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# Survey of phosphorylation near drug binding sites in the Protein Data Bank (PDB) and their effects

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## Abstract

While it is currently estimated that 40–50% of eukaryotic proteins are phosphorylated, little is known about the frequency and local effects of phosphorylation near pharmaceutical inhibitor binding sites. In this study, we investigated how frequently phosphorylation may affect the binding of drug inhibitors to target proteins. We examined the 453 non-redundant structures of soluble mammalian drug target proteins bound to inhibitors currently available in the Protein Data Bank (PDB). We cross-referenced these structures with phosphorylation data available from the PhosphoSitePlus database. 322/453 (71%) of drug targets have evidence of phosphorylation that has been validated by multiple methods or labs. For 132/453 (29%) of those, the phosphorylation site is within 12Å of the small molecule-binding site, where it would likely alter small molecule binding affinity. We propose a framework for distinguishing between drug-phosphorylation site interactions that are likely to alter the efficacy of drugs vs. those that are not. In addition we highlight examples of well-established drug targets, such as estrogen receptor alpha, for which phosphorylation may affect drug affinity and clinical efficacy. Our data suggest that phosphorylation may affect drug binding and efficacy for a significant fraction of drug target proteins.

### Keywords

drug target; data mining; crystal structure; PhosphoSitePlus; binding affinity; ligand; inhibitor

## INTRODUCTION

Current estimates indicate that approximately 40–50% of human proteins are phosphorylated. 20,266 non-redundant human proteins have been reported and reviewed in the UniProt database (http://www.uniprot.org)[1]. PHOSIDA reports 8283 (www.phosida.com)[2] non-redundant phosphorylated human proteins, Phospho.ELM reports 8698 (phospho.elm.eu.org)[3], and a search of phosphorylation sites identified in either multiple high throughput reports or in low throughput reports in PhosphoSitePlus returns

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10,062 human proteins (www.phosphosite.org)[4]. Two recent advances have greatly accelerated the pace of progress in our understanding of protein phosphorylation. First, whole-cell mass spectrometry efforts have identified large numbers of phosphorylated proteins [5]. Secondly, phosphorylation sites in mammalian proteins that have been reported in the literature are maintained in a curated database, PhosphoSitePlus [4]. The number of reported protein phosphorylation sites has increased from ~2,000 in 2003, when the database was created, to 20,000 in 2007 and over 200,000 currently [4,6].

Protein kinases, which regulate other proteins through phosphorylation, are one of the most important classes of drug targets. Kinases are commonly turned on through phosphorylation of the activation loop near the active site [7]. Small molecule inhibition of an unphosphorylated kinase can cause feedback phosphorylation of the available kinase pool [8]. In some cases where phosphorylation is known to reduce drug affinity, strategies have been developed to either lock the drug target in an inactive conformation or retain drug affinity in the phosphorylated state [9,10]. Recent studies in MEK inhibiton even suggests different upstream activators and MEK phosphorylation states have dramatic effects on clinical drug candidate efficacy [11,12]. Given that a large fraction of proteins in general, and kinases in particular are phosphorylated, it is reasonable to suggest that changes in protein phosphorylation could affect small molecule drug binding and efficacy for a significant fraction of drug target proteins. Yet, to our knowledge, no group has investigated the frequency of with which phosphorylation may affect drug binding, nor its occurrence outside of kinases. Both target and computational-based screening methods do not typically consider phosphorylation given the limited number of available phosphorylated protein structures. As a result, these screening methods may discount significant local structural effects of phosphorylation on the drug target protein [13]. Here we investigate the effects and frequency of phosphorylation near the site of drug binding for 453 drug targets in the PDB.

## METHODS

We first generated a comprehensive list of experimental drug targets in both preclinical and clinical studies, by combining those in the DrugBank database with all available PDB structures containing keywords “drug”, “inhibitor”, “agonist”, “antagonist” in the PDB. From this dataset, we removed (1) redundant targets at ≥90% sequence identity, (2) all structures that did not contain a 100–1000 Da ligand, a lenient range for drug-like molecules, (3) all non-mammalian targets, whose species is not covered in PhosphoSitePlus, (4) transmembrane protein structures lacking a cytoplasmic domain, whose extracellular domains may bind to inhibitors but are rarely phosphorylated and (5) off-target ligand-structure complexes and other proteins that do not have any known therapeutic potential based on a literature search [Fig. 1(a)]. 15,442 (79%) of the available drug target structures were redundant with other available structures, due to targets for which multiple ligand structures are available in the PDB. A ligand-bound structure was available for 2275 (55%) of unique targets. By investigating exclusively mammalian targets, we eliminated 1749 drug-bound structures, notably including a number of antibacterial agents. These drug-target interactions certainly may be affected by phosphorylation, but far fewer bacterial phosphorylation sites have been reported as the bacterial Phosphorylation Site Database contains only 2250 entries [14].

We next cross-referenced the PDB entries for the 453 non-redundant mammalian ligand-bound drug target structures with reported phosphorylation sites in PhosphoSitePlus [4]. We investigated serine, threonine, and tyrosine phosphorylation exclusively as histidine phosphorylation is rare and other post-translational modifications are rarely validated beyond mass spectrometry (MS) studies [15,16]. Data are discussed in terms of the number of phosphorylated "hits" out of 453.

In the absence of additional data, identification of a phosphorylated site by mass spectrometry alone is generally considered to be insufficient to demonstrate phosphorylation. We consider a phosphorylated site to be "valid" if multiple groups have published evidence of endogenous phosphorylation of the protein target in cells, either by mass spectrometry, biochemical and cell biological studies, or combination of those. By this criterion, 322/453 (71%) of drug targets with structures have valid evidence of phosphorylation, while 131/453 (29%) have no known validated phosphorylation sites [Figure 1(b)].

