Discovery of a new class of triazole based inhibitors of acetyl transferase KAT2A

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ABSTRACT
We have recently developed a new synthetic methodology that provided both N-aryl-5-hydroxytriazoles and N-pyridine-4-alkyl triazoles. A selection of these products was carried through virtual screening towards targets that are contemporary and validated for drug discovery and development. This study determined a number of potential structure target dyads of which N-pyridinium-4-carboxyl-5-alkyl triazole displayed the highest score specificity towards KAT2A. Binding affinity tests of abovementioned triazole and related analogs towards KAT2A confirmed the predictions of the in-silico assay. Finally, we have run in vitro inhibition assays of selected triazoles towards KAT2A; the ensemble of binding and inhibition assays delivered pyridyl-triazoles carboxylates as the prototype of a new class of inhibitors of KAT2A.

1. Introduction
1,2,3-triazoles are commonly recognised as important chemical scaffolds as well as efficient molecules in the area of medicinal chemistry. Given their versatile behaviour in acting as both Lewis acids and bases, triazoles have been used as a core structural motif in a huge variety of drug classes such as: antimicrobial, anti-inflammatory, antineoplastic, antiviral, antihypertensive and as immunomodulatory agents.

We have recently reported a novel synthetic pathway that, by reacting β-ketoesters 1 and azides 2, provided 1,2,3-triazoles 3 or 4 (Scheme 1). The reactions employing 2-unsubstituted β-ketoesters were found to provide 5-methyl-1,2,3-triazoles 4, whereas 2-alkyl-substituted β-ketoesters provided 5-hydroxy 1,2,3-triazoles 3 in high yields and as a single regioisomer. As a follow up of this work, we have posed the question of whether or not those classes of new compounds may be useful in medicinal chemistry. Triazoles were repeatedly reported as bioactive compounds and many drug candidates containing the pyridine ring were equally described. Pyridines are commonly used in medicinal chemistry because of their ability to establish hydrogen bonds either as donors or acceptors, their water solubility, small dimensions and, most importantly, their potential to act as amide bioisosteres. The latter in particular, makes pyridine displays the highest score specificity towards KAT2A. Binding affinity tests of abovementioned triazole and related analogs towards KAT2A confirmed the predictions of the in-silico assay. Finally, we have run in vitro inhibition assays of selected triazoles towards KAT2A; the ensemble of binding and inhibition assays delivered pyridyl-triazoles carboxylates as the prototype of a new class of inhibitors of KAT2A.

ACCEPTED MANUSCRIPT

ARTICLE HISTORY
Received 26 May 2022
Revised 23 June 2022
Accepted 28 June 2022

KEYWORDS
N-pyridine triazoles; KAT2A inhibitors; virtual screening; acetyl transferases; anti-cancer

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recently introduced and proved to have a higher activity and specificity compared to the natural products.

Unfortunately, most of the HATs inhibitors known showed lack of selectivity towards members of the HAT family and, therefore, selectivity for a specific enzyme, for example, KAT2A, is still an outstanding issue.

Herein we describe each stage of our research that led to the identification of compound 16 (Figure 2) as a new KAT2A inhibitor template. This study includes virtual screening, synthesis, binding studies and in vitro testing.

2. Results and discussion

2.1. Virtual screening

Compounds 11–18 were submitted to virtual screening towards a database of proteins with established structure and role in system biology using the BioGPS software in combination with the FLAP algorithm. The BioGPS workflow used for the docking screening of triazoles 11–18 consisted in 5 parts: i) Protein refinement, achieved by using an algorithm known as "Fixpdb" that enables the preparation of the protein structure obtained from the Protein Data Bank (PDB); ii) Cavity detection, achieved by using an algorithm known as "Flapsite" that allows for the identification of pocket points located within a distance of 4 Å maximum from the closest protein; iii) and iv) Cavity characterisation and comparison, achieved by the FLAP algorithm (Fingerprints for Ligands And Proteins) that enables the identification of the potential complementary ligand pharmacophoric features for a protein binding site; v) Data analysis, in which each pocket/target similarity is analysed by using the global scores that attribute to 0 no similarity and to 1 maximum similarity. This study provided docking results and score distribution of 11–18 versus the protein panel (Table 1). Importantly each of the proteins listed in Table 1 is a significant target for medicinal chemistry as for example KAT2A.

