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# Modifications of the GSK3β substrate sequence to produce substrate-mimetic inhibitors of Akt as potential anti-cancer therapeutics

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**Abstract**—Amplification, overexpression, and elevated activation of Akt have been detected in many human malignancies making it an important target for cancer therapy. The Akt substrate-binding site offers a large number of potential interactions to an appropriately designed small molecule and can form the basis for the development of selective inhibitors. Here, we report the progression of GSK3β substrate-mimetic inhibitors towards the development of a potent, small molecule substrate-mimetic inhibitor of Akt.
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Akt, or protein kinase B, is a serine/threonine protein kinase that has been found to be amplified, overexpressed, and/or activated in many human malignancies.[1–6] It exists as three separate isoforms: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, that are structurally very similar.[7] Each isoform consists of an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal regulatory tail.[7]

The events that lead to the activation of Akt have been elucidated.[8–10] Activation requires agonist-induced stimulation of phosphoinositide 3-kinase (PI3K) to generate phosphatidylinositol-3,4,5-triphosphate ($PIP_3$) that associates with the PH domain of Akt, and localizes the protein to the intracellular side of the plasma membrane. Once anchored to the plasma membrane, residues Thr308 and Ser473 are phosphorylated, inducing maximal catalytic activity. Once activated, the protein is released to target cytosolic and nuclear substrates.

Elevated activation of specific isoforms of Akt have been detected in many human malignancies including ovarian, breast, prostate, and pancreatic cancer.[1–6] Its role in malignancies is accomplished by the simultaneous promotion of cell survival, growth, and migration, and the down-regulation of apoptosis.[11–13] Overall, Akt activation down-regulates the expression of pro-apoptotic proteins and up-regulates the expression of anti-apoptotic proteins.[11,12,14] Inhibition of Akt function has been shown to stimulate apoptosis in a number of cancer cell lines.[15] These observations establish Akt as a valuable cancer target and suggest that the development of potent and selective inhibitors of Akt could lead to novel anti-cancer therapeutics.

Much effort has been applied to the development of Akt inhibitors that target the ATP-binding domain of the protein, but these have the potential to affect other kinases or the many ATP utilizing enzymes.[16–19] Recently, there has also been an emergence of allosteric inhibitors that has been met with some success.[20,21] However, as a general point of strategy, targeting inhibitors to the peptide substrate-binding site has the potential for greater selectivity since it has evolved to respond to a highly specific sequence of amino acids.[22,23]

In addition, peptide or peptidomimetic substrates conjugated to ATP mimetics have provided potent bisubstrate

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inhibitors.[24,25] The substrate-binding site offers a large number of potential interactions to a small molecule derived through mimicry of the key peptide sequence, which provides a good opportunity for development of an Akt selective inhibitor. The X-ray crystal structure of an activated Akt ternary complex with a 10-residue sequence of a cellular substrate glycogen synthase kinase 3 (GSK3β) and an ATP analogue identifies the GSK3β-peptide (GRPRTTSFAE) to be bound in an essentially extended conformation, with discrete sections of β-strand on either side of the modifiable serine residue (Fig. 1).[26] A closely related substrate, GRPRTSSF, was found to have a $K_m$ of 8 μM.[27] Extensive hydrogen bonding interactions are observed between acidic pockets of the protein and N-terminal basic residues of GSK3β, while C-terminal interactions are essentially hydrophobic. The consensus substrate for processing has been shown to be Arg-$X_{aa}$-Arg-$Y_{aa}$-$Z_{aa}$-S/T-Hyd, where $X_{aa}$ may be any amino acid, $Y_{aa}$ and $Z_{aa}$ any small amino acid other than glycine, and Hyd represents a large hydrophobic amino acid.[27]

In this paper, we report the synthesis and activity of substrate-mimetic inhibitors of Akt based on the truncated GSK3β-peptide substrate sequence GRPRTTSF.

