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New Pyrazolobenzothiazine Derivatives as Hepatitis C Virus NS5B Polymerase Palm Site I Inhibitors

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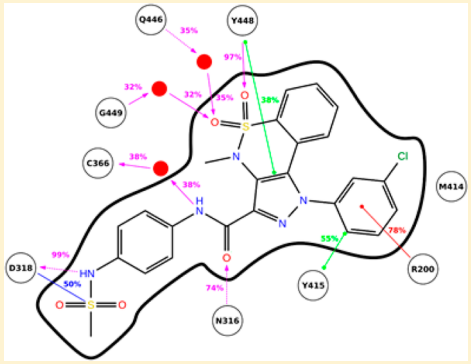
Supporting Information

ABSTRACT: We have previously identified the pyrazolobenzothiazine scaffold as a promising chemotype against hepatitis C virus (HCV) NS5B polymerase, a validated and promising anti-HCV target. Herein we describe the design, synthesis, enzymatic, and cellular characterization of new pyrazolobenzothiazines as anti-HCV inhibitors. The binding site for a representative derivative was mapped to NS5B palm site I employing a mutant counterscreen assay, thus validating our previous in silico predictions. Derivative **2b** proved to be the best selective anti-HCV derivative within the new series, exhibiting a IC_{50} of 7.9 μM against NS5B polymerase and antiviral effect (EC_{50} = 8.1 μM; EC_{90} = 23.3 μM) coupled with the absence of any antimetabolic effect (CC_{50} > 224 μM; SI > 28) in a cell based HCV replicon system assay. Significantly, microscopic analysis showed that, unlike the parent compounds, derivative **2b** did not show any significant cell morphological alterations. Furthermore, since most of the pyrazolobenzothiazines tested altered cell morphology, this undesired aspect was further investigated by exploring possible perturbation of lipid metabolism during compound treatment.



INTRODUCTION

Hepatitis C virus (HCV) infection affects approximately 180 million people worldwide and is the main cause of hepatocellular carcinoma and liver transplantation in industrialized countries.[1,2] Among several infectious diseases, chronic HCV infection is one of the hardest to treat. Currently there is no vaccine against HCV,[3] and therapy consists of a NS3/4A protease inhibitor, i.e., boceprevir[4] or telaprevir,[5] administered in combination with interferon α (pegIFN-α) and ribavirin (RBV).[6] This regimen improves the sustained virological response (SVR) to about 70% in the most prevalent HCV genotype in industrialized countries, namely, genotype 1, compared to the standard of care (pegIFN-α and RBV). However, the triple therapy still suffers from additional side effects, high cost, and increased pill burden. Thus, the development of a more adherent and efficacious therapy still remains an unmet medical need. A possibility is a combination of direct-acting antiviral agents (DAAs) targeting different HCV proteins that could eliminate the concomitant use of pegIFN-α and RBV.[7,8] Almost all the nonstructural proteins (NSs) involved in HCV replication have been extensively studied and targeted to reach the goal, with several DAAs currently in different stages of clinical trials.[9,10]

The HCV NS5B RNA-dependent RNA polymerase (RdRp) is a validated and attractive target to identify new DAAs, given its key role in viral replication and significant differences from mammalian polymerases.[11] In addition to the active site, NS5B harbors at least five allosteric sites: (i) thumb site I (TSI), (ii) thumb site II (TSII), (iii) palm site I (PSI), (iv) palm site II (PSII), and (v) palm site III (PSIII). NS5B inhibitors are classified as nucleoside inhibitors (NIs) or non-nucleoside inhibitors (NNIs) based on whether they bind to the active site or to one of the five allosteric sites, respectively.[12−14] Tremendous efforts made by both pharmaceutical companies and academic groups culminated with the identification of DAAs targeting NS5B that are now under clinical evaluation either alone or in combined therapy.[9,10] Unfortunately, the development of NS5B inhibitors suffers from high attrition rate and none of them has reached the market yet. Thus, the

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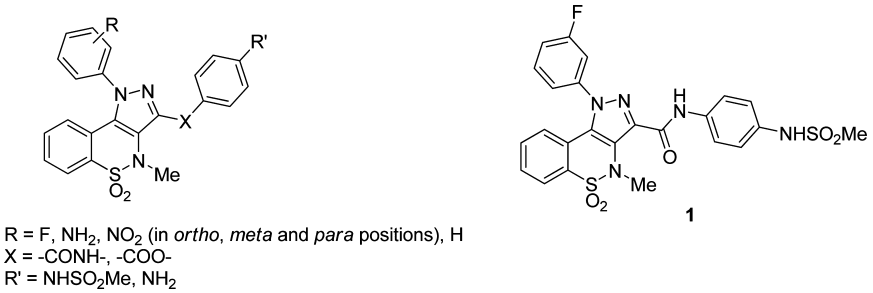
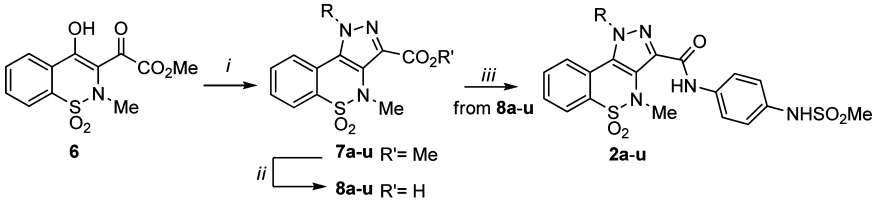


Figure 1. (Left) General formula of our first series of anti-NS5B pyrazolobenzothiazines. (Right) Hit compound **1**.