We define "hits" as ligand-bound structures with validated phosphorylated residues within 12Å of any atom in the small molecule (Table I, Supporting Information Table S1). A 12Å distance cutoff is based on past studies of several phospho-proteins, in which phosphorylation within 12Å of the site of small molecule binding could induce local conformational rearrangements that would affect ligand affinity. For example, Hsp90α is phosphorylated at Thr90, where the hydroxyl is 11.8Å away from a bound inhibitor [17]. This phosphorylation causes a decrease in affinity for ATP [18]. In cyclin-dependent kinase 2 (Cdk2), phosphorylation 9.5Å away from ATP reduces substrate peptide affinity 2.5 fold [19]. Peptide phosphorylation can similarly induce conformational changes on the order of 10Å [20]. A much more stringent cutoff is phosphorylation within 3Å of the drug binding site, which would directly affect drug binding through steric and electrostatic interactions, as shown in the case of E. coli isocitrate dehydrogenase and its natural substrate [21]. While phosphorylations greater than 12Å away from a drug binding site may induce more global conformational changes within proteins that significantly affect affinity, we cannot easily predict these effects without phosphorylated or phospho-mimetic structures, which are rarely available. As a result, our estimate of effects is likely conservative considering the changes to activity long-range phosphorylation produce [22]. The resolution of the crystal structure data for the 453 ligand-bound drug targets in our study ranges from ~1.5Å to ~3Å. Crystal structure resolution does not directly correlate with proper ligand geometry and fit to the electron density [23], therefore, differences in structural resolution were ignored in determining distances between the ligand and phosphorylation site.

## RESULTS AND DISCUSSION

### Nearly one-third of known drug targets are phosphorylated near the drug binding site

Our results show that of the 453 small-molecule bound drug targets in the PDB, 322 (71%) have at least one validated phosphorylation site. It is interesting that the frequency of phosphorylation for drug target proteins appears to significantly exceed the estimated frequency of phosphorylation for all human proteins. This may be because highly phosphorylated proteins in signaling cascades and also make good drug targets, as with

kinases. It could also be the case that proteins with measurable signaling activity, such as kinases, are more likely to be selected as drug targets. Those drug target proteins are likely to be more well-studied and validated when compared to other phospho-proteins in general.

132 of the 453 small-molecule bound drug targets in the PDB (29%) contained a validated phosphorylation site within 12Å of the drug binding site. These are the "hits" of our study, which are discussed in detail below (Table I, Supporting Information Table S1). In these tables the highest resolution drug target structures available in the PDB were used, and reported distance is the shortest from the small molecule to the phosphorylated residue. The ligand in the crystal structure is rarely the drug candidate in used clinical trials. Target "hits" are listed in Table I and Supporting Information Table S1 in order of class, validation, and stage of drug development as described below. Table I contains all proteins for which phosphorylation has known effects on target function, and Supporting Information Table S1 contains all other "hits".

The distribution of the distances between the bound ligand and the phosphorylated residue are shown in Figure 2(a). 9/132 hits contained a site within 3Å of the ligand binding site. There are large bottlenecks between MS verification of phosphorylation by multiple groups and more detailed data such as biochemical and cell based studies, as well as data establishing physiological effects of phosphorylation for the 132 hits [Fig. 2(b)]. Phosphorylation sites reported for 70/132 hits have been accompanied by functional biochemical or cellular studies, with 57/132 having demonstrated both biochemical and cellular functional relevance. To date, only 3 of the 132 hits have established clinical correlation of drug treatment outcome with target protein phosphorylation [24–26]. All of these are described in detail below.

Not surprisingly, protein kinases comprised the largest fraction of our hits. Of the 453 drug target structures, 130 are kinases. 95% (123/130) of those kinase targets are phosphorylated, with 77/130 (59%) of those phosphorylation sites occurring within 12Å of the inhibitor. The fact that phosphorylation occurs in the vast majority of kinase targets is expected given activation loop phosphorylation and involvement in signaling cascades. In addition to protein kinases, nuclear receptors were an interesting group, comprising 25 of the 453 drug target structures. 9 of those 25 had phosphorylation within 12Å of drug binding sites (36%), as phosphorylation has been established as a common mechanism of modulating the activity of these proteins [27], (Supporting Information Fig. S1).

## Classification of hits

For 70/453 of the hits, it is known whether phosphorylation activates the target, has little effect, or whether it is inhibitory [Fig. 2(b)]. These 70 hits fell into one of two classes as shown in Figure 3. Class 1 hits (27/70, 39%) have an inhibitory phosphorylation site that is close to the site of drug binding. In this situation, if phosphorylation reduces drug efficacy, it will also inactivate the target by modifying the same site. Regardless of whether the drug binds or the target is phosphorylated such that the drug cannot bind, the target is inactivated. Mineralocorticoid receptor is an example of a Class 1 hit, in which phosphorylation at Ser843 reduces the affinity for the natural agonist and inactivates the receptor [28] [(Fig. 4(a)]. Because phosphorylation occurs at the binding site for both the agonist and inhibitor of

mineralcorticoid receptor, we would predict that phosphorylation of Ser843 results in reduced drug affinity. This would not translate to a loss in efficacy, in fact one could expect the opposite effect. If a larger fraction of targets are phosphorylated, then a larger fraction of targets will be inactivated for a particular dose of a drug compound.