A simple series of peptidomimetic inhibitors was generated directly from the minimal substrate sequence by systematic replacement of the non-critical amino acids and tested for in vitro inhibition of Akt (Table 1). Inhibitors were tested utilizing a fluorescence polarization assay system.[28] Initial investigations of our substrate-mimetic inhibitors included evaluation of the contribution of a number of amino acids, starting with the phosphorylatable serine residue of the GSK3β peptide. Numerous serine substitutions were made by scanning the position with several other L-amino acids to provide weakly binding peptidic inhibitors (**1–7**). Replacement of the reactive serine with a valine residue (a non-nucleophilic surrogate) in peptide **4** provided a starting point in our design.

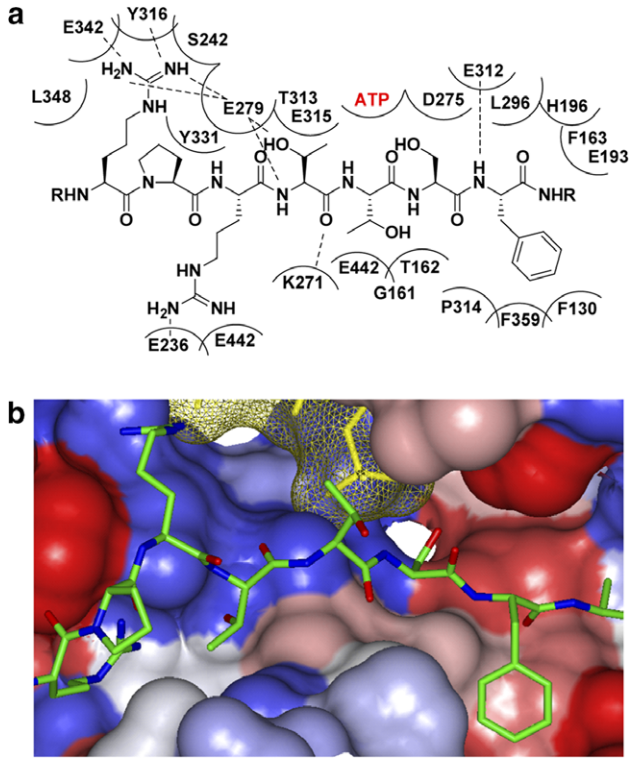



**Figure 1.** (a) Synopsis of GSK3β–Akt interactions by residue. (b) Crystal structure of the enzyme-bound conformation of GSK3β (green) and an ATP analogue (yellow) against the solvent-accessible surface area of Akt (Gradient color scale—Red, hydrophobic; blue, hydrophilic).[26]

Inclusion of a small hydrophobic group, benzyl (Bn), at the C-terminus to complement the unoccupied hydrophobic pocket afforded inhibitor **8** with a two-fold increase in potency ($IC_{50}$ of 239 μM). C-terminal coupling was conducted with benzylamine ($BnNH_2$), HATU, and DIPEA in DMF to limit the racemization of the terminal Phe residue. Both diastereomers of **10** were tested and found to have comparable activity ($IC_{50}$ of 28 and 40 μM), therefore further diastereomers of later compounds were not separated, but tested as mixtures.

Further refinement included the replacement of the internal -TT- residues, which make few interactions with the protein surface. Replacement of this dipeptide with -AA- resulted in a 2-fold decrease in activity, however, substitution with a conformationally restricted scaffold, *p*-aminobenzoic acid (Abz), afforded inhibitor **10** with a 10-fold increase in activity ($IC_{50}$ of 28 μM). Docking studies suggest that the Abz spacer can reproduce the hydrophobic interactions of the native discrete dipeptide β-strand, while reducing the entropy cost of the extended binding conformation of the unbound inhibitor.

A small screen of hydrophobic groups appended to the C-terminus (**11–17**) demonstrated similar activity to C-terminal benzyl derivative **10**. These C-terminal modifications and the reported X-ray structure suggest the hydrophobic pocket is extensive (**11, 13–14**). Further structural refinement at this position should lead to significant improvement in activity. As expected these hydrophilic peptidic inhibitors showed no cellular activity.