Scheme 1[a]



2, 7 and 8	R	2, 7 and 8	R
a	(3-Cl)Ph	**l**	(3-CF_3)Ph
b	(4-Cl)Ph	**m**	(4-CF_3)Ph
c	(2-Cl)Ph	**n**	(2-CF_3)Ph
d	(3-Br)Ph	**o**	(3,4-diF)Ph
e	(4-Br)Ph	**p**	(3,4-diCl)Ph
f	(2-Br)Ph	**q**	(3,4-diMe)Ph
g	(3-Me)Ph	**r**	(3-Cl, 4-Me)Ph
h	(4-Me)Ph	**s**	(3-CF_3, 4-Cl)Ph
i	(2-Me)Ph	**t**	Cy
j	(3-OMe)Ph	**u**	H
k	(4-OMe)Ph		

[a]Reagents and conditions: (i) ArNHNH$_2$·HCl or CyNHNH$_2$·HCl or NH$_2$NH$_2$·HCl, MeOH, reflux; (ii) 10% aq NaOH, MeOH, reflux; (iii) (a) $SOCl_2$, reflux and (b) *N*-(4-aminophenyl)methanesulfonamide, Et_3N, dry DMF, 40 °C.

discovery of new chemotypes able to inhibit NS5B remains of great interest.

In this context, we have directed our efforts toward the identification of new anti-HCV chemotypes acting as NS5B NNIs.[15−17] Employing a structure-based discovery approach, we recently reported on the identification of a novel series of pyrazolobenzothiazine-based anti-HCV compounds targeting the NS5B polymerase (Figure 1).[17] A preliminary structure−activity relationship (SAR) profiling of this class of NNIs revealed that (i) the best substituent on the N-1-phenyl group of the pyrazolobenzothiazine nucleus was the fluorine atom with the meta position exhibiting the highest activity; (ii) the methanesulfonamide moiety played an important role, since its replacement with an amino group generated inactive molecules in both the anti-NS5B and anti-HCV replicon assays; (iii) an amide spacer was strongly preferred over an ester linkage. Accordingly, compound **1** (Figure 1) emerged as the most active hit within this first series of pyrazolobenzothiazines.[17]

Specifically, docking of compound **1** within PSI revealed that (i) the benzene ring and the N-1-phenyl moiety of the pyrazolobenzothiazine nucleus filled two hydrophobic pockets in the PSI; (ii) the sulfonyl function engaged in a direct hydrogen bond with Tyr448 and in a water-mediated interaction with Gly449; (iii) the *p*-methanesulfonamide phenyl moiety at the C-3 amide group established several polar interactions with the surrounding NS5B residues.[17]

On the basis of this analysis, we describe herein the design and synthesis of novel pyrazolobenzothiazine derivatives in an attempt to better outline their SAR profile and optimize their biological activities. Furthermore, we report on the effect of these compounds on HCV NS5B polymerase inhibition and on HCV RNA replication. Employing mutant counterscreen assays, we have investigated the interaction of one of the most potent compounds with the NS5B binding pockets to validate its putative binding site, i.e., PSI. Finally, we describe molecular modeling, in vitro ADME, and drug-induced phospholipidosis investigations, aimed at gaining more detailed insight into NS5B pyrazolobenzothiazine inhibitors.

RESULTS AND DISCUSSIONS

Design of the New Pyrazolobenzothiazines. Employing pyrazolobenzothiazine derivative **1** as our reference, we designed and synthesized derivatives **2a−u** (Scheme 1 and Table 1) to explore the influence of the interaction of the lipophilic tail at the N-1 position with the hydrophobic pocket in the PSI. In particular, we replaced the *m*-fluorine atom of hit **1** with the more lipophilic chlorine (**2a**) and bromine (**2d**) substituents and also shifted these two new halogens from the meta to the para (**2b**, **2e**) and the ortho (**2c**, **2f**) positions,

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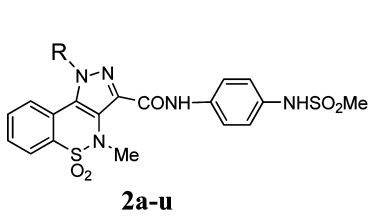
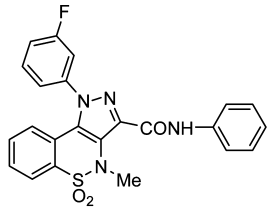
Table 1. Biological Activities of the Target Pyrazolobenzothiazines 2a–u, 3, 4, and 5



2a-u



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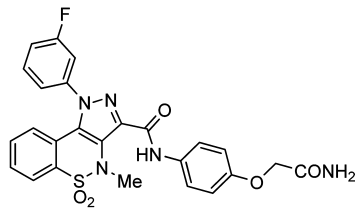

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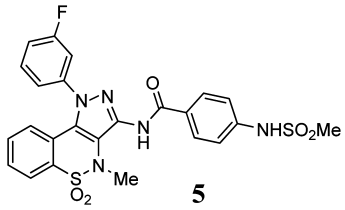