The results collated in Table 1 report the following: (1) numbers are arbitrary scores comprised between 1 and 100 and reflect the fitting of molecules 11–18 to the enzyme pocket; (2) red cells indicate a bad ligand-protein fitting; (3) green cells indicate medium ligand-protein fitting meanwhile (4) blue cells indicate a good ligand protein fitting. The results obtained showed the following: (i) triazoles 11–14, bearing a hydroxyl group at position C5, did not show significant binding scores (Table 1, except for Rab7a48 and Serine/Threonine-protein phosphatase 5 (PP5); (ii)
long chain acid 15 showed a good fitting with sixteen proteins (Table 1) hence lacked selectivity from the premises and for this reason was discarded. In conclusion, this dataset showed that the presence of a 5-hydroxy, or its ketol form, on the triazole generated a class of compounds that were not useful for further medicinal chemistry investigations with the 20 selected proteins. Equally, compound 17 (Table 1), showed a promiscuous behaviour with up to thirteen proteins and, therefore, was abandoned.

At this point we focussed our attention on compound 16, which docking with KAT2A was reported to be peculiarly higher than any of the other binders 11–15 and 17,18, reaching 80.13 (Table 1)11. Considering that many of the known ligands for KAT2A comprise a carboxylate functionality (Figure 3), we hypothesised that the binding data for compound 16 were dependent on the presence of a carboxylic function at the C4 position which favoured the interaction of triazole 16 with the desired target (Figure 4), whereas triazole 18, bearing an ester function at C4, showed a lower binding score with KAT2A (Table 1). In support of our hypothesis, polar contacts connecting 16’s OH function of the carboxylic moiety and 16’s N2-N3 atoms of the triazole ring interacted with the NH functions of KAT2A Arg-558 residue (shown with blue and light blue sticks, Figure 4).

In particular, 16’s N2 and N3 atoms of the triazole ring coordinate arginine NH number 20289 (2.2 Å and 2.1 Å distance respectively, Figure 4) while the N3 atom of the triazole ring and the OH function of the carboxylic moiety coordinate arginine NH number 20292 (2.3 Å and 2.5 Å distance respectively, Figure 4). The low binding-affinity of triazole 18 with all the 20 proteins and the interactions of 16 with KAT2A identified in this work (Figure 4), clearly indicated that this selection of proteins required triazole binders to possess hydrogen-bond donor and acceptors units at C4 and C5, which may have been a discriminator in the selection of targets from the BioGPS database46. In summary, the analysis of the docking studies identified 16 as a potential scaffold.

### Table 1. Docking scores for triazoles 11–18 found for this dataset of twenty proteins.

| Protein name | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|--------------|----|----|----|----|----|----|----|----|
| Carbonic anhydrase 2 | 51.62 | 47.49 | 50.22 | 42.64 | 53.03 | 53.65 | 48.18 | 26.41 |
| Carboxypeptidase B2 | 50.19 | 53.3 | 57.7 | 49.04 | 71.82 | 56.12 | 61.97 | 43.01 |
| Eukaryotic translational initiation factor 4E | 47.4 | 49.24 | 44.97 | 43.65 | 78.9 | 54.62 | 48.1 | 47.98 |
| Heparan sulphate glucosamine 3-O-sulfotransferase 3A1 | 43.76 | 43.09 | 44.65 | 43.65 | 78.9 | 54.62 | 48.1 | 47.98 |
| Histone acetyltransferase KAT2A | 44.25 | 54.84 | 47.54 | 47.97 | 76.47 | 80.13 | 72.29 | 44.2 |
| Inositol-tetrakisphosphate 1-kinase | 45.49 | 47.46 | 42.28 | 46.08 | 68.44 | 55.84 | 55.52 | 47.76 |
| Lysine-specific demethylase 4C | 47.58 | 46 | 54.22 | 47.21 | 65.45 | 58.89 | 70.85 | 44.04 |
| Methionine aminopeptidase 1 | 52.43 | 50.44 | 51.82 | 46.12 | 76.33 | 63.55 | 59.14 | 45.68 |
| Methionine aminopeptidase 2 | 46.83 | 48.25 | 45.71 | 40.11 | 43.77 | 54.88 | 50.94 | 44.54 |
| Mitofusin-1 | 48.08 | 50.98 | 52.7 | 52.65 | 74.23 | 65.94 | 59.63 | nd |
| NAD(P)H dehydrogenase [quinone] 1 | 48.01 | 52.78 | 50.22 | 48.23 | 78.42 | 62.76 | 55.66 | 45.84 |
| Phosphoethanolamine carboxykinase, cytosolic [GTP] | 49.82 | 46.77 | 50.86 | 41.29 | 67.21 | 50.96 | 63.36 | 43.08 |
| Phospholipase A2, membrane associated | 52.93 | 53.72 | 54.52 | 47.57 | 70.81 | 56.78 | 65.98 | 54.32 |
| Polypeptide N-acetylglactosaminyltransferase 2 | 50.69 | 57.76 | 55.42 | 51.96 | 76.42 | 68.87 | 65.06 | 55.17 |
| Pyruvate kinase PM | 52.34 | 53.33 | 53.53 | 50.28 | 68.36 | 49.53 | 63.46 | 51.71 |
| Ras-related C3 botulinum toxin substrate 1 | 50.34 | 56.99 | 50.44 | 68.85 | 77.54 | 71.79 | 54.26 |
| Ras-related protein Rab7a | 51.23 | 51.73 | 58.5 | 52 | 67.27 | 44.8 | 62 | 58.08 |
| Serine/threonine-protein phosphatase 5 | 51.42 | 46.91 | 65.49 | 50.2 | 64.87 | 50.57 | 51.54 | 53.92 |
| Sulfotransferase 1e4 | 48.97 | 50.29 | 44.49 | 48.16 | 56.55 | 51.19 | 49.17 | 44.1 |
| Sulfotransferase family cytosolic 1B member 1 | 49.44 | 52.89 | 46.34 | 45.75 | 67.73 | 58.64 | 64.66 | 51.8 |

