

Article

## The Guareschi Pyridine Scaffold as a Valuable Platform for the Identification of Selective PI3K Inhibitors

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**Abstract:** A novel series of 4-aryl-3-cyano-2-(3-hydroxyphenyl)-6-morpholino-pyridines have been designed as potential phosphatidylinositol-3-kinase (PI3K) inhibitors. The compounds have been synthesized using the Guareschi reaction to prepare the key 4-aryl-3-cyano-2,6-dihydroxypyridine intermediate. A different selectivity according to the nature of the aryl group has been observed. Compound **9b** is a selective inhibitor against the PI3K $\alpha$  isoform, maintaining a good inhibitory activity. Docking studies were also performed in order to rationalize its profile of selectivity.

**Keywords:** PI3 kinase; pyridine; Guareschi reaction; isoforms; selectivity

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## 1. Introduction

Class I phosphatidylinositol-3-kinases (PI3Ks) [1] are lipid kinases able to phosphorylate the hydroxyl group at the three position of the phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P<sub>2</sub>). They exert a pivotal role on several cellular processes such as proliferation, survival, motility, metabolism, and differentiation [2].

Class I PI3Ks are usually heterodimers consisting of a catalytic subunit and an adaptor/regulator subunit. All class I catalytic subunits share substantial homology and are referred to as p110 subunits. The contribution of each of the four isoforms of class I PI3Ks has been elucidated in detail, as their tissue localization [3]. For example, in mammals, whereas the expression of PI3K $\delta$  is restricted to the immune system, PI3K $\alpha$  and PI3K $\beta$  are ubiquitously expressed, and the p110 $\gamma$  catalytic isoform is mainly expressed in leukocytes. For these reasons, the identification of PI3K inhibitors with a different profile of selectivity can be useful for the treatment of different diseases [4].

Over the last decades, pan-PI3K inhibitors [5,6] were extensively investigated for the treatment of cancer; however, the use of isoform-selective PI3K inhibitors seems to be a better strategy in cancer therapy in order to reduce the side effects associated with pan-PI3K inhibitors [7,8]. Alterations in the PI3K pathway, such as PI3K $\alpha$  mutations, over-expression, or gene amplification, and PTEN (phosphatase and tensin homologue) phosphatase deletion, have been identified in several types of cancer (mammary gland, colon, prostate, brain) [5,6]. Moreover, recent evidence demonstrated that PI3K $\alpha$  selective inhibitors [7] are more effective against tumors with mutations in the PI3K $\alpha$  gene. PI3K $\beta$ -selective inhibitors have been proposed in the treatment of PTEN-deficient tumors [9–11], while PI3K $\delta$  inhibitors are currently under evaluation in clinical trials for the treatment of some specific lymphoproliferative diseases including chronic lymphocytic leukemia (CLL), multiple myeloma, and Hodgkin's lymphoma [12–15]. In this context, a more specific targeted therapy instead of the use of pan-PI3K inhibitors could help to maximize the antitumor effects, reducing the potential side effects (hematological complications, insulin resistance) associated with an indiscriminate inhibition of all the PI3K isoforms.

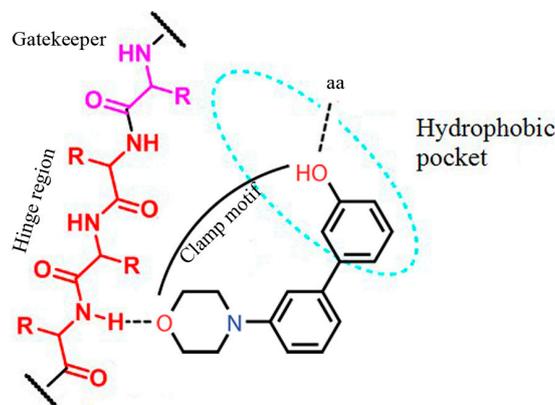
The identification of selective PI3K inhibitors is still a topic of research both in industry and academia and, to date, several potent PI3K inhibitors, able to compete with the ATP binding site, have been reported [16–19].