5

			cell-based replicon assay			
compd	R	anti-NS5B assay, IC_{50} [a] (μM)	EC_{50} [b] (μM)	EC_{90} [c] (μM)	CC_{50} [d] (μM)	SI [e]
2a	(3-Cl)Ph	4.3 ± 0.2	9.8 ± 3.2	41 ± 11	>179	>18
2b	(4-Cl)Ph	7.9 ± 0.3	8.1 ± 4.3	23.3 ± 11.4	>224	>28
2c	(2-Cl)Ph	17.8 ± 0.5	7.4 ± 2.1	20.8 ± 3.9	>224	>30
2d	(3-Br)Ph	7.7 ± 0.2	8.3 ± 0.5	26 ± 5	>166	>20
2e	(4-Br)Ph	12.1 ± 0.5	7.4 ± 0.8	21 ± 5	>166	>22
2f	(2-Br)Ph	26.5 ± 3.3	12.0 ± 0.4	ND[g]	107 ± 34	8.9
2g	(3-Me)Ph	21.2 ± 4.5	5.1 ± 0.6	11.8 ± 1.2	>233	>46
2h	(4-Me)Ph	14.7 ± 0.2	4.8 ± 1.7	19.6 ± 11.8	>186	39
2i	(2-Me)Ph	30.8 ± 3.8	14.3 ± 1.1	ND[g]	>233	>16
2j	(3-OMe)Ph	NA[f]	11 ± 2	ND[g]	>181	>16
2k	(4-OMe)Ph	NA[f]	8.1 ± 1.5	25.0 ± 7.3	>226	>28
2l	(3-CF_3)Ph	23.7 ± 0.9	7.7 ± 1.9	33.2 ± 24.9	>211	>28
2m	(4-CF_3)Ph	36.3 ± 4.2	7.3 ± 0.8	31.2	>169	>23
2n	(2-CF_3)Ph	NA[f]	25.0 ± 3	ND[g]	>169	>7
2o	(3,4-diF)Ph	25.4 ± 3.8	13.9 ± 0.4	ND[g]	138 ± 24	9.9
2p	(3,4-diCl)Ph	2.7 ± 0.3	9.7 ± 1.8	ND[g]	>168	>17
2q	(3,4-diMe)Ph	NA[f]	6.2 ± 0.3	20 ± 2	>181	>29
2r	(3-Cl,4-Me)Ph	8.0 ± 1.0	7.0 ± 2.0	37 ± 4	>175	>25
2s	(3-CF_3,4-Cl)Ph	NA[f]	6.4 ± 1.7	ND[g]	>160	>25
2t	Cy	43.0 ± 0.7	4.5 ± 0.6	11 ± 2	44 ± 38	9.7
2u	H	NA[f]	88.2 ± 42.8	65.2	155 ± 50.5	2.5
3		NA[f]	4.5 ± 0.6	ND[g]	56	12
4		NA[f]	15 ± 2	34 ± 7	>192	>13
5		NA[f]	80.8 ± 9.4	ND[g]	160 ± 18.2	2.3
1[h]	(3-F)Ph	21.0 ± 2.8	7.5 ± 2.5	42.0 ± 2.0	>370	>49

[a] IC_{50} = concentration of compound that inhibits 50% enzyme activity in vitro. The reported values represent the mean ± SD of data derived from two independent experiments performed in duplicate. [b] EC_{50} = the effective concentration that is required to inhibit virus replication by 50% as quantified by luciferase reporter signal. The reported values represent the mean ± SD of data derived from at least three dose–response curves. [c] EC_{90} = the effective concentration that is required to inhibit virus replication by 90% as quantified by luciferase reporter signal. The reported values represent the mean ± SD of data derived from at least three independent dose–response curves. [d] CC_{50} = concentration that is required to reduce the bioreduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) into formazan by 50% and is representative of the antimetabolic effect of the compound on the host cell. The reported value represents the mean ± SD of data derived from at least three dose–response curves. [e] SI = selectivity index (ratio of CC_{50} to EC_{50}). [f] NA = not active. These compounds were not able to reach ≥50% inhibition of NS5B RdRp activity at 50 μM. [g] ND = not determined. These compounds were not able to reach 90% inhibition of HCV replication at any concentration employed. [h] Data from ref 17.

respectively. The fluorine atom of **1** was also replaced by other groups with varying degrees of lipophilicity such as methyl (**2g**), methoxy (**2j**), or a trifluoromethyl (**2l**). These groups were also placed in para (**2h**, **2k**, and **2m**) and ortho (**2i**, **2n**) positions, with the exception of the *o*-methoxy group because in this case the requisite starting material was not available by our common suppliers. Further modifications carried out on the N-1 tail of the pyrazolobenzothiazine scaffold entailed the introduction of an additional fluorine atom to obtain the meta/para-disubstituted fluorine derivative (**2o**). Other disubstitution patterns, selected on the basis of commercially available phenylhydrazines as starting material, were also investigated by designing meta/para-disubstituted derivatives such as dichloro (**2p**), dimethyl (**2q**), chloro/methyl (**2r**), and trifluomethyl/chloro (**2s**) compounds. Furthermore, the phenyl ring at the N-1 position was either replaced by a cyclohexyl fragment to obtain compound **2t** or completely removed in order to realize the N-1 hydrogen derivative **2u**. In order to

