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Discovery of multi-target antivirals acting on both the dengue virus NS5-NS3 interaction and the host Src/Fyn kinases

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Abstract

This study describe the discovery of novel dengue virus inhibitors targeting both a crucial viral protein-protein interaction and an essential host cell factor as a strategy to reduce the emergence of drug-resistance. Starting from known c-Src inhibitors, a virtual screening was performed to identify molecules able to interact with a recently discovered allosteric pocket on the dengue virus NS5 polymerase. The selection of cheap-to-produce scaffolds and the exploration of the biologically relevant chemical space around them suggested promising candidates for chemical synthesis. A series of purines emerged as the most interesting candidates able to inhibit virus replication at low micromolar concentrations with no significant toxicity to the host cell. Among the identified antivirals, compound **16i** proved to be ten times more potent than ribavirin, showed a better selectivity index and represents the first-in-class DENV-NS5 allosteric inhibitor able to target both the virus NS5-NS3 interaction and the host kinases c-Src/Fyn.

Introduction

Dengue fever (DF) or break-bone fever is the most widespread arthropod-borne disease in the world and, each year, accounts for more than 50-100 million patients, 99% of all reported cases

of viral hemorrhagic fever, and around 20.000 deaths.¹ The causative agent is the dengue virus (DENV), which belongs to the *Flaviviridae* family and is divided into five serotypes (DENV 1-5). DENV is predominantly transmitted through the bite of infected *Aedes aegyptii*, although other mosquito species can be carrier of the virus as well. Considering the expanding geographical distribution of both the virus and the mosquito vector,² the increasing frequency of epidemics, and the fact that multiple serotypes are co-circulating in many regions, WHO has classified dengue as a major international public health concern.³ While infection with a single serotype is often mild and believed to induce life-long immunity to that serotype, cross-protection to other serotypes only lasts for a few weeks and subsequent infection with another serotype has been associated with the clinically more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Although vaccines are already available for different flaviviruses (e.g., yellow fever virus, tick-borne encephalitis, and Japanese encephalitis virus), the development of a vaccine for DENV proves to be very cumbersome because of the existence of multiple serotypes and the underlying mechanism that leads to DHF/DSS (i.e., antibody-dependent enhancement of infection). Past clinical trials with tetravalent vaccines have not delivered their promises and a few are still ongoing.⁴ Moreover, a drug for the treatment or prevention of DENV infection is not yet available and the management of patients is limited to symptomatic treatment and supportive care. Therefore, the development of new small-molecule antivirals against DENV is of significant interest.

A direct correlation has been demonstrated between a high DENV viral load in the blood and the development of the more severe, life-threatening form of the disease.⁵ Thus, a drug that would reduce the viral load is expected to have a significant impact on the number of patients that will progress to DHF/DSS. Furthermore, a lower viraemia should also result in a decrease in

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3 the number of uninfected mosquitoes that become carrier after feeding on a viraemic patient, and
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5 thus would also slow down transmission. Therefore, an efficient and safe drug, delivered early in
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7 the course of the disease or even taken prophylactically, will not only save lives but will also
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9 curb epidemics.
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13 Conventional antiviral drugs are typically designed to directly inhibit virus replication by
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15 targeting viral proteins. Among the ten known DENV proteins, the atomic structures of the
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17 capsid, envelope, NS3 (protease domain, helicase domain, and full-length NS3), NS5
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19 (methyltransferase and RNA-dependent RNA polymerase RdRp) have been solved and have
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21 provided a solid basis for the development of such inhibitors (Figure 1).^{6,7}
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25 However, because RNA viruses are known to mutate quite rapidly, viral protein alterations that
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27 induce resistance to drugs are expected to emerge, thus jeopardizing the long-term clinical use of
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29 such compounds. Different approaches can be pursued to increase the barrier to resistance: (i) the
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31 implementation of combination treatments with drugs that target different viral proteins, (ii) the
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33 development of compounds that target host cell factors essential for viral replication but
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35 dispensable for host cell survival, and (iii) the design of multi-target inhibitors that interfere with
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37 two (or more) targets at the same time,⁸ preferably a viral and a host cell factor. Drugs developed
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39 according to the latter two strategies may also offer a therapeutic option in an outbreak setting of
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41 viral pathogens of unknown etiology, or combat those that depend on the same host factor for
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43 replication and against which no drug has been developed so far. Even though the targeting of
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45 host cell proteins is considered a risky approach in the context of antiviral drug development,
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47 many, if not most of the drugs that are on the market do target host cell proteins and the risk of
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49 side effects is well-accepted. Because viruses heavily rely on host cell proteins for their
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51 replication, many efforts are ongoing to identify proteins that can be targeted with a minimal
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3 effect on host cell wellbeing. In this context, pursuing the development of drugs with a dual
4 mechanism of action is expected to allow treatment with a significantly lower dose, which will
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6 reduce the risk of side effects while retaining antiviral efficacy. Furthermore, the use of a single
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8 multi-target drug would simplify ADME-TOX and PK studies, and the risk of drug-drug
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10 interactions may be avoided.⁸
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15 Host cell kinases have been proven to be essential for DENV assembly and secretion. In
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17 particular, c-Src has been identified as one of the kinases that is required for DENV replication.⁹
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19 Src kinase inhibitors Dasatinib and Saracatinib (AZD0530) have been shown to inhibit virion
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21 assembly of DENV 1-4, thus validating the Src family of tyrosine kinases as potential drug
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23 targets and their inhibitors as promising lead therapeutics for the development of a treatment for
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25 dengue virus infection (Figure 1).⁹
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30 Viral polymerases have been extensively studied and proven to be suitable targets for the
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32 development of antiviral drugs. Despite the fact that most nucleoside polymerase inhibitors are
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34 characterized by low selectivity and severe side effects, recent mutagenesis studies pointed
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36 towards an allosteric pocket on DENV-NS5 (hereafter named Cavity B) that could be exploited
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38 for the development of safer non-nucleoside inhibitors. In fact, mutation of few residues within
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40 this allosteric pocket (Leu328, Lys330, Trp859, Ile863) has proven to interfere with the initiation
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42 of RNA synthesis and with the formation of the functional NS5-NS3 complex.¹⁰ To the best of
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44 our knowledge, no inhibitor targeting this site on the DENV NS5 protein has been reported so
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46 far. However, compounds targeting a similar site on the thumb domain of HCV NS5B
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48 polymerase have recently been shown to have a promising antiviral profile and are currently in
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50 clinical trial for the treatment of HCV-infected patients.¹¹
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3 In this study, we aimed to explore the possibility to develop multi-target compounds acting on
4 both the host c-Src kinase and the allosteric site on the viral NS5 polymerase as a complementary
5 strategy to block DENV replication and escape from the insurgence of drug-resistance. In fact,
6 although drugs targeting host cell kinases will not constitute a selective pressure on the virus to
7 select host mutants, in a few cases, prolonged treatment with kinase inhibitors may result in the
8 selection of alternative pathways³⁵ that could theoretically be exploited for viral replication. As a
9 consequence, the allosteric inhibition of a viral protein by the same multi-target molecule may
10 overcome the potential selection of an alternative kinase pathway while making it more difficult
11 for the virus to develop an allosteric mutation. The known host kinase involved in DENV
12 replication (c-Src) and the only druggable allosteric pocket on a DENV protein (cavity B on
13 NS5) were thus chosen as ideal targets of a multi-target DENV inhibitor.
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29 Considering the fact that the majority of DENV patients live in under-privileged regions, our
30 effort was specifically aimed at the identification of antiviral compounds that would be cheap to
31 produce/optimize by selecting appropriate, easy-to-synthesize chemical scaffolds.
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39 Results and discussion

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42 The identification of cost-effective dual NS5/Src inhibitors was accomplished by combining a
43 structure-based virtual screening on the allosteric site of NS5 and a virtual library generation,
44 starting from a library of known Src active scaffolds. Structures of compounds with known
45 bioactivity data against tyrosine-protein kinase Src were obtained from databases of bioactive
46 molecules (ChEMBL,¹² Binding DB¹³) and from an internal collection of kinase inhibitors. Only
47 compounds with an $IC_{50} \leq 100 \mu M$ were retained. While this activity cutoff may also include
48 weak Src inhibitors, the higher number of compounds may increase the chance of identifying
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NS5-binding scaffolds that could be cheap to synthesize and easily modified in a virtual library expansion to reach the optimal binding efficiency. Moreover, the synergistic effect of a dual inhibitor may lead to an improved activity profile. The Glide¹⁴ Standard Precision (SP) docking protocol and Autodock Vina¹⁵ were used to dock Src ligands (about 3.000 compounds) to the allosteric pocket of the DENV NS5 RdRp.¹⁶ This pocket corresponds to the above-mentioned cavity B and is made up by residues Leu327, Leu328, Lys330, Thr858, Trp859, Asn862, Ile863, and Ala866 (the corresponding numbering of the used DENV-3 RdRp crystal structure is Leu326, Leu327, Lys329, Thr858, Trp859, Asn862, Ile863, and Ala866). Unless specified otherwise, DENV-2 RdRp numbering will be used hereafter. Leu328, Trp859, and Ile863 are highly conserved among flaviviruses. Moreover, alanine scanning experiments have shown that L327A, W859A, and I863A mutations remarkably reduce the *de novo* RNA synthesis, while the K330A mutation inhibits the NS3-NS5 interaction. On the basis of this mutagenesis analysis, it was hypothesized that compounds that are able to bind to this cavity could act as allosteric NS5 inhibitors or NS3-NS5 protein-protein interaction inhibitors. Despite the challenge of targeting protein-protein interfaces with small-molecules, state-of-the-art structure-based design methods have proven to be successful in the identification of small-molecule protein-protein interaction modulators.¹⁷ To select scaffolds for chemical synthesis, the most recurrent scaffolds were identified by clustering compounds on the basis of the Tanimoto similarity with the Canvas Similarity and Clustering tool available in the Schrödinger Suite 2011. Clusters were analyzed by considering first of all the synthetic accessibility of each scaffold. Binding modes within the NS5 allosteric pocket and docking scores further aided in the selection. Finally, compounds **4a** and **13a,b** were selected from Autodock Vina docking results, while compounds **16a-c** were selected on the basis of Glide docking results (see Table 1). Starting from the identified scaffolds, the

biologically relevant chemical space was sampled by docking to the NS5 allosteric site a virtual library designed by the chemical expansion of the three identified scaffolds (**4**, **13**, and **16**). Based on the synthetic approaches reported for the preparation of these scaffolds (Schemes 1-4), a virtual library of synthetically accessible derivatives was designed using the software SmiLib v2.0.¹⁸ In particular, a series of building blocks available in our stockroom were combined to generate ~10,000 virtual compounds that were docked to cavity B using the same docking protocols/parameters previously applied to the ChEMBL/Binding DB compounds (see the experimental section for details). Compounds were selected by considering (i) binding modes within the NS5 allosteric pocket and (ii) docking scores. The set of compounds listed in Table 1 was thus selected and freshly synthesized for evaluation in a virus-cell-based assay.

2-Pyridone derivatives were synthesized by a two-step procedure by reacting 4-hydroxy-6-methyl-2H-pyran-2-one (**1**) with the amines **2a-d** in refluxing water, followed by alkylation of the C4 hydroxy group with substituted benzylbromides to give the desired compounds **4a-e** (Scheme 1). For the synthesis of the triazole derivatives **6a,b**, the approach described above proved to be unsuccessful either using the required triazolo-amines directly or by trying to build the triazole ring in two steps through the propargyl derivative **5**. In the latter case, the reaction of 2-pyranone **1** with propargylamine in refluxing water gave the side product **7** with no trace of the desired derivative **5**. It is likely that compound **7** derives from the nucleophilic attack of **5** in the enol form to the triple bond. This was indeed avoided by blocking the phenolic OH by O-alkylation as reported in Scheme 2. As an alternative strategy to prepare compounds **6a,b**, 2-pyranone **1** was first converted into the corresponding pyridin-2-one by reaction with concentrated ammonia, followed by C4 O-alkylation to give the intermediate **8a** (Scheme 2). The latter intermediate was selectively *N*-propargylated by treatment with NaH/LiBr in a mixture of

DMF and DME to give the desired compound **9a** in good yields. Final compounds **6a,b** were quickly obtained by reacting **9a** with substituted benzylbromides under microwave-assisted Click-chemistry conditions.¹⁹