Another example of a Class 1 hit is the androgen receptor (AR, Supporting Information Table S1). Approved antiandrogens like bicalutamide act as antagonists to the androgen receptor in hormonal dysfunction diseases such as prostate cancer. AR Ser790 is phosphorylated *in vitro* and *in vivo* by Akt [29]. Phosphomimetic substitution of Ser790 to aspartate nearly abolishes the ability of AR to bind androgen and localize to the nucleus [30]. While some research suggests Akt inhibition/activation of AR is not critical for progression of prostate cancer [31,32], clinical studies indicate that high phosphorylation of Ser790 is associated to a longer time to death from recurrence in castration-resistant prostate cancer [26].

For Class 2 hits (43/70, 61%), phosphorylation does not significantly inhibit target function or in some cases it may actually increase activity. As phosphorylation can reduce drug affinity without inhibiting activity, Class 2 proteins may avoid inhibition by small molecule drugs when phosphorylated. Nearly all kinase hits fall into this category (33/41), including insulin-like growth factor 1 receptor [IGF-1R, Fig. 4(b)]. One study on IGF-1R showed that despite high membrane permeability, lack of efflux transporters, and nanomolar affinity to the unphosphorylated state, inhibitors failed to show efficacy in cell-based assays [33]. The inhibitors used in this study were “DFG-out” inhibitors that bind only to the inactive conformation when the DFG loop is out, away from the active site. This causes the activation loop to occlude the nucleotide pocket and prevent ATP binding [Fig. 4(b), dark gray/red structure]. While “DFG-out” inhibitors can keep the protein in an inactive conformation, they do not bind well to the active, phosphorylated enzyme. The phosphorylated crystal structure of IGF-1R shows large rearrangements of the activation loop. The DFG sequence is “in”, while the activation loop itself is out and away from the nucleotide pocket, enabling ATP binding [Fig. 4(b), cyan/green structure, [34]]. Therefore it is plausible that *in vivo* phosphorylation causes a reduction in drug affinity. In the case of the BCR-Abl “DFG-out” inhibitor imatinib, there is a 200-fold reduction in affinity when the kinase is phosphorylated [9].

Among the examples of Class 2 proteins listed in Table I is Estrogen Receptor Alpha (ERα), a well-established drug target in the treatment of hormone-responsive breast cancer. The alpha carbon of tyrosine 537 is 11.3 Å away from the binding site for 4-hydroxytamoxifen. Tyr537 makes several key interactions that stabilize the inactive conformation [35], and is phosphorylated both *in vitro* and *in vivo* by Src-family kinases [36]. Phosphorylation of Tyr537 activates the ERα receptor, possibly by facilitating dimerization and interactions with other binding partner proteins[37,38], although the precise mechanism is disputed [39,40]. Phosphomimetic mutation of Tyr537 to glutamate reduces the affinity of estradiol to ERα by 10-fold [41] and results in ligand-independent activation of the receptor [42]. Recent clinical evidence associated high levels of Tyr537 phosphorylation, as studied in breast cancer, with poor overall survival of patients treated with tamoxifen [25]. Furthermore, it is intriguing that in the vast majority of tamoxifen-resistant, ERα-positive cancers, ERα does not contain any

mutations [43,44]. These data together suggest that aberrant phosphoregulation of ERα, rather than mutation of the receptor, can cause poor drug response.

The emerging cancer drug target Proliferating Cell Nuclear Antigen (PCNA) also falls under Class 2. When Tyr211 is phosphorylated, PCNA is activated and localized to the nucleus where it is involved in DNA replication/repair and cell cycle progression [45]. Because of its function in these proliferative processes, it is being investigated as a drug target and biomarker in cancer [46,47]. The hydroxyl group of Tyr211 is 11.4Å away from the inhibitor and makes several interactions with a flexible loop [48]. This loop moves to accommodate the inhibitor and phosphorylation would likely alter PCNA inhibitor binding. Clinically, phosphorylation at Tyr211 is correlated with poor survival in breast cancer patients [24], and blocking Tyr211 phosphorylation using PCNA peptide mimetics reduces tumor growth *in vivo* [49]. These data together suggest that for Class 2 hits, including kinase targets of "DFG-out" inhibitors, ERα, and PNCA, phosphorylation of the target protein may block the action and efficacy of drug inhibitors.

## CONCLUSIONS

In this comprehensive bioinformatics study, we found that 132/453, or 29% of proteins analyzed in this study are known to have phosphorylation occurring within 12 Å of a drug binding site. For 70 of the 132 phosphorylated targets, it is known whether phosphorylation activates or inhibits the target. 39% (27/70) of these were classified as Class 1 hits, for which the drug and phosphorylation have similar effects on activity, while 61% (43/70) were Class 2 hits, for which the drug and phosphorylation have opposing effects on activity. These results suggest that phosphorylation can alter drug efficacy for a rather large fraction of target proteins. Kinases and nuclear receptors represented a large fraction of the hits in our study, and some of the reason for their high representation is likely selection bias in the available data. For example numerous studies of kinase activation by phosphorylation in or near the active site have led to inhibitor design, as in the case of the "DFG-out" inhibitors described above.

Clinical studies examining the effects of specific target phosphorylation sites on specific therapies were only performed for 3 drug target proteins in our study. For the Class 1 hit AR, phosphorylation near the drug binding site was correlated with good outcome/drug sensitivity. In contrast, for the Class 2 hits ERα and PCNA, phosphorylation near the drug binding site was correlated with poor sensitivity to the drug. Our speculation is that in the case of Class 1 hits, a large fraction of the target proteins may be inactivated via phosphorylation, such that a lower concentration of drug may be required to cause sufficient inhibition of the remaining active targets. In Class 2 hits, phosphorylation of the target protein can directly cause drug resistance in the absence of any target protein mutations.