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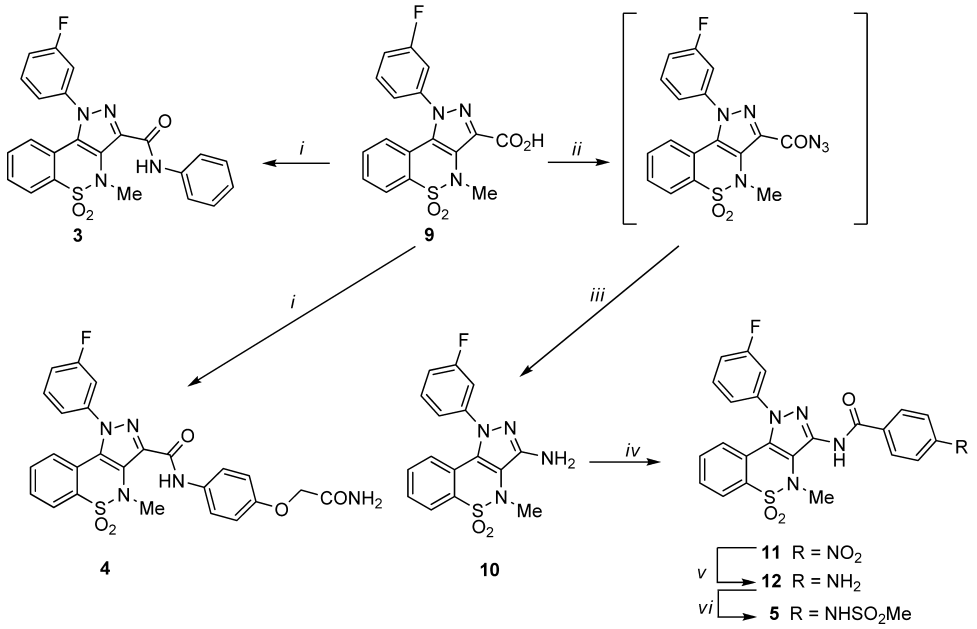
Scheme 2[a]



[a]Reagents and conditions: (i) (a) $SOCl_2$, reflux and (b) $ArNH_2$, Et_3N, dry DMF, 40 °C; (ii) (a) $SOCl_2$, reflux and (b) 1 M aq NaN_3, acetone, 0 °C to rt; (iii) 37% HCl/toluene (1:1), 100 °C; (iv) 4-nitrobenzoyl chloride, dry py, 40 °C; (v) H_2/Raney Ni, DMF, rt and atm pressure; (vi) MsCl, dry py, dry CH_2Cl_2, 0 °C to rt.

definitively confirm the importance of the methanesulfonamide moiety as an essential pharmacophoric element,[17] it was removed (derivative **3**) or replaced by an oxyacetamide fragment (derivative **4**) (Scheme 2 and Table 1); the latter substituent has been reported as a suitable group for anti-HCV activity and in vivo properties in the well-known anti-NS5B benzothiadiazine series described in the literature.[18] Finally, with the aim of exploring the influence of the amide on the inhibitory activity of our series of pyrazolobenzothiazine derivatives, it was inverted to give derivative **5** (Scheme 2 and Table 1).

Chemistry. The synthetic pathways utilized in the preparation of the desired pyrazolobenzothiazines **2a−u**, and **3−5** are outlined in Schemes 1 and 2, respectively. Through a regioselective condensation[17] of key synthon **6**[19] with the appropriate hydrazine hydrochloride, the N-1 phenylpyrazolobenzothiazine esters **7a−c**,[19] **7d−g**, **7h**,[19] **7i−k**, **7l**,[19] **7m−s**, the N-1 cyclohexyl pyrazolobenzothiazine **7t**, and the N-1 unsubstituted pyrazolobenzothiazine **7u** were prepared and subsequently hydrolyzed under basic conditions. The 3-carboxylic acids **8a−k**, **8l**,[19] and **8m−u** thus obtained were then converted to the corresponding acyl chloride, under reflux of $SOCl_2$, and immediately reacted with *N*-(4-aminophenyl)-methanesulfonamide,[20] using Et_3N as scavenger, to obtain the target amide derivatives **2a−u** (Scheme 1). Next, by use of the latter procedure, the acid **9**[17] (Scheme 2) was reacted with aniline or 2-(4-aminophenoxy)acetamide[21] to give the target **3** or **4**, respectively.

The synthesis of the inverse amide **5** (Scheme 2) entailed the conversion of the 3-carboxylic acid **9**[17] into the corresponding unstable azide intermediate which was immediately decomposed to the 3-aminopyrazolobenzothiazine **10** through a Curtius reaction. Derivative **10** was then reacted with *p*-nitrobenzoyl chloride in dry pyridine to afford the nitro intermediate **11**, which was reduced under catalytic hydrogenation to the corresponding amino derivative **12**. Finally, mesylation of the latter compound gave the desired target derivative **5**.

