Synthesis, X-ray crystallographic analysis, DFT studies and biological evaluation of triazolopyrimidines and 2-anilinopyrimidines

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Abstract

Inspired by the reported antiviral activity of pyrimidines and triazolopyrimidines, two series of 2-anilinopyrimidines (**5a-e**) and 1-aryl-[1,2,4]triazolo[4,3-*a*]pyrimidines (**14a-k**) were designed and synthesized as potential antiviral agents. X-ray crystallographic study of compounds (**14d**) and (**14k**) confirmed the structure of the desired isomer and revealed the coplanarity of the fused [1,2,4]triazolo[4,3-*a*]pyrimidine rings with the aryl side group. DFT studies revealed insights into the mechanism of the micro-reversible cyclisation step using DFT [B3LYP-D3(BJ)/6-31++G(d,p)]. The pharmacokinetic properties and calculation of drug likeness scores (DLS) of (**5a-e)** and (**14a-k**) suggested good traditional drug-like properties and led to the synthesis of derivatives (**14a-k**) which were evaluated for their anti-viral activity with the most potent derivatives subjected to cytotoxicity screening. Compounds (**14a**)**,** (**14c**)**,** (**14e**)**,** (**14f**) and (**14k**) showed moderate to strong antiviral activity with EC_{50} values 38 - 186 μM. Compound (**14e**) (DLS = 0.29) showed the best anti-CHIKV activity (EC_{50} = 38 μM) and lowest cytotoxicity (CC_{50} > 300 μg/ml) against against breast cancer cell lines, MCF-7 and MD-AMB-231 and normal cell line EA.hy926. Simplification of [1,2,4]triazolo[4,3-*a*]pyrimidine ring, led to series (**5a-e**) **(**DLS = 0.03 - 0.77). Derivatives (**5a-d**) showed fair anti-CHIKV activity (EC_{50} > 200 μM), while (**5e**) emerged as the most active antiviral agent, however the most cytotoxic.

Keywords: Triazolopyrimidine, Antiviral, DFT, Cytotoxicity, SCXRD.

1 Introduction:

Recent reports by the World Health Organization (WHO) on neglected tropical diseases highlight the importance of discovering new lead compounds to treat infection by chikungunya virus (CHIKV).[1,2] Strategies to discover antiviral agents have been described previously,[3-5] however our limited knowledge of the virus has impeded drug development and therefore the availability of a clinical anti-CHIKV drug. Currently there is no vaccine or approved drug for CHIKV-infection prophylaxis or treatment and current management is limited to supportive measures.[6] Strategies to discover anti-CHIKV agents include vaccine development, the development of small molecule drug-like ligands discovered through virtual screening, in vitro whole cell phenotypic screening, and genome-wide loss of function screens.[7-14] Advances in the anti-CHIKV drug discovery have been reviewed.[3,4,15]

Repurposing of other drugs as anti-CHIKV agents has emerged as a popular approach to overcome the lack of selective anti-CHIKV agents. The broadly anti-viral agent ribavirin (**I**), a triazole guanosine analogue, slowed the CHIKV replication (EC_{50} = 10 μM) (**Figure 1)**, but complete suppression of CHIKV particles was not observed.[16,17] Sofosbuvir (**II**) (**Figure 1**)., is a uridine nucleoside prodrug approved for treatment of Hepatitis C virus in combination with other antiviral drug.[18] Evaluation of its antiviral activity against CHIKV infection unveiled the potency and selectivity of sofosbuvir over ribavirin in human liver cells infected with CHIKV [18] It also showed *in vivo* prevention of CHIKV-induced paw oedema at a dose of 20 mg/kg/day and with decreased mortality in neonatal mice at doses of 40-80 mg/kg/day.[18] Arbidol (**III**), (EC_{50} = 12 μM) (**Figure 1**) is an approved antiviral drug in China and Russia for treatment of acute respiratory tract infections.[19] It showed a broad-spectrum antiviral activity and inhibited arthropod-borne flaviviruses and alphaviruses.[20] Arbidol inhibited CHIKV entry by targeting the E2 viral envelope protein, which is presumably involved in binding to host receptors.[21] Amongst synthetic compounds, the purine derivatives and triazolo[4,5-*d*]pyrimidin-7(6*H*)ones (**Figure 1**) have emerged as promising and potent anti-CHIKV agents.[12,13,22] The purine derivative **PU** (**IV**), showed a promising anti-CHIKV activity (EC_{50} = 12 μM) but with low selectivity index (SI = 6.2).[22] The [1,2,3]triazolo[4,5-*d*]pyrimidine derivative **TP** (**V**) emerged as a promising candidate for the development of the first clinical anti-CHIKV with EC_{50} = 3 μM and a selectivity index greater than 600.[12]

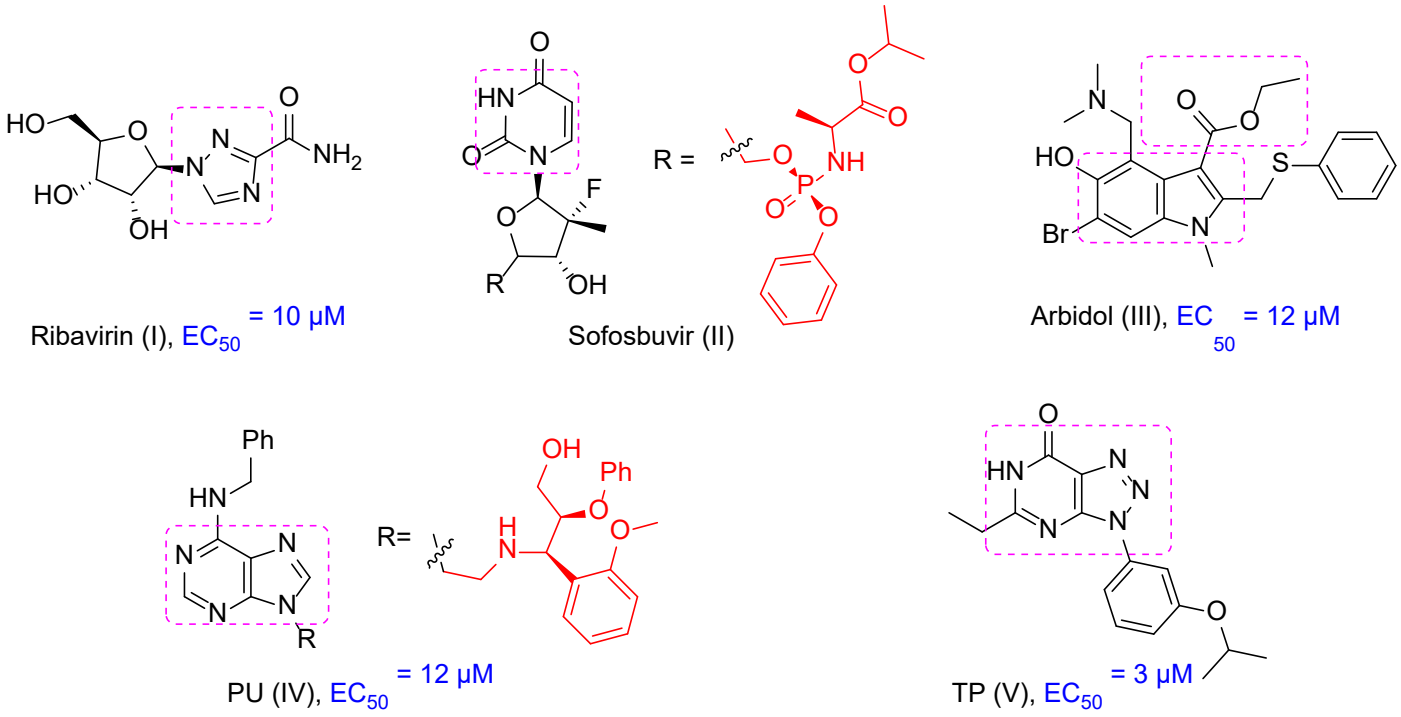


Figure 1: Examples of some anti-CHIKV agents **I-V**

Among the main scaffolds that have been reported in anti-CHIKV agents, are the triazole (in ribavirin **I**), pyrimidine (in sofosbuvir **II**), and fused pyrimidine rings (in PU **IV** and TP **V**). Motivated by these observations, our approach was to apply robust medicinal chemistry approaches to reported inhibitors, including simplification of [1,2,3]triazolo[4,5-*d*]pyrimidine (**V**) to 2-anilinopyrimidine derivatives (**5a-e**) and bioisosteric substitution of (**V**) with [1,2,4]triazolo[4,3-*a*]pyrimidine and incorporation of different groups (R^2) to position 3 of the new triazolopyrimidine system, such as ester present in Arbidol or acetyl group (**14a-k**) (Figure 2). Synthesis was followed by gaining insight into the mechanism of formation of [1,2,4]triazolo[4,3-*a*]pyrimidines (**14a-k**) and biological evaluation of the 2-arylpyrimidines (**5a-e**) [1,2,4]triazolo[4,3-*a*]pyrimidines (**14a-k**).

