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Kaushik Panigrahi
University of Nebraska - Lincoln

David L. Nelson
University of Nebraska - Lincoln

David B. Berkowitz
University of Nebraska - Lincoln, dberkowitz1@unl.edu

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Unleashing a "True" pSer-Mimic in the Cell

Kaushik Panigrahi[1], **David L. Nelson**[1], and **David B. Berkowitz**[1,*]

[1]Department of Chemistry, University of Nebraska, Lincoln, NE 68588-0304, USA

Abstract

In this issue of *Chemistry & Biology*, Arrendale and coworkers demonstrate a new prodrug strategy for intracellular delivery of an α, α-(difluoromethylene)phosphonate phosphoserine mimic. The deprotected pseudo-phosphopeptide releases the pro-apoptotic FOXO3a-transcription factor from its 14-3-3-adaptor protein complex, resulting in leukemic cell death.

Signal transduction in nature often involves protein phosphorylation. The combination of kinetic stability and thermodynamic lability of the phosphate ester renders it an excellent switch. Much of the kinetic stability to nucleophilic attack presumably derives from the negatively charged phosphoryl oxygens in its predominantly dianionic form (pK_{a2} ~6.5). Because of the ubiquity of phosphorylation as a signaling mechanism, chemical biologists have long sought to synthesize compounds that structurally and functionally mimic phosphate ester functionality but are resistant to phosphatase cleavage. Replacing the bridging oxygen with carbon confers such resistance, but simple CH_2-bridged phosphonates have an elevated second pK_a (7–7.5), exhibiting considerable monoanionic character. However, upon α-fluorination, the second pKa successively decreases in going from a CHF- (pK_a ~6–6.5) to a CF_2-bridge (pK_a ~5–5.5). At the same time, C-C(H)(F)-P bond angle increases [e.g., from 112° (CH_2) to 113° (CHF) to 117° (CF_2) in a series studied by Nieschalk et al. (1996)], reaching an angle very close to the native C-O-P angle (119°) for the difluorinated phosphonate.

Such considerations led (Caplan et al. (2000) and others to regard α, α-(difluoromethylene)phosphonates as "isopolar" and "isosteric" phosphate analogs. While the CF_2-bridge clearly projects a larger van der Waals radius than the native bridging O, the fact that it expands the C-CF_2-P bond angle lends some merit to the label "isosteric." The "isopolar" nature of this functionality, on the other hand, is supported by DFT calculations on simple model compounds (Figure 1). The partial charge on the bridging phosphate ester oxygen (−0.453) is mimicked by the bridging CF_2-phosphonate carbon (−0.395), especially when one considers the additional charge density on the pendant fluorines (−0.172 each).

Accordingly, in the 1990s, our group developed CF_2-phosphonate analogs of pSer and pThr (Berkowitz et al., 1996, 1999) as tools for the study of protein phosphorylation (abbreviated pCF_2-Ser; Panigrahi et al., 2009). Recent years have seen the very creative application of these tools. One particularly compelling use is to modulate protein-protein interactions

*Correspondence: dberkowitz1@unl.edu.

(PPIs) that depend upon Ser/Thr phosphorylation. Paramount among these are the interactions of 14-3-3 adaptor proteins, ubiquitous “chaperones” that bind to phosphorylated partner proteins to modulate signal transduction, the cell cycle, intracellular trafficking and transcription.

Obsil et al. (2001) solved a prototypical 14-3-3-adaptor/phosphorylated-partner protein complex structure. The structure reveals in beautiful molecular detail how the phosphothreonine residue of the AANAT is tightly bound at a groove in the 14-3-3 dimer interface that is rich in H-bond donating residues, Y128, R86, and R127, resulting in a “molecular Velcro”-like interaction. The two arginine residues also appear to electrostatically pair with the pThr dianion, making this an ideal site to target with the CF_2-phosphonate functionality.

With the appearance of the paper in this issue of *Chemistry & Biology* by Arrendale et al. (2012), now two complementary approaches exist that, taken together, attest to the abililty of pCF2-Ser-bearing ligands to modulate 14-3-3-mediated PPIs. In earlier work, Zheng et al. (2005) modified the native protein itself, AANAT, using expressed protein ligation (EPL) technique to graft the phosphatase-inert pCF_2-Ser residue onto position 205 of the wild-type protein. This effectively leads to a “constitutively phosphorylated” AANAT, remaining closely associated with its 14-3-3-zeta binding partner, and thereby stabilizing the protein to proteasomal degradation. In fact, using microinjection into CHO cells, Zheng et al. (2005) saw the persistence of this tight pCF_2-Ser/14-3-3 interaction under intracellular conditions.

Very recently, Tarrant et al. (2012) have shown that this same strategy can be exploited to stabilize the important signal-transducing casein kinase II (CK2) kinase involved in cell proliferation. In this case, the PPI being targeted involves the CK2 interaction with the regulatory protein, Pin1. The CK2-Pin1 association is promoted by phosphorylation of Thr-344. Again utilizing EPL to incorporate pCF_2-Ser at this site, Tarrant et al. (2012) were able to show that the semisynthetic CK2 binds tightly to its Pin1 regulatory protein partner, and that this interaction both stabilizes the protein to proteasomal degradation and alters its polypeptide substrate specificity.

Herein, Arrendale et al. (2012) first reports a new, efficient stereocontrolled synthesis of the pCF_2-Ser building block, utilizing a nice stereo-retentive aziridine-opening. Arrendale et al. (2012) then truly open up a new vista in the application of this chemical biological tool by installing one of their trademark prodrug functionalities onto the fluorinated phosphonate and using this to deliver a carefully crafted pCF_2-Ser-containing dipeptide ligand for the 14-3-3 adaptor protein into the cell.

