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Letter

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In Silico Identification and Biological Evaluation of Novel Selective Serum/Glucocorticoid-Inducible Kinase 1 Inhibitors Based on the Pyrazolo-Pyrimidine Scaffold

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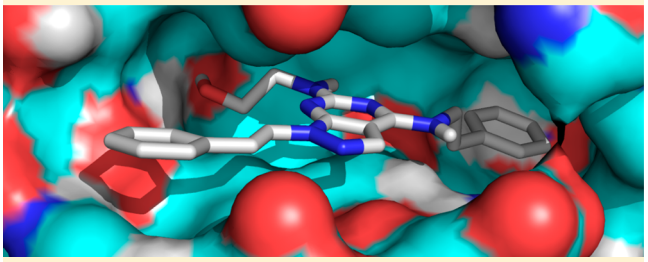
Supporting Information



ABSTRACT: The serum/glucocorticoid-inducible kinase 1 (Sgk1) has demonstrated antiapoptotic function and the capability to regulate cell survival, proliferation, and differentiation. A pivotal role of Sgk1 in carcinogenesis and in resistance to anticancer therapy has been suggested. With the aim of identifying new Sgk1 modulators, 322 pyrazolo-pyrimidine derivatives have been virtually screened with respect to a crystallographic model of Sgk1. The top five ranked compounds have been evaluated demonstrating Sgk1 inhibition in vitro and selectivity compared to RAC-alpha serine/threonine-protein kinase (Akt1).

Sgk1 is a kinase that has recently gained the attention in the field of molecular oncology. It was originally described as a key enzyme in the hormonal regulation of sodium absorption by the amiloride sensitive sodium channel (ENaC). The activity of EnaC, through Sgk1, is regulated by aldosterone, glucocorticoids, insulin, and vasopressin at the transcriptional and post-translational level.[1,2] PDK1 binds phospho-Ser422 in the hydrophobic motif (H-motif) of Sgk1 to phosphorylate Thr256. mTOR is the H-motif kinase that phosphorylates Sgk1 at Ser422.[3] More recently, Sgk1 has been implicated in mediating insulin and IGF1 dependent survival signals.[4,5] The antiapoptotic function of Sgk1 suggests a possible involvement in human carcinogenesis.[6] Ectopic expression of wild type Sgk1 is able to revert apoptosis mediated by growth factor withdrawal,[7] moreover Sgk1 mediates glucocorticoid dependent antiapoptotic signals in mammary epithelial cells and breast cancer[8,9] and IL2 dependent antiapoptotic signals in kidney cancer cells.[10] Interestingly, the active Sgk1 kinase regulates cell survival, proliferation, and differentiation through MDM2, that directs p53 to ubiquitylation and proteosomal degradation,[11] and, through RanBP1, affects mitotic stability and taxol sensitivity of RKO colon carcinoma cells in culture.[12] Taken together, these observations suggest an important role of Sgk1 in carcinogenesis and in resistance to hormonal therapy and chemotherapy. Sgk1 may thus be considered as a new molecular target in the therapy of some human tumors and, in fact, Sgk1 antagonists are considered in the experimental therapy of prostate and colon cancer.[13,14] The level of Sgk1 expression may have a central role in tumors unresponsive to Akt inhibition.[15] One of the main features of Sgk1, as compared with its analogue kinase Akt, is in fact the ability to be regulated by steroids. Glucocorticoid receptor antagonism has been recently proposed as a promising therapy in triple negative breast cancer,[16] and Sgk1 inhibition is expected to be at least as effective as glucorticoid receptor antagonism.