Anti-NS5B Activity. The anti-NS5B activity of the target compounds **2a−u** and **3−5** (Table 1) was determined using recombinant NS5BCΔ21 1b in a primer dependent elongation assay as described previously (see also Supporting Information).[16,17,22−24]

All compounds were first screened at 50 μM to identify NS5B inhibitor candidates exhibiting ≥50% inhibition of NS5B RdRp activity at this concentration. This investigation led to the identification of 15 compounds (**2a−i**, **2l**, **2m**, **2o**, **2p**, **2r**, **2t**) satisfying this criterion, while nine compounds (**2j**, **2k**, **2n**, **2q**, **2s**, **2u**, **3−5**) exhibited at the same concentration lower inhibition of NS5B RdRp activity; the latter compounds were thus considered as inactive.

The fifteen selected compounds were further screened for their NS5B inhibition potency and yielded IC_{50} values ranging from 2.7 to 43 μM.

Compounds **2a** (IC_{50} = 4.3 μM), characterized by the presence of a N-1 *m*-chlorophenyl residue, exhibited the highest NS5B inhibitory activity within the monosubstituted derivatives, displaying about 5-fold higher activity than the parent compound **1** (IC_{50} = 21.0 μM). Further analysis of the monosubstituted miniseries (compounds **2a−n**) clearly showed that the meta and para substitutions at the N-1 phenyl ring of the pyrazolobenzothiazine were preferred over the ortho substitution irrespective of the nature of the substituent. In particular, the *m*-chlorine (**2a**) and *p*-chlorine (**2b**) derivatives were about 4-fold and 2-fold more active than the corresponding *o*-chlorine derivative **2c**, respectively. From a head to head comparison between the *m*-chloro derivative **2a** and hit compound **1**, it was clear that chlorine was a good replacement for fluorine. This pattern was consistent in the N-1 bromophenyl substituted derivatives **2d−f**. The presence of a methyl group (derivatives **2g−i**) or a trifluoromethyl group (derivatives **2l** and **2m**) yielded less active compounds,

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although the impact of their positions on the activity was less evident. The presence of a more polar methoxyl group (**2j** and **2k**) resulted in inactive compounds, highlighting the importance of a lipophilic substituent at the N-1 phenyl ring. The importance of the lipophilic and bulky nature of the N-1 substituent was also confirmed by the very low activity shown by N-1 cyclohexyl derivative **2t** and by the totally inactive N-1 unsubstituted derivative **2u**.

The meta/para-disubstituted derivatives designed with the objective to better fill the hydrophobic cavity on NS5B polymerase showed variable activities. In particular, the dichloro and the chloro/methyl N-1 phenyl substituted derivatives **2p** and **2r** exhibited enhanced activity (IC_{50} of 2.7 and 8.0 μM, respectively), with derivative **2p** representing the best hit in anti-NS5B functional assay. By contrast, the difluoro N-1 phenyl substituted derivative **2o** was less active than compound **1**. These data further demonstrated that chlorine is a good replacement for fluorine at the meta and/or para position.

We previously reported that replacement of the amide linkage with an ester function was detrimental for the anti-NS5B activity.[17] Consistent with this trend, the reverse amide of starting hit **1** yielded the inactive derivative **5**, thus emphasizing the crucial role of this linker for this series of NNIs. In a SAR study on some benzothiazine and benzothiadiazine PSI-NNIs, it was observed that replacement of the methanesulfonyl moiety with the oxyacetamide chain conferred better pharmacological properties while maintaining potent anti-NS5B inhibition.[18] Paradoxically though, derivative **4** bearing an oxyacetamide moiety on the phenyl ring was inactive. Furthermore, as expected because of our previous observations,[17] deletion of the sole methanesulfonamide fragment on the hit compound **1** also yielded the inactive compound **3**.

Mapping the Inhibitor Binding Site on NS5B. In our previous studies, we reported that pyrazolobenzothiazine-based inhibitors should bind NS5B PSI based on molecular docking studies.[17]

With the objective of confirming this in silico prediction, we carried out the mutant counterscreen assay[25−27] with **2a** as a representative member of this class of NS5B inhibitors. In this assay, recombinant NS5BCΔ21 mutant proteins P495L, M423T, and M414T are employed as screens for TSI, TSII, and PSI binders, respectively.[25−27]

Each of these proteins carries a mutation at an amino acid that is critical for the binding of inhibitors to each of the aforementioned allosteric sites, thus impairing potency of the inhibitor if these residues are involved. Consistent with our hypothesis, the inhibitory potency of compound **2a** was impaired by mutation at M414T residue of NS5B but not with P495L or M423T mutants (Table 2). This was evident from the ∼30-fold higher IC_{50} value against M414T NS5B relative to wild-type NS5B. By contrast, the IC_{50} value of **2a** either remained unchanged or exhibited an approximate 2-fold increase with NS5B mutant P495L or M423T, respectively. These data thus validated our computational predictions and provided evidence for **2a** binding at PSI of NS5B.

Table 2. NS5B Mutant Inhibition Parameters for Derivative 2a[a]

binding site	NS5B mutant	IC_{50} (μM)	fold change
PSI	M414T	127.7 ± 5.5	29.7
TSI	P495L	5.1 ± 0.3	1.2
TSII	M423T	9.3 ± 1.5	2.2

[a]The IC_{50} values were evaluated against the indicated NS5B mutants as described for the wild-type NS5B and represents an average ± SD of two independent experiments in duplicate. Fold change is indicated relative to NS5B WT (IC_{50} = 4.3 μM).