Our model in Figure 3 depicts phosphorylation either directly or allosterically inhibiting drug binding. Phosphorylation should inhibit drug binding if it causes a direct structural clash with the drug in its binding site or causes unfavorable structural rearrangements. For the three proteins with known clinical effects of phosphorylation described above, phosphorylation does appear to reduce drug efficacy, consistent with inhibition of drug

binding. However, for the majority of our hits (127/132), it is not known whether phosphorylation inhibits or enhances drug binding, and examples of phosphorylation enhancing drug binding do exist. In phosphodiesterase-5, phosphorylation of Ser102 increases the enzymatic activity 1.6-fold [50]. Through allosteric rearrangements, phosphorylation increases the affinity of cGMP to PDE5, causing activation[50,51]. The inhibitor tadalafil is able to overcome this by showing a 3-fold increase in binding affinity when Ser102 is phosphorylated [52]. We did not identify PDE5 in our screen because the domain containing Ser102 has not been crystallized in an inhibitor-bound structure. In another example, phosphorylation of phenylalanine dehydroxylase (PAH) at Ser16 increased substrate-dependent enzymatic activation [53]. The site is in a disordered region of the protein and there is no electron density present in crystal structure. Small-molecule activators of PAH are being pursued for treatment of phenylketonuria (PKU). While both phosphorylation[54] and drug candidates[55] stabilize protein folding and maintain the activity of PAH, the combined effects have yet to be investigated and are difficult to determine through crystallography.

Our study is likely to have under-reported the phenomenon of phosphorylation affecting drug inhibition of targets, for several reasons. First, because drug-bound structures are often kept confidential during drug development while phosphorylation sites are continually being identified and verified, we are under-reporting the actual occurrence of phosphorylated, drug-bound proteins. Second, other post-translational modifications like acetylation are reported to have critical roles in both upregulating[56] and downregulating[57] activity, but are rarely investigated to the extent that phosphorylation is. Third, we were unable to assess targets with phosphorylation in unstructured domains. Extremely flexible protein regions are commonly phosphorylated but are rarely seen in crystal structures. Fourth, given the difficulties of predicting long-distance allosteric effects of phosphorylation on protein activity or drug binding, we did not assess the 131/453 drug target proteins with phosphorylation sites that were not within 12 Å of the drug binding site in the crystal structures. Some of these long-range phosphorylation sites even have known physiological effects. For example, phosphorylation of PPAR-γ at Ser112 reduces drug agonist binding by 10-fold despite being nearly 40 Å away [58]. In addition to the site at Tyr537 described above, ERα also contains a long distance phosphorylation site that affects antagonist binding. When ERα is phosphorylated at Ser305, tamoxifen still binds, but fails to induce an inactive conformation. Based on intramolecular FRET experiments, it appears phosphorylation of Ser305 causes tamoxifen to exhibit agonist behavior towards ERα, leading to tamoxifen resistance [59]. This critical conformational rearrangement is not reflected in published crystal structures, most of which contain only the ligand-binding domain [60]. Lastly, it should also be noted that phosphorylation sites are enriched for mutations in cancer cells [61]. The effects of these mutations on target protein phospho-regulation are highly clinically relevant, but they are not reported in PhosphoSitePlus, and hence are not included in our study.

In target-based drug discovery, target proteins are often initially produced and assayed *in vitro* or in cultured cells where the set of post-translational modifications occurring on the target protein could be vastly different than *in vivo*. Similarly, structure-based *in silico* efforts can only seldom investigate the structural changes induced by phosphorylation

because phosphorylated structures are rarely available (1,475/97,180 structures in the PDB, 2%). Except in the case of kinase auto-phosphorylation, structural information about these phospho-proteins is extremely limited and can require partial chemical synthesis to achieve a high percentage of phosphorylated protein [62]. Given the findings of this study, we encourage researchers and clinicians alike to consider the possibility of protein post-translational modification as they approach rational drug design and evaluate potential reasons for drug treatment outcomes that are either much better or worse than expected.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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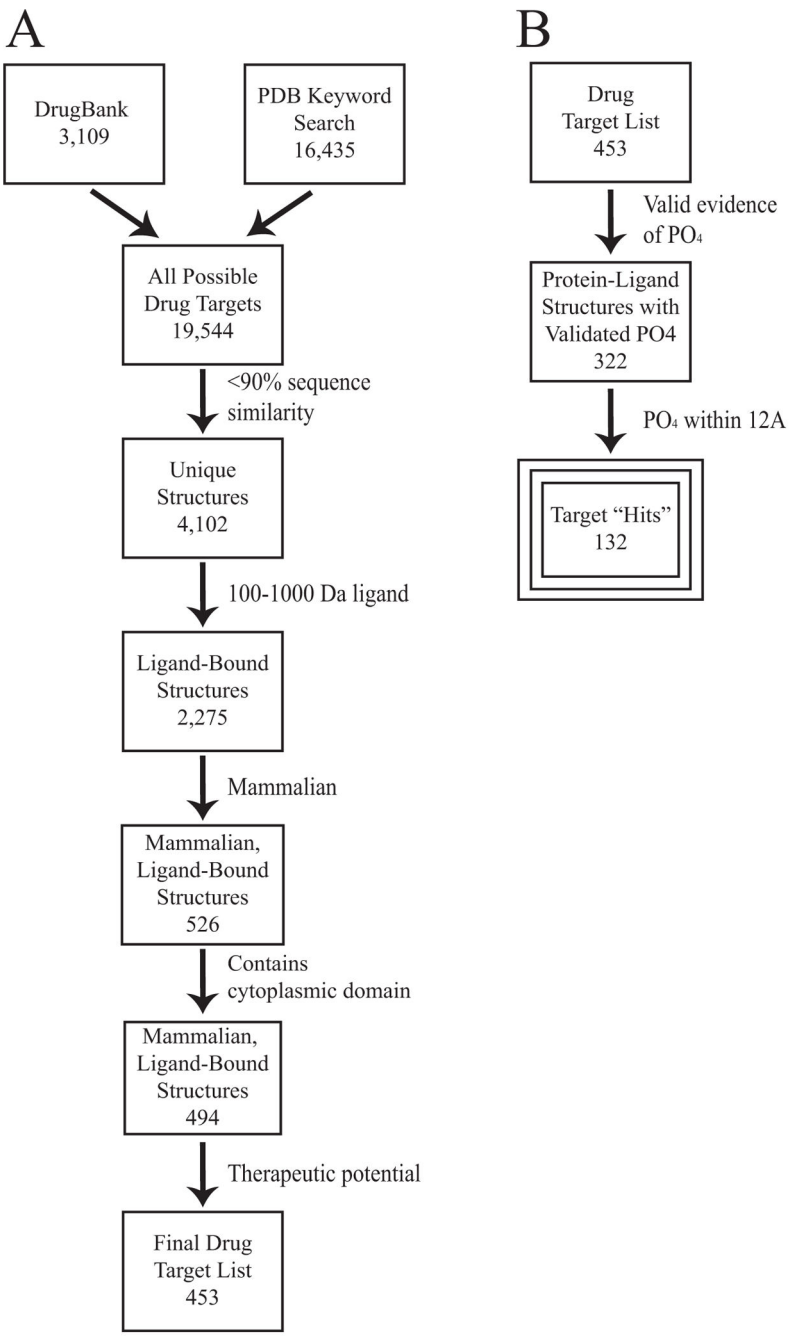