The intellectual property of ATP-competitive inhibitors is highly over-crowded, rendering the identification of novel molecular entities a daunting task for medicinal chemists. One important feature present in most of the ATP-competitive PI3K inhibitors is the presence of the so-called clamp motif [20] formed by a morpholine, a hinge-binder typical for lipid kinases, and a meta-substituted phenol (or one of its bioisosteres) grafted onto a heteroaromatic ring (Figure 1).

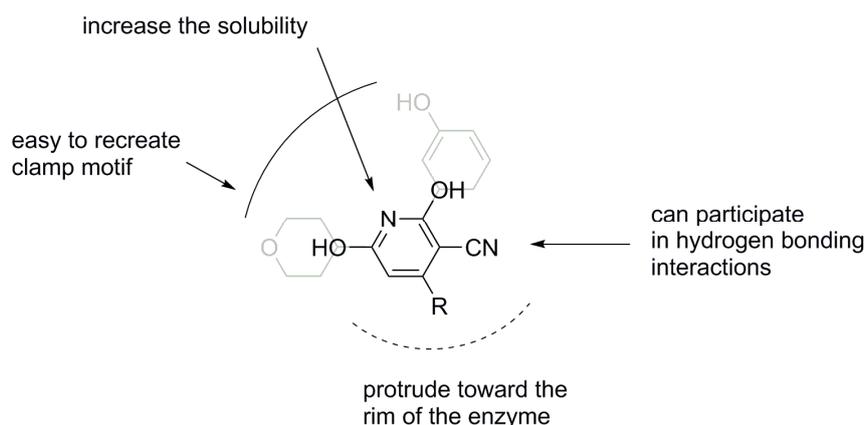
The strategy of the clamp motif grafted to a heteroaromatic ring has been utilized by several research groups. For example, cyanuric chloride was used as the starting material, exploiting the different reactivity of chlorine atoms in the nucleophilic aromatic substitution to add different hinge-binders [21].

With this in mind, looking for novel PI3K inhibitors, we focused our attention on the neglected 3-cyano-2,6-dihydropyridine scaffold, which can be obtained via Guareschi reaction [22], as a valuable chemical platform. Indeed, it contains all the chemical functions to build the clamp motif and

a group pointing towards the rim of the binding pocket. Furthermore, the pyridine ring can increase the solubility, while the cyano group is an added bonus as it might play an active role in the kinase inhibition [23] or it can be considered as a handle for further chemical manipulations (Figure 2). As far as we know, this is the first attempt to use this chemical scaffold in the kinase inhibitor field.



**Figure 1.** The clamp motif strategy for the identification of novel PI3K inhibitors (in the case of PI3K $\gamma$ , the phenol group interacts with Tyr867, while morpholine interacts with Val882).



**Figure 2.** The Guareschi pyridine scaffold can be advantageously used for the design of novel PI3K inhibitors.

As the amino acid residues located at the entrance to the catalytic site are not conserved in the different PI3K isoforms [24], we decided to maintain the morpholine and phenol clamp motifs, exploring the C<sub>4</sub>-position of the pyridine ring introducing different aromatic groups. It would be expected that these groups, which protrude outside the ATP-binding pocket, might give additional contacts with the less conserved amino acid residues, altering the profile of selectivity.