In silico simulations were then performed to confirm the binding mode previously proposed for this class of PSI-NNIs.[17] Toward this end, derivative **2a** was submitted to molecular modeling studies. In a recent paper we rationalized the influence of water molecules and protein flexibility on docking studies, putting particular emphasis on the role of specific water molecules found to mediate hydrogen bond interactions of NNIs with residues Gln446 and/or Gly449 in several experimental structures.[28]

Pyrazolobenzothiazine derivative **2a** was docked into NS5B polymerase (PDB code 3SKE)[29] by means of the Glide software,[30] yielding a bound pose that was very similar (heavy-atom rmsd of 1.9 Å) to the one we obtained in our previous paper for derivative **1** (Figure 1),[17] a strict analogue of **2a**. The 3SKE crystal structure was chosen as target because in its waterless form it was found to be one of the best performing in docking-based virtual screening simulations.[28] We considered the absence of explicit waters a good premise to avoid biasing in the study of water−ligand−protein interactions. The obtained 3SKE/**2a** complex was then submitted to a 10 ns long molecular dynamics (MD) study[31] with the aim of investigating the binding mode dynamically. From the heavy-atom rmsd analysis, the system was equilibrated after the first 4 ns of simulation, and thus, this equilibration time was not considered in the binding mode analysis.

The binding of **2a** to NS5B is characterized by several interactions (Figure 2). The sulfone group of the exocyclic sulfonamide is engaged in an ion−dipole interaction with the side chain of Asp318, which in turn accepts an H-bond from the sulfonamidic nitrogen. One of the two endocyclic sulfonamide oxygens establishes hydrogen-bonding with the backbone nitrogen of Tyr448, while the other one is engaged in water-mediated interactions with Gln446 and/or Gly449. The amide group of **2a** is involved in two H-bond interactions: a water-mediated one between the backbone oxygen of Cys366 and the amidic nitrogen and a direct one between the amide oxygen and the side chain of Asn316. Furthermore, compound **2a** is involved in three π interactions: the tricyclic system stacks with the aromatic ring of Tyr448, while the *m*-chlorophenyl moiety interacts with the aromatic ring of Tyr415 and the cationic side chain of Arg200.

Replicon System Assays. The target compounds **2a−u** and **3−5** were evaluated for their anti-HCV activity (EC_{50}, EC_{90}) in Huh-5-2 cells carrying a genotype 1b HCV subgenomic replicon (Table 1). The antimetabolic effect (CC_{50}) of the compounds was investigated in parallel employing the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay. Only compounds that showed ≥70% inhibition of virus RNA replication without exhibiting any adverse antimetabolic effect at the concentration tested were considered to be endowed with selective anti-HCV activity. In fact, any compound that can induce a cytostatic effect on cell growth could result indirectly in inhibition of HCV subgenomic replicon replication.[32,33] The selectivity index (SI) was calculated as well to estimate the therapeutic potential of the compounds in this system. Experimental details for the cellular

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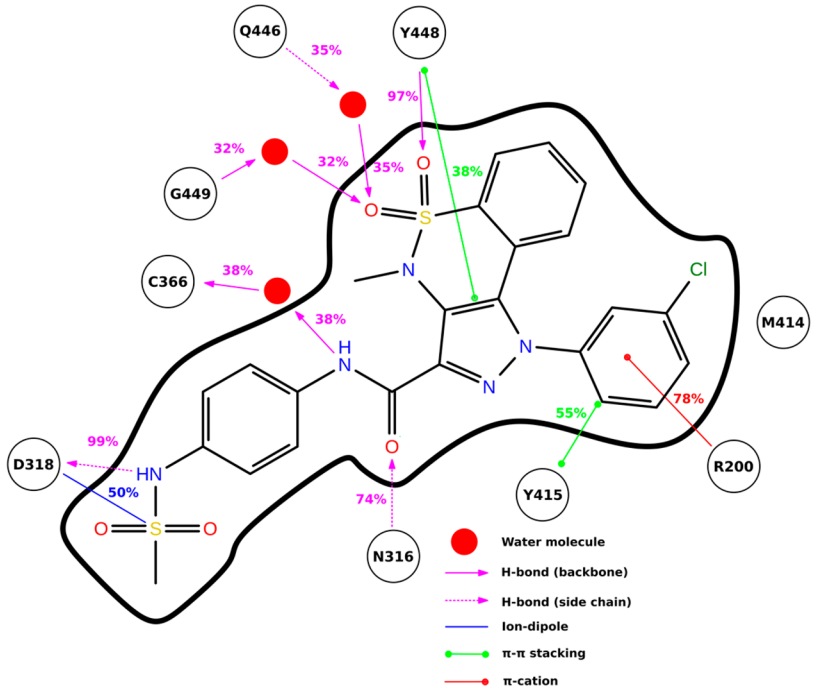

Figure 2. Binding mode analysis of pyrazolobenzothiazine derivative **2a**, depicted together with interacting NS5B residues and water molecules. Main interactions are represented schematically with their occupancies calculated in the time window 4−10 ns.

assays are according to procedures described previously (see also Supporting Information).[15−17,34]

Furthermore, to identify possible pleiotropic effects exerted by the compounds, the wells were subjected to microscopic examination to discern subtle changes in cellular phenotypes that may have resulted from compound treatment and that could be considered as a hallmark for off-target effects of the compounds.