**Figure 1.**

Flow diagrams. Each criterion is listed along with the number of targets meeting it. **A:** Dataset preparation. **B:** Identification of the 132 validated hits, as defined in text.

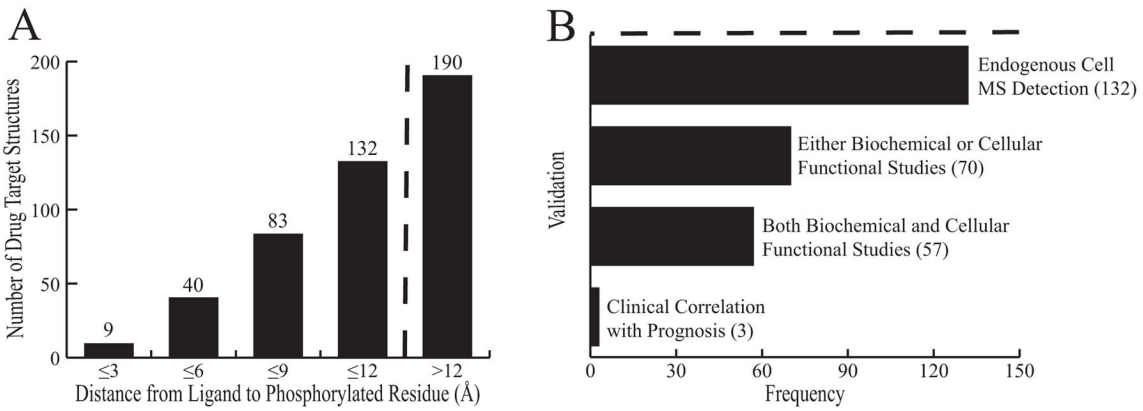


**Figure 2.**

**A:** Distribution of distance from phosphorylated residue to the target. **B:** Validation of the 132 phosphorylation sites within 12Å of drug binding sites. The dashed line indicates the cutoff for investigation in our study. Each criterion is listed along with the number of targets meeting it. Each level is inclusive of the one below.

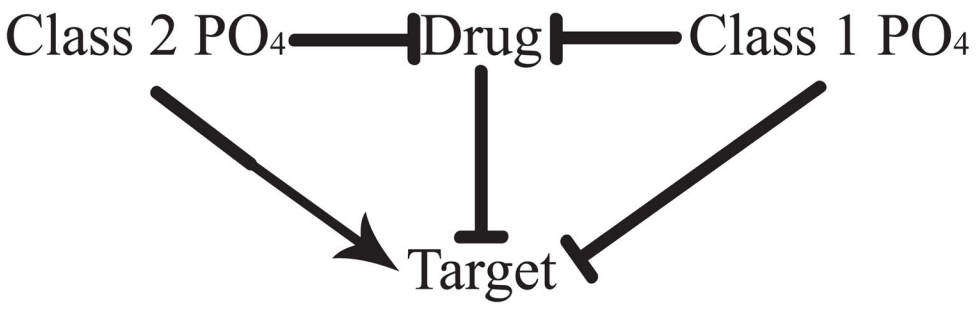


**Figure 3.**

Mechanisms by which phosphorylation may affect drug efficacy. For Class 1 hits, phosphorylation inhibits (⊤) both drug binding and target activity, whereas for Class 2 hits, phosphorylation inhibits drug binding while activating (→) the target. While this schematic depicts phosphorylation inhibiting drug binding, it may in some cases enhance binding as discussed in the text.