Quinoline-3-carbonitrile derivatives **13a-c** were synthesized following a previously reported procedure (Scheme 3): the tandem condensation/cyclization reaction of anilines **10a,b** with ethyl 2-cyano-3-ethoxyacrylate under microwave irradiation led to 4-quinolones **11a,b** in good yields with no need of any chromatographic purification.²⁰ The C4-chlorination of **11a,b** with phosphorus oxychloride gave 4-chloroquinolines **12a,b** that were directly submitted to a nucleophilic substitution with selected aniline derivatives to afford the final compounds **13a-c**. Compounds **13a,b** have been previously reported as potent c-Src inhibitors.²¹ Purine derivatives **16a-m** were easily obtained by developing a fast and practical two-step microwave-assisted protocol starting from commercially available 2,6-dichloro purine **14** (Scheme 4). In the first step, regioselective C4 nucleophilic substitution with different anilines in the presence of triethylamine led to the monosubstituted intermediates **15a-d** that were finally submitted to a C2 nucleophilic substitution with a wide range of different amines to give final compounds **16a-m** (see Table 1). Compounds **16a-c** were previously reported as potent c-Src inhibitors.²² The antiviral activity of all synthesized compounds was evaluated in either a CPE reduction assay or a virus yield reduction assay (for details on the assays, see the experimental section). In both assays, DENV serotype 2 was used and the assay was performed in a clone of Vero (African Green Monkey kidney) cells that has been selected for high susceptibility to dengue virus infection. The EC₅₀ values (the concentration at which 50% inhibition of virus-induced CPE or viral RNA replication in infected, treated cells is observed), as well as CC₅₀ values (the concentration at which 50% reduction of metabolic activity in uninfected, treated cells is

observed) were determined. Microscopic evaluation-based eligibility criteria were also applied in the identification of promising “active compounds”: (i) no alteration of normal cell morphology in treated, uninfected cells and (ii) a significant reduction of virus-induced CPE or viral RNA replication. Moreover, the selectivity index (defined as CC_{50}/EC_{50}) should at least be greater than 10 for a compound to be even considered as a selective antiviral.²³ Results are reported in Table 1 in comparison with ribavirin, which was used as a reference compound. While 2-pyridones (**4a-e**; **6a,b**) and quinoline-3-carbonitrile derivatives (**13a-c**) were characterized by a high cellular toxicity, the purine derivatives (**16a-m**) showed promising antiviral activity. Known Src-inhibitors **16a-c** showed no antiviral activity, and no or little toxicity (only for **16c**). Purines bearing a 4-acetylaniline moiety at the C6 position (**16d-f**) were characterized by a high micromolar activity and a low selectivity index independent of the C2 substituent. Purines bearing a 3-hydroxyaniline moiety at C6 showed the most interesting anti-DENV profile, with the only exception of the C2-benzyl derivative **16g**: compounds **16h-m** showed better EC_{50} values than the broad-spectrum inhibitor ribavirin and a promising selectivity index. A typical dose-response curve is depicted in Figure 4 for the best anti-DENV compound **16i**. All the synthesized compounds were then tested in a recently developed AlphaScreen assay to evaluate their ability to block the formation of the functional NS3/NS5 complex.²⁴ A preliminary AlphaScreen assay was performed with 500 μ M compound: only purine derivatives (**16a-m**) showed inhibition of the NS5-NS3 interaction at this high concentration.²⁵ The previously unpublished purines **16d-m** were subsequently tested at lower concentrations (50 μ M for **16d-m** and 25 μ M for **16e,f**): compound **16i** emerged as the only hit able to inhibit the NS3-NS5 interaction by 33% at 50 μ M and represents the first-in-class inhibitor with such a mechanism of action (Figure 2). Our previous study on the establishment of an AlphaScreen-based NS3-NS5

interaction assay³³ has demonstrated that 25 - 100 nM of recombinant NS3 and NS5 is needed to obtain a significant level of luminescent interaction signals with a robust *Z'* factor, and also showed that an excess amount of competitor molecules was required to block the NS3-NS5 interaction in the AlphaScreen assay. Since NS3-NS5 protein concentrations in DENV-infected cells are considered to be lower than those used in the *in vitro* AlphaScreen assay, it is reasonable to assume that the concentration of small molecules that is required to disrupt the *in vitro* NS3-NS5 interaction may be lower than that used in the AlphaScreen assay and, hence, its contribution to the potency of the antiviral activity may be underestimated. Although **16i** was not very potent in inhibiting the NS3-NS5 protein-protein interaction, possible underestimation in the AlphaScreen assay and the lack of inhibitors endowed with this novel mechanism of action for comparison purposes, make this compound a promising candidate for further optimization/investigation. In addition, the AlphaScreen assay can only indicate allosteric compounds that disrupt the NS3-NS5 interaction by interfering with the Lys330 functions, as suggested by previous mutagenesis studies,¹⁰ while allosteric antivirals that block the initiation of RNA synthesis cannot be detected. Nonetheless, the *in vitro* antiviral effect of DENV replication may be the result of its action on multiple targets, as per our design strategy. Consequently, the most active inhibitors **16h-m** and selected compounds from all synthesized chemical classes (**4**, **6**, **13** and **16**) were subsequently analyzed for their activity on kinases known to be involved in DENV replication. Interestingly, after the start of this work, the Yang group reported that Fyn kinase has a more pronounced role (compared to Src) in the replication of DENV: Fyn inhibition by treatment with Dasatinib and Saracatinib (nanomolar inhibitors of both Fyn and Src)^{33,34} was proposed as the main mechanism responsible for their anti-DENV activity (EC₅₀ around 1.0 and 6.0 μ M, respectively) without excluding the possibility that these

compounds may affect processes beyond RNA replication.²⁶ A mutation in the viral NS4B protein (T108I) was in fact selected after repeated treatments with Dasatinib.²⁶ Although c-Src inhibitors showed low micromolar ID_{50s} in our experimental conditions (see Table 2, entries 4-5, 7-9), a few general considerations can be drawn: *i*) 2-pyridone derivatives **4** and **6** were inactive against c-Src/Fyn and highly cytotoxic in cell-based assays; *ii*) known c-Src inhibitors **13a,b** were also active against Fyn while **13c** was completely inactive. All these quinolone derivatives are however highly cytotoxic in cell-based assays; *iii*) all purine derivatives tested showed a similar range of activity against c-Src and Fyn but only compounds **16d-m** were able to inhibit DENV replication. Overall, these data indicate that the dual c-Src/Fyn inhibition may not be a sufficient prerequisite for the identification of an effective DENV inhibitor and many different factors, still unidentified and difficult to predict, may play a key complementary role in the replication of dengue virus. At antiviral concentrations, Dasatinib and Saracatinib are in fact reported to inhibit many different kinases and further experiments are needed to clearly understand the mechanism of action of these drugs that exhibit an antiviral potency comparable to that of our compounds. At the present however, we cannot exclude that our compounds (but also Dasatinib/Saracatinib) could potentially exploit a different mechanism of action, as the one reported for the c-Src inhibitor herbimycin A, which inhibits HCV replication by blocking the interaction between c-Src and the viral proteins NS5/NS3.³⁶

Finally, the binding mode of the dual compound **16i** was further confirmed with Induced Fit Docking (IFD), i.e., a docking protocol that takes into account protein flexibility, thus allowing the retrieval of optimized binding modes. Protein flexibility, which always plays a pivotal role in all protein-ligand recognition events, may be particularly important in the case of the solvent-exposed cavity B that is part of a protein-protein interface.¹⁷ Compound **16i** was characterized by

a good steric and electronic complementarity with the cavity B of DENV polymerase (interacting with six out of the eight conserved residues that line cavity B) and was the top-scoring hit in terms of Glide SP ligand efficiency among the tested compounds (Figure 3). The 6-amine NH of **16i** makes a hydrogen bond with the backbone carbonyl oxygen of Leu327 (DENV-3 Leu326), while the ligand hydroxyl group forms a hydrogen bond with the side-chain carboxylate of Asp333 (DENV-3 Asp332). Moreover, **16i** makes favorable hydrophobic contacts with Leu327 (DENV-3 Leu326), Trp859, Ile863, Ala866, and the alkyl chains of Lys330 (DENV-3 Lys329) and Asn862. The binding mode is further stabilized by a cation-pi interaction between the purine ring and the side chain of Lys330 (DENV-3 Lys329). As is evident from the above-described binding mode, **16i** interacts with Lys330, Trp859, and Ile863, i.e., three out of the four residues that have been shown to be critical for viral replication. Notably, the ability of **16i** to disrupt the NS3-NS5 interaction may be ascribed to the hydrophobic contacts and the cation-pi interaction with Lys330 that is involved in the recognition of NS3.

Conclusions

In summary, we have described a multidisciplinary approach for the discovery of dengue virus inhibitors that act on the viral RdRp (NS5) as well as on host cell kinases previously shown to be involved in DENV replication (c-Src, Fyn). Docking of known c-Src active scaffolds to the allosteric pocket of DENV NS5 RdRp allowed the selection of three cheap-to-produce chemical scaffolds that were subsequently expanded in a virtual library of highly substituted derivatives to identify the most promising compounds for chemical synthesis. The synthesized compounds were evaluated for their: (i) antiviral activity in a virus-cell-based assay for DENV-2, (ii) ability to block the NS3-NS5 interaction, and (iii) activity on c-Src and Fyn kinases. Purine derivatives

16h-m inhibited DENV-2 replication and kinase activity at low micromolar concentrations, with an antiviral profile comparable to that of Dasatinib and Saracatinib. Notably, compound **16i** was discovered as the first multi-target antiviral blocking the formation of the viral NS3-NS5 complex and inhibiting the activity of host c-Src/Fyn kinases. This compound can therefore be considered as a promising starting point for further optimization and development.

Experimental section

Molecular modeling

Glide docking. ChEMBL¹² and Binding DB¹³ databases were searched for tyrosine-protein kinase Src inhibitors. Collected inhibitors from the two databases were merged and only compounds whose IC₅₀ was ≤ 100 μ M were retained. The Schrödinger Suite 2011²⁷ Virtual Screening Workflow (VSW) tool was used to i) remove duplicates and compounds with a molecular weight higher than 600 g/mol; ii) generate stereoisomers and retain up to 4 stereoisomers for compounds with unspecified stereocenters; and iii) generate tautomers and ionization states at a pH range of 6-8 using Epik and opting for the removal of high-energy tautomers and ionization states. Virtual library compounds were prepared using the same protocol. The dengue virus DENV-3 RNA-dependent RNA polymerase (RdRp) catalytic domain structure was downloaded from the Protein Data Bank (PDB code 2J7U). The structure was then processed with the Schrödinger Suite 2011 Protein Preparation Wizard (PPW) tool. Crystallization additives and water molecules were removed, while the magnesium and zinc ions were retained. PPW automatically adjusted the ionization and tautomerization state of the protein at a neutral pH, set the orientation of any misoriented groups (Asn, Gln, and His residues) as well

as charges and atom types for metal atoms, and optimized the hydrogen bond network. Moreover, side chains that were missing, even if away from the region of interest, were added and optimized by running a Prime structure refinement job through the PPW graphical interface. Finally, the protein structure was refined to relieve steric clashes with a restrained minimization with the OPLS2005 force field till a final RMSD of 0.30 Å with respect to the input protein coordinates. Docking was performed with Glide 57114¹⁴ (Schrödinger Suite 2011). The protein structure, prepared as described above, was used to build the energy grid. The enclosing box was centered at the centroid of residues Leu326, Leu327, Lys329, Thr858, Trp859, Asn862, Ile863, and Ala866 (the numbering of amino acids is based on DENV-3 RdRp), which define cavity B as reported by Malet *et al.*²⁸ Box dimensions and ligand diameter midpoint box sides were set to 30 Å × 30 Å × 30 Å and 10 Å × 10 Å × 10 Å, respectively. The Glide Standard Precision (SP) docking protocol was used. Ligands were docked flexibly, the sampling of ring conformations and nitrogen inversions was included, and non-planar amide conformations were penalized. Epik state penalties were added to docking scores. All SP parameters were set to their default values. Docking results were filtered to remove compounds that did not contact residues Leu326, Leu327, Lys329, Thr858, Trp859, Asn862, Ile863, and Ala866 by using the Schrödinger Suite 2011 Pose Filter tool with a contact maximum distance of 3.5 Å. Remaining compounds were clustered with the Canvas Similarity and Clustering tool that is available through the Schrödinger Suite 2011 Maestro graphical interface by using the MolPrint2D fingerprints, the Tanimoto similarity metric, and the Average linkage method. Virtual library compounds were docked with the same Glide SP docking protocol. Binding modes of purine derivatives were re-generated with Induced Fit Docking.²⁹ An initial Glide SP docking of the ligand was performed by using a softened potential, i.e., a van der Waals radius scaling factor of 0.50 for receptor atoms with a

partial atomic charge (absolute value) less than 0.25 and 0.50 for ligand atoms with a partial atomic charge (absolute value) less than 0.15. A maximum number of fifty poses were saved and submitted to the subsequent Prime side-chain orientation prediction of residues within a shell of 5 Å around the ligand. After the Prime minimization of the selected residues and the ligand for each pose, a Glide SP re-docking of each protein-ligand complex structure within 30 kcal/mol of the lowest energy structure was performed. Finally, the binding energy (IFDScore) for each output pose was calculated.

Autodock Vina docking. Docking studies were performed with Autodock Vina¹⁵ through PyRx,³⁰ while PyMol³¹ was used to visualize the results. Ligands were prepared by first generating an energy minimized 3D structure in Openbabel and then processed with Autodock Tools 1.5.4 to assign Gasteiger charges, merge nonpolar hydrogens, and set torsional bonds. Docking runs were performed within a 30 Å × 30 Å × 30 Å cubic box (grid spacing = 0.375 Å) surrounding the above described allosteric pocket. A search exhaustiveness of 8 was used and output modes were ranked according to the binding affinity.

Chemistry

General. All commercially available chemicals were purchased from both Sigma - Aldrich and Alfa Aesar and, unless otherwise noted, used without any previous purification. Solvents used for work-up and purification procedures were of technical grade. TLC was carried out using Sigma-Aldrich TLC plates (silica gel on Al foils, SUPELCO Analytical). Where indicated, products were purified by silica gel flash chromatography on columns packed with Merck Geduran Si 60 (40-63 μm). ¹H and ¹³C NMR spectra were recorded on BRUKER AVANCE 300 MHz and BRUKER AVANCE 400 MHz spectrometers. Chemical shifts (δ scale) are reported in

parts per million relative to TMS. ¹H-NMR spectra are reported in this order: multiplicity and number of protons; signals were characterized as: *s* (singlet), *d* (doublet), *dd* (doublet of doublets), *ddd* (doublet of doublet of doublets), *t* (triplet), *m* (multiplet), *bs* (broad signal). ESI-mass spectra were recorded on an API 150EX apparatus and are reported in the form of (m/z). Elemental analyses were performed on a Perkin-Elmer PE 2004 elemental analyzer, and the data for C, H, and N were within 0.4% of the theoretical values. Melting points were taken using a Gallenkamp melting point apparatus and were uncorrected. All target compounds possessed a purity of ≥95% as verified by elemental analyses by comparison with the theoretical values.

Microwave Irradiation Experiments. Microwave reactions were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC). The machine consists of a continuous focused microwave power delivery system with an operator-selectable power output from 0 to 300 W. The temperature inside the reaction vessel was monitored using a calibrated infrared temperature control mounted under the reaction vessel. All experiments were performed using a stirring option whereby the reaction mixtures were stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a Teflon-coated magnetic stir bar in the vessel.