The cell-based assays (Table 1) revealed that with the exception of derivatives **2n**, **2u**, and **5**, all remaining compounds were active against HCV, displaying EC_{50} values of ≤15 μM. However, because of either a significant toxic effect as apparent from the MTS assay or low SI values (i.e., ≤10), compounds **2f**, **2o**, **2t**, and **3** were considered as false positives (EC_{50} not reliable). After dose−response curve analysis, derivatives **2c**, **2i**, **2m**, **2p**, **2r**, **2s**, and **4** were also deemed as unsuitable, as they displayed antimetabolic effect (data not shown). Hence, only derivatives **2a**, **2b**, **2d**, **2e**, **2g**, **2h**, **2k**, **2l** produced selective anti-HCV activity without any antimetabolic effect. After microscopic analysis aimed at visualizing minor changes in the cellular morphology, only pyrazolobenzothiazines **2b**, **2k**, and to less extent **2h** proved to be true anti-HCV agents, as they did not display any significant effects on cell morphology after 72 h of compound treatment. Interestingly, all three of these derivatives were characterized by the presence of a para-substituent at the N-1 phenyl ring. Target compound **2b**, bearing a *p*-chlorine atom on the N-1 phenyl ring, was endowed with comparable inhibitory activity both against NS5B (IC_{50} = 7.9 μM) and in cell based HCV replication assay (EC_{50} = 8.1 μM and EC_{90} = 23.3 μM). In the case of derivative **2h**, the anti-HCV activity was better than the anti-NS5B activity, but this result can be easily explained by the presence of a slight pleiotropic effect that alters the cellular environment and negatively affects HCV replication. Strangely, compound **2k**, which did not display anti-NS5B activity (≤50% inhibition at 50 μM), showed good anti-HCV activity, as is evident from its EC_{50} value of 8.1 μM in a cell based assay.

In a head to head comparison, the best selective anti-HCV derivative within this new series (compound **2b**) exhibited equivalent activity as reference compound **1**. Significantly, it is important to note that, unlike the parent compound, derivative **2b** did not exhibit any cell morphological alterations, thus emerging as a more promising antiviral agent.

Selective HCV Inhibition Assessment and Physico-chemical and ADME Properties Characterization. Any compound tested on a HCV replicon system can inadvertently inhibit heterologous elements such as the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) or the neomycin phosphotransferase (neo) that is required for the proper translation of the HCV polyprotein or to keep the HCV subgenomic replicon persistently in cells, respectively.[35] To explore this unspecific effect in our pyrazolobenzothiazine series, compound **2a** was selected as a representative member because it was one of the best derivatives in both enzymatic and cellular assays and also available in large quantities. Thus, compound **2a** was tested in different replicon systems. On the one hand, we used Huh-9-13 HCV replicons that have the same genetic makeup as Huh-5-2 replicons but differ in (i) their composition of cell culture adaptive mutations, (ii) the absence of a luciferase reporter gene, and (iii) the higher HCV RNA content compared to Huh-5-2 cells. In addition, we employed a subgenomic replicon contained in HuH6 cells, where the replicon has a similar genetic makeup as in Huh-5-2 cells, but (i) it replicates in a different cell line (HuH6 cells instead of Huh-7 cells) and (ii) it carries different adaptive mutations. Moreover, in HCV subgenomic replicon containing HuH6 cells, the replicon replication is not inhibited by IFN-γ and viral RNA replication is independent from cell proliferation.[32] Compound **2a** proved to be active in both Huh-9-13 and HuH6 HCV subgenomic replicon bearing cells, exhibiting EC_{50} values of 7.6 and 14 μM, respectively, as determined by RT-

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Table 3. In Vitro Physicochemical and ADME Properties of Derivative 2a

		water solubility, log S (S in mol L^{-1})		PAMPA-GI,[b] $P_{app} \times 10^{-6}$ (cm/s) (% MR)[c]		
compd	log P[a]	thermodynamic	kinetic	5 h	22 h	HLM[d] (%)
2a	2.11	−8.40	−6.56	2.30 (7.3)	4.20 (3.2)	99.9

[a]Lipophilicity was measured through the shake flask method. [b]Permeability assay at different incubation times in gastrointestinal model. [c]% MR: percent membrane retention. [d]Metabolic stability after 60 min in presence of human liver microsomes expressed as percentage of unmodified parent drug.

