Structural Chemistry (2022) 33:769–793
https://doi.org/10.1007/s11224-022-01889-0

ORIGINAL RESEARCH

Synthesis and inverse virtual screening of new bi-cyclic structures towards cancer-relevant cellular targets

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Received: 6 January 2022 / Accepted: 25 January 2022 / Published online: 11 February 2022

Abstract

We report here synthetic approaches to access new classes of small molecules based on three heterocyclic scaffolds, i.e. 3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione, 1,8-naphthyridin-4(1*H*)-one and 4H-pyrido[1,2-a]pyrimidin-4-one. The bi-cyclic structure 3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione is a new heterocycle, described here for the first time. In silico methodologies of inverse virtual screening have been used to preliminary analyse the molecules, in order to explore their potential as hits for chemical biology investigations. Our computational study has been conducted with 43 synthetically accessible small molecules towards 31 cellular proteins involved in cancer pathogenesis. Binding energies were quantified using molecular docking calculations, allowing to define the relative affinities of the ligands for the cellular targets. Through this methodology, 16 proteins displayed effective interactions with distinct small molecules within the matrix. In addition, 23 ligands have demonstrated high affinity for at least one cellular protein, using as reference the co-crystallised ligand in the X-ray structure. The evaluation of ADME and drug score for selected hits also highlights that these new molecular series can serve as sources of lead candidates for further structure optimisation and biological studies.

Keywords Inverse virtual screening · Heterocycles · Scaffold diversity · Bi-cyclic scaffold · ADME assessment

Introduction

Heterocyclic chemistry is a key source of compounds for drug discovery due to the capability of the resulting molecules to imitate the structure of endogenous ligands and bind to a variety of biological targets [1, 2]. In medicinal chemistry, heterocyclic structures offer the advantage of generating molecular libraries based on a specific core scaffold, permitting screening tests and structure–activity relationship (SAR) studies for the targets of interest [3]. In particular, fused heterocycles can be generated with nearly boundless combinations, to result into novel bi- or polycyclic frameworks with diverse physico-chemical and biological properties. Overall, the combination of rings leads to rigid and sterically well-defined structures, ensuring a high degree of functional specialisation, as well as the capability to arrange substituents in three-dimensional space as required by nature for interactions with biological targets [4]. From cancer therapy to the treatment of infectious, parasitic and metabolic illnesses, heterocyclic drugs are often used to interfere with the actions of hormones, the neuronal transmission or the activity of enzymes, to name a few.

Scaffold diversity (i.e. variation of the nature of core scaffolds), appendage diversity (i.e. variation in structural moieties around a common scaffold), functional group diversity (i.e. variation of the functional groups present in the molecules) and stereochemical diversity (i.e. variation in the three-dimensional orientation of macromolecule-interacting residues) are determinant for late state chemical functionalisation in drug discovery [5]. Undoubtedly, the more diverse a molecule is, the more likely it can interact with a specific biological macromolecule in a distinctive way [6–9]. In particular, scaffold diversity demonstrates a key element

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to reach increased structural variation and investigate new areas of the chemical space for biological investigations [10]. In this regard, it has been reported that the overall shape diversity of small molecules is formally subordinate to the nature of the particular molecular scaffold, with the peripheral substituents having a lower impact [7]. Therefore, the exploitation of scaffold diversity represents a valid strategy to implement the whole structural diversity of small molecule libraries [10–12].

In the context of exploring scaffold diversity in biology and medicine, heterocycles can lead to increase molecular functionalisation, due to their potential in terms of structural isomerism. This can enhance the overall diversity within libraries, as well as the possibility of identifying hit candidates. Additionally, heterocycles containing nitrogen, oxygen or sulphur that can form hydrogen bonds with functional groups within the protein binding site offer wide opportunities to carry out drug optimisation strategies, aimed at tuning the affinity for a particular target, as well as the solubility, lipophilicity, polarity and the overall drug-like properties.

Computational methods such as structure-based and ligand-based screening are widely used in various drug discovery contexts, spanning from hit identification to lead optimisation stages [13, 14]. However, these virtual screening approaches are somehow limited to the analysis of only one target at a time [15, 16]. In contrast, inverse virtual screening (iVS) is a computational technique where libraries of compounds are simultaneously tested towards multiple potential targets, in order to identify hits with pharmacological activity [17–19]. Overall, iVS allows for a quicker analysis of target identification, potential side effects, toxicity, drug repositioning, receptor design and multitarget therapy.

Herein, we report the synthesis and iVS analysis of 43 new heterocyclic small-molecules towards a scaffold-guided structural diversity approach, to identify new hits for biological studies in the field of cancer therapy. Specifically, 31 cellular proteins that are responsible for promoting growth, proliferation, differentiation and metabolism in cancer cells [20, 21] were screened against the 43 novel heterocyclic small molecules based on pyrimido[4,5-d]pyridazine-4,8-dione, 1,8-naphthyridin-4-one and pyrido(1,2-a)pyrimidine scaffolds (Fig. 1). The pyrimido[4,5-d]pyridazine-4,8-dione is a new bi-cyclic structure, whose synthesis was not reported in the literature to date. This study indicates that most of the compounds within the library have potential to interact with the examined targets, representing a valid starting point to drive biological evaluation in a rapid and cost-effective fashion. The molecules were also screened via physiologically based pharmacokinetic (PBPK) analysis, to further assess permeability and absorption features as drug candidates.

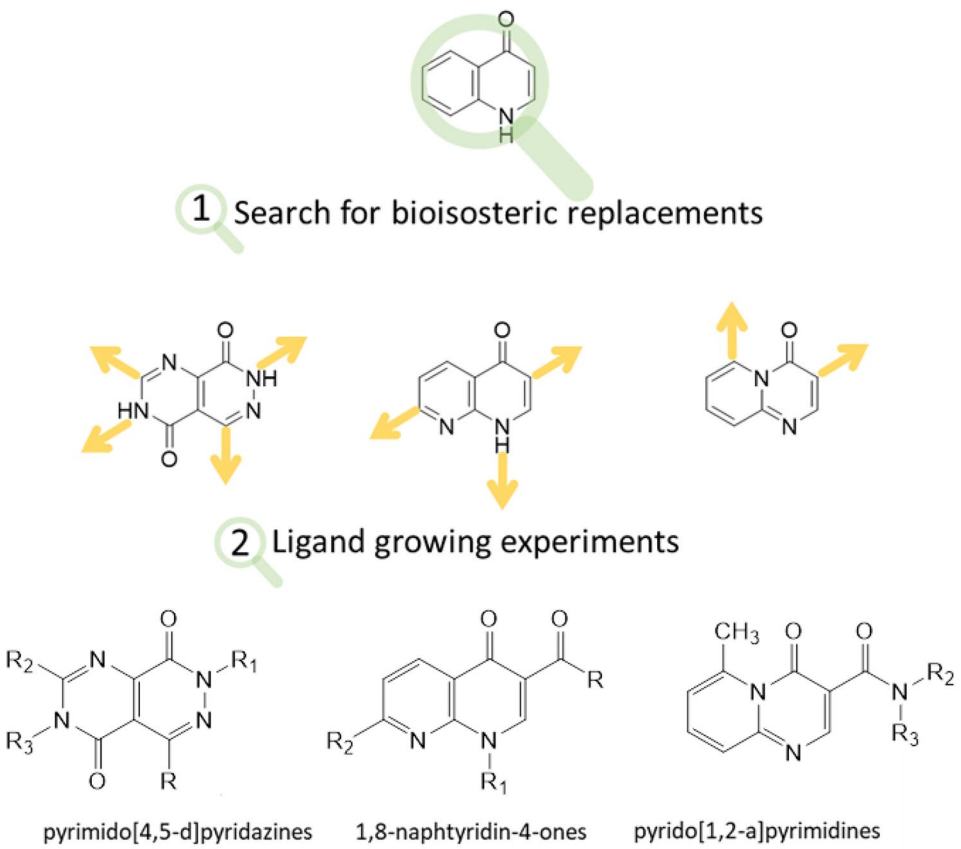


Fig. 1 Schematic representation of the two-step design

Materials and methods

General remarks

Reagents and starting materials were obtained from commercial sources. Extracts were dried over Na_2SO_4 and the solvents were removed under reduced pressure. All reactions were monitored by thin layer chromatography (TLC) using commercial plates (Merck) pre-coated with silica gel 60 F-254. Visualisation was performed by UV fluorescence (λmax = 254 nm) or by staining with iodine or potassium permanganate. Chromatographic separations were performed on silica gel columns by gravity (Kieselgel 40, 0.063–0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. When reactions were performed in anhydrous conditions, the mixtures were maintained under nitrogen atmosphere. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom 2000 (4.01.305) or CA Index Name. The identity and purity of intermediates and final compounds were ascertained through TLC chromatography, NMR and mass spectrometry. ^{1}H NMR spectra were recorded on the Avance 400 instrument (Bruker Biospin Version 002 with SGU). Chemical shifts (δ) are reported in ppm to the nearest 0.01 ppm, using the solvent as internal standard. Coupling constants (*J* values) are given in Hz and were calculated using 'TopSpin 1.3' software rounded to the nearest 0.1 Hz. Data are reported as follows: chemical shift, multiplicity (exch, exchange; br, broad; s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; sext, sextet; sept, septet; m, multiplet; or as a combination of these (e.g. dd, dt etc.)), integration, assignment and coupling constant(s). All melting points were determined on a microscope hot stage Büchi apparatus and are uncorrected.

Chemistry

General procedure for compounds 5c, 5e Hydrazine hydrate (3 mmol) was added to a cooled (0 °C) and stirred suspension of commercially available isoxazole derivatives **4c,e** (2 mmol) and PPA (50 mmol) in ethanol (5 mL), and the mixture was heated under stirring for 2–5 h at 80–90 °C. After dilution with cold water (20–30 mL), pure compounds **5c** and **5e** were recovered by filtration under vacuum.

- *4-(3-Chlorophenyl)isoxazolo[3,4-d]pyridazin-7(6H)-one (**5c**)*

  Yield = 96%, (475 mg); mp = 241–244 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.55–7.65 (m, 2H, Ar), 7.90 (d, 2H, Ar, *J* = 8.0 Hz), 10.35 (s, 1H, isoxazole), 12.90 (exch br s, 1H, NH). Anal. Calcd. for $C_{11}H_6ClN_3O_2$: C 53.35, H 2.44, N 16.97. Found: C 53.21, H 2.44, N 16.92.
- *4-Pyridin-3-yl-isoxazolo[3,4-d]pyridazin-7(6H)-one (**5e**)*

  Yield = 69%, (463 mg); mp = 227–228 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.55–7.65 (m, 1H, Ar), 8.25 (d, 1H, Ar, *J* = 8.4 Hz), 8.75 (d, 1H, Ar, *J* = 8.2 Hz), 9.05 (s, 1H, Ar), 10.35 (s, 1H, isoxazole), 12.90 (exch br s, 1H, NH). Anal. Calcd. for $C_{10}H_6N_4O_2$: C 56.08, H 2.82, N 26.16. Found: C 56.23, H 2.81, N 26.23.

General procedure for compounds 6a-e A suspension of isoxazolo pyridazinones **5a-e** (0.7–1.2 mmol) in 33% NH_4OH (2.0–2.5 mL) was stirred at 60 °C for 1–2 h. After cooling, the precipitate was recovered by filtration under vacuum to give the pure intermediates **6a-d**. For compound **6e**, the mixture was diluted with water, the suspension was extracted with CH_2Cl_2 (3 × 15 mL) and the solvent was evaporated in vacuo to afford the compound.

- *5-Amino-6-oxo-3-phenyl-1,6-dihydropyridazine-4-carboxylic acid amide (**6a**)*

  Yield = 46% (74 mg); mp ≥ 300 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 6.40 (exch br s, 2H, NH_2), 7.30–7.45 (m, 5H, Ar), 7.45–7.50 (exch br s, 2H, $CONH_2$), 12.80 (exch br s, 1H, NH). Anal. Calcd. for $C_{11}H_{10}N_4O_2$: C 57.39, H 4.38, N 24.34. Found: C 57.55, H 4.37, N 24.26.
- *5-Amino-3-(3-fluorophenyl)-6-oxo-1,6-dihydropyridazine-4-carboxylic acid amide (**6b**)*

  Yield = 50% (119 mg); mp = 285–287 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 6.45 (exch br s, 2H, NH_2), 7.20–7.30 (m, 2H, Ar), 7.35 (d, 1H, Ar, *J* = 7.8 Hz), 7.45 (m, 1H, Ar), 7.50 (exch br s, 2H, $CONH_2$), 12.10 (exch br s, 1H, NH). Anal. Calcd. for $C_{11}H_9FN_4O$: C 53.23, H 3.65, N 22.57. Found: C 53.06, H 3.66, N 22.62.
- *5-Amino-3-(3-chlorophenyl)-6-oxo-1,6-dihydropyridazine-4-carboxylic acid amide (**6c**)*

  Yield = 63% (183 mg); mp = 257–260 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 6.45 (exch br s, 2H, NH_2), 7.40–7.50 (m, 4H, Ar), 7.55 (exch br s, 2H, $CONH_2$), 11.90 (exch br s, 1H, NH). Anal. Calcd. for $C_{11}H_9ClN_4O_2$: C 49.92, H 3.43, N 21.17. Found: C 49.79, H 3.42, N 21.25.
- *5-Amino-6-oxo-3-m-tolyl-1,6-dihydropyridazine-4-carboxylic acid amide (**6d**)*

  Yield = 60% (144 mg); mp = 263–265 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.30 (s, 3H, CH_3), 6.40 (exch br s, 2H, NH_2), 7.15–7.35 (m, 4H, Ar), 7.40 (exch br s, 2H, $CONH_2$), 12.80 (exch br s, 1H, NH). Anal. Calcd. for $C_{12}H_{12}N_4O_2$: C 59.01, H 4.95, N 22.94. Found: C 59.18, H 4.94, N 22.89.

- *5-Amino-6-oxo-3-pyridin-3-yl-1,6-dihydropyridazine-4-carboxylic acid amide (**6e**)*

  Yield = 50% (139 mg); mp = 244–247 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 6.55 (exch br s, 2H, NH_2), 7.40 (m, 1H, Ar), 7.50 (exch br s, 1H, $CONH_2$), 7.55 (exch br s, 1H, $CONH_2$), 7.85 (m, 1H, Ar), 8.55 (m, 1H, Ar), 8.65 (s, 1H, Ar), 12.95 (exch br s, 1H, NH). Anal. Calcd. for $C_{10}H_9N_5O_2$: C 51.95, H 3.92, N 30.29. Found: C 51.82, H 3.92, N 30.37.

General procedure for compounds 7b-d and 7 g-k A mixture of the suitable 5-amino-1,6-dihydropyridazine-4-carboxylic acid amide derivatives **6a**, **6c-e** (0.5 mmol), K_2CO_3 (1 mmol) and the appropriate alkyl(aryl) halide (0.75–1.25 mmol) was stirred in anhydrous DMF (1–2 mL) at 80 °C for 0.5–3 h. For compound **7j,** the reaction was carried out at 100 °C for 7 h. After cooling, the precipitate was recovered by filtration under vacuum to give the pure intermediates **7b-d**, **7 g-i**. For compounds **7j,k**, the mixture was diluted with water, the suspension was extracted with CH_2Cl_2 (3 × 15 mL), and the solvent was evaporated in vacuo to afford the crude compounds. Subsequently, **7j,k** were purified by column chromatography using $CHCl_3$/MeOH 9:1 as eluent.