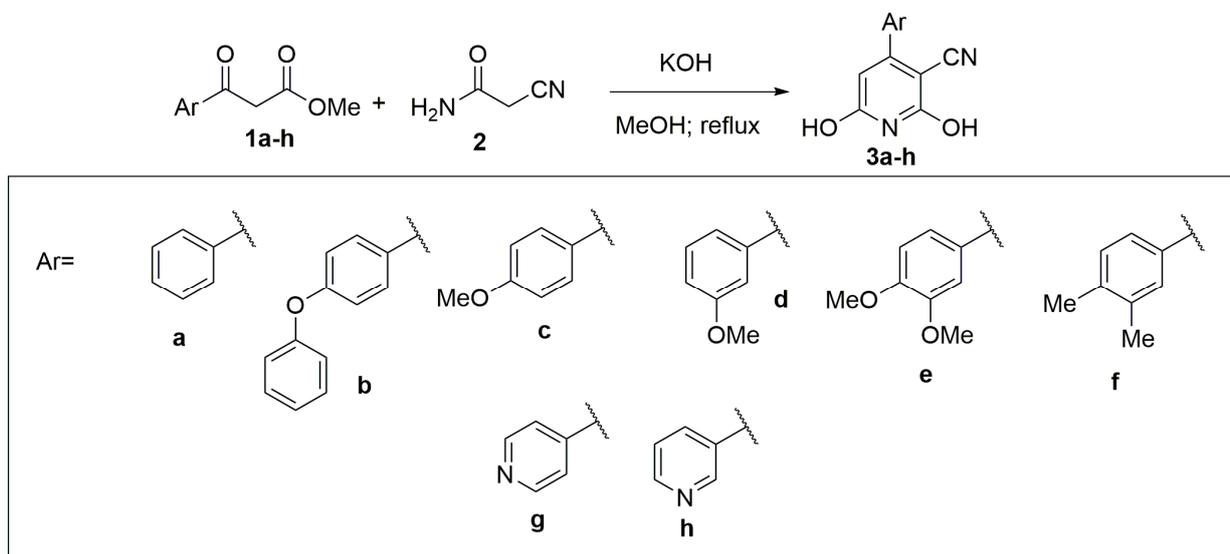
The synthesis, the biological activity, and the PI3K subtype selectivity of the compounds synthesized are presented herein.

## 2. Results and Discussion

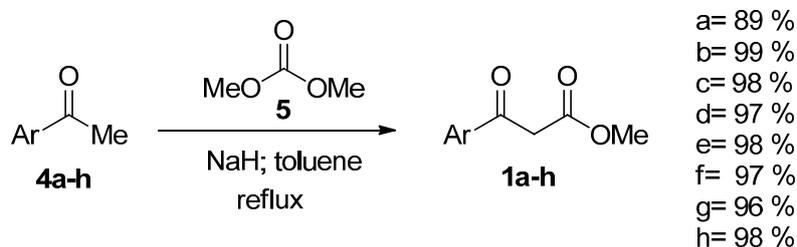
The 4-aryl-3-cyano-2,6-dihydropyridines (**3a–h**) were synthesized using the improved Guareschi pyridine synthesis reported by Bobbit and Scola [25]. This methodology consists of the condensation

of aryl  $\beta$ -keto esters (**1a–h**) and cyanoacetamide (**2**) in potassium hydroxide at reflux using methanol as solvent (Scheme 1).

Aryl  $\beta$ -keto esters **1a–h** were prepared from the corresponding commercially available aryl methyl-ketones **4a–h** by deprotonation with sodium hydride and quenching with dimethylcarbonate (**5**) [26] (Scheme 2).



**Scheme 1.** The Guareschi pyridine reaction.



**Scheme 2.** Synthesis of aryl  $\beta$ -keto esters.

Condensation of these crude dicarbonyl compounds with cyanoacetamide under basic conditions afforded the corresponding 4-aryl-3-cyano-2,6-dihydroxypyridines (**3a–h**) (Scheme 1). Despite our effort to optimize this reaction, the compounds were obtained in modest yields (Table 1).

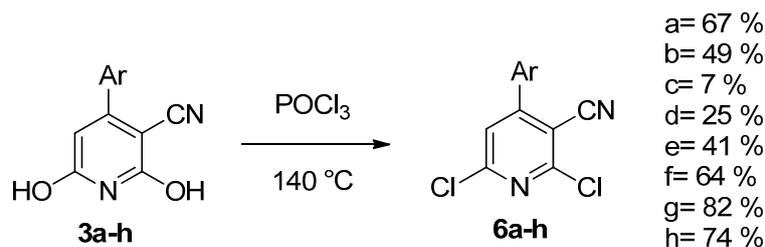
**Table 1.** Yields of 4-aryl-3-cyano-2,6-dihydroxypyridines.