Modification of the N-terminal GRPR sequence represents a challenge in the stepwise development of non-peptidic inhibitors, as previous alanine scanning had demonstrated a strict requirement for conservation of both arginines.[27,30] The presence of these polar residues would also likely hinder cell penetration and useful in vivo activity. Having introduced hydrophobic contacts (Abz, Hyd) to inhibitors **10–17**, with a concomitant improvement in Akt affinity, we reexamined the dependence of N-terminal hydrophilic contacts using an alanine scan (**18–20**, Table 1). From this we concluded that only one arginine residue was necessary to maintain activity, with **18** and **19** possessing similar potency to **10**. This series also suggests that the arginine residue adjacent to the hydrophobic spacer contributes more significantly to binding.

**Table 1.** Initial peptidic inhibitors and their contributions to binding

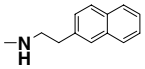
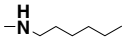
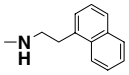
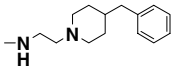
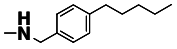
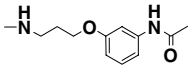
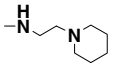
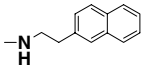
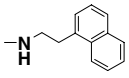
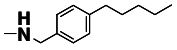
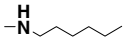
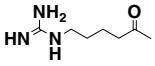
| Compound | Structure | | | | | | | | | | $IC_{50}$[a] (μM) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | Ac- | G | R | P | R | T | T | W | F | –OH | >500 |
| **2** | Ac- | G | R | P | R | T | T | F | F | –OH | >500 |
| **3** | Ac- | G | R | P | R | T | T | H | F | –OH | >500 |
| **4** | Ac- | G | R | P | R | T | T | V | F | –OH | 445 |
| **5** | Ac- | G | R | P | R | T | T | A | F | –OH | 409 |
| **6** | Ac- | G | R | P | R | T | T | C | F | –OH | 241 |
| **7** | Ac- | G | R | P | R | T | T | N | F | –OH | 229 |
| **8** | Ac- | G | R | P | R | T | T | V | F |  | 239 |
| **9** | Ac- | G | R | P | R | A | A | V | F |  | 438 |
| **10** | Ac- | G | R | P | R | Abz | | V | F |  | 28 |
| **11** | Ac- | G | R | P | R | Abz | | V | F |  | 36 |
| **12** | Ac- | G | R | P | R | Abz | | V | F |  | 52 |
| **13** | Ac- | G | R | P | R | Abz | | V | F |  | 62 |
| **14** | Ac- | G | R | P | R | Abz | | V | F |  | 94 |
| **15** | Ac- | G | R | P | R | Abz | | V | F |  | 96 |
| **16** | Ac- | G | R | P | R | Abz | | V | F |  | 322 |
| **17** | Ac- | G | R | P | R | Abz | | V | F |  | >500 |
| **18** | Ac- | G | A | P | R | Abz | | V | F |  | 41 |
| **19** | Ac- | G | R | P | A | Abz | | V | F |  | 79 |
| **20** | Ac- | G | A | P | A | Abz | | V | F |  | >500 |
| **21** | | | | Ac- | R | Abz | | V | F |  | 51 |
| **22** | | | | Ac- | R | Abz | | V | F |  | 89 |
| **23** | | | | Ac- | R | Abz | | V | F |  | 104 |
| **24** | | | | Ac- | R | Abz | | V | F |  | 119 |

**Table 1** (*continued*)

| Compound | Structure | | | | | | $IC_{50}$[a] (μM) |
|---|---|---|---|---|---|---|---|
| **25** | Ac- | R | Abz | V | F |  | 163 |
| **26** |  | | Abz | V | F |  | 193 |

[a] Reported values are an average of three independent-binding curves utilizing an IMAP® Akt Assay Kit (Molecular Devices). Reactions were conducted in wells with 20.0 μL of 10 mM Tris–HCl (pH 7.2), 10 mM $MgCl_2$, 0.1% BSA, 0.05% $NaN_3$, 1 mM DTT, 100 nM 5FAM-GRPRTSSFAEG-COOH, 5 μM ATP, 0.067 U/mL of Akt1, and inhibitor. Reaction mixtures were incubated for 1 h at room temperature and then quenched with 60 μL of the IMAP-binding solution. The reactions were equilibrated for 1 h at room temperature then data points were collected and analyzed.