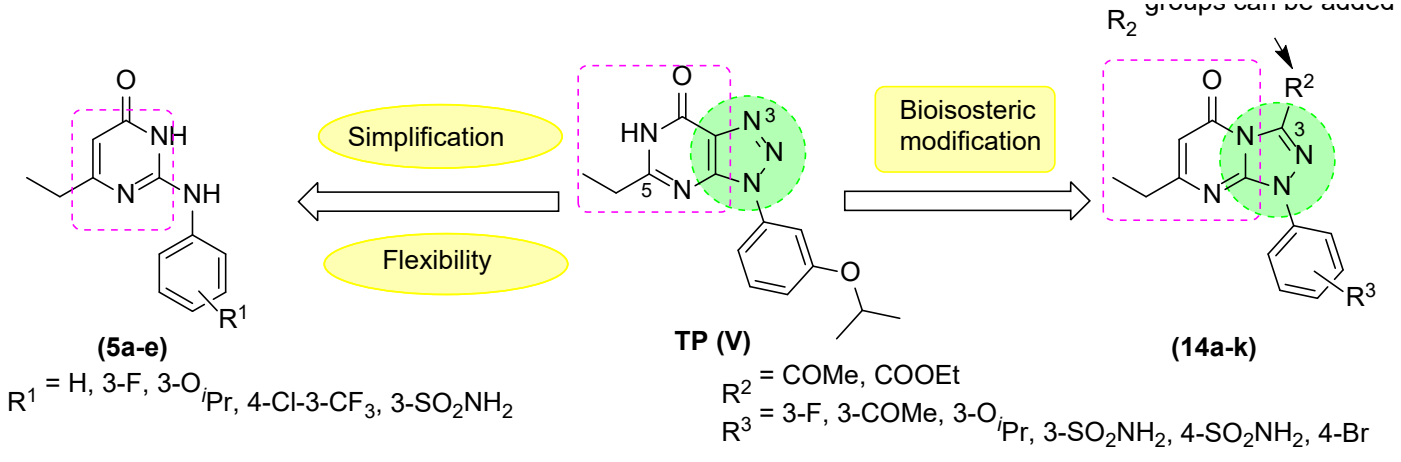


Figure 2: Application of medicinal chemistry concepts to current CHIKV inhibitor towards new potential anti-CHIKV agents.

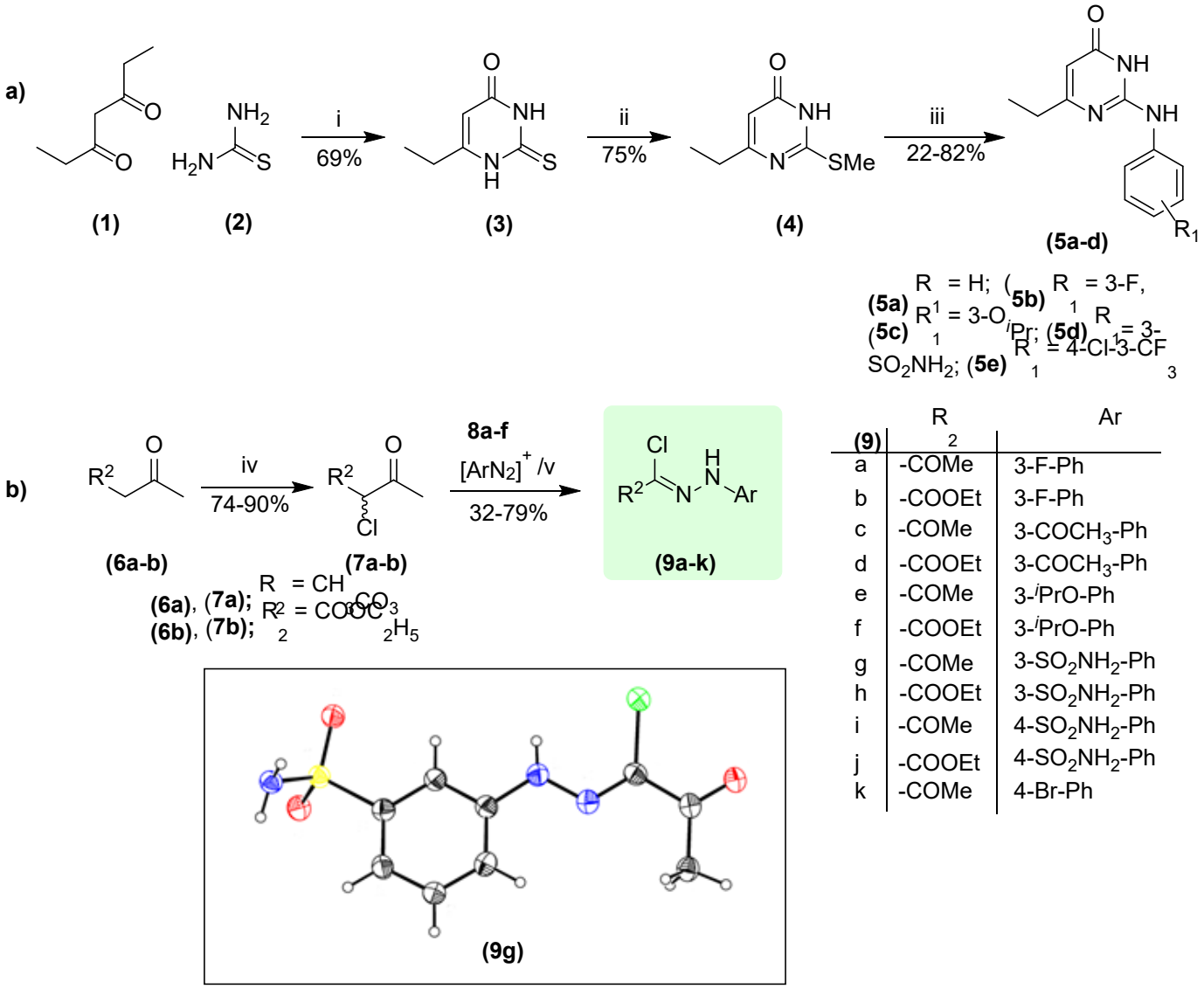
2 Results and discussion

2.1 Chemistry

The required 6-ethylthiouracil (**3**) was obtained by heating thiourea (**2**) with the β-ketoester (**1**) in the presence of sodium ethoxide in 69% yield (**Scheme 1**).[23] The synthesis of the 2-anilinopyrimidine series (**5a-e**) started

with methylation of (**3**) using methyl iodide and sodium hydroxide as reported.[24] The reaction between (**4**) and anilines was investigated using different conditions, including using a neat reaction, and using catalytic amounts of bases or acids. The best conditions were heating pivalic acid at 130 °C with *S*-methylthiouracil (**4**) and the corresponding aniline (**Scheme 1a**),[25] and afforded the 2-anilinopyrimidine derivatives (**5a-e**).

Hydrazonoyl chlorides (green in **Scheme 1b**) are the main building blocks in this synthesis of the triazolo[4,3-*a*]pyrimidine derivatives and can be made *via* several routes from inexpensive and readily available precursors. Compounds (**9a-k**) were synthesised starting with chlorination of the active methylene group of acetylacetone and ethyl acetoacetate using sulfuryl chloride as previously described (**Scheme 1b**).[26] Conventional coupling of the α-chloroacetyl derivatives (**7a-b**) with the readily prepared aryl diazonium salts (**8a-f**) afforded compounds (**9a-k**) (**Scheme 1**) in yields ranging from 32 - 79%.[27] Analysis of the single crystal X-ray diffraction (**Scheme 1** and Figs S3, S41-S44, in supporting information (SI)) of derivatives (**9b**), (**9g**) and (**9k**) was investigated and confirmed their structures and the presence of the (*Z*) configuration.