Compound	Yield (%)
<b>3a</b>	41
<b>3b</b>	15
<b>3c</b>	14
<b>3d</b>	28
<b>3e</b>	9
<b>3f</b>	27
<b>3g</b>	26
<b>3h</b>	10

This result can be ascribed to the behavior of  $\beta$ -ketoesters which decarboxylate at high temperatures, and then they underwent ester hydrolysis to  $\beta$ -ketoacids under these basic conditions. In confirmation of this situation, we recovered aryl methyl-ketones from the reaction mixture.

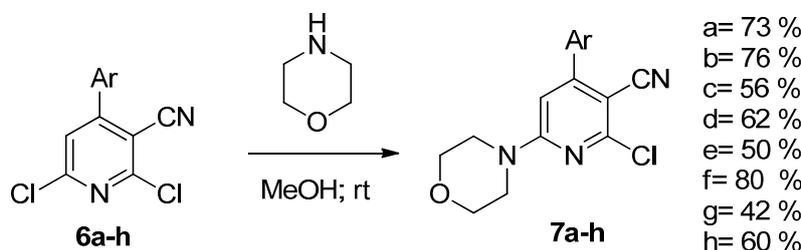
Unfortunately, under room temperature, the Guareschi reaction did not take place and the use of a milder reported procedure (1,4-diazabicyclo[2.2.2]octane or DABCO<sup>®</sup>, r.t.) failed to afford the desired products [27].

The key intermediates (**3a–h**) were then converted to the corresponding dichlorides (**6a–h**) with phosphorus oxychloride (Scheme 3).



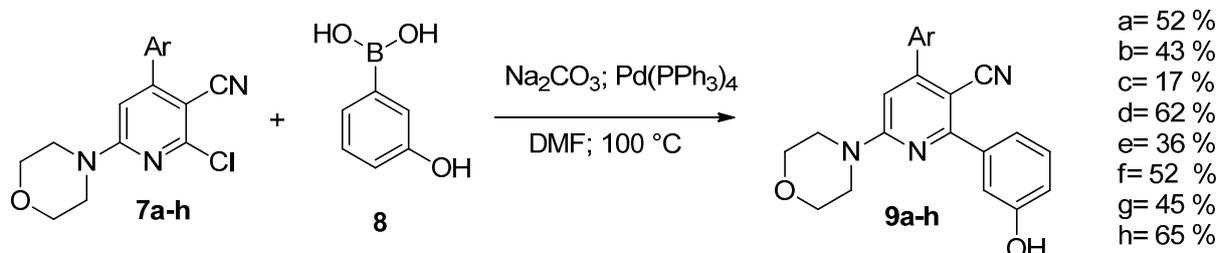
**Scheme 3.** Synthesis of the dichloride analogues.

The regioselective reaction with morpholine in methanol afforded the derivatives **7a–h** (Scheme 4). A recent paper demonstrated that regioselectivity at the six position can be easily achieved using a polar solvent like methanol [28].



**Scheme 4.** Regioselective addition of morpholine at the six position.

Finally, the desired tetrasubstituted pyridines **9a–h** were obtained via Suzuki coupling with 3-hydroxyphenylboronic acid (**8**) (Scheme 5).



**Scheme 5.** Synthesis of compounds **9a–h**.

All the synthesized compounds were then tested *in vitro* for their ability to inhibit the kinase activity of the PI3K $\alpha$ , PI3K $\beta$ , PI3K $\gamma$ , and PI3K $\delta$  isoforms in a luminescent kinase assay (ADP-Glo™ kinase assay) using purified, recombinant human enzymes and ATP. IC<sub>50</sub> values were subsequently determined.

As shown in Table 2, the compounds display an activity at low micromolar concentrations against the four isoforms, changing their profile of selectivity according to the type of aryl group on the four position of pyridine. In particular, for this series of compounds, the molecule **9b** stands over all the others, showing a remarkable profile of selectivity toward the PI3K $\alpha$  isoform.