- *5-Amino-1-(4-nitrobenzyl)-6-oxo-3-phenyl-1,6-dihydropyridazine-4-carboxylic acid amide (**7b**)*

  Yield = 95% (173 mg); mp = 213–217 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.95 (exch br s, 1H, $CONH_2$), 5.35 (exch br s, 1H, $CONH_2$), 5.40 (s, 2H, CH_2), 7.50 (s, 5H, Ar), 7.60 (d, 2H, Ar, *J* = 8.2 Hz), 8.20 (d, 2H, Ar, *J* = 8.1 Hz), 8.40 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{18}H_{15}N_5O_4$: C 59.18, H 4.14, N 19.17. Found: C 59.35, H 4.15, N 19.11.
- *4-(5-Amino-4-carbamoyl-6-oxo-3-phenyl-6H-pyridazin-1-ylmethyl)-benzoic acid ethyl ester (**7c**)*

  Yield = 97% (190 mg); mp = 162–164 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 3H, CH_2CH_3, *J* = 7.2 Hz), 4.35 (q, 2H, CH_2CH_3, *J* = 7.2 Hz), 4.95 (exch br s, 1H, $CONH_2$), 5.35 (exch br s, 1H, $CONH_2$), 5.40 (s, 2H, CH_2), 7.50–7.60 (m, 7H, Ar), 8.05 (d, 2H, Ar), 8.10 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{21}H_{20}N_4O_4$: C 64.28, H 5.14, N 14.28. Found: C 64.11, H 5.16, N 14.24.
- *5-Amino-1-(4-hydroxymethylbenzyl)-6-oxo-3-phenyl-1,6-dihydropyridazine-4-carboxylic acid amide (**7d**)*

  Yield = 77% (135 mg); mp = 193–198 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 4.45 (d, 2H, CH_2OH, *J* = 5.6 Hz), 5.15 (exch br t, 1H, CH_2OH, *J* = 5.6 Hz), 5.25 (s, 2H, NCH_2), 6.55 (exch br s, 2H, $CONH_2$), 7.25–7.50 (m, 9H, Ar), 7.65 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{19}H_{18}N_4O_3$: C 65.13, H 5.18, N 15.99. Found: C 65.30, H 5.17, N 15.94.
- *5-Amino-3-(3-chlorophenyl)-1-cyclopropylmethyl-6-oxo-1,6-dihydropyridazine-4-carboxylic acid amide (**7 g**)*

  Yield = 60% (96 mg); mp = 187–189 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.45–0.60 (m, 4H, cC_3H_5), 1.35–1.45 (m, 1H, cC_3H_5), 4.05 (d, 2H, CH_2 cC_3H_5, *J* = 7.2 Hz), 5.00 (exch br s, 1H, $CONH_2$), 5.40 (exch br s, 1H, $CONH_2$), 7.40–7.50 (m, 3H, Ar), 7.60 (s, 1H, Ar), 7.85 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{15}H_{15}ClN_4O_2$: C 56.52, H 4.74, N 17.58. Found: C 56.33, H 4.75, N 17.62.
- *5-Amino-3-(3-chlorophenyl)-1-cyclohexylmethyl-6-oxo-1,6-dihydropyridazine-4-carboxylic acid amide (**7 h**)*

  Yield = 60% (108 mg); mp = 186–188 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.05–1.30 (m, 5H, cC_6H_{11}), 1.60–1.80 (m, 5H, cC_6H_{11}), 2.00 (m, 1H, cC_6H_{11}), 4.05 (d, 2H, CH_2, *J* = 7.2 Hz), 4.95 (exch br s, 1H, $CONH_2$), 5.45 (exch br s, 1H, $CONH_2$), 7.40–7.50 (m, 3H, Ar), 7.60 (s, 1H, Ar), 8.10 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{18}H_{21}ClN_4O_2$: C 59.91, H 5.87, N 15.53. Found: C 59.75, H 5.85, N 15.57.
- *5-Amino-3-(3-chlorophenyl)-1-cyclohexyl-6-oxo-1,6-dihydropyridazine-4-carboxylic acid amide (**7i**)*

  Yield = 30% (52 mg); mp = 244–245 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.10–1.25 (m, 1H, cC_6H_{11}), 1.35–1.45 (m, 2H, cC_6H_{11}), 1.60–1.90 (m, 7H, cC_6H_{11}), 4.80 (m, 1H, cC_6H_{11}), 6.50 (exch br s, 2H, $CONH_2$), 7.40–7.50 (m, 4H, Ar), 7.55–7.65 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{17}H_{19}ClN_4O_2$: C 58.87, H 5.52, N 16.16. Found: C 58.71, H 5.51, N 16.21.
- *5-Amino-1-cyclohexylmethyl-6-oxo-3-m-tolyl-1,6-dihydropyridazine-4-carboxylic acid amide (**7j**)*

  Yield = 66% (112 mg); mp = 166–167 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.95–1.30 (m, 5H, cC_6H_{11}), 1.55–1.75 (m, 5H, cC_6H_{11}), 1.90 (m, 1H, cC_6H_{11}), 2.35 (s, 1H, CH_3), 3.95 (d, 2H, CH_2 cC_6H_{11}, *J* = 7.2 Hz), 6.45 (exch br s, 2H, $CONH_2$), 7.15–7.35 (m, 4H, Ar), 7.35–7.45 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{19}H_{24}N_4O_2$: C 67.04, H 7.11, N 16.46. Found: C 67.22, H 7.13, N 16.42.
- *5-Amino-1-cyclohexylmethyl-6-oxo-3-pyridin-3-yl-1,6-dihydropyridazine-4-carboxylic acid amide (**7 k**)*

  Yield = 28% (112 mg); mp = 233–236 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.95–1.25 (m, 5H, cC_6H_{11}), 1.55–1.75 (m, 5H, cC_6H_{11}), 1.90 (m, 1H, cC_6H_{11}), 3.95 (d, 2H, CH_2 cC_6H_{11}, *J* = 7.2 Hz), 6.60 (exch br s, 2H, $CONH_2$), 7.45 (m, 1H, Ar), 7.55 (exch br s, 2H, NH_2), 7.85 (m, 1H, Ar), 8.60 (d, 1H, Ar), 8.65 (s, 1H, Ar). Anal. Calcd. for $C_{17}H_{21}N_5O_2$: C 62.37, H 6.47, N 21.39. Found: C 62.22, H 6.48, N 21.44.
- *Acetic acid 4-(5-amino-4-carbamoyl-6-oxo-3-phenyl-6H-pyridazin-1-ylmethyl)-benzyl ester (**7e**)*

  A mixture of **7d** (0.28 mmol) and acetyl chloride (0.28 mmol) in anhydrous CH_2Cl_2 (2 mL) was stirred

at room temperature for 3 h. After washing with water, the organic layer was evaporated in vacuo to afford the pure **7e** as a precipitate. Yield = 90% (99 mg); mp = 164–168 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.10 (s, 3H, CH_3), 4.95 (exch br s, 1H, $CONH_2$), 5.10 (s, 2H, CH_2COO), 5.30 (exch br s, 1H, $CONH_2$), 5.45 (s, 2H, NCH_2), 7.30–7.60 (m, 9H, Ar), 8.35 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{21}H_{20}N_4O_4$: C 64.28, H 5.14, N 14.28. Found: C 64.01, H 5.15, N 14.24.

General procedure for compounds 1a-j, 8 A mixture of **7a-c** and **7e-k** (**7a** and **7f** are previously described) [22] (0.25–0.55 mmol), triethylorthoformate (or triethylorthopropionate for compound **1a**) (7.5–30 mmol) and a catalytic amount of concentrated sulfuric acid (0.05 mmol) was heated at 130 °C for 1–5 h in a sealed tube. After cooling, the precipitate was recovered by filtration under vacuum. For compound **1i** the crude precipitate was purified by flash column chromatography using CH_2Cl_2/MeOH 9:1 as eluent. For compound **1a**, after dilution with cold water, the suspension was extracted with ethyl acetate (3 × 15 mL). Subsequently, the solvent was evaporated in vacuo and the residue was purified by flash column chromatography using cyclohexane/ethyl acetate/MeOH 1:2:0.1 as eluent.

- *7-Ethyl-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**8**)*

  Yield = 51% (55 mg); mp > 300 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.30 (t, 3H, CH_2CH_3, J = 7.2 Hz), 4.15 (q, 2H, CH_2CH_3, J = 7.2 Hz), 7.35–7.45 (m, 5H, Ar), 8.45 (s, 1H, Ar), 13.00 (exch br s, 1H, NH). Anal. Calcd. for $C_{14}H_{12}N_4O_2$: C 62.68, H 4.51, N 20.88. Found: C 62.77, H 4.50, N 20.94.
- *2,7-Diethyl-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1a**)*

  Yield = 56% (58 mg); mp = 257–260 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.25–1.40 (m, 6H, N-CH_2CH_3 and C-CH_2CH_3), 2.70 (q, 2H, C-CH_2CH_3, J = 7.2 Hz), 4.20 (q, 2H, N-CH_2CH_3, J = 7.2 Hz), 7.35–7.50 (m, 5H, Ar), 12.90 (exch br s, 1H, NH). Anal. Calcd. for. $C_{16}H_{16}N_4O_2$: C 64.85, H 5.44, N 18.91. Found: C 64.68, H 5.43, N 18.96.
- *7-(4-Nitrobenzyl)-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1b**)*

  Yield = 69% (130 mg); mp > 300 °C dec. (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.50 (s, 2H, CH_2Ar), 7.35–7.45 (m, 5H, Ar), 7.60 (d, 2H, Ar, J = 8.2 Hz), 8.20 (d, 2H, Ar), 8.50 (s, 1H, Ar), 13.15 (exch br s, 1H, NH). Anal. Calcd. for. $C_{19}H_{13}N_5O_4$: C 60.80, H 3.49, N 18.66. Found: C 60.94, H 3.48, N 18.71.
- *4-(4,8-Dioxo-5-phenyl-4,8-dihydro-3H-pyrimido[4,5-d]pyridazin-7-ylmethyl)-benzoic acid ethyl ester (**1c**)*

  Yield = 59% (95 mg); mp = 272–274 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.30 (t, 3H, CH_2CH_3, J = 7.2 Hz), 4.30 (q, 2H, CH_2CH_3, J = 7.2 Hz), 5.45 (s, 2H, N-CH_2), 7.35–7.45 (m, 5H, Ar), 7.50 (d, 2H, Ar, J = 8.4 Hz), 7.95 (d, 2H, Ar, J = 8.1 Hz), 8.45 (s, 1H, Ar), 13.15 (exch br s, 1H, NH). Anal. Calcd. for. $C_{22}H_{18}N_4O_4$: C 65.66, H 4.51, N 13.92. Found: C 65.45, H 4.52, N 13.96.
- *Acetic acid 4-(4,8-dioxo-5-phenyl-4,8-dihydro-3H-pyrimido[4,5-d]pyridazin-7-ylmethyl)benzyl ester (**1d**)*

  Yield = 44% (53 mg); mp = 240–243 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.05 (s, 3H, CH_3), 5.05 (s, 2H, CH_2OCO), 5.35 (s, 2H, N-CH_2), 7.30–7.45 (m, 9H, Ar), 8.45 (s, 1H, Ar), 13.10 (exch br s, 1H, NH). Anal. Calcd. for. $C_{22}H_{18}N_4O_4$: C 65.66, H 4.51, N 13.92. Found: C 65.49, H 4.51, N 13.95.
- *7-Ethyl-5-(3-fluorophenyl)-3,7-dihydro-pyrimido[4,5-d]pyridazine-4,8-dione (**1e**)*

  Yield = 72% (93 mg); mp > 300 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.30 (t, 3H, CH_2CH_3, J = 7.2 Hz), 4.20 (q, 2H, CH_2CH_3, J = 7.2 Hz), 7.20–7.30 (m, 3H, Ar), 7.40–7.50 (m, 1H, Ar), 8.45 (s, 1H, Ar), 13.10 (exch br s, 1H, NH). Anal. Calcd. for. $C_{14}H_{11}FN_4O_2$: C 58.74, H 3.87, N 19.57. Found: C 58.88, H 3.88, N 19.51.
- *5-(3-Chlorophenyl)-7-cyclopropylmethyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1f**)*

  Yield = 38% (69 mg); mp > 300 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.35–0.55 (m, 4H, cC_3H_5), 1.30 (m, 1H, cC_3H_5), 4.00 (d, 2H, CH_2 c cC_3H_5, J = 6.8 Hz), 7.35–7.50 (m, 4H, Ar), 8.45 (s, 1H, Ar), 13.15 (exch br s, 1H, NH). Anal. Calcd. for. $C_{16}H_{13}ClN_4O_2$: C 58.46, H 3.99, N 17.04. Found: C 58.65, H 3.98, N 17.08.
- *5-(3-Chlorophenyl)-7-cyclohexylmethyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1 g**)*

  Yield = 20% (40 mg); mp = 271–274 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.95–1.25 (m, 5H, cC_6H_{11}), 1.55–1.70 (m, 5H, cC_6H_{11}), 1.90 (m, 1H, cC_6H_{11}), 4.00 (d, 2H, CH_2 cC_6H_{11}, J = 7.2 Hz), 7.35–7.50 (m, 4H, Ar), 8.45 (s, 1H, Ar), 13.10 (exch br s, 1H, NH). Anal. Calcd. for. $C_{19}H_{19}ClN_4O_2$: C 61.54, H 5.16, N 15.11 Found: C 61.37, H 5.18, N 15.16.
- *5-(3-Chlorophenyl)-7-cyclohexyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1 h**)*

  Yield = 58% (73 mg); mp = 289–291 °C (Et_2O). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.20 (m, 1H, cC_6H_{11}), 1.40 (m, 2H, cC_6H_{11}), 1.60–1.90 (m, 7H, cC_6H_{11}), 4.85 (t, 1H, cC_6H_{11}, J = 7.0 Hz), 7.35–7.50 (m, 4H, Ar), 8.45 (s, 1H, Ar), 13.10 (exch br s, 1H, NH). Anal. Calcd. for. $C_{18}H_{17}ClN_4O_2$: C 60.59, H 4.80, N 15.70. Found: C 60.81, H 4.79, N 15.32.

- *7-Cyclohexylmethyl-5-m-tolyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1i**)*

  Yield = 20% (40 mg); mp = 219–222 °C (Et_2O). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.95–1.30 (m, 5H, cC_6H_{11}), 1.60–1.75 (m, 5H, cC_6H_{11}), 1.90 (m, 1H, cC_6H_{11}), 2.35 (s, 3H, CH_3), 4.00 (d, 2H, CH_2 cC_6H_{11}, J = 7.2 Hz), 7.15–7.30 (m, 4H, Ar), 8.40 (s, 1H, Ar), 13.05 (exch br s, 1H, NH). Anal. Calcd. for. $C_{20}H_{22}N_4O_2$: C 68.55, H 6.33, N 15.99 Found: C 68.41, H 6.35, N 15.95.
- *7-Cyclohexylmethyl-5-pyridin-3-yl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1j**)*

  Yield = 20% (30 mg); mp > 300 °C (Et_2O). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.95–1.30 (m, 5H, cC_6H_{11}), 1.55–1.75 (m, 5H, cC_6H_{11}), 1.90 (m, 1H, cC_6H_{11}), 4.00 (d, 2H, CH_2 cC_6H_{11}, J = 7.2 Hz), 7.45 (m, 1H, Ar), 7.80 (d, 1H, Ar, J = 8.2 Hz), 8.45 (s, 1H, Ar), 8.60 (m, 2H, Ar), 13.05 (exch br s, 1H, NH). Anal. Calcd. for. $C_{18}H_{19}N_5O_2$: C 64.08, H 5.68, N 20.76. Found: C 64.27, H 5.66, N 20.69.

General procedure for compounds 1 k,l Compounds **1 k,l** were obtained from 8 by adopting the general procedure described for intermediate of type 7. After dilution with cold water, compound **1 k** was recovered by filtration under vacuum. For compound **1 l**, the suspension was extracted with CH_2Cl_2 (3 × 15 mL) and the solvent was evaporated in vacuo.

- *7-Ethyl-3-methyl-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1 k**)*

  Yield = 26% (37 mg); mp = 237–239 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.45 (t, 3H, CH_2CH_3, J = 7.2 Hz), 3.55 (s, 3H, N-CH_3), 4.40 (q, 2H, N-CH_2CH_3, J = 7.2 Hz), 7.40–7.50 (m, 5H, Ar), 8.40 (s, 1H, Ar). Anal. Calcd. for. $C_{15}H_{14}N_4O_2$: C 63.82, H 5.00, N 19.85. Found: C 63.96, H 5.01, N 19.91.
- *3-Benzyl-7-ethyl-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1 l**)*

  Yield = 46% (83 mg); mp = 217–219 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.45 (t, 3H, CH_2CH_3, J = 7.2 Hz), 4.40 (q, 2H, CH_2CH_3, J = 7.2 Hz), 5.15 (s, 2H, CH_2Ph), 7.30–7.50 (m, 10H, Ar), 8.45 (s, 1H, Ar). Anal. Calcd. for. $C_{21}H_{18}N_4O_2$: C 70.38, H 5.06, N 15.63. Found: C 70.54, H 5.05, N 15.68.

General procedure for compounds 1 m,n A mixture of **1c** or **1d** (0.12 mmol), ethanol (1.5 mL) and 6 N NaOH (0.5 mL) was stirred at room temperature for 1–2 h. After evaporation of the solvent and dilution with cold water the suspension was acidified with 6 N HCl. For compound **1 m**, the mixture was extracted with ethyl acetate (3 × 15 mL) and evaporation of the solvent afforded desiderated compound. For compound **1n**, the solid was recovered by filtration under vacuum.

- *4-(4,8-Dioxo-5-phenyl-4,8-dihydro-3H-pyrimido[4,5-d]pyridazin-7-ylmethyl)-benzoic acid (**1 m**)*

  Yield = 25% (15 mg); mp = 295–298 °C dec. (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.45 (s, 2H, CH_2), 7.35–7.50 (m, 7H, Ar), 7.90 (d, 2H, Ar, J = 8.4 Hz), 8.45 (s, 1H, Ar), 13.15 (exch br s, 1H, NH). Anal. Calcd. for. $C_{20}H_{14}N_4O_4$: C 64.17, H 3.77, N 14.97. Found: C 64.36, H 3.78, N 15.02.
- *7-(4-Hydroxymethylbenzyl)-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1n**)*

  Yield = 65% (30 mg); mp = 170–174 °C dec. (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 4.45 (d, 2H, CH_2OH, J = 5.6 Hz), 5.15 (exch br t, 1H, CH_2OH, J = 5.6 Hz), 5.35 (s, 2H, CH_2), 7.25–7.45 (m, 9H, Ar), 8.45 (s, 1H, Ar), 13.10 (exch br s, 1H, NH). Anal. Calcd. for $C_{20}H_{16}N_4O_3$: C 66.66, H 4.48, N 15.55. Found: C 66.82, H 4.49, N 15.49.
- *5-Amino-6-oxo-3-pyridin-3-yl-1,6-dihydropyridazine-4-carboxylic acid methyl ester (**9b**)*

  A mixture of isoxazolopyridazinone 5e (0.46 mmol) and piperidine (0.23 mmol) in anhydrous methanol (6 mL) was stirred at 65 °C for 1.5 h. After evaporation of the solvent, the mixture was diluted with cold water (5 mL), neutralised with 2 N HCl and extracted with CH_2Cl_2 (3 × 15 mL). Evaporation of the solvent afforded the final compound **9b**. Yield = 46% (52 mg); mp = 210–212 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.55 (s, 3H, CH_3O), 6.50 (exch br s, 2H, NH_2), 7.25–7.45 (m, 1H, Ar), 7.65 (m, 1H, Ar), 8.60 (m, 2H, Ar), 11.90 (exch br s, 1H, NH). Anal. Calcd. for $C_{11}H_{10}N_4O_3$: C 53.66, H 4.09, N 22.75. Found: C 53.83, H 4.08, N 22.71.

General procedure for compounds 10a-d Compounds **10a-d** were obtained from **9a,b** adopting the general procedure described intermediate of type 7. After dilution with water, the suspension was extracted with CH_2Cl_2 (3 × 15 mL). The organic layer was recovered, dried on sodium sulfate and evaporated to afford the pure compounds **10a** and **10c**, while compounds **10b,d** were purified by flash column chromatography using cyclohexane/ethyl acetate 1:2 as eluent.