Many kinase inhibitors are available, based on the ability to compete with ATP in the ATP binding domain of the enzyme, a region that shares significant sequence homology between kinases. The human genome encodes more than 500 protein kinases, most of which are members of the same superfamily. Recent observations proved that a number of alleged "specific" kinase inhibitors affect in reality many kinases, so that it is sometimes impossible to define the role of a single kinase by the use of these inhibitors. Indolinone inhibitors, such as SU6656, have been widely used for Src family protein kinases[17] although they were later found to inhibit many other kinases, including serine and threonine protein kinases. Compounds such as CGP57380, D4476, and SP600125 inhibit several kinases, including Sgk1 although with low specificity and an IC_{50} in the micromolar range.[18] Some pyrazolo-pyrazine sulfonamide[19] derivatives, GSK650394,[20] a pyrrolo-pyridine compound, and, recently, EMD638683,[21] a phenylbenzohydrazide structure based inhibitor, have demonstrated Sgk1 selectivity with respect to other kinases. A family of dual Src/

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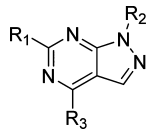
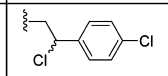
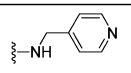
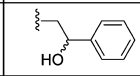
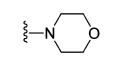
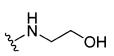
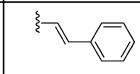
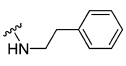
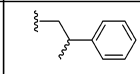
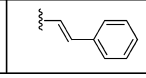
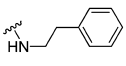
Abl inhibitors, characterized by a substituted pyrazolo[3,4-*d*]pyrimidine scaffold, has been reported to be active against several tumor cell lines.[22−26]

In order to identify novel potential Sgk1 inhibitors, following our previously reported work against another target,[27] we virtually screened one of our own molecular libraries with respect to Protein Data Bank[28] (PDB) Sgk1 crystal structures. The virtual library consisted of 322 pyrazolo[3,4-*d*]pyrimidine derivatives, with 268, 7, and 2 compounds reporting 1, 2, or 3 chiral centers respectively, characterized by molecular weight ranging from 239.28 to 578.74 a.m.u. and theoretical logP[29] from 1.44 to 7.85.

Some of the library compounds have been previously proven to maintain the original dual Src/Abl activity.[23,24] With the aim to take into account the Sgk1 active site conformation variability, the interaction of our compounds has been evaluated by means of Glide[30] ligand flexible docking simulation (Supporting Information) carried out on all PDB X-ray available models, such as 2R5T,[31] 3HDM,[32] and 3HDN.[33] For estimating the target recognition of our derivatives, the average value of the Glide scores (AS), computed for each compound against the 3 PDB models, has been adopted. For chiral compounds, both enantiomers have been taken into account and their corresponding final score has been considered as the average of (*R*)- and (*S*)-isomer values (Table 1). See the Supporting Information, Table S1, for the detailed list of the docking scores.

Table 1. Chemical Structures and Average Docking Scores of the Top Five Ranked Compounds



Id	R_1	R_2	R_3	AS[a]
1	—H			-7.41
2	—H			-7.11
3				-6.74
4	—H		—NH_2	-6.86
5	—H			-5.69

[a]Average docking score in kilocalories per mole.

Among the top five docking ranked compounds, derivatives **1**−**3** and **5** have been already reported by us,[25,28] whereas derivative **4** has been synthesized following a different procedure, reported in the Supporting Information. Compounds **1**−**5** have been submitted to inhibition in vitro tests with respect to both Sgk1 and Akt1 (Supporting Information). Asymmetric derivatives have been tested as racemic mixtures. Experimental results revealed that **1**−**5** were similarly effective in inhibiting Sgk1 kinase activity, whereas they were much less effective toward Akt1 (Figure 1).

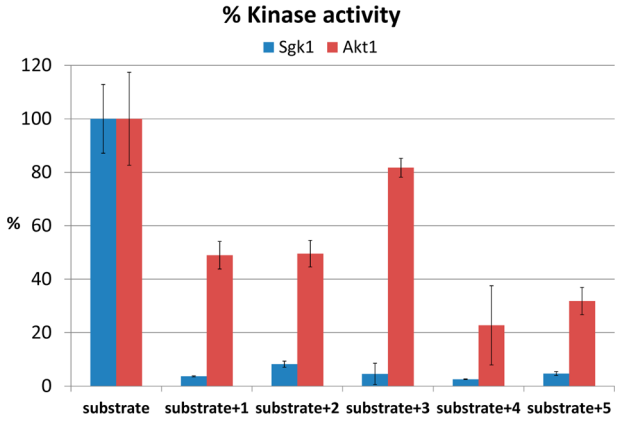


Figure 1. Percentage of Sgk1 and Akt1 kinase activity after in vitro administration of compounds **1**−**5**. Each experiment was carried out in triplicate. Error bars report ± SD.