**Table 2.** IC<sub>50</sub> inhibitory profile and standard error (SE).

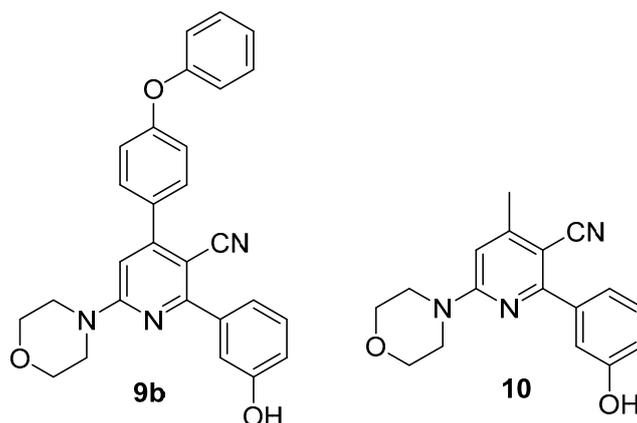
Compound	IC <sub>50</sub> Values ( $\mu$ M)			
	PI3K $\alpha$	PI3K $\beta$	PI3K $\gamma$	PI3K $\delta$
<b>9a</b>	0.13 (SE $\pm$ 0.03)	1.99 (SE $\pm$ 0.79)	>10	1.22 (SE $\pm$ 0.47)
<b>9b</b>	0.63 (SE $\pm$ 0.13)	>10	>10	>10
<b>9c</b>	1.79 (SE $\pm$ 0.47)	>10	2.18 (SE $\pm$ 0.51)	1.37 (SE $\pm$ 0.37)
<b>9d</b>	0.63 (SE $\pm$ 0.15)	2.34 (SE $\pm$ 0.54)	1.64 (SE $\pm$ 0.29)	1.09 (SE $\pm$ 0.19)
<b>9e</b>	0.83 (SE $\pm$ 0.15)	6.48 (SE $\pm$ 1.85)	2.91 (SE $\pm$ 0.56)	0.90 (SE $\pm$ 0.11)
<b>9f</b>	>10	>10	4.43 (SE $\pm$ 0.69)	1.33 (SE $\pm$ 0.19)
<b>9g</b>	1.23 (SE $\pm$ 0.14)	>10	1.56 (SE $\pm$ 0.27)	0.85 (SE $\pm$ 0.14)
<b>9h</b>	0.98 (SE $\pm$ 0.12)	>10	2.43 (SE $\pm$ 0.41)	0.71 (SE $\pm$ 0.13)

Subsequently, compound **9b** was analyzed on cell-based assay in order to evaluate its ability to inhibit the PI3K $\alpha$  signaling pathway on cells. It is well established that insulin signaling is mostly regulated by PI3K $\alpha$  [29]. The activated insulin receptor triggers PI3K $\alpha$  activity mainly by binding and phosphorylating adaptor proteins of the IRS (insulin receptor substrate) family. Once activated, PI3K $\alpha$  produces the PtdIns(3,4,5)P<sub>3</sub> lipid product, which in turn mediates the activation of downstream effectors such as Akt. Thus, phosphorylation of Akt results as a key event of the PI3K $\alpha$  activation. In order to define whether compound **9b** was able to inhibit PI3K $\alpha$  activity and, consequently, the insulin signaling, NIH3T3 cells were stimulated with insulin in the presence or absence of different concentrations of compound **9b**. A dose response curve and an IC<sub>50</sub> value were determined. Compound **9b** showed an IC<sub>50</sub> value of 2.8  $\mu$ M (SE  $\pm$  0.6), thus demonstrating its ability to inhibit PI3K $\alpha$  in a cellular context as well.

Molecular modeling studies were performed in order to investigate and rationalize this interesting selectivity. A simple search in the Protein Data Bank reveals over 50 releases spanning over all four PI3K isoforms, with only isoforms  $\alpha$  and  $\gamma$  available from human sources. Docking compounds to rationalize little differences in activity still remains a challenge [30]. However, in our case, compound **9b** shows a relevant difference in activity against PI3K $\alpha$  and PI3K $\gamma$ .

In order to verify the importance of the aryl group at the C<sub>4</sub> position to impart isoform selectivity, analogue **10**, where the aryl group has been replaced with a methyl group, was also considered and docked (Figure 3) (for the synthesis of **10** see supporting information).