General Procedures for the Synthesis of intermediates 3a-d: To an aqueous suspension (16 mL) of 4-hydroxy-6-methyl-2-pyrone (**1**) (500 mg, 3.96 mmol) the proper amine (3.96 mmol) (**2a-d**) was added dropwise. The resulting reaction mixture was heated at reflux for 2-7 hours, then cooled down to room temperature. A precipitate was obtained and separated by filtration over a Buchner funnel. The solid was purified by flash chromatography using the proper eluent.

3a: CHCl₃/MeOH 99/1-97/3; **3c**: CHCl₃/MeOH 99/1; **3d**: CHCl₃/MeOH 98/2. Compound **3b** was used in the following step without any further purification.

1-(4-chlorobenzyl)-4-hydroxy-6-methylpyridin-2(1H)-one (3a). Yield: 55%. MS (ESI) [M-H]⁻: 248.1 m/z; ¹H-NMR (DMSO-*d*₆ 300 MHz): δ 2.16 (*s*, 3H), 5.16 (*bs*, 2H), 5.59 (*d*, 1H, *J* = 3.58 Hz), 5.80 (*dd*, 1H, *J* = 2.64, 0.81 Hz), 7.12 (*d*, 2H, *J* = 8.64 Hz), 7.39 (*d*, 2H, *J* = 8.58 Hz), 10.50 (*bs*, 1H).

4-hydroxy-1-(4-methoxybenzyl)-6-methylpyridin-2(1H)-one (3b). Yield: 34%. MS (ESI) [M+H]⁺: 246.5 m/z; [M+Na]⁺: 268.4 m/z; ¹H-NMR (DMSO-*d*₆ 300 MHz) δ 2.17 (*s*, 3H), 3.71 (*s*, 3H), 5.10 (*bs*, 2H), 5.58 (*d*, 1H, *J* = 2.61 Hz), 5.77 (*dd*, 1H, *J* = 2.62, 0.76 Hz), 6.88 (*d*, 2H, *J* = 8.76 Hz), δ = 7.04 (*d*, 2H, *J* = 8.82 Hz), 10.48 (*bs*; 1H).

4-hydroxy-6-methyl-1-(4-(trifluoromethyl)benzyl)pyridin-2(1H)-one (3c). Yield: 40%. MS (ESI) [M+H]⁺: 284.1 m/z; [M+Na]⁺: 306.2 m/z; ¹H-NMR (DMSO-*d*₆ 300 MHz): δ 2.16 (*s*, 3H), 5.27 (*bs*, 2H), 5.62 (*d*, 1H, *J* = 2,61 Hz), 5.84 (*dd*, 1H, *J* = 2.64, 0.81 Hz), 7.30 (*d*, 2H, *J* = 7.98 Hz), 7.69 (*d*, 2H, *J* = 8.07 Hz), 10.59 (*bs*, 1H).

1-([1,1'-biphenyl]-4-ylmethyl)-4-hydroxy-6-methylpyridin-2(1H)-one (3d). Yield: 50%. MS (ESI) [M+H]⁺: 292.5 m/z; ¹H-NMR (DMSO-*d*₆ 300 MHz): δ 2.21 (*s*, 3H), 5.23 (*bs*, 2H), 5.62 (*d*, 1H, *J* = 2.43 Hz), 5.82 (*d*, 1H, *J* = 1.98 Hz), 7.19 (*d*, 2H, *J* = 8.13 Hz), 7.35 (*t*, 1H, *J* = 7.27 Hz), 7.45 (*t*, 2H, *J* = 7.48 Hz), 7.62 (*m*, 4H), 10.55 (*bs*, 1H).

General Procedures for the Synthesis of Compounds 4a-e: In a three-way necked round-bottom flask, intermediates **3a-d** (0.706 mmol) and K₂CO₃ (196 mg, 1.41 mmol) were stirred at room temperature for 15 minutes in dry DMF (7 mL). The proper benzylbromides (0.85 mmol), diluted in dry DMF (3 mL), were added dropwise to the reaction mixture. The resulting suspension was stirred at room temperature for 2 hours, after which H₂O and ethyl acetate were

added to the reaction mixture. The organic phase was washed with an aqueous solution of LiCl (5%), brine, dried over Na₂SO₄ and concentrated under vacuum. The crude was purified by flash chromatography using the proper eluent. **4a**: petroleum ether/ethylacetate 8/2-1/1; **4b**: petroleum ether/ethylacetate 8/2-0/10; **4c** and **4d**: CHCl₃/MeOH 99/1; **4e**: petroleum ether/ethylacetate/MeOH 73/20/2-70/20/5.

1-(4-chlorobenzyl)-4-((3-fluorobenzyl)oxy)-6-methylpyridin-2(1H)-one (4a). Yield: 55%. Mp 99-100 °C. MS (ESI) [M+H]⁺: 358.00 m/z. ¹H-NMR (CDCl₃ 300 MHz): δ 2.20 (s, 3H), 4.99 (s, 2H), 5.22 (s, 2H), 5.86 (d, 1H, *J* = 1.47 Hz), 5.96 (d, 1H, *J* = 2.04 Hz), 7.82 (m, 8H). ¹³C-NMR (CDCl₃ 75 MHz): δ 19.95, 45.51, 68.57, 95.31, 101.19, 113.84, 114.74, 122.45, 127.38, 128.38, 129.80, 132.55, 134.88, 137.47, 145.93, 160.79, 164.36, 165.80. Anal. (C₂₀H₁₇ClFNO₂) C, H, N.

4-((3-fluorobenzyl)oxy)-1-(4-methoxybenzyl)-6-methylpyridin-2(1H)-one (4b). Yield: 42%. Mp 102-103 °C. MS (ESI) [M+H]⁺: 354.3 m/z, [M+Na]⁺: 376.3 m/z. ¹H-NMR (CDCl₃ 400 MHz): δ 2.23 (s, 3H), 3.76 (s, 3H), 4.99 (s, 2H), 5.21 (s, 2H), 5.84 (s, 1H), 5.96 (s, 1H), 6.83 (d, 2H, *J* = 8 Hz), 7.03 (t, 1H, *J* = 8.4 Hz), 7.11 (d, 2H, *J* = 8.4 Hz), 7.15 (d, 2H, *J* = 8 Hz), 7.35 (bq, 1H, *J* = 6.4 Hz). ¹³C-NMR (CDCl₃ 100 MHz): δ 20.53, 46.08, 55.27, 69.03, 95.88, 101.39, 114.16, 114.36, 115.24, 122.9, 127.84, 128.90, 130.28, 138.08, 146.69, 158.83, 161.75, 164.20, 165.29, 166.13. Anal. (C₂₁H₂₀FNO₃) C, H, N.

4-((2,4-difluorobenzyl)oxy)-6-methyl-1-(4-(trifluoromethyl)benzyl)pyridin-2(1H)-one (4c). Yield: 55%. Mp 136-137 °C. MS (APCI) [M+H]⁺: 410.0 m/z. ¹H-NMR (CDCl₃ 300 MHz): δ 2.22 (s, 3H), 5.03 (s, 2H), 5.34 (s, 2H), 5.86 (dd, 1H; *J* = 2.73, 0.78 Hz), 6.03 (d, 1H, *J* = 2.73 Hz), 6.91 (m, 2H), 7.28 (d, 2H, *J* = 8.01 Hz), 7.43 (dt, 1H, *J* = 8.4, 6.66, 6.66 Hz), 7.58 (d, 2H, *J* = 8.1 Hz). ¹³C-NMR (CDCl₃ 75 MHz): δ 19.95, 45.51, 54.70, 68.46, 95.27, 100.89, 113.56,

113.59, 113.78, 114.64, 122.41, 127.25, 128.33, 129.75, 137.56, 146.24, 158.25, 160.74, 164.38, 165.64. Anal. (C₂₁H₁₆F₅NO₂) C, H, N.

1-([1,1'-biphenyl]-4-ylmethyl)-4-((2,4-difluorobenzyl)oxy)-6-methylpyridin-2(1*H*)-one

(4d). Yield: 50%. Mp 134-135 °C (with decomposition). MS (ESI) [M+H]⁺: 418.3 m/z, [M+Na]⁺: 440.4 m/z. ¹H-NMR (CDCl₃ 400 MHz): δ 2.28 (*s*, 3H), 5.04 (*s*, 2H), 5.34 (*s*, 2H), 5.86 (*s*, 1H), 6.05 (*d*, 1H, *J* = 2 Hz), 6.91 (*m*, 2H), 7.41 (*m*, 10H). ¹³C-NMR (CDCl₃ 100 MHz): δ 20.60, 46.40, 63.35, 63.26, 95.73, 101.45, 104.16, 111.58, 118.78, 126.85, 127.04, 127.32, 127.53, 128.78, 131.15, 135.84, 140.5, 146.64, 160.8, 163.29, 164.48, 165.28. Anal. (C₂₆H₂₁F₂NO₂) C, H, N.

4-((2,4-difluorobenzyl)oxy)-1-(4-methoxybenzyl)-6-methylpyridin-2(1*H*)-one (4e). Yield:

34%. Mp 121 °C. MS (ESI) [M+H]⁺: 372.1 m/z. ¹H-NMR (CDCl₃ 400 MHz): δ 2.24 (*s*, 3H), 3.78 (*s*, 3H), 5.01 (*s*, 2H), 5.22 (*s*, 2H), 5.81 (*d*, 1H, *J* = 2.16 Hz), 6.00 (*d*, 1H, *J* = 2.44 Hz), 6.84 (*d*, 2H, *J* = 8.48 Hz), 6.90 (*m*, 2H), 7.12 (*d*, 2H, *J* = 8.48 Hz), 7.42 (*q*, 1H, *J* = 8.28 Hz). ¹³C-NMR (CDCl₃ 100 MHz): δ 20.52, 46.07, 55.27, 63.26, 95.70, 101.27, 104.12, 111.56, 114.14, 118.80, 127.84, 128.90, 131.11, 146.69, 158.82, 160.75, 163.24, 165.25, 166.07. Anal. (C₂₁H₁₉F₂NO₃) C, H, N.

5-methyl-2-methylene-2,3-dihydro-7*H*-oxazolo[3,2-*a*]pyridin-7-one (7). To an aqueous

suspension (16 mL) of 4-hydroxy-6-methyl-2-pyrone (**1**) (500 mg, 3.96 mmol) propargylamine (253 μL, 3.96 mmol) was added portionwise in three subsequent additions every 60 minutes. The resulting mixture was refluxed for 3 hours, then cooled down to room temperature; a precipitate was obtained and separated by filtration over a Buchner funnel. The solid was purified by flash chromatography using (CHCl₃/MeOH 95/5-9/1). Yield: 50%. MS (APCI) [M+H]⁺: 164.4 m/z. ¹H-NMR (DMSO-*d*₆ 300 MHz): δ 2.20 (*s*, 3H), 4.63 (*dt*, 1H, *J* = 2.18, 2.22, 3.45 Hz), 4.88 (*dt*,

1H, $J = 2.55, 2.55, 3.45$ Hz), 4.97 (*t*, 2H, $J = 2.37, 2.43$ Hz), 5.56 (*d*, 1H, $J = 2.19$ Hz), 5.82 (*dd*, 1H, $J = 2.13, 2.16$ Hz). ^{13}C -NMR (CDCl_3 75 MHz): δ 17.43, 47.55, 86.95, 91.33, 113.83, 143.40, 152.42, 157.99, 179.33.

Synthesis of 4-hydroxy-6-methylpyridin-2(1H)-one. 4-hydroxy-6-methyl-2-pyrone (5g, 39.7 mmol) and concentrated ammonia (35 mL) were heated at reflux for 4 hours. After cooling down to room temperature, the reaction mixture was concentrated under vacuum until a brown precipitate was obtained. The solid was separated by filtration over a Buchner funnel and washed with H_2O . Yield: 90%. MS (APCI) $[\text{M}+\text{H}]^+$: 126.3 *m/z*. ^1H -NMR ($\text{DMSO}-d_6$ 300 MHz): δ 2.08 (*s*, 3H), 5.35 (*s*, 1H), 5.61 (*s*, 1H), 10.53 (*bs*, 1H), 11.03 (*bs*, 1H).

Synthesis of 4-((2,4-difluorobenzyl)oxy)-6-methylpyridin-2(1H)-one (8a). 4-hydroxy-6-methylpyridin-2(1H)-one (500 mg, 3.99 mmol) and K_2CO_3 (552 mg, 3.99 mmol) were stirred at room temperature for 15 minutes in dry DMF (20 mL) until the suspension turned green. 1-(bromomethyl)-2,4-difluorobenzene (513 μL , 3.99 mmol), in dry DMF (10 mL), was added dropwise to the reaction mixture. The resulting suspension was stirred at room temperature for 6 hours and then H_2O and ethyl acetate were added. The organic phase was washed with an aqueous solution of LiCl (5%), brine, dried over Na_2SO_4 and concentrated under vacuum. The crude was purified by flash chromatography using chloroform/methanol (99/1-95/5) as eluent. Yield: 50%. MS (APCI) $[\text{M}-\text{H}]^-$:

^1H -NMR ($\text{DMSO}-d_6$ 400 MHz): δ 2.09 (*s*, 3H), 5.03 (*s*, 2H), 5.69 (*s*, 1H), 5.73 (*s*, 1H), 7.13 (*t*, 1H, $J = 8.26, 8.26$ Hz), 7.30 (*t*, 1H; $J = 8.9, 8.9$ Hz), $\delta = 7.60$ (*q*, 1H; $J = 8.04, 8.04, 8.04$ Hz), $\delta = 11.16$ (*bs*, 1H).