In order to investigate the selectivity of **3**, all Sgk1 receptor models previously reported and a selection of high X-ray resolution human Akt1 models, available in PDB with codes 3CQW,[33] 3MV5,[34] 3MVH,[34] 4EKK,[35] and 4GV1,[36] have been characterized using SiteMap[37] program. In all cases, the common ATP binding site, occupied by the cocrystallized ligands, has been recognized and its overall surface and hydrophobic and hydrogen bond donor/acceptor areas have been computed (Supporting Information). The characterization analysis revealed similarities among the target active sites: the ratio between average Sgk1 and Akt1 values has indicated differences in the range of 10% for all measurements, except for the hydrophobic one. In fact, it highlighted that the Sgk1 lipophilic area was 47% larger than that of Akt1. As a consequence, the hydrophobic interaction could play a key role for explaining the preference of our compounds in Sgk1 recognition and inhibition with respect to Akt1. In order to better clarify the experimentally demonstrated Sgk1 selectivity, docking simulations of our most selective compound **3** against all previously selected Akt1 models were also performed. Theoretical results have been in qualitative agreement with the experimental data, actually Akt1 compound **3** AS was equal to −5.12 kcal/mol, indicating a better predicted interaction with the Sgk1 models (AS = −6.74 kcal/mol). Notably, in all Akt1 cases, the docking score was worse than the worst Sgk1 result. The graphical inspection of the most stable complexes of **3** into Sgk1 and Akt1 models, 3HDN (AS = −7.21 kcal/mol) and 3CQW (AS = −5.83 kcal/mol), respectively, highlighted a quite different recognition of the kinase active sites and confirmed the stabilizing role of the hydrophobic contribution. Actually, considering the Sgk1 cleft, the pyrazolo-pyrimidine core was located in a lipophilic area delimited by Ile104, Val112, Leu114, Ala125, Leu176, Ile179, Leu229, and Thr239, while the R_3 branch was able to recognize a lipophilic cage surrounded by Phe109, Tyr220, Phe241, and Leu243.

The Sgk1 binding conformation of **3** was flat; the R_1 substituent, by means of its alcoholic group, was involved in donating one hydrogen bond to the backbone of the Asp177 and accepting a second one from the backbone of Ile179, while the R_3 phenyl ring established cation-π and t-shape $\pi-\pi$ interactions to Lys127 and Phe241 side-chains, respectively. In Akt1 the substitution of Sgk1 Leu176, Ile179, and Leu229 with the corresponding Met227, Ala230, and Met281 has strongly restricted the first lipophilic area (Supporting Information,

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Figure S2). The subsequent configuration of **3** into Akt1 showed one cation-π interaction, between the Arg4 sidechain and the ligand R_3 phenyl ring, but the molecule was unable to establish any other productive interaction to the target, except unspecific van der Waals contacts (Figure 2). After rationalizing