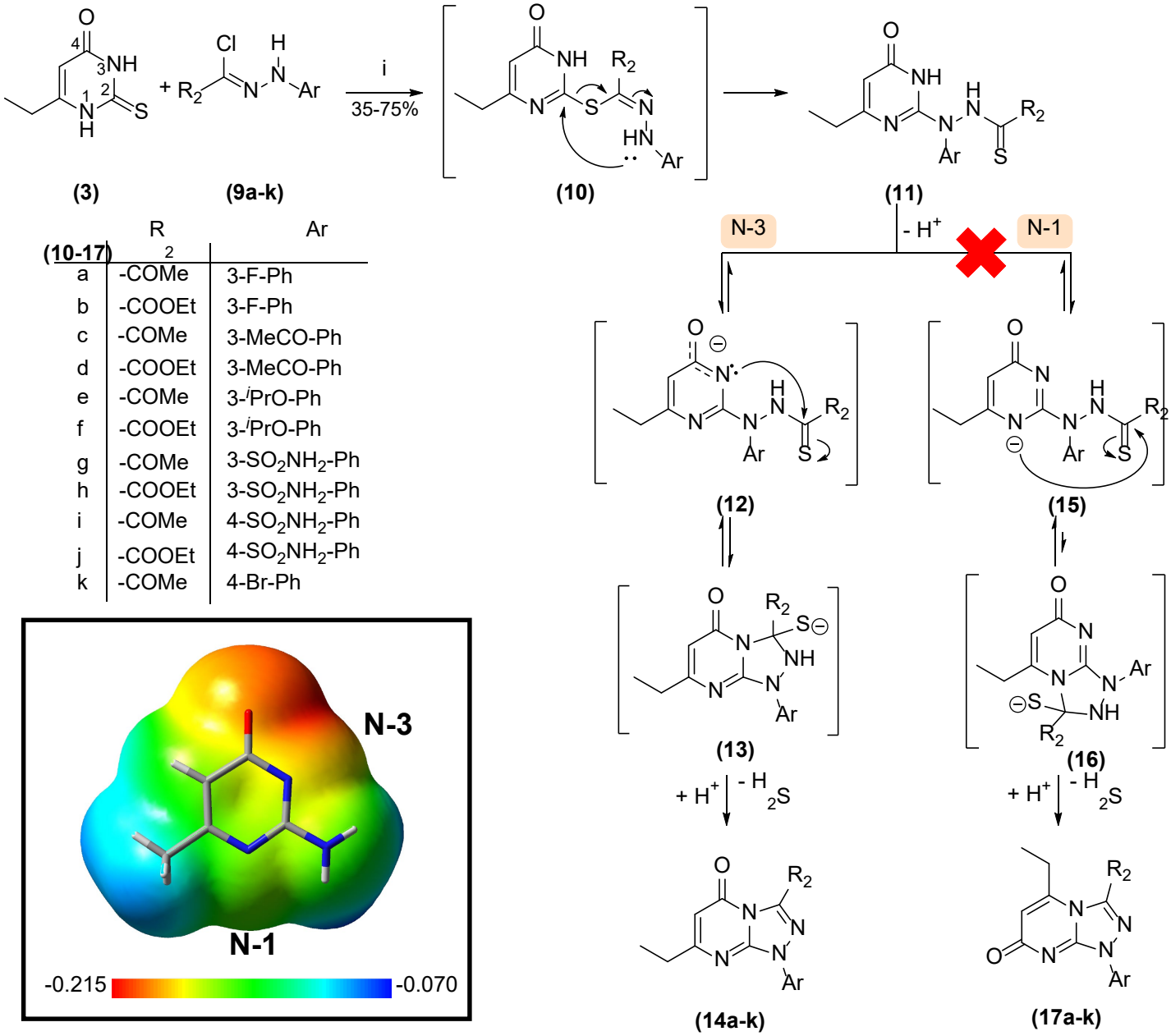


(9)	R2	Ar
a	-COMe	3-F-Ph
b	-COOEt	3-F-Ph
c	-COMe	3-$COCH_3$-Ph
d	-COOEt	3-$COCH_3$-Ph
e	-COMe	3-*i*PrO-Ph
f	-COOEt	3-*i*PrO-Ph
g	-COMe	3-SO_2NH_2-Ph
h	-COOEt	3-SO_2NH_2-Ph
i	-COMe	4-SO_2NH_2-Ph
j	-COOEt	4-SO_2NH_2-Ph
k	-COMe	4-Br-Ph

Scheme 1: Reagents and conditions: i) NaOEt, EtOH, 6 h, 78 °C; ii) NaOH, EtOH, MeI, 3 h, 50 °C; iii) Aniline, pivalic acid, 130 °C; iv) SO_2Cl_2, 0 °C; v) CH_3COONa/ 0 °C.

The reaction of the thiouracil derivatives and hydrazonoyl halides (**Scheme 2**) has been previously studied[28-30] with an almost exclusive preference for product isomers (**14**) over (**17**) (**Scheme 2**). The reaction mechanism was

postulated to start with *S*-alkylation of the thiouracil derivative *via* a Japp-Klingmann reaction to afford the thiohydrazonates (**10**). In the presence of base, the nucleophilicity of the terminal hydrazone nitrogen increases and the compound undergoes Chapman-like rearrangement to give the corresponding thiohydrazide (**11**), which cyclises *in situ* to afford (**14**) or (**17**).[28] Triethylamine as the base did not perform well but DIPEA provided good reaction outcomes with high yields in short reaction times. Analysis of the ^{13}C NMR spectra of each compound showed a resonance in the range 172-173 ppm, assigned to carbonyl of the pyrimidine ring which might indicate that isomer (**17**) was preferentially obtained as reported.[31] However, SCXRD of two compounds confirmed the presence of the other isomer (**14d)** and (**14k),** which was initially required for the antiviral testing **(Figure 3)**. X-ray analysis of compounds (**14d**) and (**14k**) showed the planarity of the fused bicyclic and the terminal aryl rings (**Figure 3**).



(10-17)	R2	Ar
a	-COMe	3-F-Ph
b	-COOEt	3-F-Ph
c	-COMe	3-MeCO-Ph
d	-COOEt	3-MeCO-Ph
e	-COMe	3-^{i}PrO-Ph
f	-COOEt	3-^{i}PrO-Ph
g	-COMe	3-SO_2NH_2-Ph
h	-COOEt	3-SO_2NH_2-Ph
i	-COMe	4-SO_2NH_2-Ph
j	-COOEt	4-SO_2NH_2-Ph
k	-COMe	4-Br-Ph

Scheme 2: Reagents and conditions: i) DIPEA, dioxane, 100 °C. Inset: DFT calculated electrostatic potential of model compound mapped onto the molecular surface (electron density = 4×10^{-4}) showing greater negative potential associated with N-3 relative to N-1.

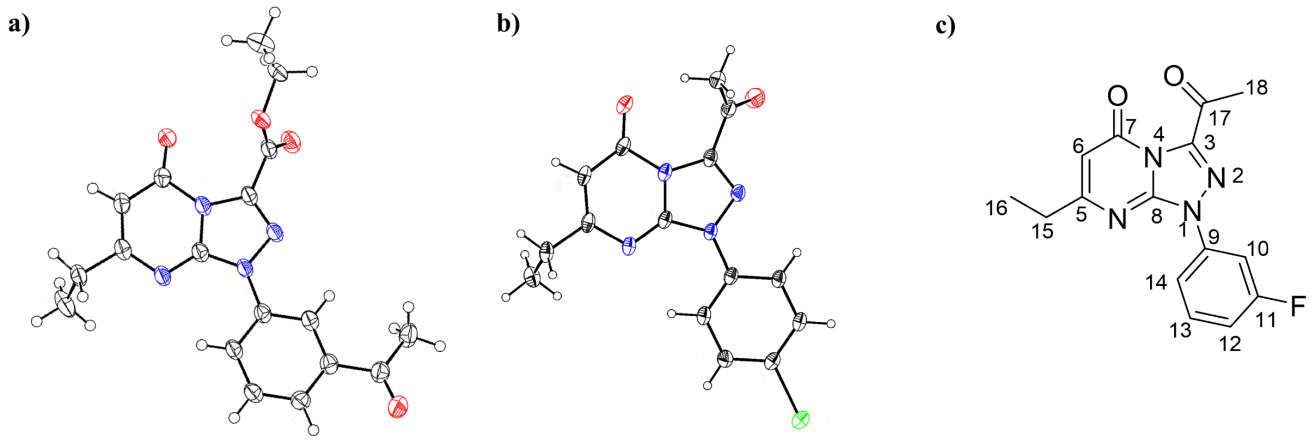


Figure 3: a) and b) ORTEP depiction of compounds (**14d**) and (**14k**), respectively. c) Atom numbering of compound (**14a**).

In a typical example, analysis of the ^{1}H NMR spectra of (**14a**) revealed a triplet at 1.31 ppm and a quartet at 2.70 assigned to the CH_3 and CH_2 of the ethyl group, respectively and a resonance at 2.81 ppm assigned to the methyl of the acetyl group. H6 was assigned to the singlet at δ 6.05 in the ^{1}H NMR spectra. To the corresponding ArH12 and ArH13 were assigned the triplet at 7.09 ppm and the doublet of doublet at 7.50 ppm, respectively, while the multiplet at 8.08-8.12 ppm was assigned to ArH10 and ArH14. The ^{13}C NMR spectrum of the fluorine containing compound (**14a**) showed a typical splitting pattern of the fluorine and carbon with coupling constants $J^{1}_{C-F} = 248$ Hz, at resonance 161.9 ppm assigned to C11, $J^{2}_{C-F} = 28$ Hz at resonance 108.1 ppm assigned to C10, $J^{2}_{C-F} = 21$ Hz at resonance 114.5 assigned to C12, $J^{3}_{C-F} = 9$ Hz, at resonance 103.8 assigned to C13, $J^{3}_{C-F} = 10$ Hz at resonance 137.8 assigned to C9 and $J^{4}_{C-F} = 4$ Hz at resonance 115.9 ppm assigned to C14.

2.2 Computational studies

As the cyclisation step requires base, the involvement of anionic species was suspected and thus as a preliminary and qualitative test for the N-3 over N-1 regioselectivity was examined using Density-Functional Theory (DFT) calculated electrostatic potential of a simplified model compound mapped onto the molecular surface (electron density isosurface) as shown in the **Scheme 2** (inset); the relatively greater negative potential associated with N-3 over N-1 being consistent with the empirical findings.

A more rigorous quantitative approach followed where the mechanism of the micro-reversible cyclisation step for the (acetyl,6-methyl) model system (**18**) was examined in detail using DFT [B3LYP-D3(BJ)/6-31++G(d,p)] (**Figure 4**). All permutations of isomeric *cis*- and *trans*-thioamide and mercaptoimine tautomers were considered at all *reaction-productive* conformations (see SI) for both neutral and conceivable anionic species. Since only relative concentrations of the reaction-productive conformations informs this analysis, mapping the full conformational space was not required. The analysis yielded ten elementary (concerted) neutral reactions (NR1 – NR10) and ten elementary (concerted) anionic reactions (AR1 – AR10). As shown in **Table S1** for all neutral reactions the forward reaction barriers are moderately high (≈100 ± 20 kJ mol^{-1}) and highly endothermic resulting in low calculated rate ratios ($K = k_1/k_{-1}$) shown as $\mathrm{Log}_{10}\, K \leq -6.0$.

In contrast to the neutral reactions, the anionic reactions shown in **Table S2** exhibit a greater diversity of energetics. The most acidic proton of the neutral reactant is the thioamide proton, however, all reactions involving a thioamidate anion (AR7 – AR10) are characterised by very high forward reaction barriers (>150 kJ mol^{-1}) and, additionally, are the most highly endothermic. As such these reactions are not expected to contribute to the mechanism. AR5 and AR6 involve cyclisation of imidothioic acid tautomers, have moderate reaction barriers (102 and 111 kJ mol^{-1}, respectively) and have relatively low endothermicities (Log_{10} K = -2.7 and -4.8, respectively). Of these two pathways, AR5, which leads to the N-3 cyclised product, is the most facile though neither reaction is expected to contribute significantly to the overall process as in each case the relative reactant concentrations are extremely low (≈10^{-7} M). For AR1 – AR4 the forward reaction barriers are the lowest of all cyclisation reactions examined (44 – 76 kJ mol^{-1}) and, additionally, represent some of the least endothermic reactions as indicated by their relatively high calculated rate ratios, in particular, those for AR1 and AR3 shown as $Log_{10}K$ = -2.8 and -1.9, respectively. Considering NR1 – NR10 and AR1 – AR10, from the relative reactant concentrations, forward reaction barriers and calculated rate ratios, AR1 involving N-3 cyclisation from a *trans*-thioamide and AR3 involving N-3 cyclisation from a *cis*-thioamide emerge as the dominant processes and shown in **Figure 4**. Reprotonation of the resulting thioloate species results in a barrierless loss of HS^- and subsequent loss of H^+ from the five membered ring for a net loss of H_2S to give (**18**).