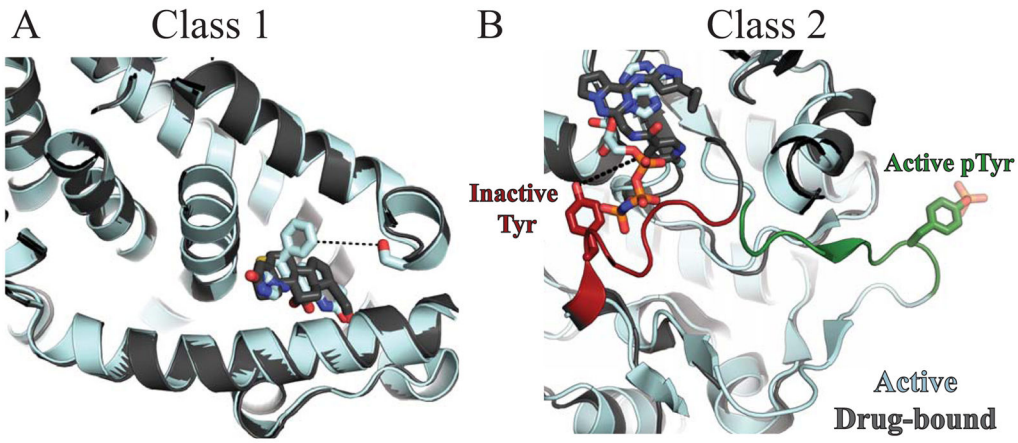


**Figure 4.**

Structural views example Class 1 and Class 2 hits. Active targets are shown in cyan, inactive targets in grey. Small molecule ligands are shown in sticks. **A:** Overlapped structures of active (2AA2) and inactive (3VHV) forms of Class 1 target mineralocorticoid receptor. When phosphorylated, Ser843 (red) inactivates the receptor by preventing agonist binding. As shown, the inhibitor binds at the same site as the agonist. Inhibitor affinity would likely be reduced by phosphorylation of Ser843 6.4Å away (dashed lines). **B:** Overlapped structures of active, phopshorylated insulin receptor (1IR3) and inactive insulin-like growth factor 1 receptor (3NW7), a Class 2 target. The two proteins share 91.3% sequence similarity within the crystallized constructs and 100% sequence identity within the activation loop. In the inactive conformation, the activation loop is red. Tyr1161 (sticks) is 7.1Å away from the “DFG-out” inhibitor (dashed lines). In the active, phosphorylated conformation, the activation loop is green. Phosphorylation simultaneously activates insulin-like growth factor 1 receptor and can reduce inhibitor affinity.

**TABLE I**

Target "hits" with cellular, biochemical, or clinical evidence of phosphorylation that can be classified by function. Entries are listed in order of class, validation, and stage of development. For validation, an entry of 1 indicates validation of phosphorylation by biochemical studies only, 2 indicates that validation by cellular functional studies, 3 indicates that both biochemical and cellular functional studies have been done to validate the phosphorylation sites, 4 indicates that there is a clinical correlation between phosphorylation of the drug target at a particular site and patient prognosis, and a (*) indicates there are less than 2 MS studies listed on PhosphoSitePlus. Evidence for kinesin-5 phosphorylation is based on our laboratory's unpublished data.

| Target Protein | Uniprot Sequence ID | Stage of Drug Development | PDB ID | PDB Ligand ID | Phosphorylated Residue(s) | Distance (Å) | Validation, References | Hit Class |
|---|---|---|---|---|---|---|---|---|
| Androgen Receptor (AR) | P10275 | Approved | 1Z95 | 198 | Ser791 | 10.0 | 3, 4* [26,29,30] | Class 1 |
| Liver X receptor beta (LXRβ) | P55055 | Preclinical | 1UPV | 444 | Ser426 | 9.5 | 3 [63] | Class 1 |
| SH2 domain containing protein tyrosine phosphatase-2 (SHP2) | Q06124 | Phase I | 3O5X | JZG | Tyr279 | 3.9 | 3 [64] | Class 1 |
| Angiopoietin-1 receptor (Tek/Tie2) | Q02763 | Phase II | 3L8P | 0CE | Tyr879 | 9.0 | 3 [65] | Class 1 |
| Cyclin-dependent kinase 2 (Cdk2) | P24941 | Phase II | 3LFQ | A28 | Thr14 | 5.8 | 3 [66] | Class 1 |
| Cyclin-Dependent Kinase 6 (Cdk6) | Q00534 | Phase III | 4AUA | 4AU | Tyr24 | 9.1 | 3 [67] | Class 1 |
| Retinoid X Receptor Alpha (RXRα) | P19793 | Approved | 1G5Y | REA | Tyr249 | 9.4 | 3 [68,69] | Class 1 |
| Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) | Q13526 | Preclinical | 3TC5 | 3T5 | Ser71 | 5.3 | 3* [70,71] | Class 1 |
| Ectonucleotide pyrophosphatase/ phosphodiesterase 2 (ENPP2) | Q9R1E6 | Preclinical | 3WAY | DWY | Thr210 | 2.4 | 3* [72,73] | Class 1 |
| Tyrosine-protein phosphatase non-receptor type 22 (PTPN22) | Q9Y2R2 | Preclinical | 4J51 | N75 | Ser35 | 10.6 | 3* [74] | Class 1 |
| Endothelial PAS domain-containing protein 1 (HIF2a/EPAS1) | Q99814 | Preclinical | 4GHI | 0X3 | Thr324 | 7.6 | 3* [75] | Class 1 |
| Glyoxylase 1 (GLO1) | Q04760 | Preclinical | 3W0T | HPU | Thr107 | 10.5 | 3* [76] | Class 1 |
| Farnesoid X receptor (FXR) | Q96RI1 | Phase II | 3P89 | 89P | Thr456 | 10.6 | 3* [77] | Class 1 |
| Phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) | Q8NEB9 | Phase II | 3LS8 | AJZ | Ser71 | 6.3 | 3* [78] | Class 1 |
| Neuronal nitricoxide synthase (nNOS) | P29476 | Phase II | 3N5W | XFJ | Tyr609 | 12.0 | 3* [79] | Class 1 |
| Heat Shock Protein 90-alpha (HSP90α) | P07900 | Phase III | 4BQJ | XKL | Ser52, Thr90 | 3.9, 12.7 | 3* [18,80] | Class 1 |
| Janus Kinase 1 (Jak1) | P23458 | Approved | 3EYG | MI1 | Tyr940 | 8.7 | 3* [81] | Class 1 |
| Janus Kinase 2 (Jak2) | O60674 | Approved | 3UGC | 046 | Tyr913 | 7.9 | 3* [81] | Class 1 |

