA/C isozymes[a,b]



	K_d[c] (nM)					
	1	**1a**	**1b**	**1c**	**1d**	**1e**
PKA	5.9 ± 0.5	7.9 ± 0.5	6.9 ± 0.4	6.4 ± 0.1	9.2 ± 0.8	43 ± 4
PKCδ	4.5 ± 0	17 ± 1	15 ± 1.5	4.9 ± 0.4	19 ± 0.5	18 ± 0.5
PKCε	0.73 ± 0.06	19 ± 8	24 ± 3.5	0.4 ± 0.02	110 ± 19	38 ± 9.5
PKCη	14 ± 0.5	38 ± 3.5	16 ± 2.5	13 ± 0	19 ± 0.5	12 ± 2
PKCθ	25 ± 6	120 ± 15	140 ± 10	24 ± 0.5	580 ± 60	850 ± 80

[a] Each shaded square indicates the ratio between the K_d of each balanoid for a particular enzyme over that of balanol. [b] Affinity gain or loss under 1.5 fold is considered no change (within error) as the error margin is between 0–41%. [c] Standard error of the mean (SEM) calculated from duplicate experiments.[21]

in enhancing both potency and selectivity for PKCε. This high positional sensitivity of PKCε towards single fluorine substitution (*i.e.* ~50–60 fold relative difference in affinity between **1a**/**b** and **1c**) is either absent or subdued in the other enzymes. For **1a** and **1b**, the relative selectivity ranking, even for this small test panel of enzymes, is altered by single fluorination, from PKCε ≫ PKC*δ*/PKA > PKCη/θ to PKA > PKC*δ*/ε/η ≫ PKCθ; however, the relative selectivity ratio (*i.e.* the ratio between the highest and lowest affinity case) did not improve for **1a** and **1b**. The PKC isozymes consistently exhibit higher sensitivity toward monofluorination at the C6 position than PKA, although the fluorine effect is not stereochemistry-dependent at this position as all five enzymes showed comparable affinities for **1a** and **1b**.

For the multiply fluorinated balanoids **1d** and **1e**, the affinities in most cases are retained in the low nM range, suggesting that fluorination, even in multiple positions, can be well tolerated. Isozymes PKC*δ* and PKCη, along with PKA, appear to be particularly tolerant of fluorine substitution (loss under 10 fold). The trifluorinated balanoid **1e** also switched the isozyme preference from PKA to PKCη. However, PKCε and PKCθ exhibited a much higher level of sensitivity toward **1d** and **1e** with an affinity loss of 20–150 fold. Interestingly, with an additional C5(*S*)-fluorine, **1e** again improves the binding affinity to PKCε by nearly 3 fold compared to that of **1d**, a case analogous to that of the monofluorinated balanoid **1c** in which the C5(*S*)-fluorine substituent improves the binding affinity by nearly two fold.

This affinity profile of the fluoro-balanoids opens the next question on the likely structural basis of this divergent protein response that results in improved affinity and selectivity of **1c** for PKCε. With no X-ray structure currently available for PKCε, the binary structure of mouse PKA bound with balanol (1BX6)[21] and another of human PKCη with naphthyridine (3TXO)[24] were used to construct a structure homology model

		Asp 493	Asp 536	Lys 416
1	N1-H$^+$	–	HB (1.83Å)	–
1a	N1-H$^+$	HB (2.37Å)	HB (2.34Å)	–
1c	C5-F			C=O--F (2.80Å)
	N1-H$^+$	HB (2.50Å)	HB (2.16Å)	HB (2.16Å)

*HB = H-bond. Interactions are to the amino acid sidechain unless in bold to indicate protein backbone interaction