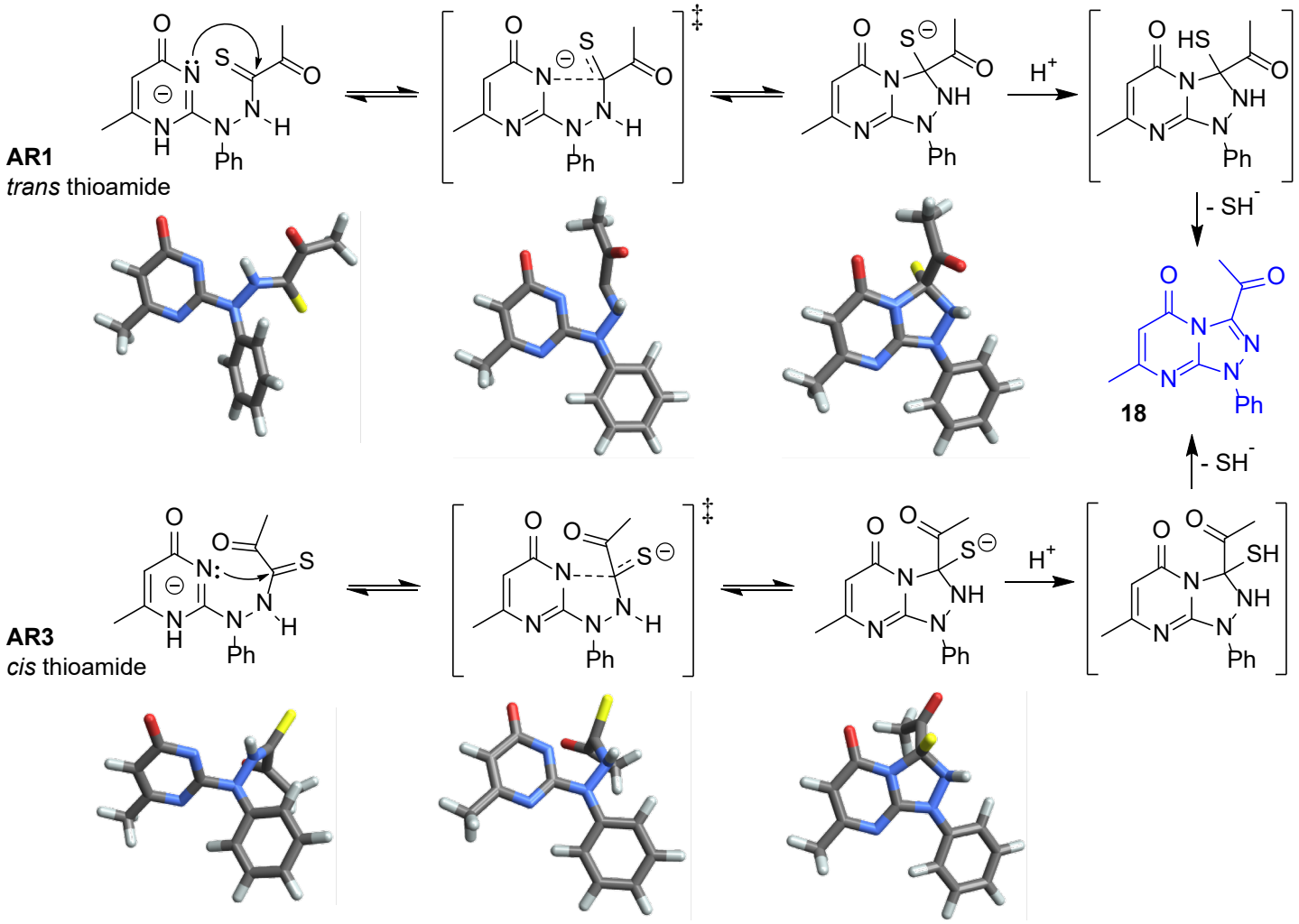


Figure 4: Detail of the two predominant contributing concerted reactions leading to the cyclised product (**18**) (blue); AR1 from a *trans* thioamide anionic intermediate and AR3 from a *cis* thioamide anionic intermediate. After reprotonation of the thioloate species the loss

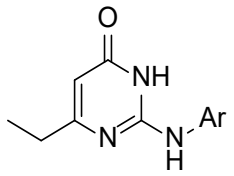
of HS^- with aromatisation of the five-membered ring was calculated to be barrierless. Contributions from neutral intermediates were not found to be competitive.

2.3 Pharmacokinetic evaluation

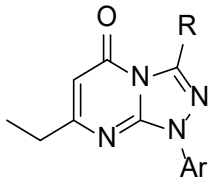
In drug development, *in silico* pharmacokinetic properties prediction provides a tool to minimize attrition and to reduce the cost of drug discovery process.[31] Lipinski guidelines fulfilment, number of rotatable bonds (nrot), topological polar surface area (TPSA), and percentage of the absorbed drugs (%ABS), which is calculated using the formula %ABS = 109 ± 0.345 × TPSA, provide indications to sustain good oral bioavailability of drug candidates.[32,33] Oral bioavailability is greatly linked to complying with Lipinski guidelines, reduced molecular flexibility as indicated with the number of rotatable bonds less or equal to ten, and TPSA (**Table 1**).

The physicochemical calculation indicated that the compounds (**5a-e**) and (**14a-k**) fulfilled the Lipinski guidelines, possess less than ten rotatable bonds (3 - 7 rotatable bond) and have decent %ABS ranging from 61.1 to 89.1 which enable them to be potential oral bioavailable leads (**Table 1**). The drug likeness score (DLS) is calculated using MolSoft software and is based on physicochemical properties, pharmacokinetics and pharmacodynamics of tested compounds.[34] Drug candidates presumably show positive DLS values, while non-drug candidates might elicit zero or negative DLS values. DLS of all the compounds (**5a-e**) and **(14a-k)** was calculated and (**14e**), (**14f**), (**14i**), (**14k**), (**5a-e**) received positive DLS ranging from 0.03 - 0.77 (Table 1), while rest of compounds showed zero or negative DLS (**Table 1**).

Table 1: Drug likeness calculations and Lipinski parameters of the compounds (**5a-e**) and (**14a-k**).



(5a-e)



(14a-k)

	R	Ar	M. Wt[a]	clog P[b]	HBA[c]	HBD[d]	N violation[e]	Rule of 5	Nrot[f]	TPSA[g]	%ABS[h]
5a	-	-Ph	215	1.9	2	2	0	pass	3	57.8	89.1
5b	-	3-F-Ph	233	2.2	2	2	0	pass	3	57.8	89.1
5c	-	3-^{i}PrO-Ph	273	2.7	3	2	0	pass	5	67.0	85.9
5d	-	3-SO_2NH_2-Ph	294	0.7	5	4	0	pass	4	118.0	68.3
5e	-	4-Cl-3-CF_3Ph	317	3.7	2	2	0	pass	4	57.8	89.1
14a	$COCH_3$	3-F-Ph	300	1.8	4	0	0	pass	3	69.3	85.1
14b	$COOC_2H_5$	3-F-Ph	330	2.6	5	0	0	pass	5	78.5	81.9
14c	$COCH_3$	3-MeCO-Ph	324	1.3	5	0	0	pass	4	86.4	79.2

14d	$COOC_2H_5$	3-MeCO-Ph	354	2.1	6	0	0	pass	6	95.6	76.0
14e	$COCH_3$	3-^{i}PrO-Ph	340	2.4	5	0	0	pass	5	78.5	81.9
14f	$COOC_2H_5$	3-^{i}PrO-Ph	370	3.2	6	0	0	pass	7	87.7	78.7
14g	$COCH_3$	3-SO_2NH_2-Ph	361	0.4	7	2	0	pass	4	129.4	64.4
14h	$COOC_2H_5$	3-SO_2NH_2-Ph	391	1.2	8	2	0	pass	6	138.7	61.1
14i	$COCH_3$	4-SO_2NH_2-Ph	361	0.4	7	2	0	pass	4	129.4	64.4
14j	$COOC_2H_5$	4-SO_2NH_2-Ph	391	1.2	8	2	0	pass	6	138.7	61.1
14k	$COCH_3$	4-Br-Ph	360	2.4	4	0	0	pass	3	69.3	85.1

[a] Molecular weight. [b] Calculated lipophilicity. [c] Number of hydrogen bond acceptors. [d] Number of hydrogen bond donors. [e] Number of violations. [f] Number of rotatable bonds. [g] Topological polar surface area. [h] Percentage absorption.

2.4 Biological evaluation

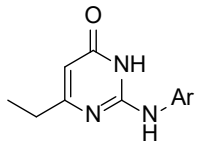
Compounds 2-anilino pyrimidin-4(3*H*)-one (**5a-e**) and [1,2,4]triazolo[4,3-*a*]pyrimidin-7(1*H*)-ones (**14a-k**) were screened for their antiviral activities in a viral-cell based assay against Chikungunya virus (Indian Ocean strain 899) (**Table 2**). The most active compounds were further tested for their cytotoxic activity against one normal cell line, Eahy926, the endothelial human sapiens cell line, and two breast cancer cell lines, namely, MCF7 and MDAMB231. Compound, 3-acetyl-5-ethyl-1-(3-isopropoxyphenyl)-[1,2,4]triazolo[4,3-*a*]pyrimidin-7(1*H*)-one (**14e**), emerged as the most promising antiviral agents among the tested series (**14a-k**) with EC_{50} = 38 µM, and CC_{50} > 300 µM against breast cancer cell lines, MCF7 and MDAMB231 and normal cell line Eahy926 (**Table 1**). Replacement of acetyl group with ester group gave compound (**14f),** with moderate antiviral activity (EC_{50} = 100 µM), but emerged as the most cytotoxic agent, among (**14a**)**,** (**14c**)**,** (**14e**)**,** (**14f**) and (**14k**)**,** against MCF7 (CC_{50} = 68 µM), MDAMB231 (CC_{50} = 65 µM) and Eahy926 (CC_{50} = 73 µM) (**Table 2**).