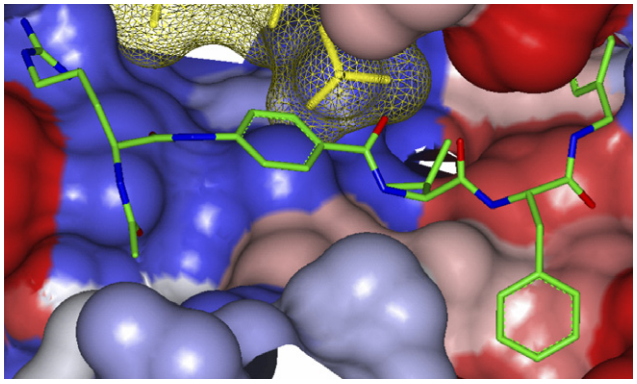


**Figure 2.** Flexible ligand docking GOLD[29] of **24** (green) against the solvent-accessible surface area of Akt (Gradient color scale—Red, hydrophobic; blue, hydrophilic).

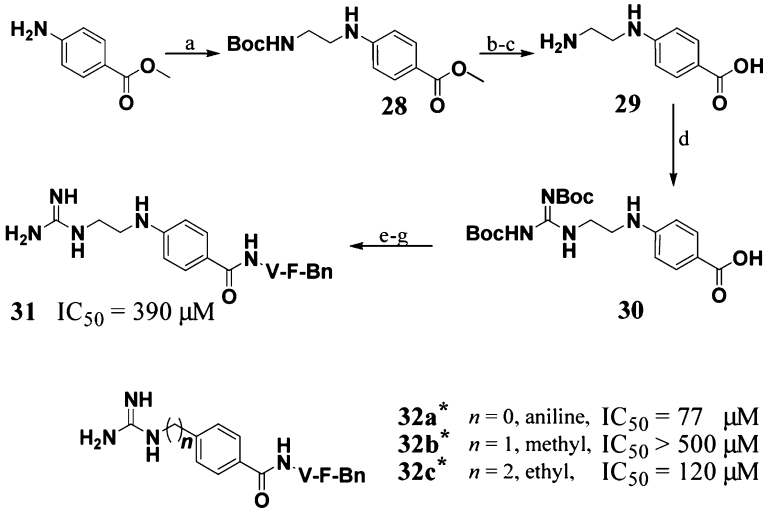
Evaluation of the role of the arginine residues led us to truncate the inhibitors, thereby eliminating the N-terminal -GRP- tripeptide sequence. This produced a set of inhibitors with only three amino acids and with the optimal hydrophobic substituents coupled to the C-terminus of AcR-Abz-V-F-OH (**21–25**). These truncated inhibitors are significantly more hydrophobic than peptidomimetics **1–20**, but retain almost identical inhibition potency. Further truncation of the N-terminal acylated amine **26** resulted in an almost two-fold loss of activity when compared to **24**, highlighting the importance of hydrophobic interactions and the interaction of the carbonyl or amide proton with an adjacent residue (Fig. 2). Docking studies suggest that this acylated amine could occupy a hydrophobic pocket of Akt previously occupied by one of the Thr residues of the GSK3β peptide or it could be hydrogen bonding to residues within the active site of Akt: E315, E279, D275, or K277. The Boc-protected modified arginine residue lacking the α-amine **27** was synthesized by guanidinylation of 5-aminovaleric acid with *N*,*N*′-bis(*tert*-butoxycarbonyl)-1H-pyrazole-1-carboxyamidine (BisBocPCH) and $Et_3N$ in $CH_3OH$.