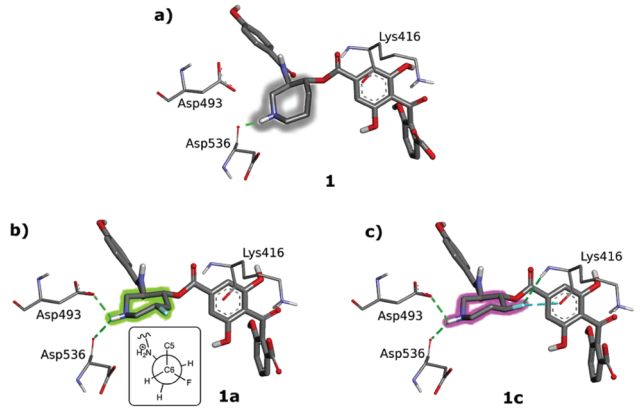


Fig. 1 Docking analysis showing PKCε-ligand interactions in the azepane-binding region for (a) **1** (grey), (b) **1a** (green), and (c) **1c** (pink). The full images and interaction comparison list for **1**, **1a**, and **1c** are in the ESI.†

of PKCε, due to the high sequence homology between these proteins in the catalytic domain (40% for PKA/PKCε and 71% for PKCη/PKCε). Ligands **1**, **1a** and **1c** were then each docked into the PKCε model, with the calculated binding energies showing an excellent linear correlation with the measured affinity values.[21]

The comparison of the bound conformations of balanol **1** (Fig. 1a) and C5(*S*)–F balanoid **1c** (Fig. 1c) identified new interactions in the azepane-binding subsite of PKCε for **1c** (Fig. 1 table, full comparison list of interactions in the ESI†). The C5(*S*)–fluorine induces azepane conformational change in **1c**, which satisfies the small fluorine–oxygen *gauche* preference,[25] facilitates H-bonding from the protonated nitrogen to the carboxylate of Asp493, and adds direct H/X-bonding interactions to Lys416.[26] Also, in the benzophenone and *p*-hydroxybenzamide binding subsites, the key ligand–protein contacts in **1c**, in particular the benzophenone contact made with the protein by moving the flexible glycine-rich loop, are preserved. For **1c** in PKCε, the chemical and conformational change, instigated by stereospecific fluorination, is synergistic such that the gain from new interactions in the azepane subsite is not accompanied by loss from ligand conformation perturbation or interactions from other subsites. Thus, C5(*S*)-fluorination effectively uncovers a highly selective ligand–protein pairing between **1c** and PKCε with stronger affinity that is unavailable for the other isozymes.

As for **1a**, the C6(*S*)–fluorine is too far away to interact with Lys416, even though the azepane ring conformation in **1a** is similar to that of **1c** (Fig. 1b). Also, the C6-fluorination of **1a**, in the bound conformation, does not satisfy the strong F/N$^+$ *gauche* preference in the azepane ring.[5] Our earlier conformational analysis of **1a** as a free ligand identified three major azepane ring conformations in equal distribution.[16] One of these three conformations is similar to the bound azepane ring conformation of **1a** here, except that the puckering of the protonated nitrogen is inverted in the free ligand that does satisfy the F/N$^+$ *gauche* preference. In the presence of the protein, the nitrogen puckering of the ligand is flipped in order to H-bond to Asp493 and Asp536, however at the cost of violating the F/N$^+$ *gauche* preference (Fig. 1b, Newman projection). This ligand conformational perturbation in **1a** is absent in **1c**. For **1a**, a mild interaction gain in the azepane-binding region, combined with ligand conformational perturbation and some contact loss in the benzophenone subsite, renders an overall affinity loss of 26 fold for **1a**. Thus, the differential affinity profile of **1a** and **1c** for PKCε is due to the congruent chemical and conformational control conferred specifically by C5-(*S*), but not C6-(*S*), monofluorination.