Substitution of 3-isopropoxy group in (**14e**) with 3-fluoro and 3-acetyl gave compounds (**14a**) and (**14c**) that demonstrated modest antiviral activities with EC_{50}= 183 µM and EC_{50}= 136 µM, respectively (**Table 2**). Both (**14a**) and (**14c**) showed weak cytotoxic effect against MCF7, with CC_{50} > 300 µM, against MDAMB231, with CC_{50} = 250 and 198 µM, and against Eahy926 CC_{50} > 300 and 207 µM respectively (**Table 2**). Generally, acetyl derivatives (**14a**), (**14c**) and (**14e**) (EC_{50} = 38 – 183 µM) were more active than the corresponding ester derivatives (**14b**) (EC_{50} > 200 µM), (**14d**) (EC_{50} > 200 µM) and (**14f**) (EC_{50} = 100 µM). The *para*-bromo derivative (**14k**) showed fair antiviral activity with EC_{50} = 186 µM and CC_{50} = 128, 81 and 147 µM against MCF7, MDAMB231 and Eahy926, respectively (**Table 1**).

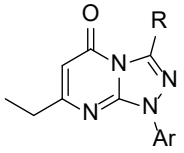
Simplification of the **TP** (**V**) was performed to increase the flexibility of the aryl side chain in **TP** (**V**) and this might provide more points of interaction with the viral proteins and glycoproteins. Compound (**5a**) bearing an unsubstituted phenyl substituent showed a weak antiviral activity with and EC_{50} value > 200 µM. Grafting fluorine, isopropoxy or sulfonamide groups in the aryl *meta*-position (**5b-5d** respectively) had no major change of the EC_{50} (EC_{50}> 200 µM), while addition of the *meta*-3-chloro-4-trifluoromethyl resulted in compound (**5e**) (DLS = 0.68)**,** which was the most active antiviral compound among all the tested compounds with an EC_{50} value

less than 3 µM. However, it exhibited the most cytotoxic activity against the cancer cell lines MCF7 and MDAMB231 (CC_{50} = 10 and 4 µg/ml, respectively), and Eahy926 (CC_{50} = 6 µg/ml).

Table 2: *In vitro* antiviral and cytotoxic activities of compounds (5a-e) and (14a-k).



(5a-e)



(14a-k)

	R	Ar	% inhibition	at [ ] / µg mL^{-1}	EC_{50} (µM)	CC_{50}(µM)			DLS[b]
					CHIKV	MCF-7[a]	MDA-MB-231[a]	EA.hy926[a]	
5a	-	-Ph	6	75	>200	**258**	**45**	NT	**0.69**
5b	-	3-F-Ph	30	75	>200	**160**	**106**	NT	**0.41**
5c	-	3-^{i}PrO-Ph	26	20	>200	**95**	**119**	NT	**0.77**
5d	-	3-SO_2NH_2-Ph	<1	75	>200	NT	NT	NT	**0.27**
5e	-	4-Cl-3-CF_3Ph	59.5	0.8	**<3**	**10**	**4**	**6**	**0.68**
14a	COMe	3-F-Ph	80	100	**183**	**>300**	**250**	**>300**	**0.00**
14b	COOEt	3-F-Ph	9	100	>200	NT	NT	NT	-0.05
14c	COMe	3-(CH_3CO)-Ph	100	100	**136**	**>300**	**198**	**207**	-0.37
14d	COOEt	3-(CH_3CO)-Ph	<1	20	>200	NT	NT	NT	-0.45
14e	COMe	3-^{i}PrO-Ph	69	20	**38**	**>300**	**>300**	**>300**	**0.29**
14f	COOEt	3-^{i}PrO-Ph	90	75	**100**	**68**	**65**	**73**	**0.29**
14g	COMe	3-SO_2NH_2-Ph	<1	100	>200	NT	NT	NT	-0.39
14h	COOEt	3-SO_2NH_2-Ph	1	100	>200	NT	NT	NT	-0.42
14i	COMe	4-SO_2NH_2-Ph	2	20	>200	NT	NT	NT	**0.07**
14j	COOEt	4-SO_2NH_2-Ph	2	100	>200	NT	NT	NT	-0.10
14k	COMe	4-Br-Ph	54	75	**186**	**128**	**81**	**147**	**0.03**

NT: not tested, [a] Mean from three replicates, (errors were in the range ± 5-10 % of the reported values), [b] Drug-likeness model score

3 Conclusion

Two series of 2-anilinopyrimidine (**5a-e)** fused triazolopyrimidine (**14a-k**) were synthesized and evaluated for their antiviral activities using a viral-cell based assay against Chikungunya virus (Indian Ocean strain 899).

SCXRD of derivatives (**14a**) and (**14k**) confirmed the structure of the synthesized derivatives (**14a-k**). Tested compounds showed promising *in silico* pharmacokinetic properties calculation and drug likeness scores. Computational studies gave insights into the cyclization mechanism. It revealed the involvement of the anionic species in the cyclization step and the greater negative potential associated with N-3 over N-1 which eventually resulted in the formation of the desired isomer (**14a-k**) over (**17a-k**). To further assess cytotoxicity of the newly synthesized compounds, the most active antivirals were subjected to cytotoxicity studies against one normal cell line, Eahy926, the endothelial human sapiens cell line, and two breast cancer cell lines, namely, MCF7 and MDAMB231. Derivative (**14e**) emerged as the most active antiviral agent (EC_{50} = 38 μM) and the least cytotoxic against the three tested cell line (IC_{50} > 300 μM). The *p*-bromophenyl and *m*-fluorophenyl derivatives with acetyl side chain (**14k**, EC_{50} = 186 μM) and (**14a**, EC_{50} = 183 μM) respectively, retained the antiviral activity, however, less than *m*-acetylphenyl (**14c**, EC_{50} = 136 μM), *m*-isopropoxyphenyl (**14e**, EC_{50} = 38 μM). Among tested compounds, derivatives (**14f**) (IC_{50} = 65 - 73 μM) and (**14k**) (IC_{50} = 81 - 147 μM) showed the most cytotoxic effect, while (**14a**) and (**14c**) (IC_{50} = 198 - >300 μM) showed less cytotoxicity. Series (**5a-e**) demonstrated distinctive pattern of antiviral activity. Compounds (**5a-d**) showed fair antiviral activity (EC_{50} >200 μM), while the *m*-chloro-*p*-trifluoromethylphenyl derivative (**5e**) was the most active antiviral agent (EC_{50} = <3 μM), but the most toxic (IC_{50} = 4 - 10 μM) against the tested cell line. The best compound (**14e**) will be subjected to a further cycle of optimization to understand more about the structure antiviral activity relationship of these scaffolds and to eventually develop the best candidates into potentially novel antiviral agents.

4 Experimental section

4.1 Chemistry

All reagents and solvents were purified and dried by standard techniques. Melting points were measured and were uncorrected. Reactions were monitored by TLC analysis using silica gel GF/UV 254. NMR spectra were recorded on Varian Gemini-300BB 500 MHz FT-NMR spectrometers (Varian Inc., Palo Alto, CA). ^{1}H spectra were measured at 500 MHz and ^{13}C spectra at 126 MHz, in deuterated dimethylsulfoxide (DMSO-d_6), $(CD_3)_2CO$ and $CDCl_3$. Chemical shifts are reported relative to TMS as internal standard and coupling constant (*J*) values are reported in Hertz. The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Electrospray (ESI single quadrupole) mass spectra were performed using a Shimadzu QP-5050 spectrometer. Ion mass to charge values (*m/z*) stated with their relative abundances as a percentage in parentheses. Peaks assigned to the molecular ion are denoted as [M+H] or [M+Na]. Column chromatography was performed using silica gel 60 (0.063-0.200 mm).

6-Ethyl-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one (3)

After preparing a sodium ethoxide solution by dissolving sodium (4.6 g, 0.1 mole) in absolute ethanol (150 mL), the thiourea (**1**) (3.8 g, 0.05 mole) was added with stirring with complete dissolution. The ethyl 3-oxopentanoate ester (**2**) was then added (7.21 g, 0.05 mole) and the reaction mixture was heated at 78 °C for 4 hours. After cooling the reaction mixture, water was added dropwise until complete dissolution of the formed white precipitate. Ice was added, and neutralization of the alkaline solution was accomplished using HCl (5 M). The

reaction flask left overnight at 4 °C and the resulting white precipitate was filtered, washed with water (3 x 50 mL) and diethyl ether (2 x 30 mL) and dried to give (**3**) (5.38 g, 69%) as a white powder. m.p 230 °C; ^{1}H NMR (DMSO-d_6, δ): 1.07 (t, J = 7.5 Hz, 3H, C<u>H</u>$_3$), 2.33 (q, J = 7.5 Hz, 2H, C<u>H</u>$_2$), 5.33 (s, 1H, pyrimidine-H), 12.30 (br s, 2H, 2 x -NH); ^{13}C NMR (CD_3OD, δ): 13.4, 27.8, 103.9, 162.0, 165.8, 179.4; MS (ESI): 157 (20%, M+H), 179 (100%, M+Na).