Synthesis of 4-((2,4-difluorobenzyl)oxy)-6-methyl-1-(prop-2-yn-1-yl)pyridin-2(1H)-one (9a). Compound **8a** (200 mg, 0.80 mmol) was suspended in a mixture of dry DME and dry DMF

(4/1 in volume). The suspension was cooled to 0 °C, NaH (60% dispersion in mineral oil) (35 mg, 0.86 mmol) was added and the reaction mixture was stirred at room temperature for 15 minutes. LiCl (67 mg, 1.6 mmol) was added and the mixture was stirred for 15 minutes. Then, propargyl bromide (177 μ L, 1.6 mmol) was added and the reaction mixture was stirred at 65 °C for 26 hours, after which H₂O and ethyl acetate were added. The organic phase was washed with an aqueous solution of LiCl (5%), brine, dried over Na₂SO₄ and concentrated under vacuum. The crude was purified by flash chromatography using ethyl acetate/methanol (98/2-9/1) as eluent. Yield: 73%. MS (APCI) [M+H]⁺: 290.3 m/z. ¹H-NMR (CDCl₃ 300 MHz): δ 2.25 (*t*, 1H, *J* = 2.50, 2.50 Hz), 2.47 (*s*, 3H), 4.83 (*d*, 2H, *J* = 2.52 Hz), 4.99 (*s*, 2H), 5.84 (*dd*, 1H, *J* = 2.76, 0.84 Hz), 6.90 (*m*, 2H), δ = 7.40 (*m*, 1H).

General Procedures for the Synthesis of Compounds 6a-b: In a microwave tube, sodium azide (5 mg, 0.076 mmol) and the opportune benzylbromide (0.076 mmol) were suspended in mixture 1/1 of H₂O/*t*-BuOH (3 mL) and stirred at room temperature for 10 minutes. Sodium ascorbate (1.34 mg, 0.0076 mmol) and CuSO₄ (0.121 mg, 0.00076 mmol) were added to the tube, the mixture was stirred at room temperature for 10 minutes, **9a** (22 mg, 0.076 mmol) was then added and the tube was heated at 125 °C for 10 minutes in a microwave oven (max μ W power input: 250 W; ramp time: 1 minute; power max: off; maximum pressure: 180 psi). At the end of the irradiation, the tube was cooled down to room temperature and NaOH 0.1 M was added till pH = 14. The solid formed at the bottom of the tube was separated by filtration over a Hirsch funnel and washed with NaOH 0.1 M, H₂O and petroleum ether.

1-((1-(3-bromobenzyl)-1*H*-1,2,3-triazol-4-yl)methyl)-4-((2,4-difluorobenzyl)oxy)-6-methylpyridin-2(1*H*)-one (6a). Yield: 70%. Mp 188-189 °C (decomposition: 180 °C). MS (ESI) [M+H]⁺: 501.2 m/z, [M+Na]⁺: 523.2 m/z. ¹H-NMR (DMSO-*d*₆ 400 MHz): δ 2.44 (*s*, 3H),

5.04 (*s*, 2H), 5.13 (*s*, 2H), 5.56 (*s*, 2H), 5.86 (*bs*, 1H), 5.91 (*d*, 1H), 7.13 (*m*, 1H), 7.32 (*m*, 3H), 7.57 (*m*, 3H), 8.08 (*s*, 1H). ^{13}C -NMR (DMSO-*d*₆ 100 MHz): δ 20.42, 38.69, 52.35, 63.66, 95.22, 100.42, 104.85, 112.23, 119.70, 122.25, 124.44, 127.63, 131.41, 132.95, 139.05, 143.94, 147.73, 159.76, 162.02, 162.55, 163.80, 164.32, 166.24. Anal. (C₂₃H₁₉BrF₂N₄O₂) C, H, N.

4-((2,4-difluorobenzyl)oxy)-6-methyl-1-((1-(3-(trifluoromethyl)benzyl)-1*H*-1,2,3-triazol-4-yl)methyl)pyridin-2(1*H*)-one (6b). Yield: 61%. Mp 162-163 °C (decomposition: 160 °C). MS (ESI) [M+H]⁺: 491.4 m/z, [M+Na]⁺: 513.4 m/z. ^1H -NMR (DMSO-*d*₆ 400 MHz): δ 2.45 (*s*, 3H), 5.04 (*s*, 2H), 5.14 (*s*, 2H), 5.67 (*s*, 2H), 5.86 (*d*, 1H, *J* = 3.6 Hz), 5.92 (*d*, 1H, *J* = 2.92 Hz), 7.13 (*m*, 1H), 7.31 (*m*, 1H), 7.60 (*m*, 3H), 7.70 (*bs*, 2H), 8.12 (*s*, 1H). ^{13}C -NMR (DMSO-*d*₆ 400 MHz): δ 20.39, 38.70, 52.45, 63.67, 95.20, 100.40, 104.63, 112.26, 119.77, 123.11, 124.53, 125.17, 125.41, 125.82, 129.66, 130.42, 132.70, 132.94, 137.85, 143.98, 147.72, 159.99, 161.77, 162.47, 163.80, 164.23, 166.24. Anal. (C₂₄H₁₉F₅N₄O₂) C, H, N.

General Procedure for the Synthesis of 11a-b: Ethyl 2-cyano-3-ethoxyacrylate (275 mg, 1.62 mmol) and the proper aniline (**10a,b**) (1.62 mmol) were heated at 120 °C for 5 minutes in a microwave oven (max μW power input: 250 W; ramp time: 1 minute; reaction time: 5 minutes; power max: off; maxim pressure: 190 psi). The solid formed at the bottom of the tube was separated by filtration, suspended in 5 mL of diphenylether and irradiated at 230 °C for 7 minutes (max μW power input: 250 W; ramp time: 3 minutes; reaction time: 7 minutes; power max: off; maxim pressure: 180 psi). At the end of the irradiation, petroleum ether was added to the reaction mixture and the solid obtained was filtered over a Buchner funnel, washed thoroughly with petroleum ether and used in the following step without any further purification.

General Procedure for the Synthesis of 12a-b: Under nitrogen atmosphere intermediates **11a-b** (0.99 mmol) and freshly distilled POCl₃ (5 mL) were heated at reflux for 2 hours. The

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3 reaction mixture was cooled down to room temperature and volatile residues were removed
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5 under vacuum. The solid obtained was cooled down to 0 °C and then CH₂Cl₂, H₂O, and solid
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7 K₂CO₃ were added until pH reached 11. The aqueous phase was extracted twice with CH₂Cl₂ and
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9 the combined organic phases were dried over Na₂SO₄, filtered and dried under vacuum.
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11 Intermediates **12a-b** were used in the next step without any further purification. Quantitative
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13 yield.
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18 **General Procedure for the Synthesis of 13a-b:** 2,4-Dichloroaniline (65 mg, 0.40 mmol) was
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20 added to a suspension of NaH (60% dispersion in mineral oil) (16 mg, 0.4 mmol) in dry DMF (5
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22 mL). The reaction mixture was stirred at room temperature for 1 hour, followed by the addition
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24 of intermediate **12** or **12b** (0.2 mmol). The mixture was heated at reflux for 2 hours after which
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26 H₂O and ethyl acetate were added and the reaction mixture was cooled down to room
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28 temperature. The organic phase was washed with an aqueous solution of LiCl (5% w/w), brine,
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30 dried over Na₂SO₄ and concentrated under vacuum. The crude was purified by flash
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32 chromatography: CH₂Cl₂/acetone (100/0-98/2) for **13a**; petroleum ether/ethyl acetate (7/3) for
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34 **13b**.
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39 **4-((2,4-dichlorophenyl)amino)-7-methoxyquinoline-3-carbonitrile (13a).** Yield: 64%. Mp
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41 194-195 °C. MS (APCI) [M+H]⁺: 374.08 m/z, ¹H-NMR (CDCl₃ 400 MHz): δ 3.99 (s, 3H), 6.98
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43 (d, 1H, *J* = 8.64 Hz), 7.17 (dd, 1H, *J* = 9.3, 2.44 Hz), 7.21 (dd, 1H, *J* = 8.7, 2.0 Hz), 7.44 (d, 1H,
44
45 *J* = 2.44 Hz), 7.53 (d, 1H, *J* = 2.2 Hz), 7.71 (d, 1H, *J* = 9.28 Hz), 8.76 (s, 1H). ¹³C-NMR (CDCl₃
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47 100 MHz): δ 56.04, 56.42, 94.74, 101.13, 109.13, 115.06, 116.30, 122.12, 126.44, 127.60,
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49 129.66, 129.87, 136.67, 147.18, 147.68, 147.82, 149.67, 150.14, 154.50. Anal. (C₁₇H₁₁Cl₂N₃O)
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51 C, H, N.
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4-((2,4-dichlorophenyl)amino)-6,7-dimethoxyquinoline-3-carbonitrile (13b). Yield: 57%. Mp 243-244 °C. MS (ESI) $[M+H]^+$: 374.4 m/z. $^1\text{H-NMR}$ (CDCl_3 400 MHz): δ 3.79 (s, 3H), 4.06 (s, 3H), 6.78 (bs, 1H), 6.84 (d, 1H, $J = 8.67$ Hz), 6.89 (s, 1H), 7.17 (dd, 1H, $J = 8.64$, 2.28 Hz), 7.43 (s, 1H), 7.52 (d, 1H, $J = 2.28$ Hz), 8.71 (s, 1H). $^{13}\text{C-NMR}$ (CDCl_3 100 MHz): δ 44.52, 47.38, 53.88, 56.08, 85.68, 109.09, 113.37, 116.18, 117.71, 118.01, 127.30, 131.29, 149.38, 151.24, 152.10, 154.38, 162.34. Anal. ($\text{C}_{18}\text{H}_{13}\text{Cl}_2\text{N}_3\text{O}_2$) C, H, N.

Synthesis of 7-methoxy-4-((4-(4-methylpiperazin-1-yl)phenyl)amino)quinoline-3-carbonitrile (13c). 4-(4-methylpiperazin-1-yl)aniline (350 mg, 1.82 mmol) was added to a suspension of NaH (60% dispersion in mineral oil) (73 mg, 1.82 mmol) in dry DMF (11 mL). The reaction mixture was stirred at room temperature for 1 hour, after which intermediate **12a** was added and the mixture was heated at reflux for 2 hours. Next, the reaction mixture was cooled to room temperature, NaOH 1 M was added until pH 11 was reached. The obtained grey solid was separated by filtration over a Buchner funnel, washed with H_2O , dried and purified by flash chromatography using acetone/MeOH/ NEt_3 (90/9/1) as eluent. Yield: 35%. Mp 231-232 °C (decomposition: 225 °C). MS (ESI) $[M+H]^+$: 374.4 m/z. $^1\text{H-NMR}$ ($\text{DMSO}-d_6$ 400 MHz): δ 2.50 (s, 3H), 2.86 (bs, 4H), 3.30 (bs, 4H), 3.92 (s, 3H), 6.99 (d, 2H, $J = 8.76$ Hz), 7.18 (d, 2H, $J = 8.64$ Hz), 7.23 (dd, 1H, $J = 9.22$, 2.4 Hz), 8.41 (d, 1H, $J = 10.28$ Hz), 8.42 (s, 1H), 9.63 (s, 1H). $^{13}\text{C-NMR}$ ($\text{DMSO}-d_6$ 100 MHz): δ 44.52, 47.38, 53.88, 56.08, 85.68, 109.09, 113.37, 116.18, 117.71, 118.01, 127.30, 131.29, 149.38, 151.24, 152.10, 154.38, 162.34. Anal. ($\text{C}_{22}\text{H}_{23}\text{N}_5\text{O}$) C, H, N.

General Procedure for the Synthesis of 15a-d: In a microwave tube 2,6-dichloro-9H-purine **14** (100 mg, 0.53 mmol) and the proper aniline (2.64 mmol) were suspended in *n*-BuOH (3 mL). NEt_3 (265 μL , 1.90 mmol) was added and the tube was heated at 70 °C for 10 minutes (max μW

power input: 300 W; ramp time: 1 minute; reaction time: 10 minutes; power max: off; maximum pressure: 260 psi). At the end of the irradiation, *n*-BuOH was evaporated under vacuum. The solid obtained was isolated by filtration over a Buchner funnel and washed with *n*-hexane and cold (4 °C) ethyl acetate.

4-((2-chloro-9*H*-purin-6-yl)amino)benzenesulfonamide (15a). Yield: 75%. ¹H-NMR (DMSO-*d*₆, 200 MHz), δ 7.01 (*t*, 1H, *J* = 7.28 Hz), 7.29 (*t*, 2H, *J* = 7.40 Hz), 7.78 (*d*, 2H, *J* = 7.63 Hz), 8.25 (*s*, 1H), 10.16 (*bs*, 1H).

2-chloro-*N*-phenyl-9*H*-purin-6-amine (15b). Yield: 61%. ¹H-NMR (DMSO-*d*₆, 200 MHz), δ 7.26 (*s*, 2H), 7.73 (*d*, 2H, *J* = 8.83 Hz), 7.96 (*d*, 2H, *J* = 8.78 Hz), 8.44 (*s*, 1H), 10.75 (*bs*, 1H).

1-(4-((2-chloro-9*H*-purin-6-yl)amino)phenyl)ethan-1-one (15c). Yield: 74%. ¹H-NMR (DMSO-*d*₆, 200 MHz), 2.53 (*s*, 3H), 7.97 (*d*, 2H, *J* = 8.61 Hz), 8.04 (*d*, 2H, *J* = 8.74 Hz), 8.62 (*s*, 1H), 10.94 (*bs*, 1H).

3-((2-chloro-9*H*-purin-6-yl)amino)phenol (15d). Yield: 81%. MS (ESI) [M-H]⁺:260.2 m/z. ¹H-NMR (DMSO-*d*₆ 300 MHz), δ 6.50 (*dd*, 1H; *J* = 8.01, 1.42 Hz), 7.12 (*t*, 1H; *J* = 8.04 Hz), 7.25 (*d*, 1H; *J* = 8.19 Hz), 7.34 (*bs*, 1H), 8.29 (*s*, 1H), 9.36 (*bs*, 1H), 10.04 (*s*, 1H), 13.37 (*bs*, 1H).