- *5-Amino-1-cyclohexylmethyl-6-oxo-3-phenyl-1,6-dihydropyridazine-4-carboxylic acid methyl ester (**10a**)*

  Yield = 50% (85 mg); mp = 134–136 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.05–1.30 (m, 5H, cC_6H_{11}), 1.65–1.75 (m, 5H, cC_6H_{11}), 2.00 (m, 1H, cC_6H_{11}), 3.50 (s, 3H, CH_3O), 4.05 (d, 2H, CH_2 cC_6H_{11}, J = 7.0 Hz), 7.05 (exch br s, 2H, NH_2), 7.35–7.45 (m, 5H, Ar). Anal. Calcd. for $C_{19}H_{23}N_3O_3$: C 66.84, H 6.79, N 12.31. Found: C 66.63, H 6.81, N 12.34.

- *5-Amino-1-benzyl-6-oxo-3-phenyl-1,6-dihydro-pyridazine-4-carboxylic acid methyl ester (**10b**)*

  Yield = 77% (130 mg); mp = 145–147 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.50 (s, 3H, CH_3O), 5.35 (s, 2H, CH_2Ph), 7.05 (exch br s, 2H, NH_2), 7.30–7.50 (m, 10H, Ar). Anal. Calcd. for $C_{19}H_{17}N_3O_3$: C 68.05, H 5.11, N 12.53. Found: C 68.28, H 5.10, N 12.49.
- *5-Amino-1-cyclopropylmethyl-6-oxo-3-phenyl-1,6-dihydro-pyridazine-4-carboxylic acid methyl ester (**10c**)*

  Yield = 40% (115 mg); mp = 119–121 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.45–0.60 (m, 4H, cC_3H_5), 1.35–1.50 (m, 1H, cC_3H_5), 3.50 (s, 3H, CH_3O), 4.05 (d, 2H, $CH_2cC_3H_5$, J = 6.8 Hz), 7.05 (exch br s, 2H, NH_2), 7.40 (s, 5H, Ar). Anal. Calcd. for $C_{16}H_{17}N_3O_3$: C 64.20, H 5.72, N 14.04. Found: C 64.37, H 5.70, N 14.08.
- *5-Amino-1-(3-chlorobenzyl)-6-oxo-3-pyridin-3-yl-1,6-dihydropyridazine-4-carboxylic acid methyl ester (**10d**)*

  Yield = 75% (140 mg); mp = 197–199 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.65 (s, 3H, CH_3O), 5.30 (s, 2H, NCH_2), 7.00 (exch br s, 2H, $CONH_2$), 7.30 (s, 3H, Ar), 7.45 (s, 1H, Ar), 7.95 (m, 1H, Ar), 8.30 (m, 1H, Ar), 8.80 (m, 2H, Ar). Anal. Calcd. for. $C_{18}H_{15}ClN_4O_3$: C 58.31, H 4.08, N 15.11. Found: C 58.15, H 4.09, N 15.16.

General procedure for compounds 11a-d Compounds **11a-d** were obtained starting from **10a-d** (1.2 mmol), following the same procedure described for **1 m,n**. In this case, the mixture was heated at 60–70 °C for 1–2 h. After acidification with 6 N HCl, the final compounds were recovered by filtration under vacuum.

- *5-Amino-1-cyclohexylmethyl-6-oxo-3-phenyl-1,6-dihydro-pyridazine-4-carboxylic acid (**11a**)*

  Yield = 93% (370 mg); mp = 224–226 °C dec. (Cyclohexane). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.05–1.30 (m, 5H, cC_6H_{11}), 1.65–1.75 (m, 5H, cC_6H_{11}), 2.00 (m, 1H, cC_6H_{11}), 4.05 (d, 2H, N-CH_2, J = 7.3 Hz), 5.05 (exch br s, 1H, OH), 7.45 (s, 5H, Ar), 8.05 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{18}H_{21}N_3O_3$: C 66.04, H 6.47, N 12.84. Found: C 66.25, H 6.45, N 12.88.
- *5-Amino-1-benzyl-6-oxo-3-phenyl-1,6-dihydro-pyridazine-4-carboxylic acid (**11b**)*

  Yield = 42% (160 mg); mp = 207–211 °C dec. (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.35 (s, 2H, NCH_2), 5.15 (exch br s, 1H, OH), 7.30–7.50 (m, 10H, Ar), 7.80 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{18}H_{15}N_3O_3$: C 67.28, H 4.71, N 13.08. Found: C 67.46, H 4.70, N 13.04.
- *5-Amino-1-cyclopropylmethyl-6-oxo-3-phenyl-1,6-dihydro-pyridazine-4-carboxylic acid (**11c**)*

  Yield = 90% (310 mg); mp = 203–206 °C dec. (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.45–0.60 (m, 4H, cC_3H_5) 1.35–1.45 (m, 1H, cC_3H_5), 4.05 (d, 2H, N-CH_2, J = 7.2 Hz), 5.70 (exch br s, 1H, OH), 7.45 (s, 5H, Ar), 7.75 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{15}H_{15}N_3O_3$: C 63.15, H 5.30, N 14.73. Found: C 63.38, H 5.28, N 14.70.
- *5-Amino-1-(3-chlorobenzyl)-6-oxo-3-pyridin-3-yl-1,6-dihydropyridazine-4-carboxylic acid (**11d**)*

  Yield = 98% (440 mg); mp = 228–232 °C dec. (Et_2O). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.30 (s, 2H, N-CH_2), 7.30 (d, 1H, Ar, J = 8.2 Hz), 6.95 (exch br s, 1H, OH), 7.35–7.45 (m, 4H, Ar), 7.75 (d, 1H, Ar, J = 8.4 Hz), 8.55 (m, 2H, Ar), 8.70 (exch br s, 2H, $CONH_2$). Anal. Calcd. for $C_{18}H_{15}ClN_4O_3$: C 58.31, H 4.08, N 15.11. Found: C 58.48, H 4.07, N 15.16.

General procedure for compounds 12a-d A catalytic amount of Et_3N was added to a cold suspension of **11a-d** (0.4 mmol) in $SOCl_2$ (1.5–3 mL) and the mixture was refluxed for 1 h. The excess of $SOCl_2$ was removed in vacuo. After cooling, 33% NH_4OH (4–6 mL) was added and the suspension was stirred for 0.5–1 h. Ice-water (10–15 mL) was added to the residue and the final compounds **12b** and **12c** were recovered by filtration under vacuum. For compounds **12a** and **12d**, the mixture was extracted with ethyl acetate (3 × 15 mL) and the solvent was evaporated in vacuo to afford pure final compounds, while **12a** was purified by flash column chromatography using cyclohexane/ethyl acetate 1:3 as eluent.

- *5-Amino-1-cyclohexylmethyl-6-oxo-3-phenyl-1,6-dihydro-pyridazine-4-carboxylic acid amide (**12a**)*

  Yield = 66% (86 mg); mp = 179–181 °C (Cyclohexane). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.05–1.30 (m, 5H, cC_6H_{11}), 1.65–1.80 (m, 5H, cC_6H_{11}), 2.00–2.10 (m, 1H, cC_6H_{11}), 4.05 (d, 2H, CH_2 cC_6H_{11}, J = 7.6 Hz), 4.95 (exch br s, 1H, $CONH_2$), 5.45 (exch br s, 1H, $CONH_2$), 7.45–7.55 (m, 5H, Ar), 8.15 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{18}H_{22}N_4O_2$: C 66.24, H 6.79, N 17.17. Found: C 66.45, H 6.77, N 17.11.
- *5-Amino-1-benzyl-6-oxo-3-phenyl-1,6-dihydro-pyridazine-4-carboxylic acid amide (**12b**)*

  Yield = 30% (40 mg); mp = 218–221 °C dec. (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.95 (exch br s, 1H, $CONH_2$), 5.30 (exch br s, 1H, $CONH_2$), 5.35 (s, 2H, CH_2Ph), 7.30–7.55 (m, 10H, Ar), 8.25 (exch br s, 2H, NH_2). Anal. Calcd. for $C_{18}H_{16}N_4O_2$: C 67.49, H 5.03, N 17.49. Found: C 67.26, H 5.04, N 17.46.
- *5-Amino-1-cyclopropylmethyl-6-oxo-3-phenyl-1,6-dihydro-pyridazine-4-carboxylic acid amide (**12c**)*

  Yield = 55% (63 mg); mp = 203–206 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.45–0.60 (m, 4H, cC_3H_5), 1.35–1.50 (m, 1H, cC_3H_5), 4.05 (d, 2H, CH_2 cC_3H_5, J = 7.2 Hz), 5.00 (exch br s, 1H, $CONH_2$), 5.40 (exch br s, 1H, $CONH_2$), 7.45–7.60 (m, 5H, Ar), 7.90 (exch br s, 2H, NH2). Anal. Calcd. for $C_{18}H_{16}N_4O_2$: C 67.49, H 5.03, N 17.49. Found: C 67.26, H 5.04, N 17.46.

- *5-Amino-1-(3-chlorobenzyl)-6-oxo-3-pyridin-3-yl-1,6-dihydro-pyridazine-4-carboxylic acid amide (**12d**)*

  Yield = 72% (102 mg); mp = 235–238 °C dec. (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.30 (s, 2H, NCH_2Ar), 6.70 (exch br s, 2H, NH_2), 7.30–7.45 (m, 5H, Ar), 7.50–7.60 (exch br s, 2H, $CONH_2$), 7.85 (d, 1H, Ar, *J* = 8.2 Hz), 7.90 (exch br s, 2H, NH_2), 8.60 (d, 1H, Ar, *J* = 8.4 Hz), 8.65 (s, 1H, Ar). Anal. Calcd. for $C_{17}H_{14}ClN_5O_2$: C 57.39, H 3.97, N, 19.68. Found: C 57.55, H 3.98, N 19.62.

General procedure for compounds 1o-s Compounds **1o-s** were obtained starting from **12a-d** (0.55 mmol) following the same procedure described for **1a-j**. After cooling, the precipitate was recovered by filtration under vacuum.

- *7-Cyclohexylmethyl-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1o**)*

  Yield = 48% (90 mg); mp = 250–253 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.95–1.25 (m, 5H, cC_6H_{11}), 1.60–1.70 (m, 5H, cC_6H_{11}), 1.85–1.95 (m, 1H, cC_6H_{11}), 4.00 (d, 2H, NCH_2, *J* = 7.2 Hz), 7.40 (s, 5H, Ar), 8.45 (s, 1H, Ar), 13.00 (exch br s, 1H, NH). Anal. Calcd. for $C_{19}H_{20}N_4O_2$: C 67.84, H 5.99, N 16.66. Found: C 67.61, H 6.01, N 16.70.
- *7-Benzyl-5-phenyl-3,7-dihydro-pyrimido[4,5-d]pyridazine-4,8-dione (**1p**)*

  Yield = 65% (120 mg); mp = 289–292 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.35 (s, 2H, NCH_2), 7.30–7.40 (m, 10H, Ar), 8.45 (s, 1H, Ar), 13.10 (exch br s, 1H, NH). Anal. Calcd. for $C_{19}H_{14}N_4O_2$: C 69.08, H 4.27, N 16.96. Found: C 69.29, H 4.26, N 16.34.
- *7-Cyclopropylmethyl-5-phenyl-3,7-dihydro-pyrimido[4,5-d]pyridazine-4,8-dione (**1q**)*

  Yield = 32% (52 mg); mp = 285–287 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.35–0.50 (m, 4H, cC_3H_5), 1.25–1.35 (m, 1H, cC_3H_5), 4.05 (d, 2H, NCH_2, *J* = 7.2 Hz), 7.35–7.45 (m, 5H, Ar), 8.45 (s, 1H, Ar), 13.05 (exch br s, 1H, NH). Anal. Calcd. for $C_{16}H_{14}N_4O_2$: C 65.30, H 4.79, N 19.04. Found: C 65.49, H 4.80, N 19.09.
- *7-(3-Chlorobenzyl)-5-pyridin-3-yl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1r**)*

  Yield = 15% (30 mg); mp = 252–255 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 5.35 (s, 2H, NCH_2), 7.30 (d, 1H, Ar), 7.35–7.45 (m, 4H, Ar), 7.85 (d, 1H, Ar), 8.50 (s, 1H, Ar), 8.55–8.65 (m, 2H, Ar). Anal. Calcd. for $C_{18}H_{12}ClN_5O_2$: C 59.11, H 3.31, N 19.15. Found: C 59.24, H 3.31, N 19.08.
- *7-Cyclopropylmethyl-2-ethyl-5-phenyl-3,7-dihydro-pyrimido[4,5-d]pyridazine-4,8-dione (**1 s**)*

  Yield = 25% (45 mg); mp = 209–213 °C dec. (Et_2O). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.35–0.50 (m, 4H, cC_3H_5), 1.25 (t, 3H, CH_2CH_3, *J* = 7.2 Hz), 1.25–1.35 (m, 1H, cC_3H_5), 2.70 (q, 2H, CH_2CH_3, *J* = 7.2 Hz), 4.00 (d, 2H, NCH_2, *J* = 7.2 Hz), 7.35–7.45 (m, 5H, Ar), 13.05 (exch br s, 1H, NH). Anal. Calcd. for $C_{18}H_{18}N_4O_2$: C 67.07, H 5.63, N, 17.38. Found: C 67.28, H 5.65, N 17.43.

General procedure for compounds 1t,u Compounds **1t,u** were obtained starting from **1o** and **1q** adopting the general procedure described for compounds 7. For **1u**, the mixture was diluted with cold water, extracted with CH_2Cl_2 (3 × 15 mL) and the solvent was evaporated in vacuo. Lastly, the final compound was purified by flash column chromatography using cyclohexane/ethyl acetate 1:3 as eluent.

- *7-Cyclohexylmethyl-3-ethyl-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1t**)*

  Yield = 56% (102 mg); mp = 216–219 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.05–1.30 (m, 5H, cC_6H_{11}), 1.40 (t, 3H, CH_2CH_3, *J* = 7.2 Hz), 1.60–1.80 (m, 5H, cC_6H_{11}), 2.00–2.10 (m, 1H, cC_6H_{11}), 4.00 (q, 2H, CH_2CH_3, *J* = 7.2 Hz), 4.20 (d, 2H, $CH_2cC_6H_{11}$, *J* = 6.8 Hz), 7.40–7.50 (m, 5H, Ar), 8.40 (s, 1H, Ar). Anal. Calcd. for $C_{21}H_{24}N_4O_2$: C 69.21, H 6.64, N 15.37. Found: C 69.38, H 6.62, N 15.33.
- *7-Cyclopropylmethyl-3-ethyl-5-phenyl-3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione (**1u**)*

  Yield = 62% (100 mg); mp = 176–180 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.45–0.60 (m, 4H, cC_3H_5), 1.40 (t, 3H, CH_2CH_3, *J* = 7.2 Hz), 1.40–1.55 (m, 1H, cC_3H_5), 4.05 (q, 2H, CH_2CH_3, *J* = 7.2 Hz), 4.20 (d, 2H, $CH_2cC_3H_5$, *J* = 6.8 Hz), 7.40–7.55 (m, 5H, Ar), 8.45 (s, 1H, Ar). Anal. Calcd. for $C_{18}H_{18}N_4O_2$: C 67.07, H 5.63 N 17.38. Found: C 67.23, H 5.64, N 17.33.

General procedure for compounds 18b and 2a A mixture of the ester **15** [17] (0.06 g, 0.26 mmol), 0.04 g (0.26 mmol) of the appropriate R-benzyl chloride and K_2CO_3 (0.07 g, 0.52 mmol) in anhydrous DMF (4 mL) was heated at 50 °C for 30 min. After cooling, water was added and the mixture was extracted with CH_2Cl_2 (3 × 15 mL). The organic phase was recovered and dried on sodium sulphate. After removal of the solvent, the residue was purified by flash column chromatography using toluene/ethyl acetate 1:1 as eluent.

- *Ethyl-1,4-dihydro-1-(4-chlorobenzyl)-7-methyl-4-oxo-1,8-naphtyridine-3-carboxylate (**2a**)*

  Yield = 80% (75 mg); mp = 139–141 °C (Cyclohexane). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.28 (t, 3H, CH_2CH_3, *J* = 7.0 Hz), 2.65 (s, 3H, CH_3), 4.25 (q, 2H, CH_2CH_3, *J* = 7.0 Hz), 5.65 (s, 2H, CH_2-Ph), 7.35–7.40 (m, 5H, 1H naphthyridine H6 and 4H Ar), 8.45 (d, 1H, naphthyridine H5, *J* = 8.0 Hz), 9.00 (s, 1H, naphtyridine

H2). Anal. Calcd. for $C_{19}H_{17}ClN_2O_3$: C 63.96, H 4.80, N 7.85. Found: C 63.78, H 4.79, N 7.87.

- *Ethyl-1,4-dihydro-1-(4-fluorobenzyl)-7-methyl-4-oxo-1,8-naphtyridine-3-carboxylate (**18b**)*

  Yield 75% (67 mg); mp = 139–141 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 3H, CH_2CH_3, J = 7.0 Hz), 2.65 (s, 3H, CH_3), 4.25 (q, 2H, CH_2CH_3, J = 7.0 Hz), 5.60 (s, 2H, CH_2-Ph), 7.05 (t, 2H, Ar, J = 7.6 Hz), 7.25 (d, 1H, naphtyridine H6, J = 8.0 Hz), 7.35 (dd, 2H, Ar, J = 8.4 Hz and J = 1.2 Hz), 8.65 (d, 1H, napthyridine H5, J = 8.0 Hz), 8.70 (s, 1H, naphtyridine H2). Anal. Calcd. for $C_{19}H_{17}FN_2O_3$: C 67.05, H 5.03, N 8.23. Found: C 67.22, H 5.03, N 8.21.

General procedure for compounds 18c,d and 2b A mixture of the ester **15** [17] (0.11 g, 0.50 mmol), the appropriate R-phenylboronic acid (0.120 g, 1.0 mmol), copper acetate (0.14 g, 0.76 mmol) and triethylamine (0.14 g, 0.1 mmol) in anhydrous CH_2Cl_2 (2 mL) was stirred at room temperature for 24 h. After dilution with CH_2Cl_2 (10 mL), the suspension was sequentially washed with 33% aqueous ammonia (3 × 10 mL), 10% NH_4Cl (1 × 10 mL) and H_2O (2 × 10 mL). The organic layer was dried and evaporated in vacuo. The crude residue was purified by flash column chromatography using cyclohexane/ethyl acetate 1:2 as eluent.