6-Ethyl-2-(methylthio)pyrimidin-4(3*H*)-one (4): A mixture of 6-ethyl-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one **3** (156 mg, 1 mmol), sodium hydroxide (44 mg, 1.1 mmol) in water (1 mL) and ethanol (3 mL) were stirred for 30 min. Methyl iodide (142 mg, 1 mmol) was added dropwise to the slurry and the reaction mixture was gently heated at 50 °C with stirring for 3 h. The reaction mixture was allowed to cool to r.t and the resulting white solid was filtered out, washed with water (3 x 30 mL) and dried to give (**4**) (128 mg, 75%) as a white powder; ^{1}H NMR ($CDCl_3$, δ): 1.24 (t, J = 7.5 Hz, 3H, C<u>H</u>$_3$), 2.56 (q, J = 7.5 Hz, 2H, C<u>H</u>$_2$), 2.60 (s, 3H, SC<u>H</u>$_3$), 6.09 (s, 1H, pyrimidine-H), 9.32 (br. s, 1H, -NH); ^{13}C NMR ($CDCl_3$, δ): 12.0, 13.2, 30.8, 106.7, 161.1, 165.9, 170.7; MS (ESI$^+$): 171 (35%, M+H)$^+$, 193 (100%, M+Na)$^+$.

General procedure A for the preparation of 6-ethyl-2-(anilino)pyrimidin-4(3*H*)-ones (5a-e):[25] A mixture of 6-ethyl-2-(methylthio)pyrimidin-4(3*H*)-one (**4**) (170 mg, 1 mmol) and the corresponding aniline (1.2 mmol) in pivalic acid (918 mg, 9 mmol) was heated at 130 °C with stirring. Heating was maintained until the starting materials were fully consumed or until no further conversion was observed (as monitored by TLC analysis). The slurry was allowed to cool to 60-70 °C and hexanes (18 volumes) was added slowly with stirring and was further cooled to r.t. The resulting solid was filtered, washed with hexane, and dried.

6-Ethyl-2-(phenylamino)pyrimidin-4(3*H*)-one (5a): The compound was prepared according to general method A (170 mg, yield = 79%) as a white powder, m.p: 178-180 °C. ^{1}H NMR ($CDCl_3$, δ): 1.27 (t, J = 7.0 Hz, 3H, C<u>H</u>$_3$), 2.55 (q, J = 7.0 Hz, 2H, C<u>H</u>$_2$), 5.81 (s, 1H, pyrimidine-H), 7.05-7.13 (m, 1H, ArH), 7.36 (t, J = 8.0 Hz, 2H, ArH), 7.75 (d, J = 7.0 Hz, 2H, ArH), 9.50 (br. s, 1H, -NH), 11.62 (br. s, 1H, -NH); ^{13}C NMR ($CDCl_3$, δ): 14.5, 31.4, 101.1, 120.2, 123.4, 129.1, 138.9, 151.7, 165.5, 173.9; MS (ESI$^+$) *m/z* 216 (65%, M + H)$^+$, MS (ESI$^-$) *m/z* 214 (100%, M - H)$^-$; HRMS (ESI$^+$) calcd for $C_{12}H_{14}N_3O$: 216.1142 (M + H)$^+$, found: 216.1137.

6-Ethyl-2-((3-fluorophenyl)amino)pyrimidin-4(3*H*)-one (5b): The compound was prepared according to general method A (191 mg, yield = 82%) as a buff powder, m.p: 198-200 °C. ^{1}H NMR ($CDCl_3$, δ): 1.26 (t, J = 7.0 Hz, 3H, CH_3), 2.56 (q, J = 7.0 Hz, 2H, CH_2), 5.83 (s, 1H, pyrimidine-H), 6.76-6.80 (m, 1H, ArH), 7.20-7.31 (m, 2H, ArH), 7.81 (d, J = 10.0 Hz, 1H, ArH), 9.45 (br. s, 1H, -NH), 11.44 (br. s, 1H, -NH); ^{13}C NMR ($CDCl_3$, δ): 12.2, 31.2, 101.4, 107.1 (d, J^2_{C-F} = 26 Hz) , 109.7 (d, J^2_{C-F} = 21 Hz), 114.9, 129.9 (d, J^3_{C-F} = 10 Hz), 140.2 (d, J^3_{C-F} = 11 Hz), 151.0, 162.1 (d, J^1_{C-F} = 248 Hz), 165.1, 173.9; MS (ESI$^+$) *m/z* 234 (40%, M + H)$^+$, MS (ESI$^-$) *m/z* 232 (100%, M-H)$^-$; HRMS (ESI$^-$) calcd for $C_{12}H_{11}FN_3O$: 232.0886 (M - H)$^-$, found: 232.0879.

6-Ethyl-2-((3-isopropoxyphenyl)amino)pyrimidin-4(3*H*)-one (5c): The compound was prepared according to general method A (66 mg, yield = 24%) as a green powder, m.p: 100-102 °C. ^{1}H NMR ($CDCl_3$, δ): 1.27 (t, J = 7.0 Hz, 3H, CH_2C<u>H</u>$_3$), 1.38 (d, J = 5.0 Hz, 6H, $2CH_3$), 2.55 (q, J = 7.0 Hz, 2H, C<u>H</u>$_2CH_3$), 4.45-4.59 (m, 1H, CH), 5.82 (s, 1H, pyrimidine-H), 6.63 (d, J = 7.0 Hz, 1H, ArH), 7.12 (d, J = 8.0 Hz, 1H, ArH), 7.22 (dd, J = 7.0, 8.0

Hz, 1H, ArH), 7.60 (s, 1H, ArH), 9.43 (br. s, 1H, -NH), 11.47 (br. s, 1H, -NH); ^{13}C NMR ($CDCl_3$, δ): 12.2, 22.1, 31.2, 70.0, 101.0, 107.4, 111.2, 112.0, 129.6, 139.8, 151.4, 158.5, 165.2, 173.5; MS (ESI^+) *m/z* 274 (50%, M + H)$^+$, MS (ESI^-) *m/z* 272 (60%, M - H)$^-$; HRMS (ESI^-) calcd for $C_{15}H_{18}N_3O_2$: 272.1401 (M - H)$^-$, found: 272.1399.

3-((4-Ethyl-6-oxo-1,6-dihydropyrimidin-2-yl)amino)benzenesulfonamide (5d): The compound was prepared according to general method A (65 mg, yield = 22%) as a buff powder, m.p: 203-205 °C. ^{1}H NMR (DMSO-d_6, δ): 1.17 (t, J = 8.0 Hz, 3H, $CH_2C\underline{H}_3$), 2.44 (q, J = 8.0 Hz, 2H, $C\underline{H}_2CH_3$), 5.78 (s, 1H, pyrimidine-H), 7.33 (br. s, 2H, $-SO_2NH_2$), 7.42-7.49 (m, 2H, ArH), 7.83 (d, J = 7.0 Hz, 1H, ArH), 8.26 (s, 1H, ArH), 9.18 (br. s, 1H, -NH), 11.79 (br. s, 1H, -NH); ^{13}C NMR (DMSO-d_6, δ): 12.6, 30.5, 101.2, 116.6, 119.5, 122.4, 129.7, 140.2, 145.1, 153.7, 164.9, 171.0; MS (ESI^-) *m/z* 293 (60%, M - H)$^-$, 587 (100%, 2M - H)$^-$; HRMS (ESI^-) calcd for $C_{12}H_{13}N_4O_3S$: 293.0711 (M - H)$^-$, found: 293.0708.

2-((3-Chloro-4-(trifluoromethyl)phenyl)amino)-6-ethylpyrimidin-4(3*H*)-one (5e): The compound was prepared according to general method A (187 mg, yield = 59%) as a white powder, m.p: 195-197 °C. ^{1}H NMR (DMSO-d_6, δ): 1.16 (t, J = 7.0 Hz, 3H, $CH_2\underline{CH_3}$), 2.45 (q, J = 7.0 Hz, 2H, $\underline{CH_2}CH_3$), 5.85 (s, 1H, pyrimidine-H), 7.58 (d, J = 9.0 Hz, 1H, ArH8), 7.85 (d, J = 8.0 Hz, 1H, ArH7), 8.46 (s, 1H, ArH4), 9.41 (br. s, 1H, -NH), 11.06 (br. s, 1H, -NH); ^{13}C NMR (DMSO-d_6, δ): 12.0, 30.0, 100.3, 117.6, 119.6, 121.8, 123.6, 126.5 (q, J_{C-F} = 33 Hz, CF_3), 131.7, 139.3, 154.4, 165.5, 170.7 (CO); MS (ESI^-) *m/z* 316 (60%, M + H), 633 (100%, 2M - H); HRMS (ESI^-) calcd for $C_{13}H_{10}ClF_3N_3O$: 316.0496 (M + H), found: 316.0464.

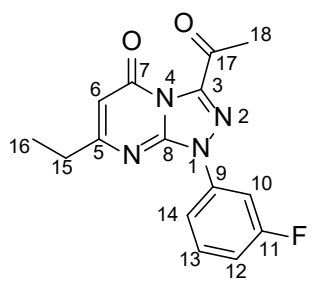
General procedure B for preparation of hydrazonyl chlorides (9a-k):[35]

The diazonium salt was prepared by slowly adding a cold sodium nitrite solution (0.7 g, 10 mmol) in deionized water (5 mL) to a cold solution of the appropriate arylamine (10 mmol) dissolved in hydrochloric acid (6 M, 6 mL). In a second beaker, sodium acetate trihydrate (4.1 g, 30 mmol) was added to a stirred solution of 3-chloro-2,4-pentanedione (1.34 g, 10 mmol) (**7a)** or ethyl 2-chloro-3-oxobutanoate (**7b**) (1.64 g, 10 mmol) in ethanol (100 mL) and the slurry was stirred for 15 min. The diazonium salt (**8a-f**) was added dropwise to the second beaker and the reaction was stirred for 2 h at 0-5 °C, then left for 8 h in a refrigerator (4 °C). The resulting solid was collected by filtration, washed thoroughly with water and dried. The crude product was crystallized from ethanol to give the corresponding hydrazonoyl chlorides (**9a-k)**. Experimental data of the synthesized hydrazonyl chlorides (**9a-k)** are deposited in the supporting information.