| Target Protein | Uniprot Sequence ID | Stage of Drug Development | PDB ID | PDB Ligand ID | Phosphorylated Residue(s) | Distance (Å) | Validation, References | Hit Class |
|---|---|---|---|---|---|---|---|---|
| Mineralocorticoid Receptor (MCR) | P08235 | Approved | 3VHV | LD1 | Ser843 | 6.4 | 3* [28] | Class 1 |
| Cytochrome P450 19A1 (CYP19A1) | P11511 | Approved | 3S7S | EXM | Ser118 | 11.8 | 3* [82] | Class 1 |
| Retinoic acid receptor alpha (RARα) | P10276 | Approved | 4DQM | LUF | Ser219 | 9.9 | 3* [83] | Class 1 |
| Deoxyribonucleoside 5′-monophosphate N-glycosidase (Rcl) | O35820 | Preclinical | 4FYH | TR5 | Ser28 | 3.5 | 1 [84] | Class 1 |
| Kinesin-5 (Eg5) | P52732 | Phase II | 3KEN | ZZD | Tyr231 | 3.5, 9.3 | 1 [85] | Class 1 |
| Calcium/calmodulin-dependent protein kinase type 4 (CAMK4) | Q16566 | Preclinical | 2W4O | DKI | Tyr940 | 9.3 | 1* [86] | Class 1 |
| Protein Mdm4 (MDMX) | O15151 | Preclinical | 3LBJ | WW8 | Tyr99 | 4.4 | 2 [87] | Class 1 |
| Ribosomal protein S6 kinase alpha-1 (Rsk1) | Q15418 | Preclinical | 2Z7R | STU | Ser154 | 9.4 | 2* [88] | Class 1 |
| BCR-Abelson murine leukemia viral oncogene homolog 1 Thr→Ala (BCR-Abl) | P00519 | Approved | 3CS9 | NIL | Tyr253 | 3.7 | 2* [89] | Class 1 |
| Proliferating Cell Nuclear Antigen (PCNA) | P12004 | Preclinical | 3VKX | T3 | Tyr211 | 9.6 | 2, 4* [24,90] | Class 2 |
| Estrogen Receptor α (ERα) | P03372 | Approved | 3ERT | OHT | Tyr537 | 9.9 | 3, 4* [25,35,36] | Class 2 |
| L-Lactate Dehydrogenase A-Chain (LDHA) | P00338 | Preclinical | 4AJJ | 88R | Tyr83 | 4.4 | 3 [91] | Class 2 |
| Tyosine Protein Phosphotase Non-Receptor Type 1 (PTP1B Phosphotase) | P18031 | Preclinical | 2CM7 | IZD | Tyr46 | 3.5 | 3 [92] | Class 2 |
| Eukaryotic Translation Initiation Factor 4E (eIF4E) | Q13541 | Preclinical | 2V8Y | MGV | Ser209 | 11.1 | 3 [93] | Class 2 |
| p-21 Activated Kinase 1 (PAK1) | Q13153 | Preclinical | 4EQC | XR1 | Tyr285 | 5.5 | 3 [94] | Class 2 |
| Deoxycytidine kinase (dCK) | P27707 | Preclinical | 4JLK | 1NO | Ser74 | 11.9 | 3 [95] | Class 2 |
| Mitogen-activated protein kinase kinase 6 (MAP2K6/Mek6) | P52564 | Preclinical | 3VN9 | ANK | Ser207 | 6.6 | 3 [96] | Class 2 |
| Interleukin-2 Inducible Kinase (Itk) | Q08881 | Preclinical | 4HCU | 13L | Tyr512 | 8.9 | 3 [97,98] | Class 2 |
| Mitogen-activated kinase 8 (MAPK8/ JNK1) | P45983 | Preclinical | 3ELJ | GS7 | Tyr185 | 6.4 | 3 [99] | Class 2 |
| Activated CDC42 kinase 1 (TNK2/ ACK1) | Q07912 | Preclinical | 3EQR | T74 | Tyr284 | 9.7 | 3 [100] | Class 2 |
| Glutathione S-transferase P (GSTP1) | P09211 | Phase II | 3GUS | N11 | Tyr8 | 4.0 | 3 [101] | Class 2 |