- *Ethyl-1,4-dihydro-7-methyl-4-oxo-1-phenyl-1,8-naphtyridine-3-carboxylate (**18c**)*

  Yield = 60% (93 mg); mp = 202–205 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.50 (s, 3H, CH_3), 4.40 (q, 2H, CH_2CH_3, J = 7.2 Hz), 7.25–7.35 (m, 1H, naphthyridine H6), 7.45–7.50 (m, 2H, Ar), 7.55–7.60 (m, 3H, Ar), 8.70–8.75 (m, 2H, naphtyridine H5 and H6). Anal. Calcd. for $C_{18}H_{16}N_2O_3$: C 70.12, H 5.23, N 9.09. Found: C 70.33, H 5.22, N 9.06.
- *Ethyl-1,4-dihydro-1-(3-fluorophenyl)-7-methyl-4-oxo-1,8-naphtyridine-3-carboxylate (**18d**)*

  Yield = 12% (20 mg); mp = 218–221 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.55 (s, 3H, CH_3), 4.45 (q, 2H, CH_2CH_3, J = 7.2 Hz), 7.30–7.70 (m, 4H, naphthyridine H6 and 3H Ar), 7.50–7.60 (m, 1H, Ar), 8.65 (s, 1H, naphthyridine H2), 8.70 (d, 1H, naphthyridine H5, J = 8.0 Hz). Anal. Calcd. for $C_{18}H_{15}FN_2O_3$: C 66.25, H 4.63, N 8.58. Found: C 66.41, H 4.64, N 8.55.
- *Ethyl 7-methyl-1-(3-nitrophenyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (**2b**)*

  Yield = 45% (80 mg); mp = 212–215 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.30 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.53 (s, 3H, CH_3), 4.44 (q, 2H, CH_2CH_3, J = 7.2 Hz), 7.30–7.35 (m, 1H, naphthyridine H6), 7.41 (s, 1H, naphthyridine H2), 7.50–7.60 (m, 3H, Ar), 8.40–8.50 (m, 2H, Ar), 8.72 (d, 1H, naphthyridine H5, J = 8.0 Hz). Anal. Calcd. for $C_{18}H_{15}N_3O_5$: C 61.19, H 4.28, N 11.89. Found: C 61.37, H 4.29, N 11.83.

General procedure for the synthesis of the carboxylic acids 19b-e Compounds **19b-e** were obtained starting from intermediate **2a** and **18b-d** (1.2 mmol) following the procedure previously described for **1 m,n**.

- *1,4-Dihydro-1-(4-chlorobenzyl)-7-methyl-4-oxo-1,8-naphtyridine-3-carboxylic acid (**19b**)*

  Yield = 95% (375 mg); mp = 252–255 °C (EtOH). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.75 (s, 3H, CH_3), 5.70 (s, 2H, CH_2-Ph), 7.25–7.35 (m, 4H, Ar), 7.45 (d, 1H, naphtyridine H6, J = 8.0 Hz), 8.70 (d, 1H, naphtyridine H5, J = 8.0 Hz), 9.00 (s, 1H, naphtyridine H2), 14.55 (exch br s, 1H, COOH). Anal. Calcd. for $C_{17}H_{13}ClN_2O_3$: C 62.11, H 3.99, N, 8.52. Found: C 62.30, H 3.98, N 8.49.
- *1,4-Dihydro-1-(4-fluorobenzyl)-7-methyl-4-oxo-1,8-naphtyridine-3-carboxylic acid (**19c**)*

  Yield = 97% (363 mg); mp = 253–256 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.80 (s, 3H, CH_3), 5.70 (s, 2H, CH_2), 7.05 (t, 2H, Ar, J = 7.6 Hz), 7.40–7.50 (m, 3H, 2H Ar and naphtyridine H6), 8.70 (d, 1H, naphtyridine H5, J = 8.0 Hz), 9.00 (s, 1H, naphtyridine H2), 14.60 (exch br s, 1H, COOH). Anal. Calcd. for $C_{17}H_{13}FN_2O_3$: C 65.38, H 4.20, N, 8.97. Found: C 65.13, H 4.21, N 8.95.
- *1,4-Dihydro-1-phenyl-7-methyl-4-oxo-1,8-naphtyridine-3-carboxylic acid (**19d**)*

  Yield = 90% (302 mg); mp = 274–276 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.60 (s, 3H, CH_3), 7.40–7.50 (m, 3H, 2H Ar and naphtyridine H6), 7.55–7.65 (m, 3H, Ar), 8.75 (d, 1H, naphtyridine H5, J = 8.0 Hz), 9.00 (s, 1H, naphtyridine H2), 14.55 (exch br s, 1H, COOH). Anal. Calcd. for $C_{16}H_{12}N_2O_3$: C 68.56, H 4.32, N 9.99. Found: C 68.41, H 4.33, N 9.94.
- *1,4-Dihydro-1-(3-fluorophenyl)-7-methyl-4-oxo-1,8-naphtyridine-3-carboxylic acid (**19e**)*

  Yield = 98% (350 mg); mp > 300 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.65 (s, 3H, CH_3), 7.20–7.30 (m, 3H, Ar), 7.40 (d, 1H, naphtyridine H6, J = 8.0 Hz), 7.50–7.60 (m, 1H, Ar), 8.70 (d, 1H, naphtyridine H5, J = 8.0 Hz), 9.00 (s, 1H, naphtyridine H2), 14.40 (exch br s, 1H, COOH). Anal. Calcd. for $C_{16}H_{11}FN_2O_3$: C 64.43, H 3.72, N, 9.39 Found: C 64.22, H 3.71, N 9.36.

General procedure for the synthesis of final compounds 2c-i The appropriate carboxylic acid of type 19 (0.24 mmol) was converted into the acyl chloride with $SOCl_2$ (6.5 mmol) and triethylamine (0.06 mL) at room temperature for 40–60 min. After removing the excess $SOCl_2$, the residue was washed 3 times with cyclohexane and used in the following step without further purification. Under stirring at

0 °C, a solution of the appropriate acyl chloride (1.3 mmol) in anhydrous THF (5–7 mL) was added dropwise to a solution of the appropriate amine (2.6 mmol) dissolved in anhydrous THF (3–7 mL). The mixture was stirred at room temperature for 2–3 h. After removed of the solvent, the residue was treated with cold water and extracted with CH_2Cl_2 (3 × 15 mL). The crude amides were purified by flash column chromatography using $CHCl_3$/MeOH 9:1 (for **2d,e**), cyclohexane/ethyl acetate 1:2 (**2f**), EtOH/$CHCl_3$/ethyl ether/petroleum ether/33% NH_4OH 4.5:18:18:45:0.25 (for **2 g,h**), or by crystallisation from ethanol (for **2c,d,i**).

- *1-Ethyl-7-methyl-4-oxo-N-(pyridin-3-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2c**)*

  Yield = 10% (10 mg); mp = 160–163 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.60 (t, 3H, CH_2CH_3, J = 7.0 Hz), 2.75 (s, 3H, CH_3), 4.60 (q, 2H, CH_2CH_3, J = 7.0 Hz), 7.30–7.35 (m, 1H, pyridine), 7.40 (d, 1H, naphthyridine H6, J = 8.0 Hz), 8.25–8.30 (m, 1H, pyridine), 8.30–8.40 (m, 1H, pyridine), 8.70 (d, 1H, naphthyridine H5, J = 8.0 Hz), 8.95 (s, 1H, pyridine), 9.05 (s, 1H, naphthyridine H2), 12.35 (exch br s, 1H, NH). Anal. Calcd. for $C_{17}H_{16}N_4O_2$: C 66.22, H 5.23, N 18.17. Found: C 66.06, H 5.25, N 18.12.
- *N'-Acetyl-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbohydrazide (**2d**)*

  Yield = 15% (10 mg); mp = 264–267 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.55 (t, 3H, CH_2CH_3, J = 7.0 Hz), 2.20 (s, 3H, $COCH_3$), 2.70 (s, 3H, CH_3), 4.60 (q, 2H, CH_2CH_3, J = 7.0 Hz), 7.35 (d, 1H, naphthyridine H6, J = 8.4 Hz), 8.05 (exch br s, 1H, NH), 8.70 (d, 1H, naphthyridine H5, J = 8.0 Hz), 9.00 (s, 1H, naphthyridine H2), 12.10 (exch br s, 1H, NH). Anal. Calcd. for $C_{14}H_{16}N_4O_3$: C 58.32, H 5.59, N 19.43. Found: C 58.47, H 5.60, N 19.48.
- *1-Ethyl-N,7-dimethyl-4-oxo-N-phenyl-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2e**)*

  Yield = 63% (50 mg); mp = 160–163 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.60 (s, 3H, CH_3), 3.50 (s, 3H, N-CH_3), 4.40 (q, 2H, CH_2CH_3, J = 7.2 Hz), 7.10–7.15 (m, 2H, Ar), 7.15–7.30 (m, 4H, 3H Ar and naphthyridine H6), 7.90–8.00 (m, 1H, naphthyridine H5), 8.40–8.50 (m, 1H, naphthyridine H2). Anal. Calcd. for $C_{19}H_{19}N_3O_2$: C 71.01, H 5.96, N 13.08. Found: C 71.17, H 5.95, N 13.12.
- *1-Ethyl-7-methyl-N-(naphthalen-2-yl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2f**)*

  Yield = 60% (50 mg); mp = 217–220 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.60 (t, 3H, CH_2CH_3, J = 7.0 Hz), 2.80 (s, 3H, CH_3), 4.65 (q, 2H, CH_2CH_3, J = 7.0 Hz), 7.40 (d, 1H, naphthyridine H6, J = 8.4 Hz), 7.55 (t, 2H, Ar, J = 7.6 Hz), 7.60–7.70 (m, 2H, Ar), 7.90 (d, 1H, Ar, J = 7.6 Hz), 8.40 (d, 1H, Ar, J = 8.0 Hz), 8.55 (d, 1H, Ar, J = 7.8 Hz), 8.80 (d, 1H, naphthyridine H5, J = 8.0 Hz), 9.15 (s, 1H, naphthyridine H2), 12.80 (exch br s, 1H, NH). Anal. Calcd. for $C_{22}H_{19}N_3O_2$: C 73.93, H 5.36, N, 11.76. Found: C 73.70, H 5.34, N 11.73.
- *1-(4-Chlorobenzyl)-N,7-dimethyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2 g**)*

  Yield = 20% (16 mg); mp = 231–233 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.70 (s, 3H, CH_3), 3.05 (d, 3H, NH-CH_3, J = 9.2 Hz), 5.65 (s, 2H, CH_2-Ph), 7.30–7.40 (m, 5H, 4H Ar and naphthyridine H6), 8.66 (d, 1H, naphthyridine H5, J = 8.0 Hz), 9.00 (s, 1H, naphthyridine H2), 9.75 (exch br s, 1H, NH). Anal. Calcd. for $C_{18}H_{16}ClN_3O_2$: C 63.25, H 4.72, N 12.29. Found: C 63.02, H 4.73, N 12.33.
- *1-(3-Fluorophenyl)-N,7-dimethyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2 h**)*

  Yield = 70% (52 mg); mp = 201–204 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.60 (s, 3H, CH_3), 3.05 (d, 3H, NH-CH_3, J = 6.0 Hz), 7.20–7.30 (m, 3H, Ar), 7.35 (d, 1H, naphtyridine H6, J = 8.0 Hz), 7.50 (t, 1H, Ar, J = 7.6 Hz), 8.70 (d, 1H, naphtyridine H5, J = 8.0 Hz), 9.00 (s, 1H, naphtyridine H2), 9.70 (exch br s, 1H, NH). Anal. Calcd. for $C_{17}H_{14}FN_3O_2$: C 65.59, H 4.53, N 13.50. Found: C 64.45, H 4.55, N 13.53.
- *N'-Acetyl-1-(3-fluorophenyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carbohydrazide (**2i**)*

  Yield = 87% (80 mg); mp = 290–293 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.15 (s, 3H, $COCH_3$), 2.58 (s, 3H, CH_3), 7.20–7.30 (m, 3H, Ar), 7.36 (d, 1H, naphthyridine H6, J = 8.0 Hz), 7.55–7.60 (m, 1H, Ar), 8.55 (exch br s, 1H, NH), 8.73 (d, 1H, naphthyridine H5, J = 8.0 Hz), 8.90 (s, 1H, naphthyridine H2), 12.15 (exch br s, 1H, NH). Anal. Calcd. for $C_{18}H_{15}FN_4O_3$: C 61.01, H, 4.27, N, 15.81. Found: C 61.15, H 4.28, N 15.86.

General procedure for the synthesis of the 3,5-dichloropyridilamides 2j,k NaH (0.43 mmol) was added to a cooled (0 °C) solution of 3,5-dichloro-4-pyridilamine (0.43 mmol) in anhydrous THF (3 mL), and the mixture was stirred for 3 h. The appropriate acyl chloride (0.43 mmol) in anhydrous THF (3 mL) was added at 0 °C. After 30 min, the solvent was removed, and the residue was sequentially washed with 6 M NaOH (2 × 10 mL), 2 N HCl (2 × 10 mL) and H_2O. The solid residue was purified by flash column chromatography using cyclohexane/ethyl acetate 2:1 as eluent.

- *N-(3,5-Dichloropyridin-4-yl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2j**)*

  Yield = 7% (10 mg); mp = 237–241 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.60 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.75 (s, 3H, CH_3), 4.63 (q, 2H, CH_2CH_3, J = 7.2 Hz), 7.39 (d, 1H, naphtyridine H6, J = 8.0 Hz), 8.60 (s, 2H, pyridine), 8.75 (d, 1H, naphtyridine H5,

$J = 8.4$ Hz), 9.00 (s, 1H, naphtyridine H2), 12.30 (exch br s, 1H, NH). Anal. Calcd. for $C_{17}H_{14}Cl_2N_4O_2$: C 54.13, H 3.74, N 14.85. Found: C 54.19, H 3.75, N 14.88.

- *N-(3,5-Dichloropyridin-4-yl)-1-(4-fluorobenzyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2 k**)*

  Yield = 7% (14 mg); mp = 266–269 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.76 (s, 3H, CH_3), 5.71 (s, 2H, CH_2-Ph), 7.06 (t, 2H, Ar, $J = 8.4$ Hz), 7.40–7.50 (m, 3H, 2H Ar and naphtyridine H6), 8.60 (s, 2H, pyridine), 8.75 (d, 1H, naphtyridine H5, $J = 8.4$ Hz), 9.10 (s, 1H, naphtyridine H2), 12.20 (exch br s, 1H, NH). Anal. Calcd. for $C_{22}H_{15}Cl_2FN_4O_2$: C 57.78, H 3.31, N, 12.25. Found: C 57.91, H 3.30, N 12.29.
- *1-Ethyl-7-methyl-3-(5-methyl-1,3,4-oxadiazol-2-yl)-1,8-naphthyridin-4(1H)-one (**2 l**)*

  The reaction was performed using $POCl_3$ on silica gel as dehydrating agent. This reagent was prepared as follows: $POCl_3$ (1.5 mL) was dissolved in anhydrous pyridine (3 mL), and the solution was diluted with CH_2Cl_2 (25 mL), then cooled at 0 °C, and the silica gel (10 g) was added portion-wise. After 1 h, the solvent was carefully removed in vacuo. 0.5 g di $POCl_3$ adsorbed on silica gel were added to compound **2d** (0.02 g, 0.07 mmol) suspended in toluene (1.5 mL), and the mixture was warmed at 80 °C for 5 h. The solvent was removed in vacuo, and the residue was extracted 5 times with CH_2Cl_2. Evaporation of the solvent afforded the crude product which was purified by flash column chromatography using $CHCl_3$/MeOH 9:1 as eluent. Yield = 68% (13 mg); mp = 188–191 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.55 (t, 3H, CH_2CH_3, $J = 7.0$ Hz), 2.65 (s, 3H, CH_3 oxadiazole), 2.70 (s, 3H, CH_3), 4.95 (q, 2H, CH_2CH_3, $J = 7.0$ Hz), 7.30 (d, 1H, naphthyridine H6, $J = 8.0$ Hz), 8.65–8.70 (m, 2H, naphthyridine H5 and H2). Anal. Calcd. for $C_{14}H_{14}N_4O_2$: C 62.21, H 5.22, N 20.73. Found: C 62.08, H 5.23, N 20.80.
- *Ethyl-1,4-dihydro-1-ethyl-7-(3-nitrophenyl)-4-oxo-1,8-naphthyridine-3-carboxylate (**2 m**)*

  A suspension of 3-nitrophenylboronic acid (0.06 g, 0.35 mmol), 2 M Na_2CO_3 (2 mL) in EtOH (2 mL) was added to a stirred mixture of the ester **20c** [23] (0.10 g, 0.35 mmol) and tetrakis(triphenylphosphine)-palladium(0) (0.020 g, catalytic amount) in toluene (2 mL). The reaction was heated at 60 °C for 90 min. After cooling, the mixture was extracted with toluene (3 × 15 mL) and dried on Na_2SO_4. The solvent was removed in vacuo, and the residue was purified by flash column chromatography using EtOH/$CHCl_3$/ethyl ether/petroleum ether/33% NH_4OH 4.5:18:18:45:0.25 as eluent. Yield = 10% (12 mg); mp = 225–228 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.45 (t, 3H, OCH_2CH_3, $J = 7.2$ Hz), 1.65 (t, 3H, N-CH_2CH_3, $J = 7.2$ Hz), 4.45 (q, 2H, OCH_2CH_3, $J = 7.2$ Hz), 4.65 (q, 2H, N-CH_2CH_3, $J = 7.2$ Hz), 7.76 (t, 1H, Ar, $J = 8.0$ Hz), 7.95 (d, 1H, naphthyridine H6, $J = 8.4$ Hz), 8.40 (d, 1H, Ar, $J = 8.0$ Hz), 8.48 (d, 1H, Ar, $J = 8.0$ Hz), 8.70 (s, 1H, naphthyridine H2), 8.90 (d, 1H, naphthyridine H5, $J = 8.0$ Hz), 9.00 (s, 1H, Ar). Anal. Calcd. for $C_{19}H_{17}N_3O_5$: C 62.12, H 4.66, N, 11.44. Found: C 62.26, H 4.64, N 11.41.