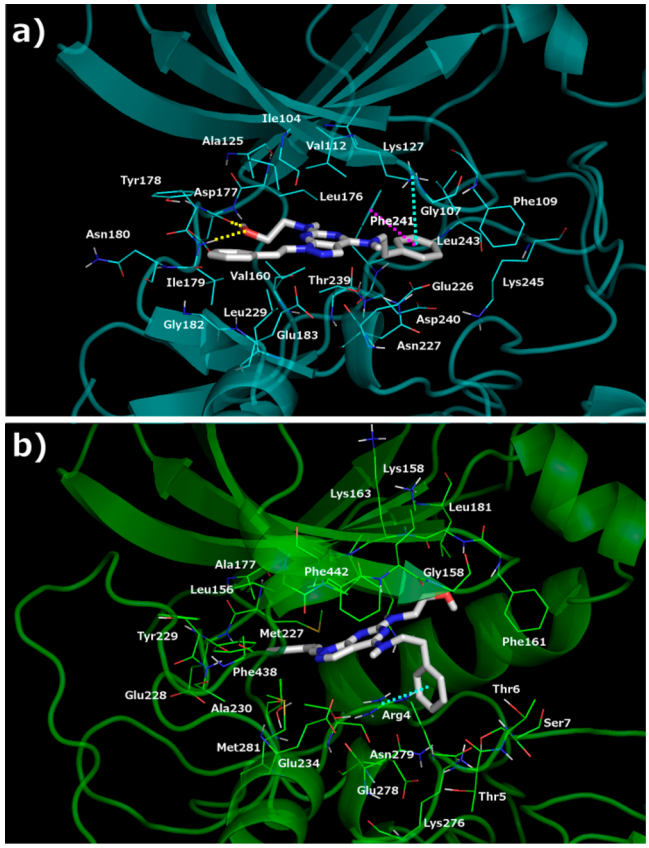

Figure 2. Compound **3** recognition of (a) Sgk1, cyan, and (b) Akt1, green. The inhibitor is depicted in polytube CPK colored, interacting residues in wireframe; the rest of the enzymes is shown as a cartoon. Yellow, cyan, or magenta dotted lines represent hydrogen bonds, cation-π, or t-shape $\pi-\pi$ interactions, respectively.

the mechanism of action of compound **3**, we focused our study onto the characterization of its biological activity that has appeared to be the least effective in Akt1 inhibition. A dose-dependent response curve of **3** inhibition on both Sgk1 and Akt1 (Figure 3) highlighted the inhibition of the Sgk1 activity with an IC_{50} value of 600 nmol/L and a selectivity, with respect to Akt1, almost 1000-fold higher. In fact, Akt1 was inhibited with an IC_{50} value equal to 50 μmol/L.

In conclusion, a new Sgk1 selective inhibitor has been identified following a multidisciplinary approach consisting of association of molecular modeling, organic synthesis, molecular biology, and cell biology skills. The present work started from the virtual screening of our own library, containing more than 300 pyrazolo-pyrimidine derivatives, with respect to Sgk1 and Akt1 PDB crystallographic models. The top five docking ranked compounds, reporting a novel pyrazolo-pyrimidine chemical scaffold with respect to the known Sgk1 binders, have been synthesized, and their inhibition properties have been experimentally investigated, by means of kinase assays, with respect to both Sgk1 and Akt1. Molecular modeling techniques have been applied to rationalize the mechanism of action of the most promising compound **3**. Further study, based on these

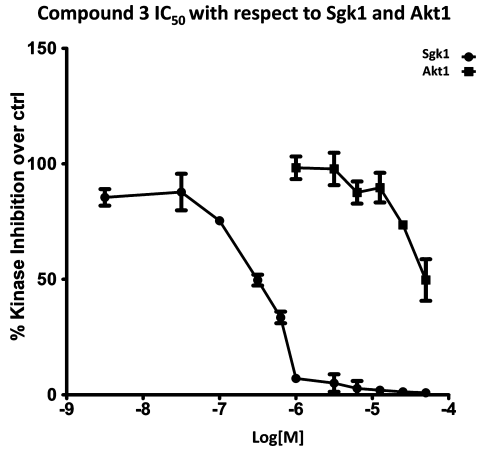

Figure 3. Compound **3** dose-dependent in vitro inhibition of Sgk1 and Akt1 kinase activity. Results expressed as mean of three experiments ± SD.

preliminary results, will be carried out for investigating the biological properties of the identified inhibitors on cell lines and in vivo models.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and characterization data, molecular modeling protocols and in vitro experimental procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Sgk1, serum/glucocorticoid-inducible kinase 1; Atk1, RAC-alpha serine/threonine-protein kinase; ENaC, amiloride sensitive sodium channel; IL2, interleukine 2

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