General Procedure for the Synthesis of 16a-l: In a microwave tube intermediates **15a-d** (50 mg, 0.19 mmol) and the opportune amine (0.47 mmol) were suspended in *n*-BuOH (1.5 mL). Trifluoroacetic acid (14.63 μL, 0.19 mmol) was added and the tube was heated in the microwave in two consecutive steps: first at 170 °C for 10 minutes and then at 150 °C for 10 minutes (STEP-1: max μW power input: 300 W; ramp time: 1 minute; reaction time: 10 minutes; power max: off; maximum pressure: 260 psi; STEP-2: max μW power input: 300 W; reaction time: 10 minutes; power max: off; maximum pressure: 260 psi).

4-((2-(4-(2-hydroxyethyl)piperazin-1-yl)-9H-purin-6-yl)amino)benzenesulfonamide (16a).

Yield: 54%. Mp > 250 °C (decomposition). ¹H-NMR (DMSO-*d*₆, 400 MHz): δ 2.42 (*t*, 2H, *J* = 6.12 Hz), 2.45-2.52 (*m*, 4H), 3.53 (*d*, 2H, *J* = 3.92 Hz), 3.68 (*s*, 4H), 4.43 (*bs*, 1H), 7.17 (*bs*, 2H), 7.73 (*d*, 2H, *J* = 8.6 Hz), 7.91 (*s*, 1H), 8.04 (*d*, 2H, *J* = 8.48 Hz), 9.88 (*bs*, 1H), 12.53 (*bs*, 1H). ¹³C-NMR (DMSO-*d*₆, 100.6 MHz): δ 44.90, 53.52 (2 x), 58.96 (2 x), 60.80, 114.13, 119.71 (2 x), 126.74 (2 x), 137.00, 137.94, 143.69, 151.46, 153.37, 158.87. Anal. (C₁₇H₂₂N₈O₃S) C, H, N.

2-(4-(6-(phenylamino)-9H-purin-2-yl)piperazin-1-yl)ethan-1-ol (16b). Yield: 44%. Mp >

250 °C (decomposition). ¹H-NMR (DMSO-*d*₆, 200 MHz): δ 2.23-2.43 (*m*, 6H), 3.44 (*s*, 2H), 3.60 (*s*, 4H), 4.40 (*bs*, 1H), 6.91 (*t*, 1H, *J* = 7.03 Hz), 7.23 (*t*, 2H, *J* = 7.72 Hz), 7.80 (*m*, 3H), 9.41 (*bs*, 1H). ¹³C-NMR (DMSO-*d*₆, 100.6 MHz): δ 44.99 (2 x), 53.69 (2 x), 59.09, 60.95, 114.01, 120.70 (2 x), 122.43, 128.91 (2 x), 137.46, 140.63, 152.02, 153.01, 159.10. Anal. (C₁₇H₂₁N₇O) C, H, N.

4-((2-morpholino-9H-purin-6-yl)amino)benzenesulfonamide (16c). Yield: 61%. Mp > 250

°C (decomposition). ¹H-NMR (DMSO-*d*₆, 200 MHz): δ 3.50 (*s*, 8H), 7.14 (*s*, 2H), 7.69 (*d*, 2H, *J* = 8.77 Hz), 7.90 (*s*, 1H), 8.00 (*d*, 2H, *J* = 8.83 Hz), 9.90 (*bs*, 1H). ¹³C-NMR (DMSO-*d*₆, 100.6 MHz): δ 45.35 (2 x), 66.43 (2 x), 117.00, 119.74 (2 x), 126.73 (2 x), 136.99, 138.08, 143.56, 151.43, 153.19, 158.90. Anal. (C₁₅H₁₇N₇O₃S) C, H, N.

1-(4-((2-(isopentylamino)-9H-purin-6-yl)amino)phenyl)ethan-1-one (16d). Yield: 66%. Mp

> 250 °C (decomposition). ¹H-NMR (DMSO-*d*₆, 200 MHz): δ 0.79-0.86 (*m*, 6H), 1.38-1.50 (*m*, 2H), 1.57-1.63 (*m*, 1H), 2.48 (*s*, 3H), 2.70-2.78 (*m*, 2H), 7.97-8.10 (*m*, 4H), 8.62 (*s*, 1H). ¹³C-NMR (DMSO-*d*₆, 100.6 MHz): δ 22.74 (2 x), 25.30, 26.06, 35.74, 41.32, 112.83, 120.18 (2 x), 129.32 (2 x), 131.11, 142.07, 143.83, 151.64, 152.08, 154.22, 196.80. Anal. (C₁₈H₂₂N₆O) C, H, N.

1-(4-((2-(methylamino)-9H-purin-6-yl)amino)phenyl)ethan-1-one (16e). Yield: 47%. Mp > 250 °C (decomposition). ¹H-NMR (DMSO-*d*₆, 200 MHz): δ 2.53 (*s*, 3H), 3.06 (*s*, 3H), 7.94 (*d*, 2H, *J* = 11.72 Hz), 8.04 (*d*, 2H, *J* = 8.76 Hz), 8.62 (*s*, 1H), 10.94 (*bs*, 1H). ¹³C-NMR (DMSO-*d*₆, 100.6 MHz): δ 25.99, 26.35, 115.30, 119.24 (2 *x*), 129.22 (2 *x*), 131.33, 141.57, 143.24, 150.18, 152.26, 153.22, 196.39. Anal. (C₁₄H₁₄N₆O) C, H, N.

1-(4-((2-((2-hydroxyethyl)amino)-9H-purin-6-yl)amino)phenyl)ethan-1-one (16f). Yield: 42%. Mp > 250 °C (decomposition). ¹H-NMR (DMSO-*d*₆, 200 MHz): δ 2.58 (*s*, 3H), 3.26 (*t*, 2H, *J* = 8.20 Hz), 3.50 (*t*, 2H, *J* = 1.80 Hz), 7.97 (*d*, 2H, *J* = 8.64 Hz), 8.05 (*d*, 2H, *J* = 9.24 Hz), 8.68 (*s*, 1H), 10.96 (*bs*, 1H). ¹³C-NMR (DMSO-*d*₆, 100.6 MHz): δ 26.05, 39.95, 62.22, 114.22, 119.90 (2 *x*), 128.53 (2 *x*), 130.99, 140.11, 142.33, 152.24, 154.83, 156.53. Anal. (C₁₅H₁₆N₆O₂) C, H, N.

3-((2-(benzylamino)-9H-purin-6-yl)amino)phenol (16g). Yield: 49%. Mp 267-268 °C (with decomposition). MS (ESI) [M+H]⁺: 333.3 m/z, [M+Na]⁺: 355.3 m/z. ¹H-NMR (DMSO-*d*₆ 300 MHz): δ 4.52 (*d*, 2H; *J* = 6.21 Hz), 6.39 (*dd*, 1H; *J* = 7.83, 1.11 Hz), 7.01 (*m*, 2H), 7.18 (*t*, 1H; *J* = 7.08 Hz), 7.28 (*t*, 2H; *J* = 7.35 Hz), 7.43 (*bs*, 2H), 7.49 (*d*, 2H; *J* = 7.14 Hz), 7.80 (*s*, 1H), 8.35 (*bs*, 1H), 9.21 (*bs*, 1H), 13.37 (*bs*, 1H). ¹³C-NMR (DMSO-*d*₆ 75 MHz): δ 45.02, 107.65, 109.37, 111.52, 126.73, 127.45, 128.50, 129.23, 136.91, 141.70, 141.83, 152.11, 157.79, 159.47. Anal. (C₁₈H₁₆N₆O) C, H, N.

3-((2-(isopentylamino)-9H-purin-6-yl)amino)phenol (16h). Yield: 70%. Mp 223-224 °C (with decomposition). MS (ESI): [M+H]⁺: 313.2 m/z, [M+Na]⁺: 335.4 m/z. ¹H-NMR (DMSO-*d*₆ 300 MHz): δ 0.90 (*d*, 6H; *J* = 6.6 Hz), 1.46 (*dd*, 2H; *J* = 14.7, 7.08 Hz), 1.67 (*e*, 1H; *J* = 6.68 Hz), 3.30 (*dd*, 2H; *J* = 14.5, 7.3 Hz), 6.37 (*m*, 2H), 7.02 (*t*, 1H, *J* = 8.08 Hz), 7.43 (*s*, 1H), 7.50 (*d*, 1H, *J* = 8.37 Hz), 7.78 (*s*, 1H), 8.14 (*s*, 1H), 9.10 (*s*, 1H), 9.18 (*s*, 1H), 12.31 (*bs*, 1H). ¹³C-

NMR (DMSO- d_6 75 MHz): δ 23.06, 25.92, 39.41, 107.60, 109.33, 111.42, 129.25, 141.94, 157.78, 159.59, 163.54. Anal. (C₁₆H₂₀N₆O) C, H, N.

3-((2-(methylamino)-9H-purin-6-yl)amino)phenol (16i). Yield: 45%. Mp 293 °C (with decomposition). MS (ESI) [M-H]⁻: 255.3. ¹H-NMR (DMSO- d_6 300 MHz): δ 2.81 (*d*, 3H, *J* = 4.32 Hz), 6.39 (*d*, 1H; *J* = 8.16 Hz), 6.5 (*d*, 1H; *J* = 4.1 Hz), 7.03 (*t*, 1H; *J* = 7.92 Hz), 7.45 (*d*, 1H, *J* = 8.22 Hz), 7.50 (*s*, 1H), 8.36 (*bs*, 1H), 9.22 (*s*, 1H), 12.10 (*bs*, 1H). ¹³C-NMR (DMSO- d_6 75 MHz): δ 28.99, 107.65, 109.32, 111.35, 129.24, 136.98, 141.98, 151.94, 153.97, 157.86, 160.19. Anal. (C₁₂H₁₂N₆O) C, H, N.

3-((2-((2-hydroxyethyl)amino)-9H-purin-6-yl)amino)phenol (16l). Yield: 69%. Mp 252-253 °C (with decomposition). MS (ESI) [M+H]⁺: 287.3 m/z, [M+Na]⁺: 309.4 m/z. ¹H-NMR (DMSO- d_6 300 MHz): δ 3.35 (*d*, 2H, *J* = 2.85 Hz), 3.58 (*d*, 2H, *J* = 2.85 Hz), 4.72 (*t*, 1H, *J* = 5.3 Hz), 6.27 (*t*, 1H, *J* = 5.5 Hz), 6.39 (*dd*, 1H, *J* = 8.01, 1.89 Hz), 7.04 (*t*, 1H, *J* = 8.02 Hz), 7.45 (*d*, 1H, *J* = 8.31 Hz), 7.50 (*bs*, 1H), 7.80 (*s*, 1H), 9.17 (*s*, 1H), 12.38 (*bs*, 1H). ¹³C-NMR (DMSO- d_6 75 MHz) δ 44.51, 60.61, 107.68, 109.34, 111.49, 114.06, 129.30, 136.43, 141.89, 152.44, 157.74, 159.56, 161.66. Anal. (C₁₃H₁₄N₆O₂) C, H, N.

3-((2-((2-(1H-imidazol-4-yl)ethyl)amino)-9H-purin-6-yl)amino)phenol (16m). In a microwave tube intermediate **15d** (50 mg, 0.19 mmol) and histamine dihydrochloride (88 mg, 0.49 mmol) were suspended in *n*-BuOH (3.0 mL). Et₃N (173 μ L, 1.24 mmol) was then added and the tube was heated in the microwave at 130 °C for 10 minutes (max μ W power input: 300 W; ramp time: 1 minute; reaction time: 10 minutes; power max: off; maximum pressure: 260 psi). At the end of the irradiation, *n*-BuOH was evaporated under vacuum and the solid obtained was purified by flash chromatography using ethyl acetate/MeOH + NEt₃ (8/2+3%) as eluent.

Yield: 35%. Mp 245-246 °C (with decomposition). MS (ESI) [M-H]⁺: 335.4 m/z. ¹H-NMR (DMSO-*d*₆ 300 MHz): δ 2.93 (*m*, 2H), 3.58 (*m*, 2H), 6.40 (*d*, 1H, *J* = 6.72 Hz), 6.73 (*bs*, 1H), 7.02 (*t*, 1H, *J* = 8.01 Hz), 7.26 (*bs*, 1H), 7.36 (*bs*, 1H), 7.45 (*d*, 1H, *J* = 8.37 Hz), 7.59 (*bs*, 1H), 7.88 (*bs*, 1H), 8.77 (*bs*, 1H), 9.36 (*bs*, 1H), 10.21 (*bs*, 1H), 12.10 (*bs*, 1H). ¹³C-NMR (DMSO-*d*₆ 100 MHz): δ 38.17, 45.82, 107.76, 109.73, 11.41, 116.31, 129.34, 132.44, 134.03, 134.78, 141.63, 151.40, 152.30, 157.91, 160.33, 162.45. Anal. (C₁₆H₁₆N₈O) C, H, N.