| Target Protein | Uniprot Sequence ID | Stage of Drug Development | PDB ID | PDB Ligand ID | Phosphorylated Residue(s) | Distance (Å) | Validation, References | Hit Class |
|---|---|---|---|---|---|---|---|---|
| Macrophage colony-stimulating factor 1 receptor (CSF1R) | P077333 | Phase II | 3DPK | 8C5 | Tyr809 | 8.9 | 3 [102,103] | Class 2 |
| Mitogen-Activated Protein Kinase Kinase (MEK1) | Q02750 | Phase II | 4LMN | EUI | Ser218, Ser222 | 5.9, 10.5 | 3 [104,105] | Class 2 |
| BDNF/NT-3 growth factors receptor (TrkB) | Q16620 | Phase II | 4AT5 | MUJ | Tyr706 | 10.3 | 3 [106,107] | Class 2 |
| Insulin-like growth factor 1 receptor (IGF1R) | P08069 | Phase II | 3NW7 | LGV | Tyr1161 | 7.1 | 3 [108,109] | Class 2 |
| Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) | Q13627 | Phase II | 4MQ1 | 2C3 | Tyr321 | 6.5 | 3 [110,111] | Class 2 |
| Hepatocyte growth factor receptor (c-Met) | P08581 | Approved | 3F66 | IHX | Tyr1234 | 10.3 | 3 [112] | Class 2 |
| Mast/stem cell growth factor kit (c-Kit) | P10721 | Approved | 1T46 | STI | Tyr823 | 10.8 | 3 [113,114] | Class 2 |
| Bruton's tyrosine kinase (Btk) | Q06187 | Approved | 3OCS | 746 | Tyr551 | 3.7 | 3 [115,116] | Class 2 |
| Kelch-like ECH-associated protein 1 (Keap1) | Q14145 | Preclinical | 4IQK | IQK | Ser602 | 2.5 | 3* [117] | Class 2 |
| Co-activator-associated arginine methyltransferase 1 (CARM1) | Q86X55 | Preclinical | 2Y1W | 849 | Ser216 | 3.9 | 3* [118] | Class 2 |
| Mitogen-activated protein kinase 1 (MAPK1/Erk2) | P28482 | Preclinical | 3QYZ | Z8B | Ser29 | 8.4 | 3* [119] | Class 2 |
| Receptor Interacting Protein 1 (RIP1) Kinase | Q13546 | Preclinical | 4NEU | Q1A | Ser161 | 9.5 | 3* [120] | Class 2 |
| PRK-like Endoplasmic Reticulum Kinase (PERK) | Q9NZJ5 | Preclinical | 4G31 | 0WH | Tyr619 | 6.4 | 3* [121] | Class 2 |
| Transforming growth factor-β activated kinase-1 (MAP3K7/TAK1) | O43318 | Preclinical | 4GS6 | 1FM | Ser192 | 9.8 | 3* [122] | Class 2 |
| NF-κβ-inducing Kinase (MAP3K14/NIK) | Q99558 | Preclinical | 4IDT | T28 | Thr559 | 10.2 | 3* [123] | Class 2 |
| Choline Kinase alpha (CHKA) | P35790 | Phase 1 | 4DA5 | 0H7 | Tyr333 | 4.4 | 3* [124] | Class 2 |
| Maternal Embryonic Zipper Kinase (MELK) | Q14680 | Phase 1 | 4BKY | 82B | Ser171 | 7.1 | 3* [125] | Class 2 |
| Polo-like kinase 1 (PLK1) | P53350 | Phase I | 4J52 | 1J3 | Ser137 | 6.5 | 3* [126] | Class 2 |
| 3-Phosphoinositide-dependent protein kinase 1 (PDK1) | O15530 | Phase I | 4AW0 | MJF | Ser160 | 3.0 | 3* [127] | Class 2 |
| Serine/Threonine kinase B-Raf (BRAF) | P15056 | Approved | 4JVG | B96 | Thr599 | 3.6 | 3* [128] | Class 2 |

| Target Protein | Uniprot Sequence ID | Stage of Drug Development | PDB ID | PDB Ligand ID | Phosphorylated Residue(s) | Distance (Å) | Validation, References | Hit Class |
|---|---|---|---|---|---|---|---|---|
| Janus Kinase 3 (Jak3) | P52333 | Approved | 3LXL | IZA | Tyr904 | 3.4 | 3* [129] | Class 2 |
| Mitogen-activated protein kinase 11 (MAPK11) | Q15759 | Approved | 3GP0 | NIL | Tyr323 | 8.1 | 3* [130,131] | Class 2 |
| Protein Kinase C iota type (PKCI) | P41743 | Approved | 3ZH8 | C58 | Tyr265 | 2.9 | 3* [132] | Class 2 |
| Insulin Receptor (IR) | P06213 | Approved | 4IBM | IR1 | Ser1033 | 7.1 | 3* [133] | Class 2 |
| Fatty acid-binding protein, adipocyte (FABP4) | P15090 | Preclinical | 3HK1 | B64 | Tyr20 | 3.4 | 1 [134] | Class 2 |
| Cell Division Cycle 7-related Kinase (Cdc7) | O00311 | Phase II | 4F9C | 0SX | Thr376 | 9.0 | 1 [135] | Class 2 |
| Caspase 1 (Casp1) | P29466 | Preclinical | 1RWX | YBH | Ser376 | 7.0 | 2 [136] | Class 2 |
| Mammalian STE20-like Kinase 4 (Mst4) | Q9P289 | Preclinical | 4FZF | DKI | Thr178 | 11.3 | 2* [137] | Class 2 |
| MEK Kinase 5 (MAP3K5/ASK1) | Q99683 | Preclinical | 4BF2 | STU | Thr842 | 9.8 | 2* [138] | Class 2 |
| Protein Kinase B (Akt1) | P31749 | Phase II | 4GV1 | 0XZ | Tyr176 | 6.5 | 2* [139] | Class 2 |
| Proto-oncogene tyrosine-protein kinase Src (c-Src) | P12931 | Approved | 3G5D | 1N1 | Tyr338 | 3.3 | 2 [140] | Class 2 |