Scores are intended as arbitrary values (on a maximum of 100) according to the Molecular Interaction Fields (MIFs) used in the calculation. Red cells indicate a bad ligand-protein fitting; Green cells indicate medium ligand-protein fitting; Blue cells indicate a good ligand protein fitting.
to be evaluated as KAT2A inhibitor. For this reason, we proceeded with the synthesis of a small library of triazole 16 analogs 26a-e and 27a-d.

### 2.2. Synthesis of pyridyl-triazoles 26a-f and 27a-d

A small library of 1,2,3-triazoles 26a-e and 27a-d bearing either a carboxylate or an ester functionality at C4 was therefore prepared (Table 2). We anticipated that the carboxylic functionality was indeed crucial to high binding, however, we also noted that compound 18, i.e., the ethyl ester of compound 16, showed a significant binding for KAT2A. Hence, both carboxylates and their esters were included in the library.

The synthesis of compounds 26a-e has been carried out following a procedure previously reported12. Pyridine azides 2a-e were reacted with alkynes 25a,b to give triazoles 26a-e via a CuAac cycloaddition protocol (Table 2). Pyridine-based triazoles 26a-d were obtained in high yields (Table 2). Considering that carboxylates were found better ligands by virtual screening, as highlighted in the docking results (Table 1), we then proceeded with the hydrolysis of esters 26a,b and 26c,e to reveal the corresponding acids 27a-d (Table 3). This entailed standard saponification using a solution of potassium hydroxide, which provided 27a-d in good to high yields.

### 2.3. Preliminary binding studies through fluorescence analysis

The binding properties, alongside the ability of 16 and 27a-d to inhibit the activity of KAT2A were evaluated using a standard protocol based upon the measurement of fluorescence51. KAT2A fluorogenic assay was developed by others to screen for inhibitors of this enzyme52. This test is based on the transfer of an acetyl group from acetyl-CoA (acetyl coenzyme A) to a peptide substrate. After incubation with acetyl-CoA and the inhibitor, the KAT2A acetylating activity at just 5 μM concentration (coloured in blue). A significant divergence was observed for the behaviour of 27d, which holds a p-CI substitution on the pyridine ring. The results collected indicated that 27d seemed to promote rather than inhibit the enzymatic activity of KAT2A, as highlighted by the increasing level of CoASH produced vs concentration (Figure 5). This result was most unexpected in lieu of the striking similarity between 27d and 27a-c and it should be looked in more details in future work.

In summary, we have demonstrated that triazoles 16, 26c and 27a-c possess inhibitory activity of KAT2A whereas only 27d proved to behave as an activator. Compounds 16, 26c and 27a-d were then subjected to in vitro assays in a cell model displaying dysfunctional activity of KAT2A to confirm the bioactivity.

### 2.4. In vitro testing of triazoles 16, 26c and 27a-d

Compounds 16, 26c and 27a-d were tested in U937 cell line (from human myeloid leukaemia, AML) in which KAT2A is known to be overexpressed53. U937 cells were stimulated with different concentrations of compounds 16, 26c and 27a-d, starting from 200 μM. Cell viability was measured by thiazoyl blue tetrazolium

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Table 2. Synthesis of pyridyl-based triazoles 26a-e

|Entry| Azide| R | R' | Alkynec| R''| Product| Yield (%)b |
|-----|------|---|----|---------|----|--------|------------|
|1    | 2a   | H | H  | 25a     | COOEt| 26a    | 90         |
|2    | 2a   | H | H  | 25b     | (CH2)m-COOH| 26b  | 89         |
|3    | 2b   | OCH3| H | 25a     | COOEt| 26c    | 78         |
|4    | 2c   | H | CH3| 25a     | COOEt| 26d    | 94         |
|5    | 2d   | H | Cl  | 25a     | COOEt| 26e    | 99         |

*Reaction conditions: 2a-d (1 equiv.), Cu(OTf)2·CH3CH2 (0.1 equiv.), tolune (0.25 M).
*Isolated yields.