Biology

Evaluation of the activity of compounds on cell metabolism and DENV replication:

DENV serotype 2 strain New Guinea C (DENV-2 NGC) was kindly provided by Dr. V. Deubel (formerly at Institute Pasteur, Lyon, France). The virus was propagated in C6/36 mosquito cells (from *Aedes albopictus*; ATCC CCL-1660) at 28 °C in Leibovitz's L-15 medium (Life Technologies, Cat N°11415049) that is supplemented with 10% FBS, 1% non-essential amino acids (Life Technologies, Cat N°11140035), 1% HEPES buffer (Life Technologies, Cat N°15630056) and 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) solution. The CPE reduction assay is automated on a Freedom EVO200 liquid handling platform (Tecan), which is set up in a custom-made bio-safety level 2 robotics enclosure. Assay setup and data acquisition are barcode-traced, and raw data are processed using a custom-designed database-coupled software package. Vero-B cells [African Green monkey kidney cells, obtained from the European Collection of Cell Cultures (ECACC)] were grown in minimum essential medium (MEM; Gibco, Merelbeke, Belgium) supplemented with 10% FBS, 1% l-glutamine and 1% sodium bicarbonate. Antiviral assays were performed in medium supplemented with 2% FBS, 1% l-glutamine and 1% sodium bicarbonate. In CPE-reduction assays (compounds **4a-16c**),

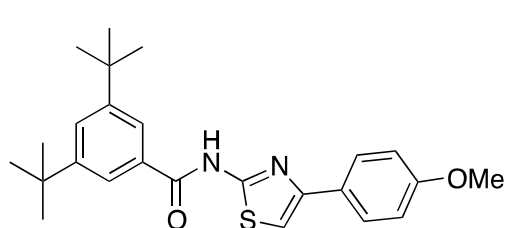
Vero-B cells were seeded at a density of 7×10^3 cells/well in 100 μ L assay medium and allowed to adhere overnight. The following day, serial compound dilutions (1:2) were added to each well (starting concentration 100 μ g/mL), following by the addition of 100 μ L assay medium containing 100 50% cell culture infectious doses (i.e., CCID₅₀) of DENV-2. After 7 days of incubation, the assay medium was discarded and cells were fixed with ethanol and stained with 1% methylene blue solution. For toxicity assays, the same protocol was followed with the exception that virus addition was omitted. The 50% effective concentration (EC₅₀) and the 50% cytotoxic concentration (CC₅₀), which are defined as the compound concentration that is required respectively to inhibit the virus-induced cytopathogenic effect (CPE) by 50% and to inhibit the cell growth by 50%, were calculated based on microscopic scoring data for each well. In virus yield reduction assays (compounds **16d-16m**), Vero-B cells were seeded at a density of 5×10^4 cells/well in 96-well plates. One day later, medium was replaced by 100 μ L virus inoculum (100 CCID₅₀) and incubated for 2 hours, after which the cell monolayer was washed 3 times with assay medium to remove non-adsorbed virus. Cells were further cultivated in 200 μ L of fresh assay medium containing the 5-fold serial dilutions of compounds (50 – 0.08 μ g/mL) for 4 days. Supernatant was harvested and viral RNA load was determined by real-time quantitative RT-PCR, as described previously.²³ The EC₅₀ value, which is defined as the compound concentration that is required to inhibit viral RNA replication by 50%, was determined using logarithmic interpolation.

AlphaScreen assay: Production of DENV-2 NS3 and NS5 proteins by a wheat germ cell-free protein production system was performed as previously described.²⁴ Briefly, *in vitro* transcription (IVT) was carried out using the pEU vector for the production of N-terminal

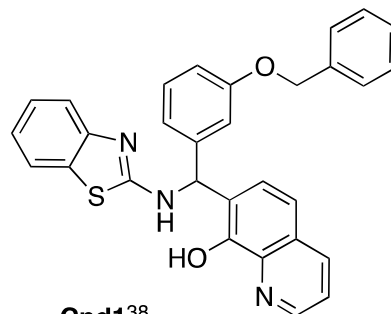
hexahistidine- and FLAG-tagged (HF) NS3 or N-terminal glutathione S-transferase-tagged and biotinylated (GB) NS5.²⁴ After IVT, cell-free expression of proteins was performed in a translation reaction using wheat germ extract according to the protocol of CellFree Sciences, Japan. Biotinylated NS5 was produced as previously described.²⁴ Recombinant protein was batch purified by Protomist DTII (CellFree Sciences) using Ni Sepharose High Performance beads (for HF-NS3, GE Healthcare) or Glutathione Sepharose Fast Flow beads (for GB-NS5, GE Healthcare), and eluted with His-tag elution buffer (20 mM Tris-HCl, pH7.5, 500 mM NaCl, 10% glycerol, 500 mM imidazole) or GST-tag elution buffer (50 mM Tris-HCl, pH8.0, 50 mM NaCl, 10% glycerol, 10 mM reduced glutathione). HF-NS3 was further purified using a HiTrap Desalting column (GE Healthcare) and desalting buffer (20 mM Tris-HCl, pH7.5, 50 mM NaCl, 10% glycerol) to remove imidazole. As a negative control protein for HF-NS3, N-terminal His- and FLAG-tagged bacterial dihydrofolate reductase (HF-DHFR) was produced and purified by performing the same procedure.²⁴ AlphaScreen assay to detect NS3-NS5 interaction was performed in 384-well OptiPlates (PerkinElmer). Twenty-five nanomolar HF-NS3 (or HF-DHFR in the control reaction) and GB-NS5 were incubated with 50 μ M compound (25 μ M for **16e,f** only) in the presence of 0.5% DMSO in 15 μ l of binding mixture (20 mM Tris-HCl, pH7.5, 50 mM NaCl, 5 mM MgCl₂, 200 μ M DTT, 1 mg/ml BSA, 0.02% Tween-20) at room temperature for 1 h. Then, 10 μ l of the detection mixture containing 0.6 mg/ml anti-FLAG mouse monoclonal IgG (1E6, Wako Pure Chemical Industries), 0.1 μ l of protein A-conjugated acceptor beads, 0.1 μ l of streptavidin-coated donor beads (AlphaScreen IgG detection kit, PerkinElmer), 20 mM Tris-HCl, pH7.5, 50 mM NaCl, 5 mM MgCl₂, 200 μ M DTT and 1 mg/ml BSA was added, followed by incubation at room temperature for 1 h. Light emission was analyzed by an EnSpire Alpha microplate reader (PerkinElmer).²⁴

In vitro Kinase inhibition assay: Active recombinant his-tagged full length Src and Fyn were purchased from Merck-Millipore. Assay conditions were as follows: Fyn reactions were performed in 50 mM MOPS/NaOH pH 7.0, 0.1 mM EDTA, 0.0013% NP40, 0.1 mM sodium orthovanadate (Na_3VO_4), 10% DMSO, 3 mM $\text{MnCl}_2/\text{MgCl}_2$, 100 μM ATP/ $[\gamma\text{-}^{33}\text{P}]\text{ATP}$, 250 μM of the Src substrate peptide KVEKIGEGTYGVVYK, and 30 ng active enzyme. Src reactions were performed in 10 mM MOPS/NaOH pH 7.0, 0.2 mM EDTA, 0.0013% NP40, 0.1 mM Na_3VO_4 , 10% DMSO, 3 mM $\text{MnCl}_2/\text{MgCl}_2$, 100 μM ATP/ $[\gamma\text{-}^{33}\text{P}]\text{ATP}$, 250 μM of the Src substrate peptide KVEKIGEGTYGVVYK, and 30 ng active enzyme. All reactions were performed in 10 μl at 30 $^\circ\text{C}$ for 10 min. To avoid proteins and peptidic substrate adsorption on the surface of the plastic ware, inhibition assays were performed using protein low binding plastic tubes (LoBind, Eppendorf) and tips (GoldenGate). Reactions were stopped by adding 5 μl of 0.8% phosphoric acid. Aliquots (10 μl) were then transferred onto a P30 Filtermat (PerkinElmer), washed five times with 75 mM phosphoric acid and once with acetone for 5 min. The filter was dried and transferred to a sealable plastic bag, and scintillation cocktail (4 mL) was added. Spotted reactions were read in a scintillation counter (Trilux, PerkinElmer). ID50 values were obtained according to Equation (1), where v is the measured reaction velocity, V is the apparent maximal velocity in the absence of inhibitor, I is the inhibitor concentration, and ID50 is the 50% inhibitory dose.

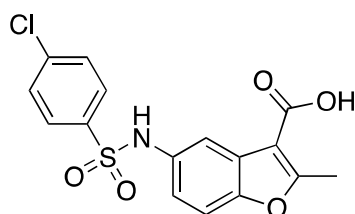
$$v = V / \{1 + (I / \text{ID50})\} \quad (1)$$



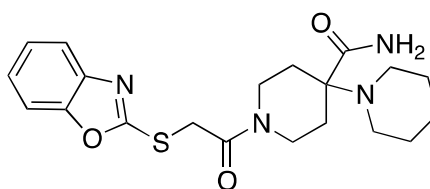
NITD-618³⁷
NS4B-inhibitor



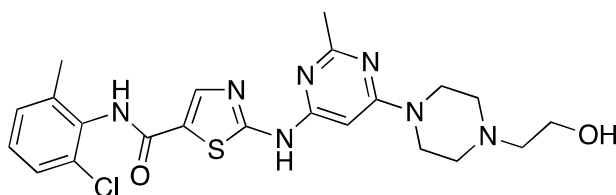
Cpd1³⁸
NS3 protease-inhibitor



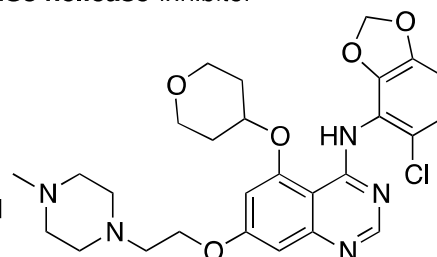
NITD-107³⁹
NS5 polymerase-inhibitor



ST-610⁴⁰
NS3 helicase-inhibitor



Dasatinib
Src/Fyn-inhibitor



Saracatinib (AZD0530)
Src/Fyn-inhibitor

Figure 1. Examples of compounds that either target a viral or host cell protein essential for DENV replication.

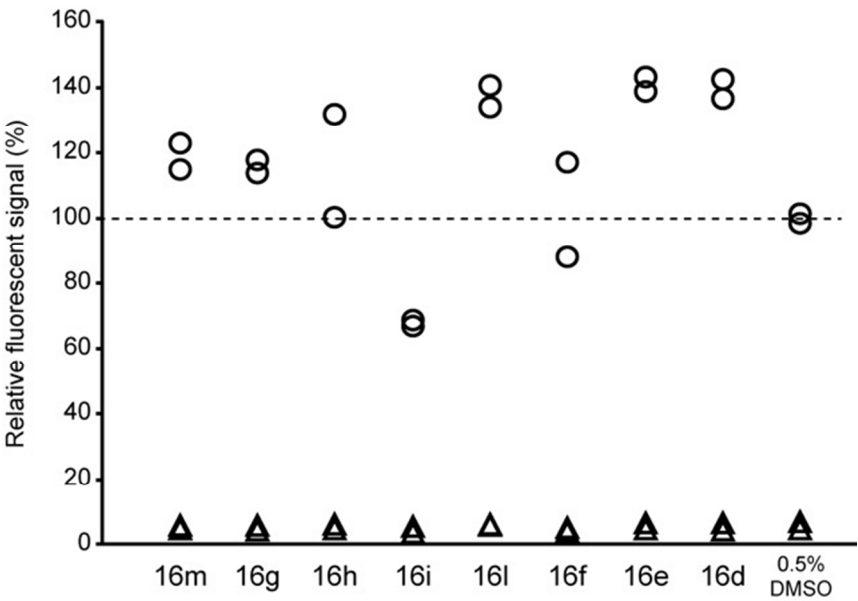


Figure 2. NS3-NS5 AlphaScreen assay for evaluation of compounds **16d-m**. Assay was performed with 25 nM protein and 50 μ M compound (25 μ M for **16e** and **16f**) in the presence of 0.5% DMSO (circles, n=2). AlphaScreen assay was also carried out with a negative control reaction containing HF-DHFR and GB-NS5 in the presence of respective compounds (triangles, n=2). Results are presented as a percentage of the luminescent signal obtained by positive control (PC) reactions (i.e., NS3-NS5 AlphaScreen assay without compounds [0.5% DMSO, 100%, dashed lines]), which was obtained by the following formula: $100 \times [(\text{luminescent count of sample} - \text{meanNC})/(\text{meanPC} - \text{meanNC})]$.

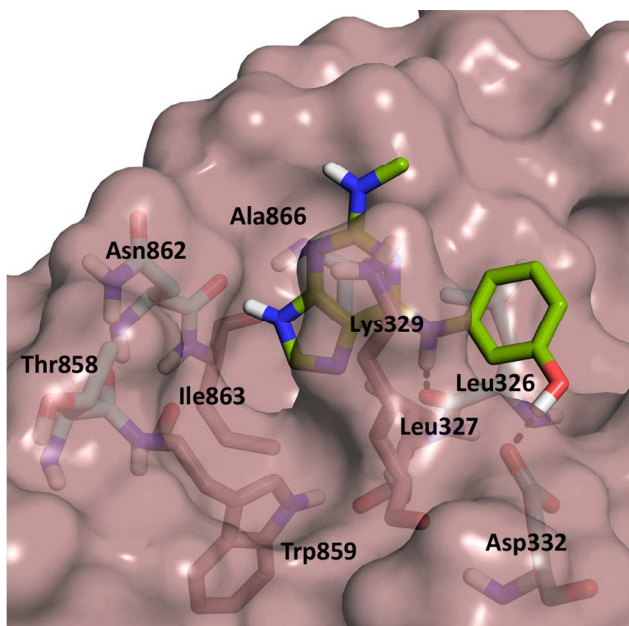


Figure 3. The IFD binding mode of 16i. The numbering of amino acids is based on DENV-3 RdRp (DENV-2 numbering of corresponding residues: Leu327, Leu328, Lys330, Asp333, Thr858, Trp859, Asn862, Ile863, and Ala866). Residues that have been shown to be critical for the de novo RNA synthesis or NS3-NS5 interaction by mutagenesis studies are shown in pink. Hydrogen bonds are represented as black dotted lines.