General procedure for the synthesis of final compounds 2n-p Compounds **2n-p** were obtained by adopting the same procedure for carboxamides **2c-i**, reacting **22a,b** with the appropriate amine. The crude compounds **2o,p** were purified by flash column chromatography using EtOH/$CHCl_3$/ethyl ether/petroleum ether/33% NH_4OH 4.5:18:18:45:0.25 as eluent, while compound **2n** was purified by crystallisation from ethanol.

- *1-Ethyl-7-methoxy-4-oxo-N-phenyl-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2n**)*

  Yield = 50% (38 mg); mp = 189–192 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.60 (t, 3H, CH_2CH_3, $J = 7.2$ Hz), 4.10 (s, 3H, OCH_3), 4.53 (q, 2H, CH_2CH_3, $J = 7.2$ Hz), 6.91 (d, 1H, naphthyridine H6, $J = 8.8$ Hz), 7.13 (t, 1H, Ar, $J = 7.2$ Hz), 7.38 (t, 2H, Ar, $J = 7.8$ Hz), 7.79 (d, 2H, Ar, $J = 8.0$ Hz) 8.66 (d, 1H, naphthyridine H5, $J = 8.4$ Hz), 8.93 (s, 1H, naphthyridine H2), 12.25 (exch br s, 1H, NH). Anal. Calcd. for $C_{18}H_{17}N_3O_3$: C 66.86, H 5.30, N, 13.00. Found: C 66.71, H 5.28, N 13.04.
- *1-Ethyl-4-oxo-N-phenyl-7-(pyrrolidin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2o**)*

  Yield = 20% (17 mg); mp = 234–237 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.50 (t, 3H, CH_2CH_3, $J = 7.0$ Hz), 1.50–1.60 (m, 2H, pyrrolidine), 1.70–1.90 (m, 4H, pyrrolidine), 2.10–2.20 (m, 2H, pyrrolidine), 4.40 (q, 2H, CH_2CH_3, $J = 7.0$ Hz), 6.50 (d, 1H, naphthyridine H6), 7.10 (t, 1H, Ar, $J = 7.2$ Hz), 7.36 (t, 2H, Ar, $J = 7.8$ Hz), 7.79 (d, 2H, Ar, $J = 8.4$ Hz), 8.41 (d, 1H, naphthyridine H5, $J = 8.8$ Hz), 8.80 (s, 1H, naphthyridine H2), 12.40 (exch br s, 1H, NH). Anal. Calcd. for $C_{21}H_{22}N_4O_2$: C 69.59, H 6.12, N 15.46. Found: C 69.78, H 6.10, N 15.51.
- *1-Ethyl-N-methyl-4-oxo-N-phenyl-7-(pyrrolidin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**2p**)*

  Yield = 50% (45 mg); oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.35 (t, 3H, CH_2CH_3), 1.50–1.60 (m, 2H, pyrrolidine), 1.60–1.75 (m, 2H, pyrrolidine), 1.75–1.80 (m, 2H, pyrrolidine), 2.00–2.15 (m, 2H, pyrrolidine), 3.50 (s, 3H, N-CH_3), 4.20 (q, 2H, CH_2CH_3), 6.40 (d, 1H, naphthyridine H6), 7.10–7.20 (m, 1H, Ar), 7.15 (s, 1H, naphthyridine H2), 7.20–7.30 (m, 4H, Ar), 8.15 (d,1H, naphthyridine H5). Anal. Calcd. for $C_{22}H_{24}N_4O_2$: C 70.19, H 6.43, N 14.88. Found: C 70.03, H 6.45, N 14.83.
- *6-Methyl-4-oxo-4H-pyrido[1,2-a]pyrimidine-3-carboxylic acid (**17**)*

  A suspension of the ethyl ester **16** [24] (0.085 mmol) in a mixture of acetic acid (5 mL) and 6 M HCl (5 mL) was refluxed for 3 h. After evaporation in vacuo, the

residue was crystallised from EtOH. Yield = 18% (15 mg); mp = 178–181 °C (EtoH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.20 (s, 3H, CH_3), 7.10 (d, 1H, pyridopyrimidine H9, J = 6.8 Hz), 7.70 (d, 1H, pyridopyrimidine H7, J = 8.8 Hz), 7.85 (t, 1H, pyridopyrimidine H8, J = 7.6 Hz), 9.15 (s, 1H, pyridopyrimidine H2), 12.65 (exch br s, 1H, COOH). Anal. Calcd. for $C_{10}H_8N_2O_3$: C 58.82, H 3.95, N 13.72. Found: C 58.69, H 3.96, N 13.75.

General procedure for the synthesis of pyridopyrimidine carboxamides 3a-f Compounds **3a-f** were obtained starting from intermediate **17** following the same procedure described for **2c-i**. After 40 min of stirring at room temperature, the solvent was removed, and the residue was purified by flash column chromatography using $CHCl_3$/MeOH 9:1 (for **3a**), EtOH/$CHCl_3$/ethyl ether/petroleum ether/33% NH_4OH 4.5:18:18:45:0.25 (for **3b,d,e**), toluene/ethyl acetate 1:2 (for **3c**) or cyclohexane/ethyl acetate 1:1 (for **3f**) as eluents.

- *6-Methyl-4-oxo-N-methyl-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (**3a**)*

  Yield = 65% (34 mg); mp = 201–203 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.05 (s, 3H, NH-CH_3), 3.10 (s, 3H, CH_3), 6.85–6.95 (m, 1H, pyridopyrimidine H9), 7.55–7.70 (m, 2H, pyridopyrimidine H7 and H8), 8.80 (exch br s, 1H, NH), 9.15 (s, 1H, pyridopyrimidine H2). Anal. Calcd. for $C_{11}H_{11}N_3O_2$: C 60.82, H 5.10, N 19.34. Found: C 60.65, H 5.12, N 19.39. [25]
- *6-Methyl-4-oxo-N-phenyl-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (**3b**)*

  Yield = 45% (30 mg); mp = 179–181 °C (Et_2O). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.20 (s, 3H, CH_3), 7.00–7.05 (m, 1H, pyridopyrimidine H9), 7.15 (t, 1H, Ar, J = 8.4 Hz), 7.35 (t, 2H, Ar, J = 7.8 Hz), 7.70–7.80 (m, 4H, 2H Ar + pyridopyrimidine H7 and H8), 9.25 (s, 1H, pyridopyrimidine H2), 11.00 (exch br s, 1H, NH). Anal. Calcd. for $C_{16}H_{13}N_3O_2$: C 68.81, H 4.69, N 15.05. Found: C 68.62, H 4.71, N 15.08. [25]
- *6-Methyl-N-(naphthalen-2-yl)-4-oxo-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (**3c**)*

  Yield = 21% (16 mg); mp = 176–179 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.30 (s, 3H, CH_3), 7.05–7.10 (m, 1H, pyridopyrimidine H9), 7.55 (t, 2H, Ar, J = 7.6 Hz), 7.60 (t, 1H, pyridopyrimidine H8, J = 8.4 Hz), 7.70 (d, 1H, pyridopyrimidine H7, J = 8.0 Hz), 7.75–7.85 (m, 2H, Ar), 7.90 (d, 1H, Ar, J = 7.8 Hz), 8.20 (d, 1H, Ar, J = 7.8 Hz), 8.50 (d, 1H, Ar, J = 7.6 Hz), 9.35 (s, 1H, pyridopyrimidine H2), 11.60 (exch br s, 1H, NH). Anal. Calcd. for $C_{20}H_{15}N_3O_2$: C 72.94, H 4.59, N 12.76. Found: C 72.75, H 4.59, N 12.73.
- *N-Benzhydryl -6-methyl-4-oxo-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (**3d**)*

  Yield = 15% (13 mg); mp = 160–164 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.15 (s, 3H, CH_3), 6.50 (d,1H, NH-*CH*, J = 8.0 Hz), 6.90 (d, 1H, pyridopyrimidine H9, J = 6.4 Hz), 7.25–7.30 (m, 2H, Ar), 7.30–7.40 (m, 8H, Ar), 7.60 (d, 1H, pyridopyrimidine H7, J = 8.0 Hz), 7.70 (t, 1H, pyridopyrimidine H8, J = 7.2 Hz), 9.20 (s, 1H, pyridopyrimidine H2), 9.80 (d, 1H, NH). Anal. Calcd. for $C_{23}H_{19}N_3O_2$: C 74.78, H 5.18, N 11.37. Found: C 74.61, H 5.16, N 11.39.
- *N,N-Dibenzyl-6-methyl-4-oxo- 4H-pyrido[1,2-a]pyrimidine-3-carboxamide (**3e**)*

  Yield = 15% (14 mg); mp = 260–263 °C (Petroleum ether). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.10 (s, 3H, CH_3), 4.50 (s, 2H, CH_2-Ph), 4.75 (s, 2H, CH_2-Ph), 6.80 (d, 1H, pyridopyrimidine H9, J = 6.4 Hz), 7.20 (d, 2H, Ar, J = 8.0 Hz), 7.30–7.40 (m, 8H, Ar), 7.50 (d, 1H, pyridopyrimidine H7, J = 7.8 Hz), 7.55 (t, 1H, pyridopyrimidine H8, J = 7.8 Hz), 8.35 (s, 1H, pyridopyrimidine H2). Anal. Calcd. for $C_{24}H_{21}N_3O_2$: C 75.18, H 5.52 N 10.96. Found: C 75.36, H 5.50, N 10.98.
- *6-Methyl-4-oxo-N,N-diphenyl-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (**3f**)*

  Yield = 40% (34 mg); mp = 121–124 °C (EtOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.45 (s, 3H, CH_3), 6.70 (d, 1H, pyridopyrimidine H9, J = 6.0 Hz), 6.95 (t, 2H, Ar, J = 7.2 Hz), 7.10 (d, 4H, Ar, J = 8.0 Hz), 7.15–7.20 (m, 4H, Ar), 7.40 (d, 1H, pyridopyrimidine H7, J = 9.2 Hz), 7.50 (t, 1H, pyridopyrimidine H8, J = 7.8 Hz), 8.55 (s, 1H, pyridopyrimidine H2). Anal. Calcd. for $C_{22}H_{17}N_3O_2$: C 74.35, H 4.82, N 11.82. Found: C 74.26, H 4.84, N 11.87.

Molecular modelling

The pairwise similarity was calculated by using ECFP4/FCFP4 circular fingerprints using Forge 10.6.0 Revision: 36,004 (Cresset Biomolecular Discovery Ltd.). Each ligand was then subjected to an optimisation process with the additions of hydrogens using YASARA (version 14.7.17, YASARA Biosciences). Using the same software, each ligand was subjected to an energy minimisation process to obtain 3D structures ready for molecular docking saved as a PDB file. Once obtained the 3D structures for all the compounds, the geometry was also optimised at semi-empirical level using the PM3 semi-empirical Hamiltonian as implemented in MOPAC 2016 package. A protein database, consisting of 31 crystallised proteins, was built through Protein Data Bank (www.rcsb.org) and then using YASARA the proteins was prepared by removing water and ions, and other co-crystallised molecules. Moreover, the protein structure was also optimised by the addition of hydrogens. A simulation cell was created in the binding pockets of the 31 selected proteins, defined as 2 Å cube around the 3D ligand. The ligand was then removed, ready for the molecular docking process as a YASARA scene file. To obtain binding energies, molecular docking calculations were done using King's Virtual Desktop client running eight cores 2.59 Ghz Intel Xenon processor and utilising the Autodock Vina software package applied in YASARA (i.e. dock_run.mcr). The highest docking calculation for each ligand was selected and imputed in a matrix.

Drug score and ADME assessment

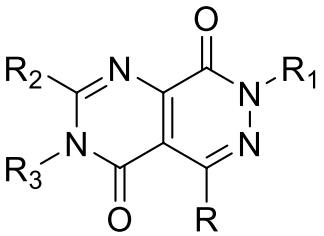
The current set of compounds was analysed with the PBPK modelling software platform PK-Sim/MoBi (Bayer Technology Services, Leverkusen, Germany) version 9. Physico-chemical properties of the compounds and their unbound fractions are presented in Table S8. A uniform 20 mg intravenous (I/V) and oral administration protocol applied to the European population (European ICRP 2002/Generic model PK-Sim-MoBi) were adopted for the compound dataset, due to the unavailability of data on minimum effective concentration, minimum toxic concentration and therapeutic window. European ICRP 2002 is a generic model structure, having the physiological database of predefined European population and its demographic features as reported in Table S10.

Results and discussion

Heterocyclic small-molecule design

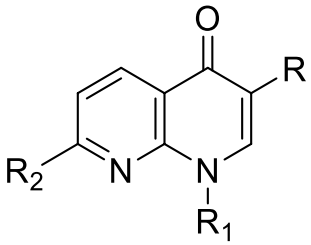
To generate new series of molecular diverse compounds, we have used a two-step computing-assisted molecular design. As showed in Fig. 1, the design focused on the search for bioisosteric-replacements/scaffold-hopping [26, 27] of the quinolinone scaffold. Quinolines and quinolinones are widely explored scaffolds in medicinal chemistry. The increase of their molecular diversity would result in expanding the array of biological targets and drug discovery applications for this chemotype [28, 29]. Our bioisosteric replacement analysis led to the selection of three nitrogen-containing heterocyclic frameworks, i.e. 3,7-dihydropyrimido[4,5-d]pyridazine-4,8-dione, 1,8-naphthyridin-4(1H)-one and 4H-pyrido[1,2-a]pyrimidin-4-one, showing features of both synthetic accessibility and molecular diversity. In the second step of the design, a ligand growing approach was chosen to enlarge the library of compounds and reach optimum physico-chemical properties, i.e. molecular weight, cLogP, cLogS, H-bond-acceptors, H-bond-donors, total surface area, relative polar surface area, shape index and flexibility [30, 31]. The results of the ligand growing experiments led to define 43 analogues reported in Tables 1, 2, and 3. To assess the molecular diversity within the compounds, the pairwise similarity was calculated by using ECFP4/FCFP4 circular fingerprints [32, 33]. The results of the calculated physico-chemical properties and the fingerprints analyses are reported in Figs. 2 and 3 and Tables S2 and S3

Table 1 Structures of the heterocyclic small-molecules with pyrimido[4,5-d]pyridazine-4,8-dione scaffold



Comp.	R	R_1	R_2	R_3
1a	Ph	C_2H_5	C_2H_5	H
1b	Ph	CH_2-(4-NO_2)-Ph	H	H
1c	Ph	CH_2-(4-$COOC_2H_5$)-Ph	H	H
1d	Ph	CH_2-(4-CH_2OCOCH_3)-Ph	H	H
1e	3-F-Ph	C_2H_5	H	H
1f	3-Cl-Ph	$CH_2cC_3H_5$	H	H
1g	3-Cl-Ph	$CH_2cC_6H_{11}$	H	H
1h	3-Cl-Ph	cC_6H_{11}	H	H
1i	3-CH_3-Ph	$CH_2cC_6H_{11}$	H	H
1j	pyridin-3-yl	$CH_2cC_6H_{11}$	H	H
1k	Ph	C_2H_5	H	CH_3
1l	Ph	C_2H_5	H	CH_2Ph
1m	Ph	CH_2-(4-COOH)-Ph	H	H
1n	Ph	CH_2-(4-CH_2OH)-Ph	H	H
1o	Ph	$CH_2cC_6H_{11}$	H	H
1p	Ph	CH_2Ph	H	H
1q	Ph	$CH_2cC_3H_5$	H	H
1r	pyridin-3-yl	CH_2-(3-Cl)-Ph	H	H
1s	Ph	$CH_2cC_3H_5$	C_2H_5	H
1t	Ph	$CH_2cC_6H_{11}$	H	C_2H_5
1u	Ph	$CH_2cC_3H_5$	H	C_2H_5

Table 2 Structures of the heterocyclic small-molecules with 1,8-naphtyridin-4-one scaffold



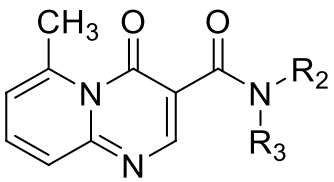
Comp.	R_1	R	R_2
2a	CH_2-(4-Cl)-Ph	$COOCH_2CH_3$	CH_3
2b	3-NO_2-Ph	$COOCH_2CH_3$	CH_3
2c	CH_2CH_3	CONH- pyridin-3-yl	CH_3
2d	CH_2CH_3	$CONHNHCOCH_3$	CH_3
2e	CH_2CH_3	$CON(CH_3)Ph$	CH_3
2f	CH_2CH_3	CONH-naphtalen-2-yl	CH_3
2g	CH_2-(4-Cl)-Ph	$CONHCH_3$	CH_3
2h	3F-Ph	$CONHCH_3$	CH_3
2i	3F-Ph	$CONHNHCOCH_3$	CH_3
2j	CH_2CH_3	CONH-(3,5-dichloropyridin)-4-yl	CH_3
2k	CH_2-(4-F)-Ph	CONH-(3,5-dichloropyridin)-4-yl	CH_3
2l	CH_2CH_3	5-methyl-1,3,4-oxadiazol-2-yl	CH_3
2m	CH_2CH_3	$COOCH_2CH_3$	3-NO_2-Ph
2n	CH_2CH_3	CONHPh	OCH_3
2o	CH_2CH_3	CONHPh	pyrrolidin-1-yl
2p	CH_2CH_3	$CON(CH_3)Ph$	pyrrolidin-1-yl

(see Supporting Information). ECFP4/FCFP4 circular fingerprints showed that the new set of compounds covers a wide range of molecular features (in line with the structural diversity investigated in this study), which can be summarised as follows: molecular weight, from 217 to 457; cLogP, from -0.83 to 3.81; cLogS, from −6.56 to −1.26; H-acceptors, between 5 and 9; H-donors, between 0 and 2; total polar surface, from 167 to 321; relative polar surface area, from 0.15 to 0.35; shape index, from 0.43 to 0.58; flexibility, from 0.28 to 0.53.