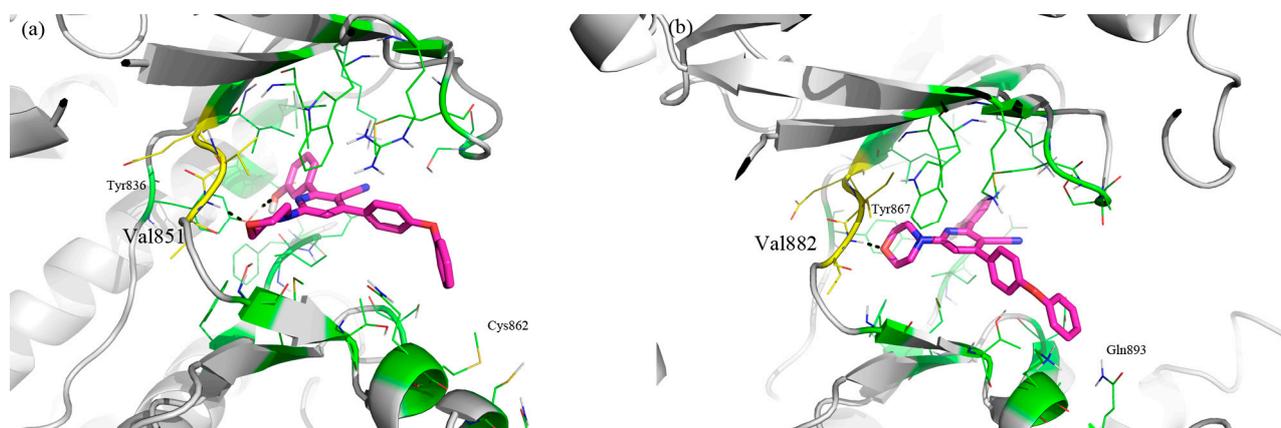
Compound **10** is indeed a pan-inhibitor against the different isoforms (PI3K $\alpha$  = 1.18  $\mu$ M  $\pm$  0.17, PI3K $\beta$  = 1.55  $\mu$ M  $\pm$  0.30, PI3K $\gamma$  = 1.05  $\mu$ M  $\pm$  0.10, and PI3K $\delta$  = 0.42  $\mu$ M  $\pm$  0.05).



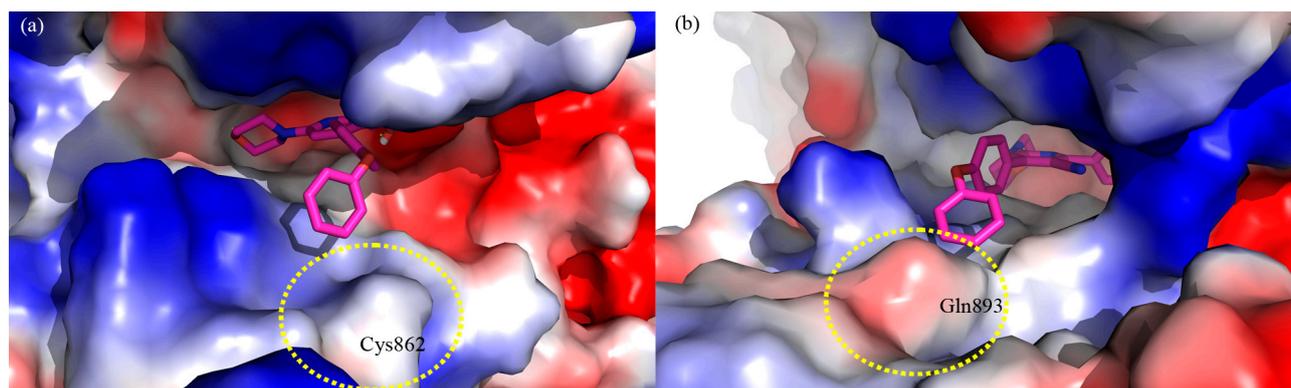
**Figure 3.** Compounds **9b** and **10** were docked on  $\alpha$  and  $\gamma$  human PI3Ks.