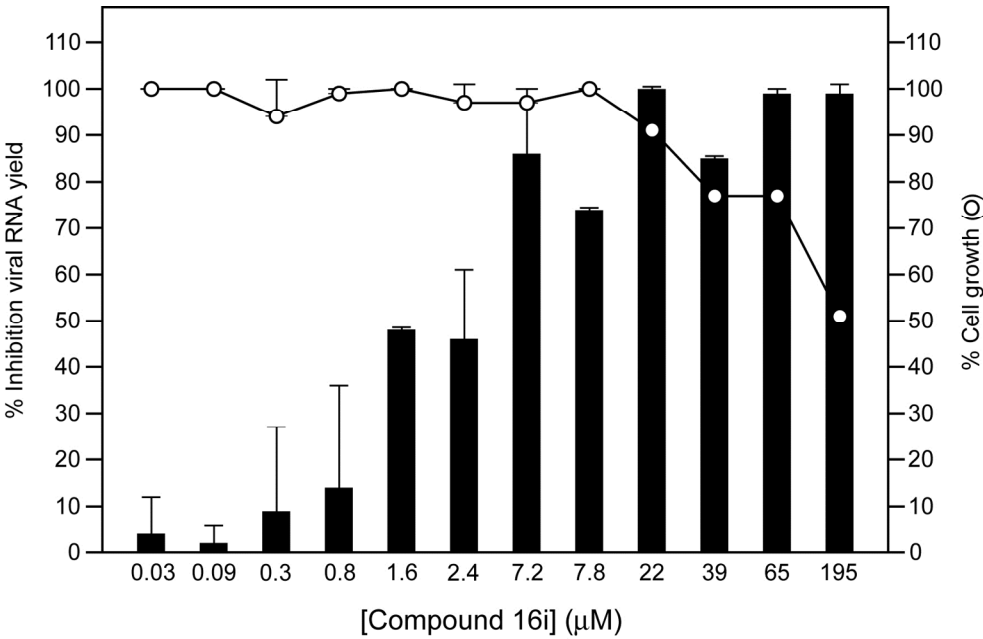
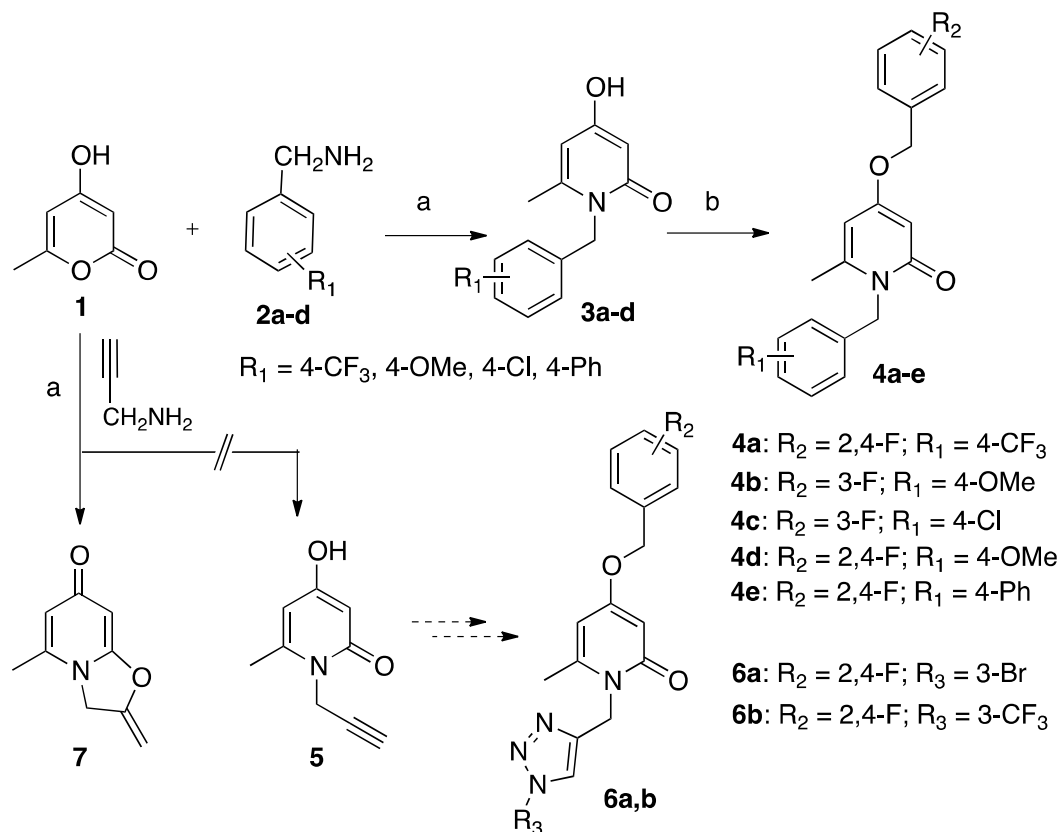
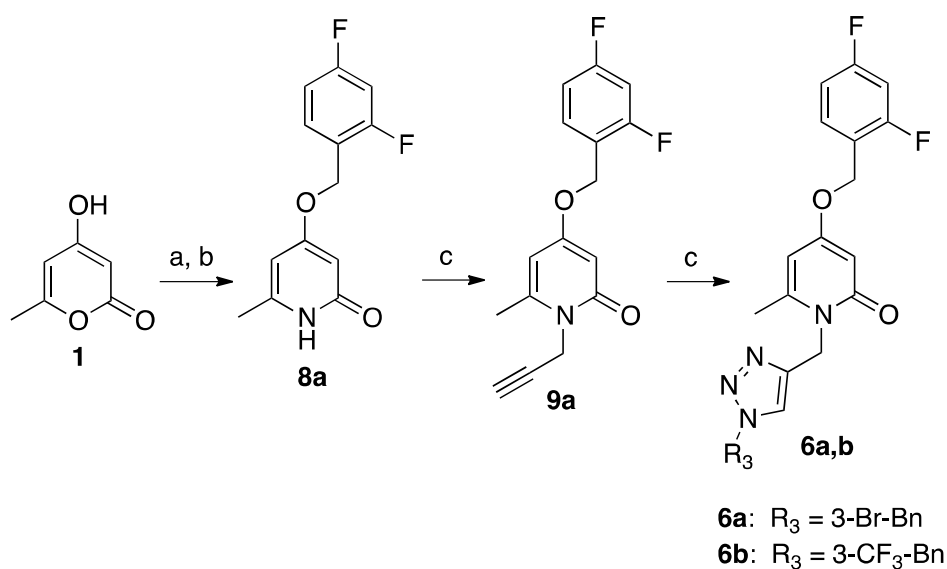


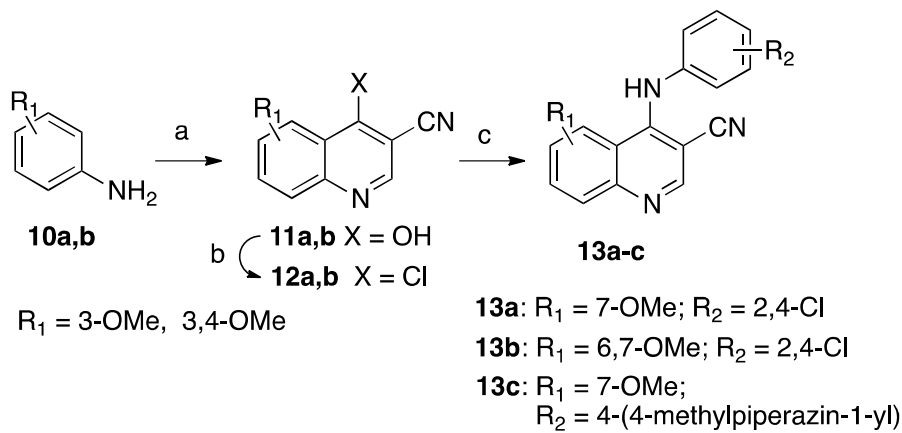
Figure 4. Dose-dependent inhibition of DENV RNA replication by compound **16i**. Vero-B cell cultures infected with DENV-2 were treated with different concentrations of **16i**. Viral RNA levels in the supernatant were quantified on day 4 p.i. by means of RT-qPCR and are expressed as percentage inhibition of untreated virus control (black bars). Mock-infected cells were treated with the same dilution series of **16i**. Cell viability was determined by means of the MTS/PMS method and is expressed as percentage of cell growth of untreated control (white circles). Data represent mean values $\pm$ standard deviations (SD) for three independent experiments.



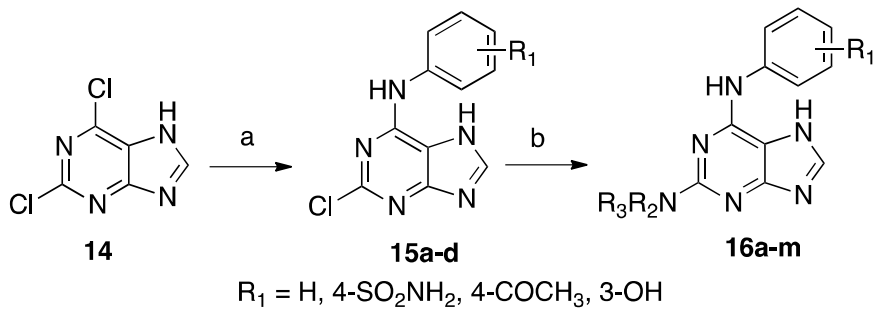
Scheme 1. Reagents and conditions. a) H_2O , reflux, 2-7 h; b) benzylbromides, K_2CO_3 , DMF, r.t., 2 h.



Scheme 2. Reagents and conditions. a) NH_4OH , reflux, 4h; b) 2,4-Difluorobenzyl bromides, K_2CO_3 , DMF, 7 h, r.t.; c) propargyl bromide, NaH, LiCl, DME:DMF (4:1), 65 °C, 26 h; d) benzylbromides, NaN_3 , CuSO_4 , sodium ascorbate, $\text{H}_2\text{O}:\text{t-BuOH}$ 1:1, MW 125 °C, 10 min.

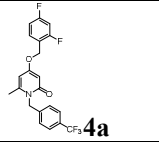
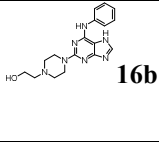
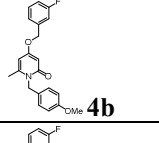
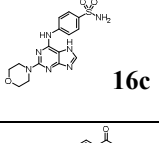
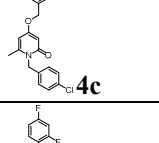
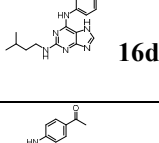
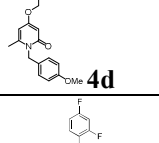
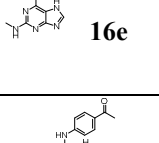
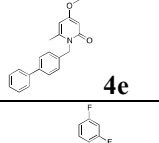
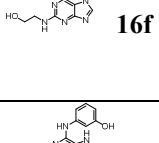
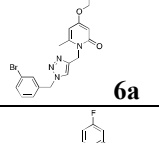
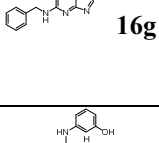
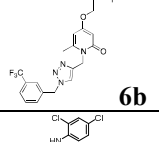
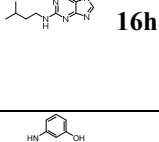
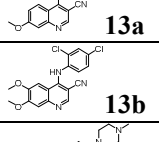
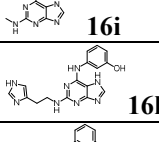
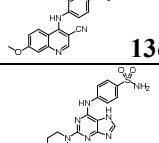
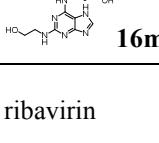
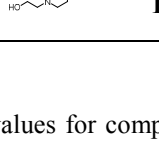
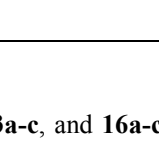
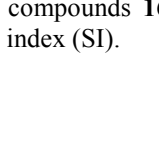


Scheme 3. Reagents and conditions. a) i. Ethyl 2-cyano-3-ethoxyacrylate, neat, MW 120 °C, 5 min; ii. $(\text{Ph})_2\text{O}$, MW 230 °C, 7 min; b) POCl_3 , reflux, 2 h; c) Anilines, DMF, NaH, 2 h, reflux.



Scheme 4. Reagents and conditions. a) anilines, n-BuOH, MW, Et_3N , 70 °C, 10 min; b) Method A (for **16a-l**): anilines, n-BuOH, TFA, MW, 170 °C, 10 min then 150 °C 10 min; Method B (for **16m**) histamine dihydrochloride, n-BuOH, Et_3N , MW, 130 °C, 10 min.

Table 1. DENV-2 replication inhibitory effect.

Cpds	EC ₅₀ (μM)	CC ₅₀ (μM)	Cpds (μM)	EC ₅₀ ^a (μM)	CC ₅₀ (μM)
 4a	<4.8	<4.8	 16b	NA	474
 4b	<6.4	<6.4	 16c	NA	373
 4c	<6.4	<6.4	 16d	59 (1) ^c	85
 4d	<4.6	<4.6	 16e	42 ± 28 (>4)	>177
 4e	NA ^b	10	 16f	72 (2)	160
 6a	NA	36	 16g	69 (>2)	>150
 6b	NA	40	 16h	20 ± 7.7 (8)	151 ± 19
 13a	NA	19	 16i	5.3 ± 6.6 (32)	168 ± 30
 13b	<5.7	<5.7	 16l	10 ± 6.0 (>15)	>149
 13c	NA	7.9	 16m	7.4±0.7 (>24)	>175
 16a	NA	437	ribavirin	42±4 (>10)	409

^a EC₅₀ values for compounds **4a-e**, **6a,b**, **13a-c**, and **16a-c** were generated using the CPE-reduction assay; EC₅₀ values for compounds **16d-i**, **16l,m** were generated using the virus-yield reduction assay; ^b NA = not active; ^c Selectivity index (SI).

Table 2. Kinase inhibitory activities

Cpds	c-Src (ID ₅₀ μM) ^a	Fyn (ID ₅₀ μM) ^a
4a	40.0	NA ^b
4b	NA	NA
6b	NA	NA
13a	24.4 (0.2) ^c	2.0
13b	0.62 (0.03) ^c	0.22
13c	NA	NA
16a	7.4 (0.26) ^d	4.8
16b	4.2 (3.14) ^d	4.2
16c	0.9 (0.02) ^d	0.5
16g	3.7	4.1
16h	1.7	1.7
16i	4.9	3.6
16l	1.1	0.7
16m	2.6	2.1

^aValues are the mean of at least two experiments. ^bNA = not active; no inhibition at 100 μM. ^cIC₅₀ values from reference 21 are reported in parentheses. ^dIC₅₀ values from reference 22 are reported in parentheses.