Chemistry

The synthetic procedures carried out to obtain the 43 target compounds of type 1–3 are reported in Schemes 1, 2, 3 and 4. The structures were confirmed on the basis of analytical and spectral data. Scheme 1 shows the synthetic pathway affording the final compounds **1a-n** containing the new bicyclic scaffold pyrimido[4,5-d]pyridazine-4,8-dione. Intermediates **5a-e** (5a [34] and 5b,5d [22]) were obtained starting from isoxazole **4a-e** (where 4c and 4e are commercially available, while compounds **4a**, **4b** and **4d** were synthesised by adopting previously reported protocols) [22, 35] using hydrazine hydrate and polyphosphoric acid (PPA) in ethanol. The 4-amino-5-amido derivatives **6a-e** were obtained by reacting analogues of type 5 with 33% NH_4OH at 60 °C. The alkylation of **6a-e** with suitable alkyl(aryl) halides in standard conditions afforded the intermediates **7a-d** and **7f-k** (where 7a,f were previously reported) [22]. Differently,

Table 3 Structures of the heterocyclic small-molecules with pyrido[1,2-a]pyrimidine scaffold



Comp.	R_2	R_3
3a	H	CH_3
3b	H	Ph
3c	H	naphtalen-2-yl
3d	H	$CH(Ph)_2$
3e	CH_2-Ph	CH_2-Ph
3f	Ph	Ph

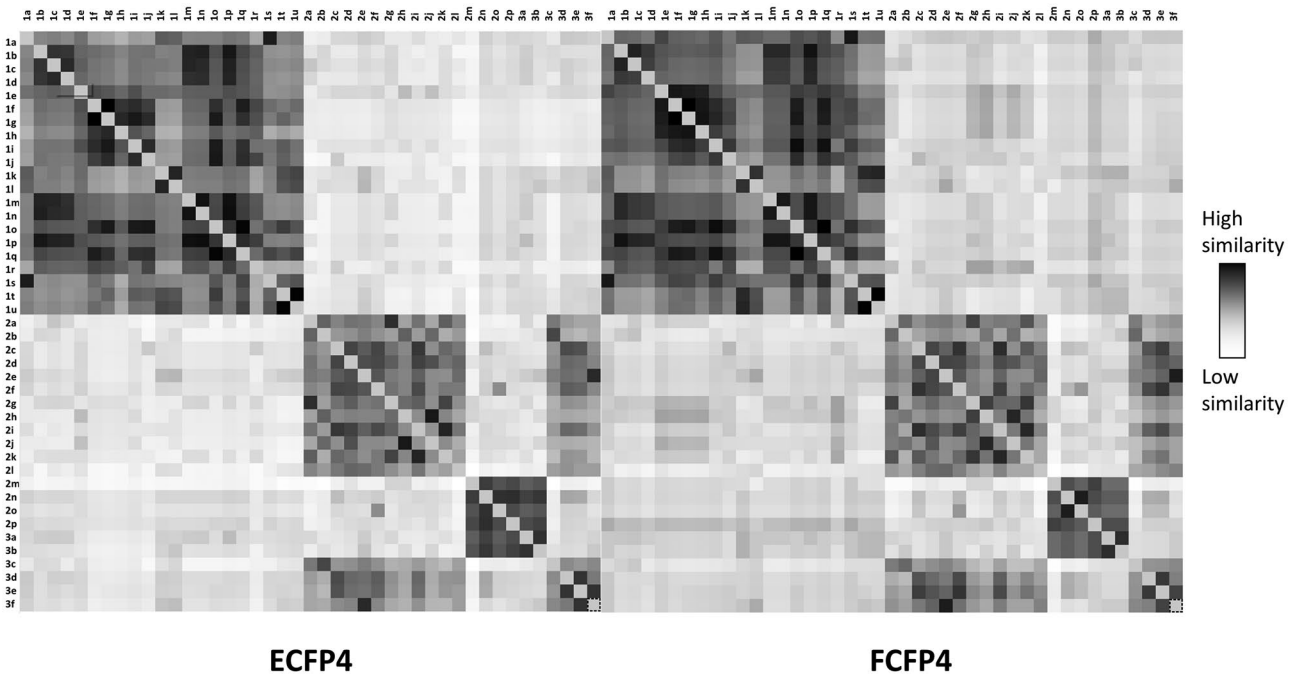


Fig. 2 ECFP4 and FCFP4 fingerprint similarity matrix

compound **7e** was obtained from **7d** by treatment with acetyl chloride in anhydrous CH_2Cl_2 at room temperature. Lastly, compounds **1a-j** and **8** were obtained by the cyclisation of intermediates of type 7 with triethylorthoformate or triethylorthopropionate in H_2SO_4. For the synthesis of **1 k,l**, compound **8** was alkylated with the appropriate alkyl halide in standard conditions, while compounds **1 m,n** were obtained starting from **1c** and **1d** through hydrolysis with NaOH in EtOH at room temperature.

Scheme 2 reports the synthesis of the final compounds **1o-u** also showing the pyrimido[4,5-d]pyridazine-4,8-dione scaffold. The pyridazinones **9a** [22] and **9b** were obtained starting from **5a** and **5e**, by reductive cleavage with piperidine in anhydrous CH_3OH at 60 °C. After alkylation in standard conditions with the appropriate alkyl halide to give **10a-d**, the 4-ammino-5-carboxyl derivatives **11a-d** were obtained through hydrolysis with 6 N NaOH in EtOH at 60–70 °C. **11a-d** were then converted by treatment with $SOCl_2$, Et_3N and 33% NH_3 into the corresponding amide derivatives **12a-d** which, in turn, were cyclised to final compounds **1o-s** with triethylorthoformate or triethylorthopropionate, by adopting the same procedure described in Scheme 1. Lastly, the alkylation of **1o** and **1q** with ethyl bromide afforded the final compounds **1t,u**.

The naphtyridone scaffold 15 (Scheme 3) was synthesised from 2-amino-6-methylpyridine **13** and diethyl-2-(ethoxymethylene)malonate (EMME), through a simultaneous and thermally induced ring closure of the diethyl-(6-methyl-2-pyridylaminomethylene)malonate intermediate **14** [36, 37]. Compound **15** was treated with the suitable alkyl/aryl halide in standard alkylation conditions (for **18a,b**, **2a**) [38], or coupled with the appropriate R-phenylboronic acid in the presence of $Cu(OAc)_2$ to obtain compounds **18c,d** and **2b**. Subsequently, the alkaline hydrolysis of the ester group afforded the carboxylic acids **19a-e** (19a [38]) which were firstly converted in the corresponding acid chlorides using $SOCl_2$ at room temperature and then treated with the appropriate amine in dry tetrahydrofuran to give the final compounds **2c-k**. The oxadiazole derivative **2 l** was synthesised from the acetyl carboxyhydrazide **2d** using $POCl_3$ on SiO_2 as cyclodehydrating agent. During the synthesis of intermediate **15**, a side-reaction following the Gould-Jacobs mechanism afforded the isomer 4H-pyrido[1,2-a]pyrimidine **16** [24]. The latter was used to synthesise the pyrido(1,2-a)pyrimidine analogues **3a-f** through the following steps: (i) treatment with HCl in acetic acid at reflux (to obtain the corresponding carboxylic acid **17**) and (ii) conversion into the final products **3a-**f using the same procedure adopted for **2c-k**.

Scheme 4 reports the synthesis of the naphthyridone-based amide derivatives **2n-p** and the 3-carbethoxy **2 m** bearing at position 7 a methoxy, a pyrrolidine or a 3-NO_2-phenyl substituent. Starting from intermediates of type **20** [23, 39, 40], the alkylation reaction with bromoethane e potassium carbonate in dry DMF afforded the derivatives **21a,b** [23, 39]. Differently, the derivative **2 m** was obtained through Suzuki coupling performed on **20c** and using 3-nitrophenylboronic acid, 2 M sodium carbonate and tetrakis(triphenylphosphine)-palladium(0) in toluene. Alkaline hydrolysis of **21a,b** generated the carboxylic acids **22a,b** [39, 41] which, in turn,

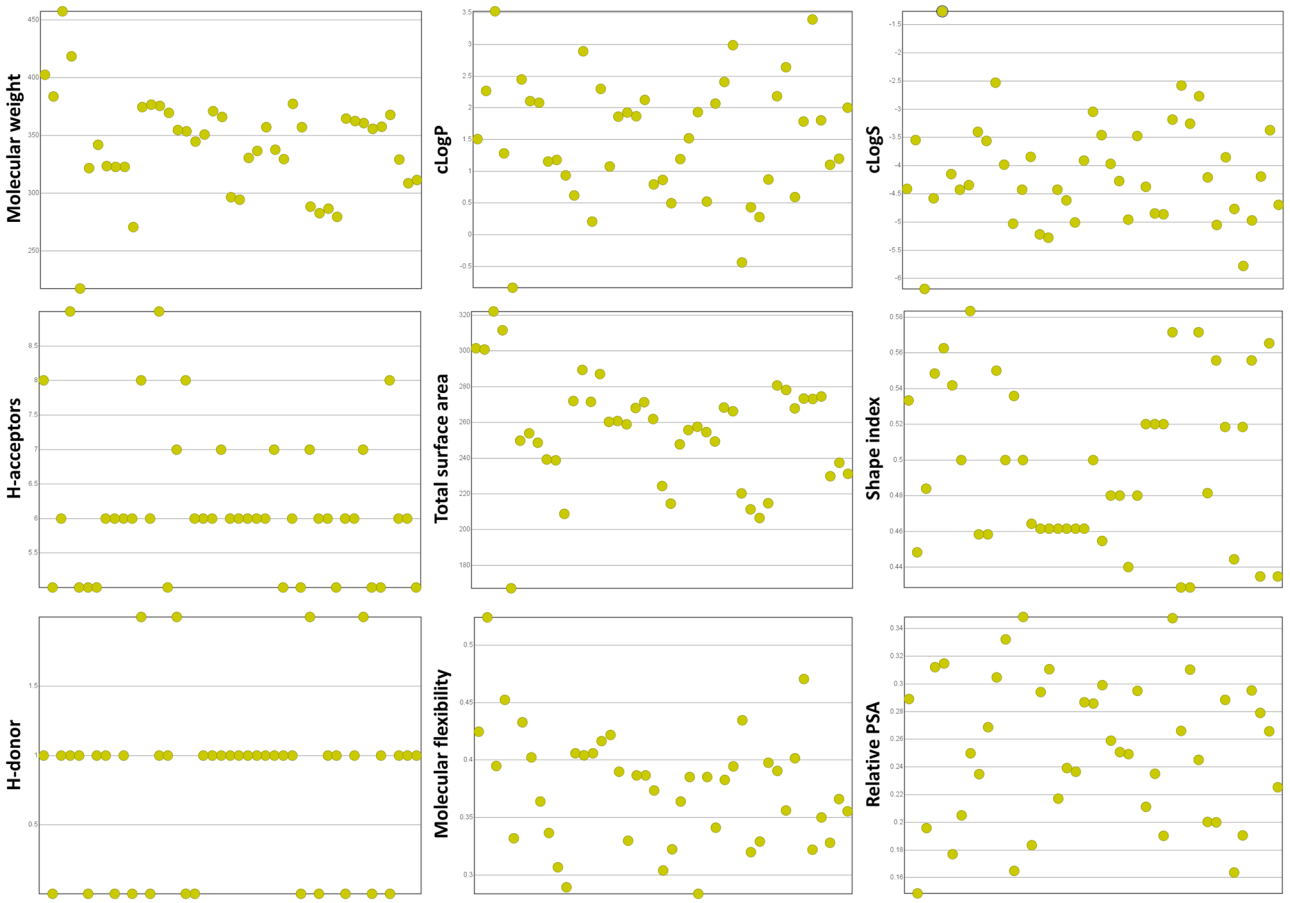


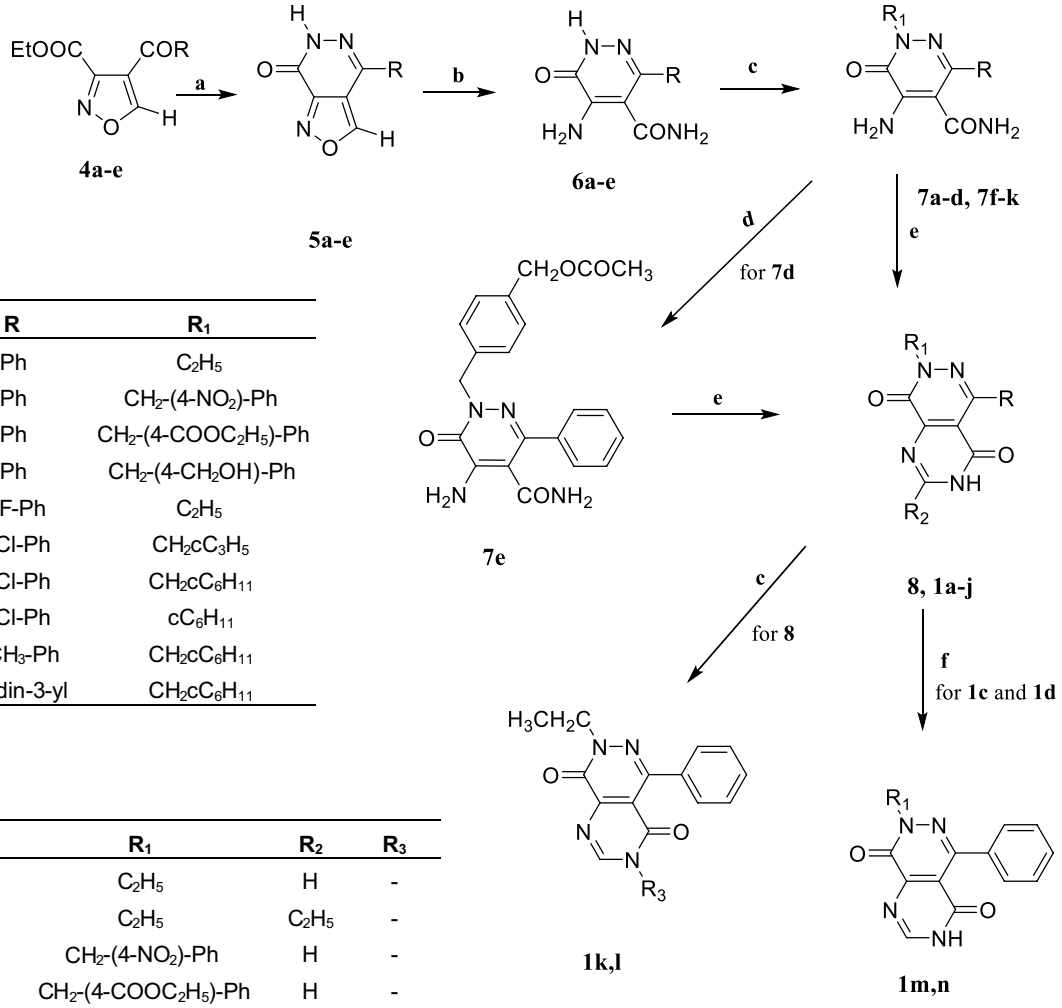
Fig. 3 Variability of physico-chemical properties within the library of 43 compounds

was converted into the final amides **2n-p** through the acid chlorides intermediates, in usual conditions.

Molecular modelling

Adopting iVS methodologies, the library of compounds reported here (43 terms) was subsequently screened against a panel of 31 cellular targets, that are involved in the development, proliferation or progression of cancer in order to find potential hits for biomedical studies (Table S1, Supporting Information). The 31 targets were selected from a previously reported dataset, where a panel of several proteins involved in cancer was successfully validated for iVS investigations [17, 20, 21]. In this study, protein targets with diverse physiological functions were selected for the screening. Specifically, we have usedkinases with various roles (phospho-CDK2/Cyclin A, B-Raf, PLK1, AKT, Pi3K, CK2, human anaplastic lymphoma kinase, PAK6, MDM2, MST3, C-SRC, MRCK, human ROS1 kinase, C-Abl kinase, PTK6, BRAF, ephrin A2 receptor protein kinase), grow factors (EGFR, C-Met, VEGFR2, FGFR1), transporters (FABP4), chaperones (HPS90), cellular enzymes (17-beta-hydroxysteroid dehydrogenase type1, PARP, matriptase, DNPH1, carbonic anhydrase IX), BCL6 (a transcription factor) and the human retinoid × receptor alpha. AutoDock Vina was used as the docking software [20, 21]. The analysis of binding energies between the new heterocyclic ligands and targets was reported in Table S4 (see Supporting Information). Docking analysis of crystallised ligands, with an established binding mode, was carried out in order to obtain a minimum energy level which has been used as the cut-off for the assessment of binding energies of the new ligands. In particular, the binding efficiency was evaluated through the ratio between the binding energies of analysed ligands and reference ligands co-crystallised in the protein, by applying Eq. (1):

$$\delta = \frac{\Delta G\text{compound}}{\Delta G\text{reference ligand}} \quad (1)$$



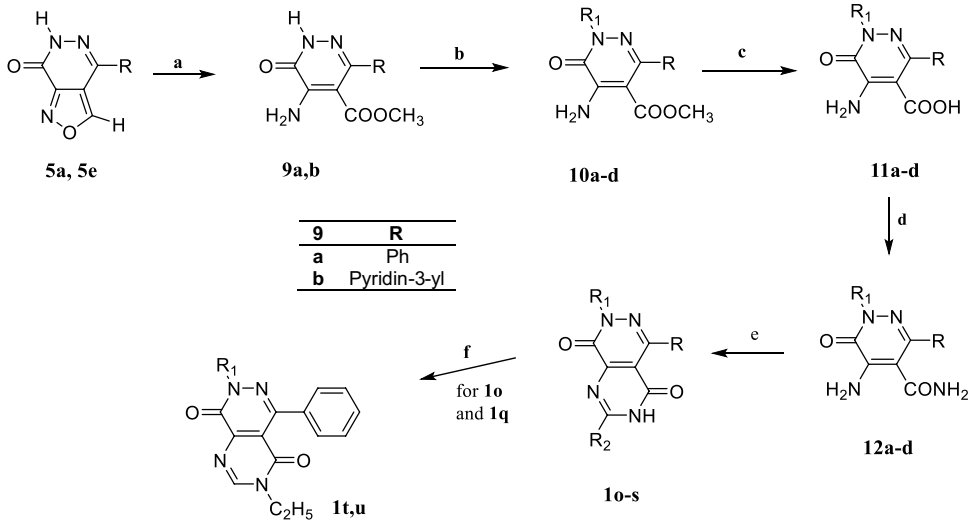
Comp. 7	R	R_1
a	Ph	C_2H_5
b	Ph	CH_2-(4-NO_2)-Ph
c	Ph	CH_2-(4-$COOC_2H_5$)-Ph
d	Ph	CH_2-(4-CH_2OH)-Ph
f	3-F-Ph	C_2H_5
g	3-Cl-Ph	$CH_2cC_3H_5$
h	3-Cl-Ph	$CH_2cC_6H_{11}$
i	3-Cl-Ph	cC_6H_{11}
j	3-CH_3-Ph	$CH_2cC_6H_{11}$
k	Pyridin-3-yl	$CH_2cC_6H_{11}$