Both compounds were prepared using Omega2 [31–33], and the docking software FRED [34,35] was used to predict their binding poses. The docking results for compound **10** (Supporting Information Table S1, Figure S1) showed a shared binding mode over the considered isoforms  $\alpha$  and  $\gamma$ . The oxygen of morpholine is able to form a hydrogen bond interaction with the -NH of PI3K $\gamma$  Val882 (PI3K $\alpha$  Val851) in the hinge region, while the phenolic group acts as hydrogen bond donor interacting with the -OH of Tyr867 in PI3K $\gamma$  (Tyr836 in PI3K $\alpha$ ). The methyl and cyano groups protrude toward the solvent-exposed region.

Poses of compound **9b** are consistent with docking results of **10** (Figure 4a,b). In this case, the aryl group protrudes toward the solvent-exposed region. The second aryl group of **9b** fits in a hydrophobic zone near Cys862 in PI3K $\alpha$ , while in PI3K $\gamma$  a more polar environment is present. This is a consequence of the presence of Gln893 (Figure 5a,b). In PI3K $\alpha$  the terminal oxydibenzene ring is positioned above an electrostatically positive area (Figure 5a), and this would be an attracting interaction with the electronegative  $\pi$ -cloud of the terminal ring of **9b**. In PI3K $\gamma$  the area below the terminal oxydibenzene ring is electrostatically negative and that would make for an unfavorable interaction (Figure 5b).



**Figure 4.** Predicted binding pose of compound **9b** (purple sticks) in the PI3K $\alpha$  (a) and PI3K $\gamma$  (b) binding sites (green sticks and white cartoon); aminoacids of hinge region are shown as yellow sticks.



**Figure 5.** Details of surface electrostatic charges in PI3K $\alpha$  (a) and PI3K $\gamma$  (b). The colors denote the electrostatic charge at the surface of the protein from negative (blue) to positive (red).

### 3. Experimental Section

#### 3.1. Molecular Modeling

All molecular modeling studies were performed on a Tesla workstation equipped with two Intel Xenon X5650 2.67 GHz processors and Ubuntu 12.04 ([www.ubuntu.com](http://www.ubuntu.com)). All structural images were prepared using PyMOL [36] with the APBS plugin [37]. Different crystal structures of human PI3K isoforms with inhibitors have been reported; we select in our study the X-ray structures showing high resolution ( $<3 \text{ \AA}$ ) and high chemical similarity of cocrystallized ligand to our compounds. The selected Protein Data Bank (PDB) id were: human PI3K $\alpha$  (PDB id: 3ZIM, resolution 2.85  $\text{\AA}$ ) [38], and human PI3K $\gamma$  (PDB id: 3DBS, res. 2.80  $\text{\AA}$ ) [39]. Water molecules and ligands were removed; the binding site was detected using the original ligand coordinates (Supporting Information Figure S2). Hydrogens were added to the protein complexes, protonation and tautomer states were set, and finally the hydrogen bond network was optimized using the MolProbity server. [40]. Ligand structures were built from a SMILES string and were minimized using Omega2 [31–33]. A conformer library was built with the program Omega2, which generated an average of 81 conformations per molecule. Default parameters were used. The docking simulations were performed using FRED [34,35]. Two constraints were applied due to the presence of hydrogen bond between the original ligands and active pocket residues (referring PI3K $\gamma$  numbering: Val882, backbone amide hydrogen, and Tyr867, phenol oxygen). Default settings were used.

#### 3.2. Chemistry

Synthetic procedures and spectral data are reported in the Supporting Information.

#### 3.3. *In Vitro* Enzyme Inhibition

To evaluate the ability of each compound of formula (9a–h) to inhibit the lipid kinase activity of each PI3K isoform, 30 ng of PI3K $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  recombinant protein (purchased from JenaBioscience—Jena, Germany; PI3K $\alpha$  #PR-335, PI3K $\beta$  #PR-344, PI3K $\gamma$  #PR-343, PI3K $\delta$  #PR-345) were incubated with at least five different concentrations (from 0.016 to 10  $\mu\text{M}$ ) of the compounds (9a–h). The

phosphorylation reaction was then started by adding 10  $\mu\text{M}$  of ATP, and 10  $\mu\text{g}$  of lipid micelles containing the appropriate substrate phosphatidylinositol and the phosphatidylserine [41,42].