The large difference between the affinities of **1c** and **1d** to PKCε (275 fold) also prompted docking analysis of **1d** in the ATP site of PKCε.[21] Comparison of the interactions of **1c** and **1d** in the ATP site of PKCε (Fig. 2a) shows that C6-bisfluorination of **1d** causes conformational changes in the azepane ring and is accompanied by rotation of the *p*-hydroxybenzamide amide bond (Fig. 2a, red highlight) that is binding in the opposite orientation compared to that of **1c** (Fig. 2a, pink highlight). As a consequence, **1d** suffers the loss of a backbone H-bonding contact deep in the adenine subsite to Val489. For **1d** in PKCε, the loss of binding interactions, particularly

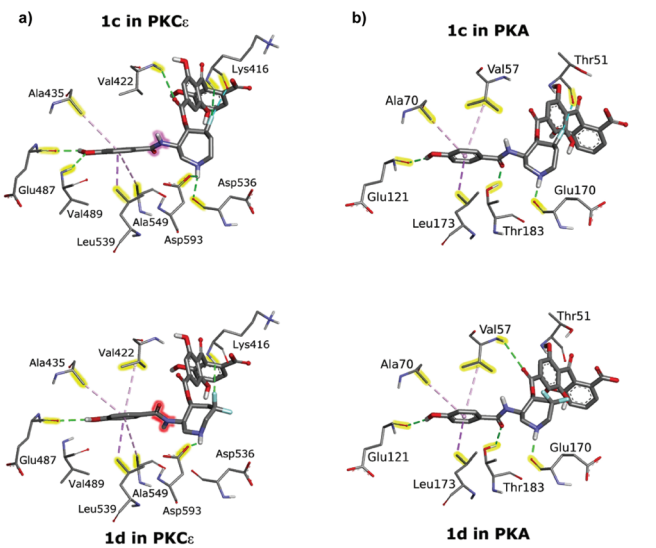


Fig. 2 (a) **1c** and **1d** in PKCε: interaction differences highlighted in yellow in the *p*-hydroxybenzamide and azepane-binding subsites. The amide group of the ligand is highlighted in pink for **1c** and red for **1d**; (b) **1c** and **1d** in PKA showing similar azepane binding conformations. The full comparison list of interactions for **1c** and **1d** in PKA/PKCε is in the ESI.†

H-bonds, is found in all three binding subsites for the *p*-hydroxybenzamide, azepane and benzophenone moieties, showing that PKCε in this case is unable to adapt to the "remote control" effect of azepane fluorine perturbation.

Contrary to the case of PKCε, PKA as a receptor is insensitive to the fluorination perturbation in either **1c** or **1d** (Fig. 2b). Comparison of **1c** and **1d** in the ATP site of PKA shows highly preserved interactions in the *p*-hydroxybenzamide and benzophenone binding regions, along with comparable binding conformations in the azepane ring.[21] This is consistent with the observed binding affinities of these two compounds that are within 1.5 fold for PKA. For **1c**, more H-bonding interactions are made to the azepane-binding subsite of PKCε than to that of PKA.[21] Effectively, the fluorine perturbation from the azepane ring in **1c** and **1d** is discernable by PKCε but not PKA, due to the ability of the PKCε pocket to specifically "solicit" a productive binding conformation from **1c** but not **1d**. Stereocontrolled fluorination here is shown as the origin of integrated chemical and conformational control that engenders recognition cooperativity between the ligand and protein for specificity enhancement (*i.e.* 275 fold diffidence in K_d for **1c** and **1d** in PKCε *vs.* 1.43 fold diffidence in K_d for **1c** and **1d** in PKA) in a protein-dependent manner.

While the perturbation investigation here utilized a small number of fluorine atoms, the divergent response from these highly homologous active sites toward stereospecific fluorination on the ligand is clearly evident. This is the first demonstration of shape-controlling fluorination that extracts additional selectivity potential in highly homologous kinase ATP sites, which complements the existing avenues of inhibitor discovery by high-throughput-screening, structure-guided protein–ligand engineering or fragment-based lead design.[27–30] Given the large number of members in this family and also other ATP-binding proteins, this new strategy will open additional avenues for addressing the selectivity issues not just for kinases but other ligand–protein interactions in general.[23,31,32] Furthermore, complex natural product ligands that are amenable to fragment-based development,[33] such as balanol, can incorporate this fluorine-moderated fragment approach by incrementally challenging the adaptability of a protein target to further enhance selectivity.

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