Comp.	R	R_1	R_2	R_3
8	Ph	C_2H_5	H	-
1a	Ph	C_2H_5	C_2H_5	-
1b	Ph	CH_2-(4-NO_2)-Ph	H	-
1c	Ph	CH_2-(4-$COOC_2H_5$)-Ph	H	-
1d	Ph	CH_2-(4-CH_2OCOCH_3)-Ph	H	-
1e	3-F-Ph	C_2H_5	H	-
1f	3-Cl-Ph	$CH_2cC_3H_5$	H	-
1g	3-Cl-Ph	$CH_2cC_6H_{11}$	H	-
1h	3-Cl-Ph	cC_6H_{11}	H	-
1i	3-CH_3-Ph	$CH_2cC_6H_{11}$	H	-
1j	Pyridin-3-yl	$CH_2cC_6H_{11}$	H	-
1k	-	-	-	CH_3
1l	-	-	-	CH_2Ph
1m	-	CH_2-(4-COOH)-Ph	-	-
1n	-	CH_2-(4-CH_2OH)-Ph	-	-

Comp. 4-6	R
a	Ph
b	3-F-Ph
c	3-Cl-Ph
d	3-CH_3-Ph
e	Pyridin-3-yl

Scheme 1 Reagents and conditions: (**a**) $NH_2NH_2.H_2O$, PPA, EtOH, 80–90 °C, 2–5 h; (**b**) 33% NH_4OH, 60 °C, 1–2 h; (**c**) suitable alkyl(aryl) halide, K_2CO_3, DMF, 80 °C, 1–3 h; (**d**) acetyl chloride, anhydrous CH_2Cl_2, rt, 3 h; (**e**) triethylorthoformate (or triethylorthopropionate), H_2SO_4 conc, 130 °C, 1–5 h; (**f**) 6 N NaOH, EtOH, rt, 1–2 h

Equation (1) was used as an inclusion criterion to assess which are the compounds worth further analysis. Only the analogues achieving a $\delta \geq 1$ were processed through a normalisation procedure. This approach allows to select and examine only the proteins showing the highest affinity for the ligands. The matrix containing δ calculations



9	R
a	Ph
b	Pyridin-3-yl

1	R	R_1	R_2
o	Ph	$CH_2cC_6H_{11}$	H
p	Ph	CH_2-Ph	H
q	Ph	$CH_2cC_3H_5$	H
r	Pyridin-3-yl	CH_2-(3-Cl)-Ph	H
s	Ph	$CH_2cC_3H_5$	C_2H_5
t	-	$CH_2cC_6H_{11}$	-
u	-	$CH_2cC_3H_5$	-

Comp. 10-12	R	R_1
a	Ph	$CH_2cC_6H_{11}$
b	Ph	CH_2-Ph
c	Ph	$CH_2cC_3H_5$
d	Pyridin-3-yl	CH_2-(3-Cl)-Ph

Scheme 2 Reagents and conditions: (**a**) anhydrous MeOH, piperidine, 65 °C, 1.5 h; (**b**) suitable alkyl halide, K_2CO_3, DMF, 80 °C, 30–90 min; (**c**) 6 N NaOH, EtOH, 60–70 °C, 1–2 h; (**d**) step1: $SOCl_2$, Et_3N, 60–70 °C, 1 h; step 2: 33% NH_4OH, 0 °C, 30–60 min; (**e**) triethylorthoformate (or triethylorthopropionate), H_2SO_4 conc., 130 °C, 2–4 h; (**f**) CH_3CH_2Br, K_2CO_3, dry DMF, 80 °C, 30 min

(Table S5, Supporting Information) highlighted 17 proteins (PDB codes: 1XKK, 2FB8, 2RKU, 2XAB, 3L3L, 3LBZ, 3PE1, 3ZXZ, 4ASE, 4KS8, 4P5E, 4Q07, 4QMZ, 4U5J, 4UAL, 4YC8 and 5I9Y) where some specific compounds of the library express good to high affinity. In addition, we found higher affinity towards most of the ligands for the two proteins 2RKU and 4Y5J, with 2RKU having higher binding energies than the co-crystallised ligand in all dockings and 4Y5J having only two results that were not higher than the co-crystallised ligand.

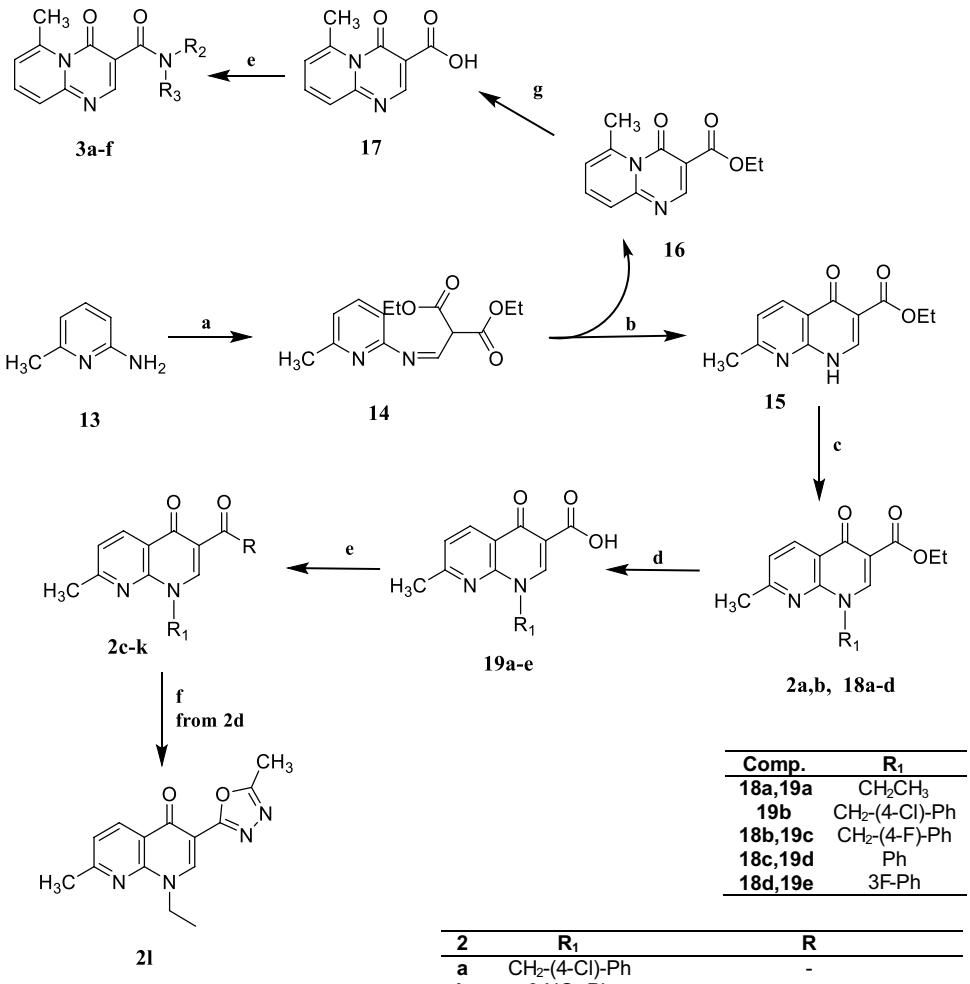
Similarly, several ligands have shown affinity for more than one cellular protein. Examining the δ calculation matrix, seven ligands have greater δ for 10 or more targets. These are the ligands **1b**, **1 g**, **1 m**, **2f**, **2j**, **2 k**, **3c**, **3d**, which can be valid hits for multitarget pharmacological approach [42]. Moreover, four ligands (i.e. **1u**, **2a**, **2d** and **2e**) show selectivity towards one single protein, hence demonstrating possible candidates for targeted therapy. In contrast, the δ calculation matrix shows that only one ligand, **3a**, did not have a $\delta \geq 1$ for any of the 31 proteins.

Normalisation of the binding energies values was conducted as proposed by Lauro et al. [20, 21] in order to overcome the lack of selectivity and occurrence of false positives, as well as to avoid systematic errors associated with the interaction of ligands and biological targets. Equation (2) was used to normalise the binding energy values in the matrix:

$$V = V0/[(ML + MR)/2] \quad (2)$$

In this formula, V is the value associated with each compound and can be used to generate a ranking system between a protein and a ligand. V0 is the value of the binding energy (kcal/mol) from the molecular docking calculations, ML is the average binding energy (kcal/mol) of each ligand on different targets and MR is the average binding energy (kcal/mol) associated with each target on various targets. Every single value in the matrix representing a single ligand versus a specific receptor was normalised taking simultaneously into account the specific averages contained in V values (Table S6, Supporting Information), which were also calculated for ligands co-crystallised in the protein in order to show significant interactions within the matrix.

The values obtained led to the identification of several compounds showing interaction with a number of proteins, highlighting 16 targets (PDB codes: 1XKK, 2FB8, 2RKU, 2XAB, 3L3L, 3PE1, 3ZXZ, 4ASE, 4KS8, 4P5E, 4Q07, 4QMZ, 4U5J, 4UAL, 4YC8 and 5I9Y) from the 17 already selected with the Eq. (1). Specifically, these cellular proteins showed a higher trend of V values for the compound dataset, in comparison to the V values of the specific co-crystallised



Scheme 3 Reagents and conditions: (**a**) EMME, toluene, 100 °C, 6 h; (**b**) diphenyl ether, 270 °C, 4 h; (**c**) for 2a and 18a,b: alkyl halide, K_2CO_3, DMF, 60 °C, 30–180 min; for 18c,d and 2b: R-arylboronic acid, $Cu(OAc)_2$, Et_3N, CH_2Cl_2, 24 h, rt; (**d**) 6 N NaOH, EtOH, 0.5–1 h, rt; (**e**) for 2c–i, step 1: $SOCl_2$ (excess), Et3N (catalytic), 2–3 h, rt; step 2: appropriate amine, anhydrous THF, 2–3 h, rt; for 2j,k, step 1: $SOCl_2$ (excess), Et3N (catalytic), 2–3 h, rt; step 2: 3,5–dichloro-4-pyridilamina, NaH, anhydrous THF, 30 min, rt; (**f**) $POCl_3$/SiO_2, toluene, 80 °C, 5 h; (**g**) 6 M HCl, AcOH, reflux, 3 h

Comp.	R_1
18a,19a	CH_2CH_3
19b	CH_2-(4-Cl)-Ph
18b,19c	CH_2-(4-F)-Ph
18c,19d	Ph
18d,19e	3F-Ph

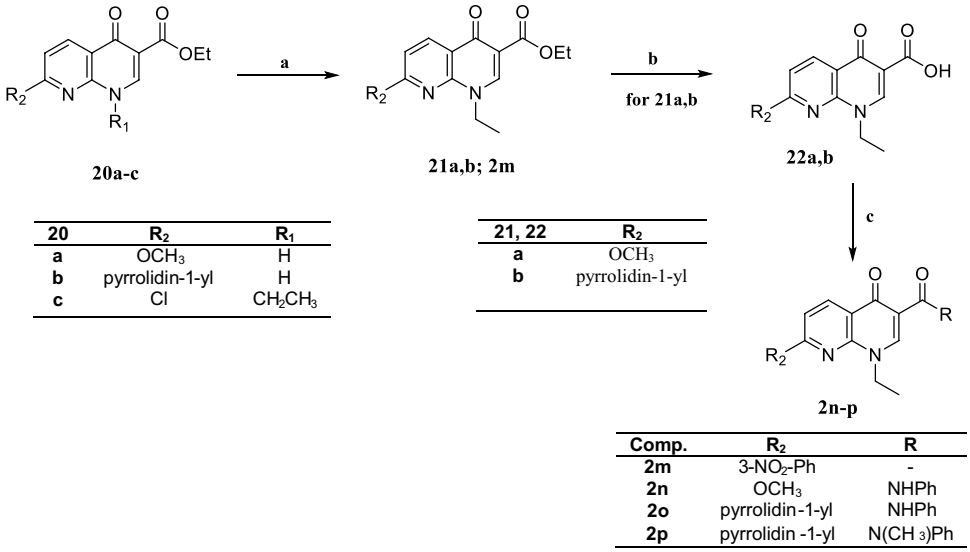
2	R_1	R
a	CH_2-(4-Cl)-Ph	-
b	3-NO_2-Ph	-
c	CH_2CH_3	NH-pyridin-3-yl
d	CH_2CH_3	$NHNHCOCH_3$
e	CH_2CH_3	$N(CH_3)Ph$
f	CH_2CH_3	NH-naphtyl-3-yl
g	CH_2-(4-Cl)-Ph	$NHCH_3$
h	3F-Ph	$NHCH_3$
i	3F-Ph	$NHNHCOCH_3$
j	CH_2CH_3	NH-(3,5-dichloropyridin)-4-yl
k	CH_2-(4-F)-Ph	NH-(3,5-dichloropyridin)-4-yl

3	R_2	R_3
a	H	CH_3
b	H	Ph
c	H	naphtalen-2-yl
d	H	$CH(Ph)_2$
e	CH_2-Ph	CH_2-Ph
f	Ph	Ph

inhibitor. V values against the selected targets are summarised in Table 4 and Fig. 4.

As mentioned earlier, all the proteins examined in this study are established targets with key roles in cancer pathogenesis. Moreover, 12 out of the 16 selected proteins which showed good affinity for the ligands are protein kinases. In this regard, the pyrimido[4,5-d]pyridazine-4,8-dione scaffold is a bi-cyclic structure containing a fused pyridazinone heterocycle [43, 44], which is a privileged structural motif for targeting the cellular proteins investigated in this study. For example, several previously reported inhibitors of VEGF-2, PDE4, c-met and EGFR inhibitors are based on the pyridazinone scaffold [45–47]. Pyridazine-based drugs such as olaparib and fuzuopali are also targeted to PARP for use in ovarian, breast, gastric and prostate solid tumours. Likewise, pyridine-containing molecules are inhibitors of B-Raf kinase and PLK 1 [48], as well as naphthyridine-based scaffolds, have been investigated for antiproliferative activity [49]. These literature evidences support our iVS study as an effective approach to identify hits from a database of ligands showing affinity for protein targets with relevance in cancer pathogenesis.

For selection and comparison of ligands which can be useful hits for biomedical studies, the molecules were



20	R_2	R_1
a	OCH_3	H
b	pyrrolidin-1-yl	H
c	Cl	CH_2CH_3

21, 22	R_2
a	OCH_3
b	pyrrolidin-1-yl

Comp.	R_2	R
2m	3-NO_2-Ph	-
2n	OCH_3	NHPh
2o	pyrrolidin-1-yl	NHPh
2p	pyrrolidin -1-yl	N(CH_3)Ph

Scheme 4 Reagents and conditions: (**a**) for 21a,b: Br-CH_2CH_3, 20a or 20b, K_2CO_3, DMF, 60 °C, 30–180 min; for 2 m: 3-nitrophenylboronic acid, 20c, tetrakis, 2 M Na_2CO_3, ethanol, toluene, 60 °C, 2–3 h; (**b**) NaOH 6 N, EtOH, 0.5–1 h, rt; (**c**) step 1: $SOCl_2$ (excess), Et_3N (catalytic), 2–3 h, rt; step 2: appropriate amine, anhydrous THF, 2–3 h, rt

ranked for each particular protein using the V value matrix (Table S6, Supporting Information). Table 4 shows that ligand **3c** was in the top three ranks between the analysed molecules of several chosen proteins, suggesting as a potential drug candidate with multitarget activity. Differently, ligand **3a** is the ligand usually found in the lowest ranks and not present in any top ranks of the protein. One ligand (**1 k**) demonstrates selective for one cellular protein, while six others (**1b**, **1q**, **2c**, **2j**, **2p** and **3f**) show affinity only for a small number of cellular targets. These results confirm the validity of Eq. 1 as an inclusion and exclusion criterion for the selection of possible hits. Subsequent pharmacological tests will conclusively confirm which are the potential hits as anticancer drug candidates. Nevertheless, the iVS analysis suggests that nine ligands (**1b, 1 k**, **1q**, **2c**, **2j**, **2p**, **3a**, **3c** and **3f**) have high binding efficacy towards several cellular proteins considered in this study.