Further refinement of inhibitor **24** toward non-peptidic, small molecule substrate-mimetics was focused on three main areas of modification: the N-terminal hydrophilic residues, the interior hydrophobic spacer, and the C-terminal hydrophobic contacts.

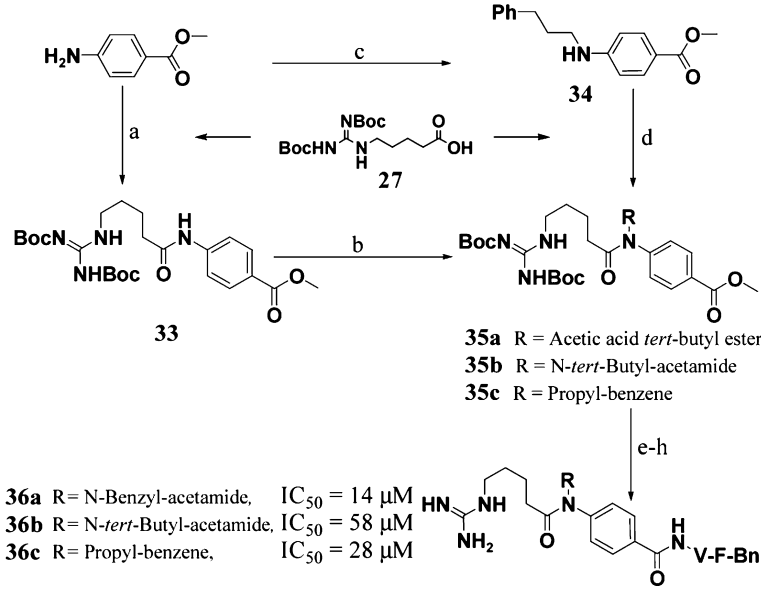
Modifications to the N-terminal hydrophilic residues were concentrated on increasing the rigidity and hydrophobicity of the inhibitors. Rigidifying modifications were focused on decreasing the length and rotational freedom of the essential guanidinium functionality to project it directly into an acidic pocket of Akt (Scheme 1). Different length linkers were explored, with 0, 1, 2, and 3 atoms separated from the aromatic spacer to afford inhibitors that showed comparable or better affinity than **24**, which contains the entire Arg residue. Inhibitors with 0–2-atom linkers, **32a–c**, respectively, were synthesized by the guanidinylation of commercially available aminobenzoic acids followed by solid-phase coupling, cleavage, and C-terminal coupling. Inhibitor **31** possesses a three-atom linker and was synthesized by the reductive amination of methyl 4-aminobenzoate with *N*-Boc-2-aminoacetaldehyde, with subsequent saponification and deprotection to afford **29**. Guanidinylation of **29**, followed by solid phase coupling, cleavage, and C-terminal coupling, provided **31.** Inhibitor **32a** provides the best affinity in this series with an $IC_{50}$ of 77 μM, suggesting that a one-atom linker is sufficient to reach the hydrophilic pocket.

Additional N-terminal modifications focused on scaffolds with a functionalizable handle to access a hydrophobic pocket previously occupied by one of the Thr residues of the GSK3β peptide. This handle was also used to incorporate a hydrogen-bonding functionality to investigate the hypothesis that the N-terminal acyl group of the Arg residue is hydrogen bonding to an active site residue. A simple series of inhibitors was synthesized to probe both interactions (**36a–c**, Scheme 2). Two derivatives were synthesized by coupling **27** and methyl 4-aminobenzoate to afford **33** which was then alkylated on the amide nitrogen with the corresponding bromide to provide **35a–b**. Reductive amination of methyl 4-aminobenzoate with 3-phenyl-propionaldehyde and subsequent coupling to **27** via an in situ acid chloride formation afforded **35c**.[31] Saponification of **35a–c** followed by solid phase coupling, cleavage, and C-terminal coupling afforded inhibitors **36a–c**.