General procedure C for preparation of target compounds (14a-k)

To a mixture of 6-ethyl-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one (**3**) (156 mg, 1 mmol) and the corresponding hydrazonoyl chloride (**9a-k**) (1 mmol) in dioxane (15.0 mL), DIPEA (174 µL, 1 mmol) was added. The reaction mixture was heated at 101 °C until the starting materials were fully consumed or until no further conversion was observed (monitored by TLC analysis). The solvent was removed under vacuum and the residue was triturated with methanol and the resulting precipitate filtered, taken up in EtOAc and washed with water (2 x 30 mL), brine (30 mL), dried ($MgSO_4$), filtered and concentrated then subjected to flash chromatography (methanol/dichloromethane, 1:10) to give (**14a-k**).

3-Acetyl-7-ethyl-1-(3-fluorophenyl)-[1,2,4]triazolo[4,3-*a*]pyrimidin-5(1*H*)-one (14a): The compound was



prepared according to method C (105 mg, yield = 35%) as a buff powder, m.p: 122-124 °C. ^{1}H NMR ($CDCl_3$, δ): 1.31 (t, J = 7.5 Hz, 3H, C$\underline{H}_3$), 2.70 (q, J = 7.5 Hz, 2H, C$\underline{H}_2$), 2.81 (s, 3H, COC$\underline{H}_3$), 6.05 (s, 1H, ArH), 7.09 (app t, J = 8.0 Hz, 1H, ArH), 7.50 (dd, J = 8.0, 14.5 Hz, 1H, ArH), 8.08-8.12 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$, δ): 12.7, 30.0, 31.4, 101.2, 108.1 (d, J^2_{C-F} = 28 Hz), 114.5 (d, J^2_{C-F} = 21 Hz), 115.9 (d, J^4_{C-F} = 4 Hz), 130.8 (d, J^3_{C-F} = 9 Hz), 137.8 (d, J^3_{C-F} = 10 Hz), 141.6, 147.8, 155.9, 161.9 (d, J^1_{C-F} = 248 Hz), 172.2, 186.6; MS (ESI$^+$) m/z 301 (80%, M + H)$^+$, 623 (100%, 2M + Na)$^+$; HRMS (ESI$^+$) calcd for $C_{15}H_{14}FN_4O_2$: 301.1101 (M + H)$^+$, found: 301.1095.

Ethyl 7-ethyl-1-(3-fluorophenyl)-5-oxo-1,5-dihydro-[1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (14b): The compound was prepared according to general method C (139 mg, yield = 42%) as a buff powder, m.p: 92-94 °C. ^{1}H NMR ($CDCl_3$, δ): 1.31 (t, J = 7.5 Hz, 3H, C$\underline{H}_3$), 1.46 (t, J = 7.0 Hz, 3H, C$\underline{H}_3$), 2.69 (q, J = 7.5 Hz, 2H, C$\underline{H}_2$), 4.58 (q, J = 7.0 Hz, 2H, C$\underline{H}_2$), 6.05 (s, 1H, ArH), 7.07 (td, J = 1.5, 8.0 Hz, 1H, ArH), 7.49 (dd, J = 8.0, 14.5 Hz, 1H, ArH), 8.06-8.11 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$, δ): 12.8, 14.0, 31.5, 64.2, 100.7, 108.0 (d, J^2_{C-F} = 28 Hz), 114.4 (d, J^2_{C-F} = 21 Hz), 115.7 (d, J^2_{C-F} = 3 Hz), 130.8 (d, J^3_{C-F} = 9 Hz), 135.8, 137.7 (d, J^3_{C-F} = 11 Hz), 147.2, 155.6, 156.7, 161.8 (d, J^2_{C-F} = 247 Hz), 172.6; MS (ESI$^+$) m/z 331 (55%, M + H)$^+$, 683 (100%, 2M + Na)$^+$; HRMS (ESI$^+$) calcd for $C_{16}H_{16}FN_4O_3$: 331.1218 (M + H)$^+$, found: 331.1206.

3-Acetyl-1-(3-acetylphenyl)-7-ethyl-[1,2,4]triazolo[4,3-*a*]pyrimidin-5(1*H*)-one (14c): The compound was prepared according to general method C (62 mg, yield = 19%) as an orange powder, m.p: 118-120 °C. ^{1}H NMR ($CDCl_3$, δ): 1.32 (t, J = 7.5 Hz, 3H, C$\underline{H}_3$), 2.68-2.71 (m, 5H, C$\underline{H}_3$ + C$\underline{H}_2$), 2.83 (s, 3H, COC$\underline{H}_3$), 6.07 (s, 1H, ArH), 7.66 (app t, J = 8.0 Hz, 1H, ArH), 7.97 (d, J = 8.0 Hz, 1H, ArH), 8.47 (d, J = 8.0 Hz, 1H, ArH), 8.90 (s, 1H, ArH); ^{13}C NMR ($CDCl_3$, δ): 12.6, 27.0, 30.0, 31.3, 101.2, 120.3, 124.7, 127.3, 129.9, 136.9, 138.1, 141.5, 147.8, 155.8, 172.1, 186.6, 197.1; MS (ESI$^+$) m/z 325 (20%, M + H)$^+$, 671 (100%, 2M + Na)$^+$; HRMS (ESI$^+$) calcd for $C_{17}H_{17}N_4O_3$: 325.1293 (M + H)$^+$, found: 325.1301.

Ethyl 1-(3-acetylphenyl)-7-ethyl-5-oxo-1,5-dihydro-[1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (14d): The compound was prepared according to general method C (152 mg, yield = 43%) as a white powder, m.p: 120-122 °C. ^{1}H NMR ($CDCl_3$, δ): 1.32 (t, J = 7.5 Hz, 3H, C$\underline{H}_3$), 1.48 (t, J = 7.5 Hz, 3H, C$\underline{H}_3$), 2.68-2.71 (m, 5H, C$\underline{H}_3$ + C$\underline{H}_2$), 4.59 (q, J = 7.0 Hz, 2H, C$\underline{H}_2$), 6.03 (s, 1H, ArH), 7.64 (app t, J = 8.0 Hz, 1H, ArH), 7.97 (d, J = 8.0 Hz, 1H, ArH), 8.46 (d, J = 8.0 Hz, 1H, ArH), 8.90 (s, 1H, ArH); ^{13}C NMR ($CDCl_3$, δ): 12.7, 14.1, 26.9, 31.5, 64.2, 100.7, 120.4, 124.6, 127.1, 129.9, 136.1, 137.2, 138.3, 147.4, 155.6, 156.7, 172.5, 197.0; MS (ESI$^+$) m/z 355 (20%, M + H)$^+$, 377 (90%, M + Na)$^+$; HRMS (ESI$^+$) calcd for $C_{18}H_{19}N_4O_4$: 355.1422 (M + H)$^+$, found: 355.1406.

3-Acetyl-7-ethyl-1-(3-isopropoxyphenyl)-[1,2,4]triazolo[4,3-*a*]pyrimidin-5(1*H*)-one (14e): The compound was prepared according to general method C (92 mg, yield = 27%) as a buff powder, m.p: 88-90 °C. ^{1}H NMR ($CDCl_3$, δ): 1.30 (t, J = 7.5 Hz, 3H, C$\underline{H}_3$), 1.40 (d, J = 5.5 Hz, 6H, 2 x C$\underline{H}_3$), 2.68 (q, J = 7.5 Hz, 2H, C$\underline{H}_2$), 2.80 (s, 1H, 3H, C$\underline{H}_3$), 4.62-4.64 (m, 1H, C$\underline{H}$), 6.04 (s, 1H, ArH), 6.91 (d, J = 7.5 Hz, 1H, ArH), 7.40 (app t, J = 7.5 Hz, 1H, ArH), 7.73-7.79 (m, 1H, ArH), 7.84 (s, 1H, ArH); ^{13}C NMR (CDCl3, δ): 12.7, 22.1, 30.0, 31.4, 70.6,

100.8, 108.3, 112.7, 115.7, 130.3, 137.6, 141.3, 148.0, 156.1, 158.7, 172.2, 186.7; MS (ESI^+) *m/z* 341 (60%, M + H)$^+$, 703 (100%, 2M + Na)$^+$; HRMS (ESI^+) calcd for $C_{18}H_{21}N_4O_3$: 341.1611 (M + H)$^+$, found: 341.1614.

Ethyl 7-ethyl-1-(3-isopropoxyphenyl)-5-oxo-1,5-dihydro-[1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (14f): The compound was prepared according to general method C (130 mg, yield = 35%) as a buff powder, m.p: 95-97 °C. ^{1}H NMR ($CDCl_3$, δ): 1.30 (t, J = 7.5 Hz, 3H, C<u>H</u>$_3$), 1.38 (d, J = 6.0 Hz, 6H, 2C<u>H</u>$_3$), 1.46 (t, J = 7.0 Hz, 3H, C<u>H</u>$_3$), 2.67 (q, J = 7.5 Hz, 2H, C<u>H</u>$_2$), 4.57 (q, J = 7.0 Hz, 2H, C<u>H</u>$_2$), 4.60-4.65 (m, 1H, C<u>H</u>), 6.00 (s, 1H, ArH), 6.89 (d, J = 8.0 Hz, 1H, ArH), 7.39 (dd, J = 8.0, 8.5 Hz, 1H, ArH), 7.74 (d, J = 8.5 Hz, 1H, ArH), 7.82 (s, 1H, ArH); ^{13}C NMR ($CDCl_3$, δ): 12.8, 14.1, 22.2, 31.6, 64.1, 70.5, 100.3, 108.0, 112.7, 115.8, 130.3, 135.7, 137.7, 147.4, 155.9, 157.0, 158.7, 172.7; MS (ESI^+) *m/z* 371 (80%, M + H)$^+$, 763 (100%, 2M + Na)$^+$; HRMS (ESI^+) calcd for $C_{19}H_{23}N_4O_3$: 371.1720 (M + H)$^+$, found: 371.1719.