Drug score and ADME assessment

Physiologically based pharmacokinetic (PBPK) models are valid tools for early stages of drug discovery to predict the pharmacokinetics of drug candidates, by integrating information on human physiology and anatomy with physic-chemical (e.g. acidity/basicity, lipophilicity, solubility) and biological (e.g. unbound fraction) properties of the molecules [50]. More in detail, PBPK modelling is a blood flow-based pharmacokinetic modelling in which each compartment represents a physiologically distinct compartment, such as an organ or tissue with a specific blood flow, describing drug concentration changes over time in each organ. The parameters and compartments are then optimised to fit the model with existing data. This approach differs from other pharmacokinetic prediction tools used in the field, such as PopPK modelling, which is also a compartment-based pharmacokinetic modelling [51]. However, in contrast to PBPK, in PopPK, the compartments are not linked to anatomic or physiologic values. In general, PopPK basically considers available pharmacokinetic information and builds a model that fits the data. PopPK modelling follows a "top-down" approach, being mainly empirical and starting with a very simple model (usually one compartment) and assessing linear kinetics. Additional peripheral compartments can then be added to interpret the data and assess statistical significance. Differently, PBPK models are based on the "bottom-up" approach, starting from the organ or at the tissue level [52]. In PBPK, simulated version of drug concentrations in various tissues versus time profiles is usually generated to carry out the analysis [53]. Using the PK-Sim/Mobi platform (Bayer Technology Services), we have processed the 43 compounds composing library through PBPK to evaluate the plasma concentration–time profile (Figs. S1–S5, Supporting Information). Pharmacokinetic parameters used in our model, including area under the curve (AUC), maximum concentration (Cmax), time to reach maximum concentration (tmax), mean residence time (MRT), clearance (CL/F) and volume of distribution (Vd), are presented in Table S7. Overall, the PBPK analysis highlights compounds **1a**, **1q** (with pyrimido[4,5-d] pyridazine-4,8-dione scaffold) and **3b** (with pyrido(1,2-a) pyrimidine scaffold) displayed the highest values of AUC, followed by **1 k** and **1f**. The respective Cmax values of these five terms were also higher than the Cmax values of the rest of the compounds (Table S8, Supporting Information). However, the value of absolute bioavailability results reduced to 18.5% for **1q**, **1 k** and **1f** when the AUC after

Table 4 Calculated V values used to rank binding affinity between ligands and proteins, for the selected proteins with $\delta \geq 1$

	Protein (PDB code)																
Ligand	**1xkk**	**2fb8**	**2rku**	**2xab**	**3l3l**	**3lbz**	**3pe1**	**3zxz**	**4ase**	**4ks8**	**4p5e**	**4q07**	**4qmz**	**4u5j**	**4ual**	**4yc8**	**5i9y**
Co-crystallised	0.98	1.05	0.85	1.02	0.95	0.72	1.12	1.09	0.93	0.90	0.87	0.91	0.94	0.81	1.04	1.03	1.03
1a	1.04	1.03	1.06	0.95	1.02	0.77	1.02	0.93	0.85	0.99	0.89	1.04	0.90	0.96	1.02	1.00	1.06
1b	1.07	1.06	1.03	0.94	1.09	0.77	1.18	1.02	0.93	1.03	0.96	0.97	0.95	1.05	1.19	0.99	0.99
1c	1.08	1.05	1.01	0.90	0.97	0.77	1.13	0.99	0.90	1.02	0.93	0.91	0.94	1.02	1.12	1.03	0.96
1d	1.09	1.09	1.14	0.87	1.03	0.82	1.17	1.07	0.94	1.03	1.02	0.91	1.01	1.01	1.06	1.08	0.99
1e	0.96	1.03	1.00	0.95	1.02	0.73	1.05	0.97	0.86	0.94	0.94	1.02	0.92	0.99	0.98	0.95	0.96
1f	0.96	1.06	0.96	0.96	1.03	0.72	1.01	1.08	0.85	0.94	0.98	1.03	0.96	0.99	0.97	0.91	0.94
1g	1.10	1.10	1.06	0.99	1.10	0.78	1.13	1.01	0.95	1.06	1.01	1.00	0.99	1.05	1.10	1.07	1.01
1h	1.04	1.03	1.11	1.02	1.04	0.75	1.01	0.99	0.88	1.05	0.99	1.00	0.97	1.00	0.97	0.99	1.05
1i	0.95	1.04	1.08	1.02	1.11	0.75	1.01	1.06	0.89	0.97	0.94	1.00	1.02	1.06	1.08	0.98	0.98
1j	1.04	0.99	1.08	0.99	1.05	0.77	0.97	0.98	0.93	0.99	1.06	0.97	1.01	1.02	1.06	0.96	1.00
1k	1.01	1.07	0.96	0.93	1.02	0.75	1.08	0.90	0.83	0.95	0.93	1.06	0.91	0.94	1.01	0.99	0.98
1l	1.09	1.15	1.06	0.96	1.07	0.74	1.07	0.92	0.89	1.03	0.96	0.99	0.96	1.04	1.16	0.96	1.01
1m	1.07	1.08	1.09	1.04	1.07	0.80	1.15	1.05	0.96	1.07	0.99	0.97	0.99	1.02	1.08	1.03	1.01
1n	1.04	1.07	1.05	1.00	1.07	0.78	1.12	1.04	0.94	1.05	0.99	0.96	0.97	1.01	1.11	1.00	1.03
1o	0.95	1.02	1.05	0.99	1.01	0.72	1.08	1.08	0.91	0.97	0.99	1.00	0.96	1.05	1.07	0.96	0.98
1p	1.11	1.08	1.06	0.99	1.08	0.78	1.12	1.01	0.95	1.05	1.00	1.01	0.96	1.03	1.10	0.98	0.99
1q	0.93	1.12	0.94	0.93	1.00	0.71	1.01	1.05	0.83	0.91	0.95	1.05	0.95	0.99	1.01	0.91	0.93
1r	1.06	1.13	1.10	0.97	1.07	0.79	1.08	0.98	0.88	1.04	1.00	1.01	1.05	1.03	1.08	0.95	1.01
1s	1.02	1.11	1.02	0.94	0.99	0.74	0.91	1.00	0.86	0.93	0.93	0.97	0.98	0.97	1.01	1.04	1.05
1t	1.11	1.11	1.06	0.97	1.09	0.79	1.08	0.97	0.98	1.02	0.93	0.96	0.98	1.03	1.14	1.01	1.02
1u	0.93	1.13	0.96	0.97	0.99	0.73	1.01	1.00	0.85	0.97	0.92	1.00	0.93	0.99	1.03	0.93	0.92
2a	0.99	0.98	1.07	0.95	1.00	0.74	0.99	1.08	0.88	0.97	0.95	0.99	0.91	0.95	1.00	0.96	0.94
2b	1.05	1.06	0.96	0.93	1.05	0.76	1.09	0.98	0.87	1.02	1.00	1.03	0.96	1.00	1.03	0.93	0.94
2c	0.98	0.96	0.99	0.98	0.99	0.74	1.07	1.12	0.84	0.98	0.92	0.93	0.93	1.00	1.05	1.00	0.98
2d	0.93	0.94	0.99	0.96	0.93	0.76	1.07	1.06	0.83	0.94	0.90	0.91	0.92	0.90	0.98	0.97	0.90
2e	0.86	0.96	1.06	1.04	1.03	0.74	1.07	1.11	0.88	0.95	0.93	0.95	1.01	1.04	1.15	1.00	0.98
2f	1.10	0.99	1.13	1.09	1.12	0.76	1.13	1.07	1.05	1.00	0.98	1.03	0.97	1.06	1.04	1.21	1.03
2g	1.02	0.99	1.13	0.92	1.02	0.74	1.04	1.11	0.92	0.98	0.99	0.98	0.94	1.00	0.99	1.00	0.96
2h	1.07	1.08	0.95	0.92	1.07	0.74	1.12	0.99	0.84	1.01	0.91	0.98	0.98	1.05	1.07	0.92	0.96
2i	0.96	0.96	0.98	0.95	1.03	0.76	0.96	1.01	1.00	0.90	0.86	0.98	0.99	1.04	0.98	1.06	1.06
2j	1.15	1.14	1.15	0.96	1.10	0.80	1.07	1.08	1.02	0.98	0.96	0.92	0.99	1.07	1.10	1.16	0.99
2k	1.15	1.08	1.15	0.95	1.02	0.79	0.96	1.14	0.99	1.01	0.93	0.95	1.03	1.08	1.14	1.14	1.07
2l	0.99	0.98	0.99	0.95	0.98	0.76	1.04	0.99	0.85	0.92	0.96	0.91	0.94	0.93	1.03	0.95	1.02
2m	1.06	1.02	1.09	0.94	0.94	0.74	0.99	0.99	0.90	0.96	1.01	0.88	0.97	1.00	0.95	1.07	1.05
2n	0.99	0.99	0.96	0.97	0.97	0.74	1.08	1.08	0.89	1.02	0.97	0.88	1.01	0.99	1.14	0.92	0.95
2o	1.00	1.00	0.97	1.01	0.99	0.78	0.98	1.08	0.88	0.95	0.99	0.91	0.98	1.08	1.13	0.97	0.96
2p	0.96	1.06	0.98	0.99	0.88	0.80	1.10	1.21	0.96	1.03	1.00	0.91	1.04	1.03	1.04	1.09	1.10
3a	0.86	0.92	0.90	0.95	0.92	0.70	0.92	0.94	0.84	0.87	0.86	0.90	0.91	0.88	0.86	0.92	0.90
3b	0.99	1.08	1.00	1.02	1.05	0.80	1.06	1.06	0.93	0.93	0.88	1.00	0.97	0.95	1.06	0.98	0.96
3c	1.17	1.13	1.17	1.09	1.08	0.85	1.05	1.00	0.99	0.98	0.99	0.93	1.02	0.97	1.00	1.11	1.08
3d	1.06	1.12	1.15	1.11	1.11	0.87	1.08	1.06	0.96	0.99	1.01	1.04	1.01	1.03	1.13	1.08	1.03
3e	1.03	1.14	1.06	1.08	1.20	0.77	1.04	1.09	0.99	1.00	0.98	0.96	0.99	0.94	1.08	1.00	1.06
3f	1.00	1.11	1.11	1.07	1.14	0.72	1.07	0.82	0.97	0.87	0.92	1.06	1.00	0.95	1.05	0.97	1.06

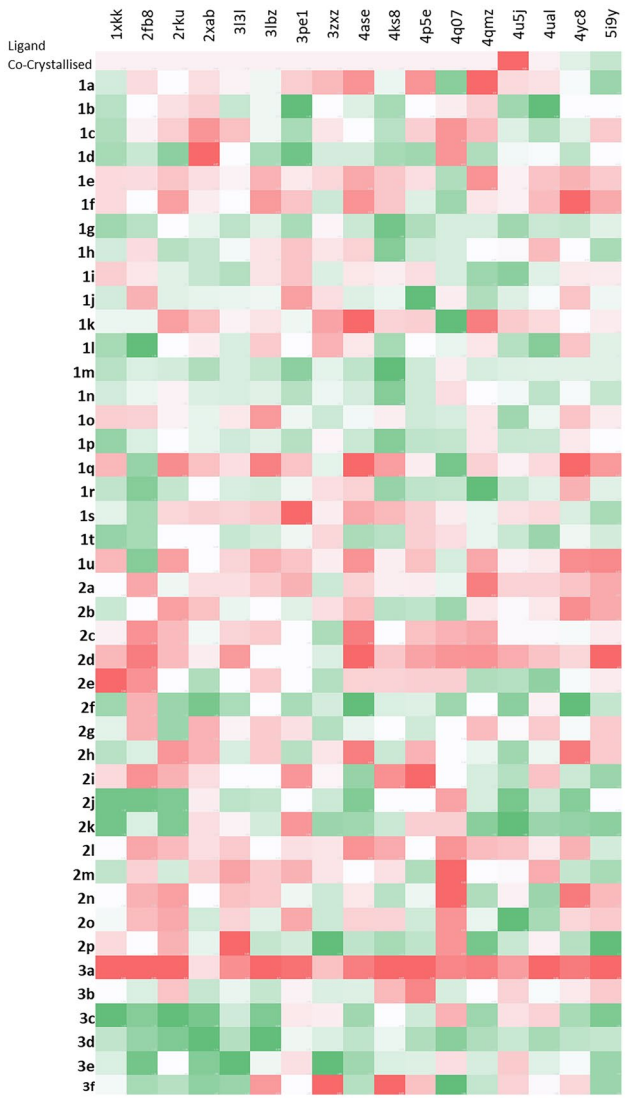


Fig. 4 Matrix of the calculated V values used to rank binding affinities between ligands and cellular targets, for selected proteins with $\delta \geq 1$. Green-high value, red-low value

oral administration is taken into consideration, while **3b** and **1a** still possess a bioavailability of 44.6 and 36.3%. Vd values for **3b**, **1q** and **1a** are 337.7, 278.6 and 442.1 mL/kg, respectively. These low values suggest that the reduced lipophilic nature of **3b**, **1q** and **1a** may be a determinant for their lower volume of distribution, influencing their lower absorption and bioavailability after oral administration. In this regard, a correlation coefficient of 0.4 was found (Fig. 5) for both cLogP (i.e. lipophilicity measure) versus Vd, and cLogP versus bioavailability, in line with the assumption that the lipophilicity of the molecules may play a role in the absorption when administrated orally.

Among the terms with pyrimido[4,5-d]pyridazine-4,8-dione scaffold, compound **1t** showed the highest value of bioavailability (95.2%) followed by compounds **1i** and **1 l**, having a bioavailability of 92.4 and 90.8%, respectively (Table S9, Supporting Information). Therefore, these compounds demonstrate optimal features for oral administration. Compound **1t** has a high cLogP (i.e. 2.18) due to the presence of the $CH_2cC_6H_{11}$ residue at R1 and the ethyl group at R3 of the pyrimido[4,5-d]pyridazine-4,8-dione *core*, in addition to the Ph at R and the H at R2 which are common to the majority of the other compounds with the same scaffold (Table 1). Similarly, also compounds **1i** and **1 l** possess relatively high values of cLogP (i.e. 1.866 and 1.9261 respectively), suggesting higher absorption in the case of oral formulations. With regard to the analogues with 1,8-naphthyridin-4-one scaffold, **2 g** and **2 h** (with 4-Cl-CH_2Ph and 3-F-C_6H^4 residues in the X position; Table 2) display the highest bioavailability (i.e. 98.1% and 98.5%) within the library (Table S9, Supporting Information). The cLogP values of **2 h** and **2 g** are also high (i.e. 2.107 and 2.0004). For the analogues with pyrido(1,2-a) pyrimidine scaffold, compound **3d** possess the highest bioavailability (i.e. 96.4%; Table S9, Supporting Information), having a cLogP of 2.29 which is most likely determined by the presence of the $CH(Ph)_2$ residue at R1 position (Table 3). However, also **3c** and **3e** have high cLogP and

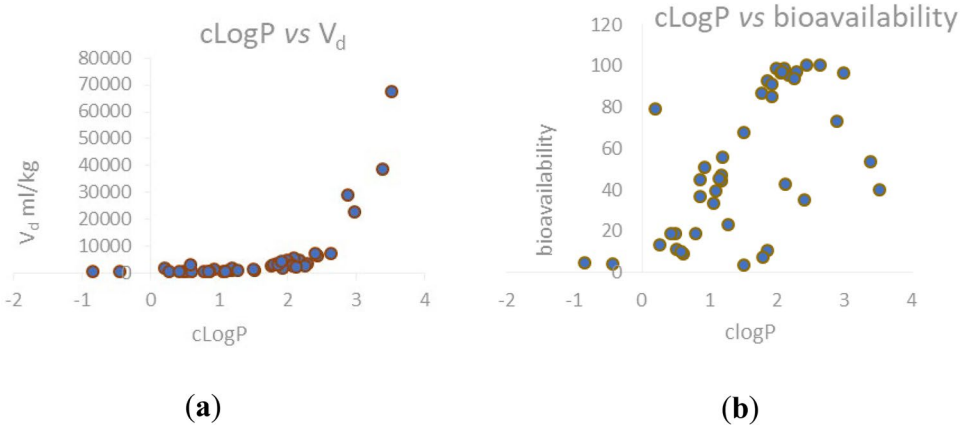


Fig. 5 Scatter plots for the correlation between cLogP and PK parameters (Vd and bioavailability). **a** Correlation of R2 = 0.4 between cLogP and Vd. **b** Correlation of R2 = 0.4 between cLogP and bioavailability (%)

bioavailability values in close range. Lastly, compounds **1c**, **1 m**, **1n**, **2 m** and **3a**, resulted in very low bioavailability values of 2.9, 8.3, 9.6, 3.1, 3.2% (Table S9, Supporting Information), suggesting that these analogues would have poor absorption after oral administration.

Conclusions

In this study, a new set of scaffold-diverse compounds was developed by means of a two-step molecular design process based on scaffold hopping/bio-isosteric replacement and automated ligand growing methodologies. We have used this approach to carry out a preliminary evaluation of a compound dataset versus a panel of cellular proteins involved in cancer cell survival and cancer progression. Standard synthetic procedures allow the ease production of the molecular series used in this work, which are based on three bi-cyclic heterocycles, i.e. pyrimido[4,5-d]pyridazine-4,8-dione, 1,8-napthyridin-4-one and pyrido(1,2-a)pyrimidine. Our data further confirm the advantages of inverse virtual screening protocols as an in silico approach to implement conventional methods in drug design and discovery, allowing the preliminary assessment of affinity between a set of ligands and suitable pharmacological targets. The normalisation of the binding energies proves valid to identify significant interactions between the ligands and several protein targets used in the study (i.e. HSP90, PARP, VEGFR2, mammalian sterile20-like kinase 3, EGFR kinase, B-Raf kinase, PLK1, protein kinase CK2, c-Met, PAK6 kinase, DNPH1, carbonic anhydrase IX, c-Src, MRCK beta, c-Abl kinase, and ephrin A2 receptor protein kinase). In parallel, the normalisation process also proposes nine ligands (**1b**, **1 k**, **1q**, **2c**, **2j**, **2p**, **3a**, **3c** and **3f**) as useful tools for biomedical investigations in the context of cancer chemotherapy. With a bioavailability higher than 90%, compounds **1t**, **2 g**, **2 h** and **3d** also demonstrate permeability and absorption features worth to be assessed in subsequent pharmacological studies.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s11224-022-01889-0.

Acknowledgements Andrew Beavil (King's College London) is gratefully acknowledged for providing software resources via King's Virtual Desktop.

Author contribution Conceptualisation, G.F., L.C. and A.C. Software, G.F and S.N. In silico methodology and experimental work, A.B., G.F. and S.N. Synthetic methodology and experimental work, C.B. and N.C. Data analysis, C.B., N.C., L.C., C.V., A.B., G.F. and S.N. Data curation, L.C., C.V., G.F. and S.N. Writing—original draft preparation, L.C and G.F. Writing—review and editing, M.P. and A.C. Supervision and project administration, M.P and A.C.

Funding S.N. was funded by the Post-Doc Fellowships Programme (3-1/PDFP/HEC/2020/856), Higher Education Commission, Pakistan. G.F. is currently a Marie Skłodowska-Curie fellow funded by the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 893784.

Availability of data and material The online version contains supplementary material and data available at https:

Declarations

Conflict of interest The authors declare no competing interests.

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