Incorporation of an amide functionality and a large hydrophobic group gave a significant increase in activity, with the benzyl derivative **36a** showing an $IC_{50}$ of 14 μM, which is significantly higher than that of the unacylated analogue **26**. Docking studies of **36a** suggest that the benzyl substituent projects into a large pocket




**Scheme 1.** Reagents: (a) i—*N*-Boc-2-aminoacetaldehyde, AcOH, $CH_3OH$, 4Å mol. sieves, ii—$NaCNBH_3$; (b) LiOH, $THF/H_2O$; (c) TFA; (d) BisBocPCH, $Et_3N$, $CH_3OH$; (e) $NH_2$-Val-Phe-Wang, HBTU, DIPEA, DMF; (f) TFA, 5% thioanisole, 1% TIPS, 1% $H_2O$; (g) $BnNH_2$, HATU, DIPEA, DMF (*Synthesized from commercially available amino benzoic acids following the sequences d–g.).




**Scheme 2.** Reagents: (a) Compound **27**, DIC, $CH_2Cl_2$, cat. DMAP; (b) R–Br, Base, DMF; (c) i—3-phenyl-propionaldehyde, AcOH, $CH_3OH$, 4Å mol. sieves, ii—$NaCNBH_3$; (d) Compound **27**, $Ph_3PCl_2$, $CHCl_3$; (e) LiOH, $THF/H_2O$; (f) $NH_2$-Val-Phe-Wang, HBTU, DIPEA, DMF; (g) TFA, 5% thioanisole, 1% TIPS, 1% $H_2O$; (h) $BnNH_2$, HATU, DIPEA, DMF.

within the active site of Akt, previously occupied by residues of the GSK3β peptide (Fig. 3). The *t*-butyl derivative **36b** was slightly less potent with an $IC_{50}$ of 58 μM. Inhibitor **36c** was synthesized with similar hydrophobic character as **36a**, but lacking the hydrogen bonding potential. Its affinity is comparable to inhibitors **36a** and **36b**, suggesting that hydrophobic contacts in the Thr pocket are the key interactions promoting increased affinity.

Many malignancies display elevated levels of specific isoforms of Akt identifying it as an attractive target for cancer therapy. Divergence in Akt isoform-specific function presumably occurs at the level of substrate selection on the basis of sequence diversity. Preliminary studies of substrate-mimetic inhibitors demonstrate that limited structural modification of the initial peptidic substrate provides inhibitors with decreasing peptidic character and increasing lipophilicity, which with future modifications should lead to in vivo activity. Modifications of these inhibitors toward increasing potency and selectivity for specific Akt isoforms will provide a useful set of molecular probes to assist in the validation of Akt as a potential target for anti-cancer drug design.

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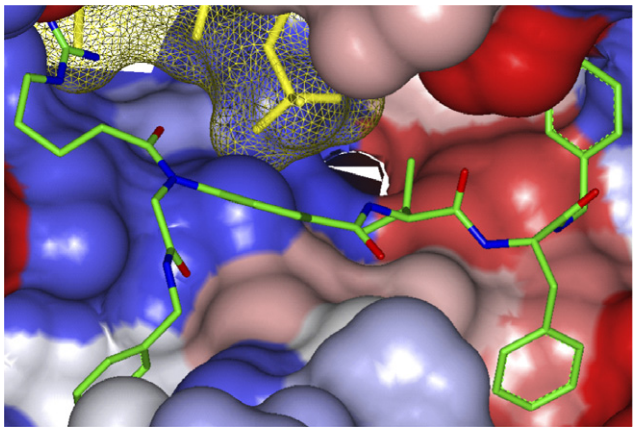

**Figure 3.** Flexible ligand docking GOLD[29] of **36a** (green) against the solvent-accessible surface area of Akt (Gradient color scale—Red, hydrophobic; blue, hydrophilic).

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.004.

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