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Table 3. Hydrolysis of triazoles 26a,b-d,e reveal corresponding carboxylates 27a-d

|Entry| Triazole| R | R'  | Product| Yield (%)b |
|-----|----------|---|-----|--------|------------|
|1    | 26a      | H | H   | 27a    | 87         |
|2    | 26b      | OCH3| H  | 27b    | 70         |
|3    | 26c      | H | CH3| 27c    | 71         |
|4    | 26e      | H | Cl  | 27d    | 64         |

*Reaction conditions: 26a,b-d,e (1 equiv.), KOH (1 equiv.), H2O (1 M).
*Isolated yields.
Figure 5. KAT2A fluorescence tests performed on triazoles 16, 26c and 27a-d.

Figure 6. MTT assay performed on triazoles 16, 26c and 27a-d. NT = non treated.
bromide (MTT) assay. This assay displayed a slight reduction (10–20%) in cell viability by all the compounds tested (Figure 6).

To investigate compounds 16, 26c and 27a-d induced inhibition of KAT2A acetylation levels of histone H3K9/14ac were analysed. To this end, U937 cells were treated with a 200 µM concentration of compounds 16, 26c and 27a-d for 24 h. SAHA (suberoylanilide hydroxamic acid), a known histone deacetylase inhibitor, was used as a positive control of acetylation at 5 µM concentration. Histone extraction and subsequent Western Blot (WB) analysis were carried out checking H3K9/14ac acetylation levels. Triazole 26c showed 40% reduction in the acetylation of H3K9/14ac (Figure 7) which was the strongest inhibition value obtained.

Considering the results obtained, it is possible that a lateral and flexible chain at C4 on the triazole ring is required to allow a tight interaction of triazoles and KAT2A. This in addition to the presence of a hydrogen-bond acceptor site which is represented by the carboxylate function.

### 3. Conclusion

In conclusion, we identified a new class of binders/inhibitors of KAT2A which comprises a pyridine and a long chain carboxylate linked via a triazole ring. The study used virtual screening to select a small library of pyridine-based 1,2,3-triazoles, including 26a-e and 27a-d. We then submitted triazoles 26a-e and 27a-d to fluorescence binding assays versus KAT2A enzyme which confirmed the binding abilities of these entities to KAT2A. Fluorescence binding assays revealed that only triazoles 27a-d and 26c interacted with KAT2A, meanwhile their correspondent ester analogs 26a,b,e,f did not show any binding. Finally, we evaluated the in vitro activity of 26c and 27a-d in U937 cell line of human AML and found out 26c to be the most active compound showing a 40% inhibition of KAT2A acetylating activity. It is noteworthy that compound 26c, having a longer chain, displayed the best inhibitory activity in vitro; meanwhile, shorter chain carboxylate, alike 16 or 27, that were demonstrated optimal binders, showed reduced activity compared to 26c. Studies are currently ongoing to (1) determine the structure-activity relationship (SAR) of 26c analogs and (2) improve potency and selectivity of this new template (26c) for KAT2A inhibition at a lower dose concentration.

### Acknowledgements

The authors acknowledge IRC GOIPG/2018/3165 for support to RP and VALERE: “Vanvitelli per la Ricerca Program: EPInhibitDRUGre (CUP B66J20000680005) and Programma Operativo Nazionale (PON) Ricerca e Innovazione 2014–2020 AIM attrazione e mobilità dei ricercatori” for supporting NDG and GB research about KAT2A biological assays.

### Disclosure statement

No potential conflict of interest was reported by the author(s).

### Funding

This work was supported by Irish Research Council (GOIPG/2018/3165), Programma Operativo Nazionale Ricerca e Competitività (Ricerca e Innovazione 2014–2020 AIM), and VALERE (EPInhibitDRUGre (CUP B66J20000680005)).

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### Figure 7

WB analysis (on the left) of 16, 26c and 27a-d showing H3K9/14Ac levels in U937 cells following 24 h treatment at the concentration of 200 µM; 5 µM SAHA treatment was used as a positive control of acetylation. Densitometric analysis of WB is shown on the right.
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