After 30 min of incubation (1 h for PI3K $\beta$  recombinant protein) at room temperature, the reaction was stopped, and the amount of ADP metabolite was measured with a luminescent kinase assay, ADP-Glo™ Kinase Assay (purchased from Promega—USA #V9101). The luminescent signal positively correlated with the formed ADP and, therefore, with the kinase activity. The percentage of kinase activity was calculated over the control samples, containing only dimethyl sulfoxide (DMSO). To derive the IC<sub>50</sub> values for each assayed compound (**9a–h**), all data were plotted on a dose response curve (Graph Pad Software) and the IC<sub>50</sub> was calculated by using the nonlinear regression fit (equation (log agonist) vs. response—Sigmoidal dose response). The IC<sub>50</sub> was representative of two different experiments performed in triplicate.

### 3.4. Cell-Based Assay

NIH3T3 cells were cultured in Eagle's minimal essential medium (DMEM) (GIBCO-Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO-Thermo Fisher Scientific Inc.), 100 units/mL penicillin (GIBCO-Thermo Fisher Scientific Inc.), and 100  $\mu\text{g}/\text{mL}$  streptomycin (GIBCO-Thermo Fisher Scientific Inc.). The day before the assay, NIH3T3 cells were seeded in a 96-well plate at a concentration of  $5 \times 10^4$  cells/mL and starved O.N. in presence of DMEM 0.5% FBS. Subsequently, cells were pre-treated for 1.5 h with the inhibitor at different concentrations (From 0.03  $\mu\text{M}$  to 20  $\mu\text{M}$ ). Then cells were stimulated for 5 min with DMEM containing 1  $\mu\text{M}$  of insulin and proteins were extracted with Laemmli buffer. The P-Akt production is detected by Western blot analysis and quantified with ChemiDoc™ XRS system. From the absolute values, a percentage of residual P-Akt is calculated for each inhibitor concentration by using the control vehicle as 100%. To derive the IC<sub>50</sub>, all data are plotted on a dose response curve (Graph Pad software) and the IC<sub>50</sub> is calculated by using the non-linear regression fit (equation (log agonist) vs. response—Sigmoidal dose response) on three different experiments.

## 4. Conclusions

In this manuscript, we described the use of 4-aryl-3-cyano-2-(3-hydroxyphenyl)-6-morpholino-pyridines as valuable starting points for the synthesis of PI3K inhibitors. We demonstrated that the modifications on the C<sub>4</sub>-position of this pyridine scaffold could impart a different profile of selectivity on the PI3K isoforms according to the pattern of substitution.

Compound **9b** was the most interesting of the series, with an activity profile selective for the PI3K $\alpha$  isoform.

Molecular modeling studies tried to rationalize this profile of selectivity, pointing out that the presence of a polar Gln893 in the PI3K $\gamma$  isoform may disrupt the binding of **9b** through unfavorable electrostatic interactions.

Given these interesting results, further medicinal chemistry efforts are in progress to synthesize more structurally diverse analogues using the Guareschi reaction with the aim to increase the potency and identify different profiles of selectivity.

## Supplementary Materials

Supplementary materials can be accessed at: <http://www.mdpi.com/1420-3049/20/09/17275/s1>.

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## Author Contributions

U.G. carried out the synthesis of the compounds and wrote synthetic procedures; E.C. and J.P.M. evaluated the biological activity; A.M. carried out the molecular docking studies; G.S. and G.C.T. designed the research; G.C.T. and E.H. wrote the paper. All the authors read and approved the final manuscript.

## Conflicts of Interest

E.H. and G.C.T. are co-founders of Kither Biotech Srl. The other authors declare that they do not have competing interests.

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*Sample Availability:* Samples of the compounds **9a–h** and **10** are available from the authors.

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