3-(3-Acetyl-7-ethyl-5-oxo-[1,2,4]triazolo[4,3-*a*]pyrimidin-1(5*H*)-yl)benzenesulfonamide (14g): The compound was prepared according to general method C (141 mg, yield = 39%) as a buff powder, m.p: 180-182 °C. ^{1}H NMR ($(CD_3)_2CO$), δ: 1.30 (t, J = 7.5 Hz, 3H, C<u>H</u>$_3$), 2.68 (q, J = 7.5 Hz, 2H, C<u>H</u>$_2$), 2.77 (s, 1H, 3H, C<u>H</u>$_3$), 6.00 (s, 1H, ArH), 6.85 (br. s, 2H, NH_2), 7.82 (dd, J = 8.0, 8.5 Hz, 1H, ArH), 7.94 (d, J = 8.0 Hz, 1H, ArH), 8.61 (d, J = 8.5 Hz, 1H, ArH), 8.79 (s, 1H, ArH); ^{13}C NMR ($(CD_3)_2CO$), *δ:* 12.2, 29.8, 31.0, 100.1, 118.1, 123.5, 124.7, 130.3, 137.5, 142.1, 145.8, 148.5, 155.9, 172.0, 188.0; MS (ESI^+) *m/z* 362 (10%, M + H)$^+$, 384 (15%, M + Na)$^+$; HRMS (ESI^+) calcd for $C_{15}H_{16}N_5O_4S$: 362.0917 (M + H)$^+$, found: 362.0923.

Ethyl 7-ethyl-5-oxo-1-(3-sulfamoylphenyl)-1,5-dihydro-[1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (14h): The compound was prepared according to general method C (215 mg, yield = 55%) as a pale yellow powder, m.p: 176-178 °C. ^{1}H NMR (DMSO-d_6, δ): 1.24 (t, J = 7.5 Hz, 3H, C<u>H</u>$_3$), 1.35 (t, J = 7.0 Hz, 3H, C<u>H</u>$_3$), 2.62 (q, J = 7.5 Hz, 2H, C<u>H</u>$_2$), 4.48 (q, J = 7.0 Hz, 2H, C<u>H</u>$_2$), 6.02 (s, 1H, ArH), 7.59 (br. s, 2H, NH_2), 7.81-7.86 (m, 2H, ArH), 8.42 (d, J = 8.0 Hz, 1H, ArH), 8.57 (s, 1H, ArH); ^{13}C NMR (DMSO-d_6, δ): 12.9, 14.2, 31.0, 64.0, 100.0, 118.1, 123.8, 124.8, 130.8, 135.8, 137.0, 145.8, 148.2, 155.5, 157.0, 172.0; MS (ESI^+) *m/z* 414 (35%, M + Na)$^+$, 805 (100%, 2M + Na)$^+$; HRMS (ESI^+) calcd for $C_{16}H_{17}N_5O_5S$: 414.0858 (M + Na)$^+$, found: 414.0848.

4-(3-Acetyl-7-ethyl-5-oxo-[1,2,4]triazolo[4,3-*a*]pyrimidin-1(5*H*)-yl)benzenesulfonamide (14i): The compound was prepared according to general method C (184 mg, yield = 51%) as a buff powder, m.p: 162-164 °C. ^{1}H NMR (DMSO-d_6, δ): 1.25 (t, J = 7.5 Hz, 3H, C<u>H</u>$_3$), 2.65 (q, J = 7.5 Hz, 2H, C<u>H</u>$_2$), 2.72 (s, 1H, 3H, C<u>H</u>$_3$), 6.07 (s, 1H, ArH), 7.49 (br. s, 2H, NH_2), 8.05 (d, J = 8.5 Hz, 2H, ArH), 8.37 (d, J = 8.5 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6, δ): 13.0, 30.6, 30.9, 100.5, 121.1, 127.6, 139.1, 141.9, 142.9, 148.4, 155.9, 171.8, 188.6; MS (ESI^+) *m/z* 384 (10%, M + Na)$^+$, 745 (15%, 2M + Na)$^+$; HRMS (ESI^+) calcd for $C_{15}H_{16}N_5O_4S$: 362.0912 (M + H)$^+$, found: 362.0923.

Ethyl 7-ethyl-5-oxo-1-(4-sulfamoylphenyl)-1,5-dihydro-[1,2,4]triazolo[4,3-*a*]pyrimidine-3-carboxylate (14j): The compound was prepared according to general method C (239 mg, yield = 61%) as a pale yellow powder, m.p: 180-182 °C. ^{1}H NMR ($(CD_3)_2CO$), δ: 1.32 (t, J = 7.5 Hz, 3H, C<u>H</u>$_3$), 1.45 (t, J = 7.0 Hz, 3H, C<u>H</u>$_3$), 2.71 (q, J = 7.5 Hz, 2H, C<u>H</u>$_2$), 4.56 (q, J = 7.0 Hz, 2H, C<u>H</u>$_2$), 6.00 (s, 1H, ArH), 6.72 (br. s, 2H, NH_2), 8.13 (d, J = 8.5 Hz, 2H, ArH), 8.51 (d, J = 8.5 Hz, 2H, ArH); ^{13}C NMR ($(CD_3)_2CO$), δ: 12.1, 13.3, 30.9, 63.5, 99.8, 120.4,

127.4, 136.2, 139.5, 142.6, 148.0, 155.1, 156.7, 172.0; MS (ESI^+) *m/z* 392 (5%, M + H)$^+$, MS (ESI^-) *m/z* 390 (100%, M – H)$^-$; HRMS (ESI^+) calcd for $C_{16}H_{18}N_5O_5S$: 392.1032 (M + H)$^+$, found: 392.1029.

3-Acetyl-1-(4-bromophenyl)-7-ethyl-[1,2,4]triazolo[4,3-*a*]pyrimidin-5(1*H*)-one (14k): The compound was prepared according to general method C (115 mg, yield = 32%) as a buff powder, m.p: 155-157 °C. ^{1}H NMR ($CDCl_3$, δ): 1.30 (t, *J* = 7.5 Hz, 3H, C$\underline{H}_3$), 2.68 (q, *J* = 7.5 Hz, 2H, C$\underline{H}_2$), 2.81 (s, 3H, COC$\underline{H}_3$), 6.05 (s, 1H, ArH), 7.66 (d, *J* = 9.0 Hz, 2H, ArH), 8.17 (d, *J* = 9.0 Hz, 2H, ArH); ^{13}C NMR ($CDCl_3$, δ): 12.6, 29.9, 31.3, 100.9, 121.3, 122.0, 132.4, 135.6, 141.5, 147.6, 155.8, 172.1, 186.5; MS (ESI^+) *m/z* 361 (10%, M + H, ^{79}Br)$^+$, 363 (10%, M + H, ^{81}Br)$^+$; HRMS (ESI^+) calcd for $C_{15}H_{14}{}^{79}BrN_4O_2$: 361.0301 (M + H)$^+$, found: 361.0300.

4.2 Biological evaluation

4.2.1 Antiviral assay [36]

CHIKV Indian Ocean strain 899 (Genbank FJ959103.1) was generously provided by Prof. S. Günther (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany) (Panning M et al., Emerging Infectious Diseases 2008). BGM cells were maintained in cell growth medium composed of minimum essential medium (MEM Rega-3, Gibco, Belgium) supplemented with 10% Foetal Bovine Serum (FBS, Integro, The Netherlands), 1% L-glutamine (Gibco), and 1% sodium bicarbonate (Gibco). The antiviral assays were performed in virus growth medium which is the respective cell growth medium supplemented with 2% (instead of 10%) FBS. Cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and 95-99% humidity. BGM cells were seeded in 96-well tissue culture plates (Becton Dickinson, Aalst, Belgium) at a density of 2.5 x 104 cells/well in 100 µL assay medium and were allowed to adhere overnight. Next, a compound dilution series was prepared in the medium on top of the cells after which the cultures were infected with 0.001 MOI of CHIKV 899 inoculum in 100 µl assay medium. On day 5 post-infection (p.i.), the plates were processed using the MTS/PMS method as described by the manufacturer (Promega, The Netherlands). The 50% effective concentration (EC_{50}), which is defined as the compound concentration that is required to inhibit virus-induced cell death by 50%, was determined using logarithmic interpolation. All assay wells were checked microscopically for minor signs of virus induced CPE or possible alterations to the cell or monolayer morphology caused by the compound.

4.2.2 Cell Viability Assays

Exactly 100 µL of cells were cultured in 96 well-plates at a seeding density of 10^5 cells/mL. After 24 h, the cells were treated with the samples (dissolved in DMSO) and incubated for 72 h. Doxorubicin at 1 µM concentration was used as a positive control with 0.1% DMSO as a negative control for each plate. At the end of incubation period the culture media were aspirated followed by the addition of 100 µL of 0.1 mg/mL Alamar blue solution (resazurin, stock solution (1 mg/mL in a freshly prepared PBS), followed by 1:10 dilution with FBS free media) was added to each well. Absorbance at 570 nm and 600 nm was measured using a BMG LABTech FLUOstar OPTIMA plate reader (Mount Eliza, Victoria, Australia). The drug were tested in triplicate with a negative control was taken as 100% cell viability. Nonlinear regression and IC_{50} calculations were performed with GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA.

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