The elegant presumptive phosphonate release mechanism is depicted in Figure 2. The 5′-nitrofuryl-2′-methyl N(4″-chlorobutyl)phosphonamido ester prodrugging strategy masks the fluorinated phosphonate as a neutral non-polar derivative, and subsequently promotes its release under reducing conditions within the cell. Reductive cleavage of the phosphonate ester, and subsequent ionization places more electron density on the amide nitrogen, permitting a Baldwin's rules-favored 5-exo-tet cyclization to a pyrrolidinium ring, also an excellent leaving group. Hydrolysis and ionization then give the free phosphonate dianion.

In the case at hand, the released modified dipeptide ligand successfully competes, in vivo, with the phosphorylated FOXO3a transcription factor for its 14-3-3 adaptor protein, thereby effectively titrating off the adaptor at micromolar ligand concentrations. Applied to leukemia cells, the authors show that this 14-3-3-sequestering strategy can be used to induce the pro-apoptotic effects of the free FOXO3a protein, with attendant anti-leukemic activity.

These studies expand the domain in which such “teflon phosphates” may be deployed to ask fundamental questions in chemical biology, allowing for their transport into the cell. Given recent exciting developments in photocaging/release of native pSer functionality from the Imperiali lab (Goguen et al., 2011), it will be interesting to see if this strategy can be expanded to include wavelength specific photolabile prodrug functionalities for pCF_2Ser. One can imagine thusly asking more finely tuned questions relating to the temporal regulation of protein function via phosphorylation. Clearly, given the abundance of 14-3-3 adaptor protein-mediated regulatory interactions, both this work and the complementary protein-grafting work in the Cole group open up important horizons for the interrogation of phosphorylation-dependent PPIs with this chemical biological tool.

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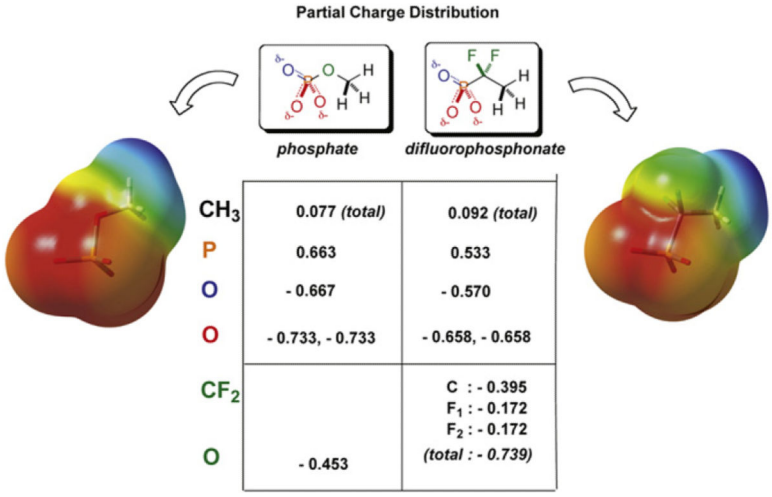

Figure 1. Fluorinated Phosphonates versus Phosphates: A Computational Comparison

Electrostatic potential (ESP) maps were generated from DFT calculations with Gaussian 03W using the B3LYP function at the 6-311+g(d,p) level. Calculations were performed on full ground state optimizations, plus frequencies. ESP maps were generated in Gaussview 3.0. Isosurfaces were generated with a total charge density of 0.0004 e/bohr. Note that the charge distribution shown is for the minimum energy conformer located. At room temperature, rapid single bond rotation should lead to equal charge distribution across the three anionic phosph(on)ate oxygens.

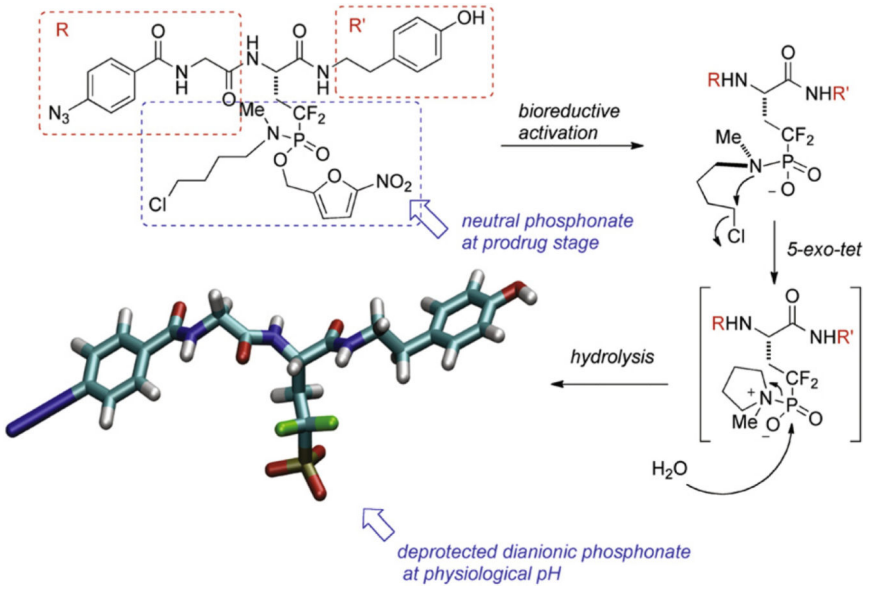

Figure 2. Prodrug Release Mechanism

Depicted is the putative mechanism of intracellular prodrug activation, under reductive conditions, for the pCF_2-Ser-containing dipeptide ligand for the 14-3-3 adapter protein.