ASSOCIATED CONTENT

Supporting Information. SMILES molecular formula strings and associated biological data (CSV). This material is available free of charge on the ACS Publications website at

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

CPE, cytopathic effect; DENV, Dengue virus; DF, Dengue fever; DHF, Dengue hemorrhagic fever; DSS, Dengue shock syndrome; GB-NS5, biotinylated NS5; IFD, induced fit docking;

HCV, Hepatitis C virus; HF-DHFR, flag-tagged dihydrofolate reductase; PK, pharmacokinetic; RdRp, RNA-dependent RNA-polymerase.

REFERENCES

(1) World health organization. Guidelines for diagnosis, treatment, prevention and control. Geneva, 2009. <http://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf>. Last accessed 21 May 2015.

(2) a) Wilder-Smith, A.; Schwartz, E. Dengue in travelers. *N. Engl. J. Med.* **2005**, *353*, 924-932. b) Jelinek, T.; Mühlberger, N.; Harms, G.; Corachán, M.; Grobusch, M.P.; Knobloch, J.; Bronner, U.; Laferl, H.; Kapaun, A.; Bisoffi, Z.; Clerinx, J.; Puente, S.; Fry, G.; Schulze, M.; Hellgren, U.; Gjørup, I.; Chalupa, P.; Hatz, C.; Matteelli, A.; Schmid, M.; Nielsen, L. N.; da Cunha, S.; Atouguia, J.; Myrvang, B.; Fleischer, K. Epidemiology and clinical features of imported dengue fever in europe: sentinel surveillance data from TropNetEurop. *Clin. Infect. Dis.* **2002**, *35*, 1047-1052.

(3) Gubler, D.; Kuno, G.; Markoff, L. (2007) In fields virology (Knipe, D. M. and Howley, P. M. eds) 5th Ed. pp. 1153-1253, Lippincott Williams & Wilkins, Philadelphia.

(4) Pugachev, K. V.; Guirakhoo, F.; Trent, D. W.; Monath, T. P. Traditional and novel approaches to flavivirus vaccines. *Int. J. Parasitol.* **2003**, *33*, 567-582.

(5) Srikiatkachorn, A.; Wichit, S.; Gibbons, R. V.; Green, S.; Libraty, D. H.; Endy, T. P.; Ennis, F. A.; Kalayanaroj, S.; Rothman, A. L. Dengue viral RNA levels in peripheral blood mononuclear cells are associated with disease severity and preexisting dengue immune status. *PLoS ONE* **2012**, *7*(12): e51335. doi:10.1371/journal.pone.0051335.

- (6) a) Wang, Q. Y.; Patel, S. J.; Vangrevelinghe, E.; Xu, H. Y.; Rao, R.; Jaber, D.; Schul, W.; Gu, F.; Heudi, O.; Ma, N. L.; Poh, M. K.; Phong, W. Y.; Keller, T. H.; Jacoby, E.; Vasudevan, S. G. A small-molecule dengue virus entry inhibitor. *Antimicrob. Agents Chemother.* **2009**, *53*, 1823-1831. b) Li, Z.; Khaliq, M.; Zhou, Z.; Post, C. B.; Kuhn, R. J.; Cushman, M. Design, synthesis, and biological evaluation of antiviral agents targeting flavivirus envelope proteins. *J. Med. Chem.* **2008**, *51*, 4660-4671. □ c) Yin, Z.; Chen, Y. L.; Kondreddi, R. R.; Chan, W. L.; Wang, G.; Ng, R. H.; Lim, J. Y.; Lee, W. Y.; Jeyaraj, D. A.; Niyomrattanakit, P.; Wen, D.; Chao, A.; Glickman, J. F.; Voshol, H.; Mueller, D.; Spanka, C.; Dressler, S.; Nilar, S.; Vasudevan, S. G.; Shi, P. Y.; Keller, T. H. N-sulfonylanthranilic acid derivatives as allosteric inhibitors of dengue viral RNA-dependent RNA polymerase. *J. Med. Chem.* **2009**, *52*, 7934-7937.
- (7) Lim, S. P.; Wang, Q. Y.; Noble, C. G.; Chen, Y. L.; Dong, H.; Zou, B.; Yokokawa, F.; Nilar, S.; Smith, P.; Beer, D.; Lescar, J.; Shi, P. Y. Ten years of dengue drug discovery: progress and prospects. *Antiviral Res.* **2013**, *100*, 500-519.
- (8) Anighoro, A.; Bajorath, J.; Rastelli, G. Polypharmacology: challenges and opportunities in drug discovery. *J. Med. Chem.* **2014**, *57*, 7874-7887.
- (9) Chu, J. J. H.; Yang, P. L. c-Src protein kinase inhibitors block assembly and maturation of dengue virus. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3520-3525.
- (10) Zou, G.; Chen, Y.-L.; Dong, H.; Lim, C. C.; Yap, L. J.; Yau, Y. H.; Shochat, S. G.; Lescar, J.; Shi, P.-Y. Functional analysis of two cavities in flavivirus NS5 polymerase. *J. Biol. Chem.* **2011**, *286*, 14362-14372.

- (11) Gentles, R. G.; Ding, M.; Bender, J. A.; Bergstrom, C. P.; Grant-Young, K.; Hewawasam, P.; Hudyma, T.; Martin, S.; Nickel, A.; Regueiro-Ren, A.; Tu, Y.; Yang, Z.; Yeung, K.-S.; Zheng, X.; Chao, S.; Sun, J.-H.; Beno, B. R.; Camac, D. M.; Chang, C.-H.; Gao, M.; Morin, P. E.; Sheriff, S.; Tredup, J.; Wan, J.; Witmer, M. R.; Xie, D.; Hanumegowda, U.; Knipe, J.; Mosure, K.; Santone, K. S.; Parker, D. D.; Zhuo, X.; Lemm, J.; Liu, M.; Pelosi, L.; Rigat, K.; Voss, S.; Wang, Y.; Wang, Y.-K.; Colonno, R. J.; Gao, M.; Roberts, S. B.; Gao, Q.; Ng, A.; Meanwell, N. A.; Kadow, J. F. Discovery and preclinical characterization of the cyclopropylindolobenzazepine BMS-791325, a potent allosteric inhibitor of the hepatitis C virus NS5B polymerase. *J. Med. Chem.* **2014**, *57*, 1855–1879.
- (12) <https://www.ebi.ac.uk/chembl/>. Accessed June 2012.
- (13) <http://www.bindingdb.org/bind/index.jsp>. Accessed June 2012.
- (14) Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S. Glide: A new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J. Med. Chem.* **2004**, *47*, 1739-1749.
- (15) Trott, O.; Olson, A. J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461.
- (16) Yap, T. L.; Xu, T.; Chen, Y. L.; Malet, H.; Egloff, M. P.; Canard, B.; Vasudevan, S. G.; Lescar, J. Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. *J. Virol.* **2007**, *81*, 4753-4765.

- (17) Falchi, F.; Caporuscio, F.; Recanatini, M. Structure-based design of small-molecule protein-protein interaction modulators: the story so far. *Future Med. Chem.* **2014**, *6*, 343–357.
- (18) Schüller, A.; Hähnke, V.; Schneider, G. SmlLib v2.0: a java-based tool for rapid combinatorial library enumeration. *QSAR Comb. Sci.* **2007**, *26*, 407–410.
- (19)a) Castagnolo, D.; Radi, M.; Dessì, F.; Manetti, F.; Saddi, M.; Meleddu, R.; De Logu, A.; Botta, M. Synthesis and biological evaluation of new enantiomerically pure azole derivatives as inhibitors of mycobacterium tuberculosis. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2203–2205. b) Castagnolo, D.; Dessì, F.; Radi, M.; Botta, M. Synthesis of enantiomerically pure α -[4-(1-substituted)-1,2,3-triazol-4-yl]-benzylacetamides via microwave-assisted click chemistry: towards new potential antimicrobial agents. *Tetr. Asymm.* **2007**, *18*, 1345–1350.
- (20) Cao, X.; You, Q.-D.; Li, Z.-Y.; Yang, Y.; Wang, X.-J. Microwave-assisted simple synthesis of substituted 4-quinolone derivatives. *Synth. Commun.* **2009**, *39*, 4375–4383.
- (21) Boschelli, D. H.; Wang, Y. D.; Ye, F.; Wu, B.; Zhang, N.; Dutia, M.; Powell, D. W.; Wissner, A.; Arndt, K.; Weber, J. M.; Boschelli, F. Synthesis and src kinase inhibitory activity of a series of 4-phenylamino-3-quinolinecarbonitriles. *J. Med. Chem.* **2001**, *44*, 822–833.
- (22) Huang, H.; Ma, J.; Shi, J.; Meng, L.; Jiang, H.; Ding, J.; Liu, H. Discovery of novel purine derivatives with potent and selective inhibitory activity against c-src tyrosine kinase. *Bioorg. Med. Chem.* **2010**, *18*, 4615–4624.

- (23) Kaptein, S. J.; De Burghgraeve, T.; Froeyen, M.; Pastorino, B.; Alen, M. M.; Mondotte, J. A.; Herdewijn, P.; Jacobs, M.; de Lamballerie, X.; Schols, D.; Gamarnik, A. V.; Sztaricskai, F.; Neyts, J. A derivate of the antibiotic doxorubicin is a selective inhibitor of dengue and yellow fever virus replication in vitro. *Antimicrob. Agents Chemother.* **2010**, *54*, 5269-5280
- (24) Takahashi, H.; Takahashi, C.; Moreland, N. J.; Chang, Y. T.; Sawasaki, T.; Ryo, A.; Vasudevan, S. G.; Suzuki, Y.; Yamamoto N. Establishment of a robust dengue virus NS3-NS5 binding assay for identification of protein-protein interaction inhibitors. *Antiviral Res.* **2012**, *96*, 305-314.
- (25) Data not shown
- (26) Wispelaere, M.; LaCroix, A. J.; Yang, P. L. Small molecules AZD0530 and dasatinib inhibit dengue virus RNA replication via fyn kinase. *J Virol.* **2013**, *87*, 7367-7381.
- (27) Schrödinger, LLC, New York, NY, 2005.
- (28) Malet, H.; Massé, N.; Selisko, B.; Romette, J. L.; Alvarez, K.; Guillemot, J. C.; Tolou, H.; Yap, T. L.; Vasudevan, S.; Lescar, J.; Canard, B. The flavivirus polymerase as a target for drug discovery. *Antiviral Res.* **2008**, *80*, 23–35.
- (29) Sherman, W.; Day, T.; Jacobson, M. P.; Friesner, R. A.; Farid, R. Novel procedure for modeling ligand/receptor induced fit effects. *J. Med. Chem.* **2006**, *49*, 534-553.
- (30) Wolf L.K. New software and websites for the chemical enterprise. *Chem. Eng. News Archive*, **2009**, *87*, 32.

- (31) The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.
- (32) Sawasaki, T.; Kamura, N.; Matsunaga, S.; Saeki, M.; Tsuchimochi, M.; Morishita, R.; Endo, Y. Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate. *FEBS Lett.* **2008**, *582*, 221–228.
- (33) Davis, M. I.; Hunt, J. P.; Herrgard, S.; Ciceri, P.; Wodicka, L. M.; Pallares, G.; Hocker, M.; Treiber, D. K.; Zarrinkar, P. P. Comprehensive analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* **2011**, *29*, 1046-1051.
- (34) Schenone, S.; Brullo, C.; Musumeci, F.; Biava, M.; Falchi, F.; Botta, M. Fyn kinase in brain diseases and cancer: the search for inhibitors. *Curr. Med. Chem.* **2011**, *18*, 2921-2942.
- (35) Sierra, J. R.; Cepero, V.; Giordano, S. Molecular mechanisms of acquired resistance to tyrosine kinase targeted therapy. *Molecular Cancer* **2010**, *9*:75, 1-13.
- (36) Pfannkuche, A.; Buther, K.; Karthe, J.; Poenisch, M.; Bartenschlager, R.; Trilling, M.; Hengel, H.; Willbold, D.; Haussinger, D.; Bode, J.G. c-Src is required for complex formation between the hepatitis C virus-encoded proteins NS5A and NS5B: a prerequisite for replication. *Hepatology* **2011**, *53*, 1127-1136.
- (37) Xie, X.; Wang, Q. -Y.; Xu, H. Y.; Qing, M.; Kramer, L.; Yuan, Z.; Shi, P. -Y. Inhibition of Dengue Virus by Targeting Viral NS4B Protein. *J. Virol.* **2011**, *85*, 11183–11195.

- (38)Lai, H.; Sridhar Prasad, G.; Padmanabhan, R. Characterization of 8-hydroxyquinoline derivatives containing aminobenzothiazole as inhibitors of dengue virus type 2 protease in vitro. *Antiviral Res.* **2013**, *97*, 74-80.
- (39)Noble, C. G.; Lim, S. P.; Chen, Y. L.; Liew, C. W.; Yap, L.; Lescar, J.; Shi, P. Y. Conformational flexibility of the Dengue virus RNA-dependent RNA polymerase revealed by a complex with an inhibitor. *J. Virol.* **2013**, *87*, 5291-5295.
- (40)Byrd, C. M.; Grosenbach, D. W.; Berhanu, A.; Dai, D.; Jones, K. F.; Cardwell, K. B.; Schneider, C.; Yang, G.; Tyavanagimatt, S.; Harver, C.; Wineinger, K. A.; Page, J.; Stavale, E.; Stone, M. A.; Fuller, K. P.; Lovejoy, C.; Leeds, J. M.; Hruby, D. E.; Jordan, R. Novel benzoxazole inhibitor of dengue virus replication that targets the NS3 helicase. *Antimicrob. Agents Chemother.* **2013**, *57*, 1902-1912.

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