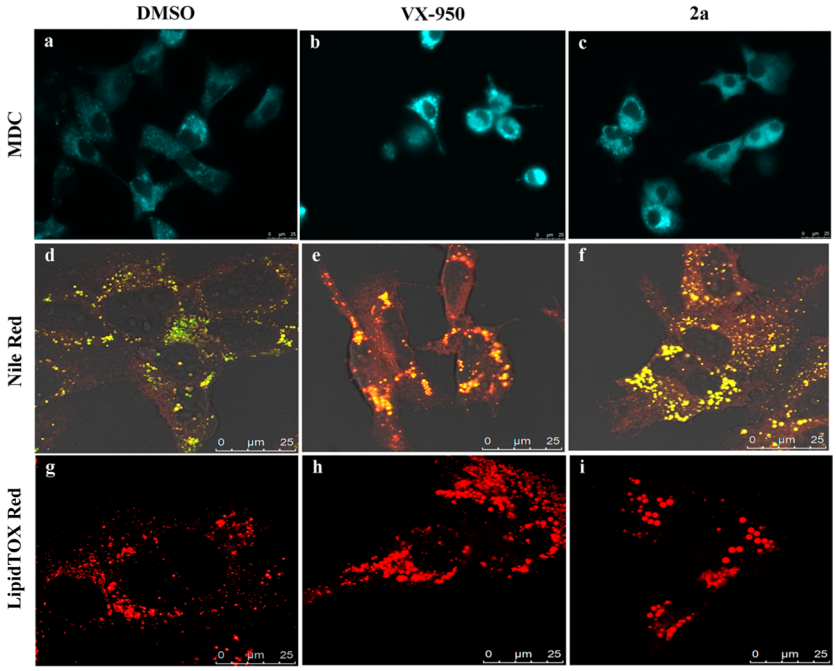

Figure 3. Huh-9-13 cells were stained with monodansylcadaverine (MDC) (a–c), Nile Red (d–f), or LipidTOX red (g–i) after mock treatment (DMSO) or treatment with either the HCV NS3 protease inhibitor VX-950 (at 15 μM) or compound **2a** (at 90 μM). Monodansylcadaverine stains multilamellar bodies and autophagic vacuoles (blue). Nile Red stains both neutral lipids (yellow/gold color) and phospholipids (orange/red color). LipidTOX red stains neutral lipids (red).

qPCR. In both replicon containing cells, **2a** did not show any effect on the proliferation and metabolism of the host cell as determined by the MTS assay (CC_{50} > 90 μM). The values obtained are in the same range as for the Huh-5-2 HCV subgenomic replicon containing cells, thus excluding any inhibitory effect on the luciferase expressed by the HCV subgenomic replicons in Huh-5-2 cells.

Derivative **2a** was also selected for additional profiling of our pyrazolobenzothiazine PSI-NNIs. Toward this end, log P, thermodynamic and kinetic water solubility (log S), parallel artificial membrane permeability (PAMPA), membrane retention (MR), and human liver microsome (HLM) stability were experimentally determined (Table 3).

The log P value turned out to be in the optimal range for druglike compounds. The thermodynamic solubility value varies with the crystal form of the solid (amorphous, crystalline, different polymorphs, hydrates, and solvates). Kinetic solubility has two distinguishing characteristics: the first one is that the compound initially is fully dissolved in an organic solvent (DMSO) and then added to the water. Desired log S for a drug candidate should be between −4 and −6, whereas log values of <−6 indicate low solubility. Both low thermodynamic and kinetic solubility values were determined for this derivative, and this behavior may be partially due to a high molecular packing effect. To evaluate membrane permeability, we have used the PAMPA assay proposed by Kansy in 1998.[36] Accordingly, several studies indicate that PAMPA permeability is correlated with both Caco-2 cell permeability and human intestinal absorption.[37,38] The PAMPA experiments on **2a** revealed a low permeability, which cannot be correlated to a high membrane retention as shown by the low MR value. Notably, **2a** turned out to be a metabolically stable NS5B inhibitor, given the high HLM stability value, indicating that no metabolites were detected after 60 min of exposure to cytochrome metabolism.

Assessment of Potential Off-Target Effects of the Compounds Studied. As reported in the previous paragraph, microscopic observations had highlighted that, with the exceptions of hit compounds **2b**, **2h**, and **2k**, most of the pyrazolobenzothiazines tested altered cell morphology. These morphological alterations may be a hallmark of potential off-target or pleiotropic effects of the compounds studied. Among the derivatives showing this undesired aspect, compound **2a** was selected as a representative analogue for further investigations.

A potential off-target effect observed during compound treatment may due to perturbation of cellular lipid metabolism,[39] e.g., inhibition of fatty acid oxidation, which may affect HCV replication.[40] Candidate drugs can perturb the lipid metabolism of a cell in different ways, for example, by inducing phospholipidosis and steatosis.[39] To assess if our pyrazolobenzothiazines could alter the cellular lipid metabolism, we used differential vital stainings that could indicate the ability of

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2a to induce phospholipidosis (Nile Red stain and monodansylcadaverine) or drug-induced steatosis (Nile Red stain and LipidTOX red stain). The aim of monodansylcadaverine staining was to screen for the presence of multilamellar bodies, a hallmark of phospholipidosis. Cell treatment with 90 μM derivative **2a** showed that the compound did not induce multilamellar bodies (Figure 3).

Finally LipidTOX red stain and Nile Red stain were performed with the aim to evaluate if compound **2a** was able to induce intracellular accumulation of neutral lipids and/or phospholipids. Overall these two assays demonstrated that derivative **2a** (at 90 μM) significantly altered the neutral lipids content of the treated cells (Figure 3) as apparent from the increase of neutral lipid stain (yellow/gold color) in Nile Red stained cells and the size of the lipid droplets in LipidTOX red stain. These observations suggest that the alteration of cell morphology induced by **2a**, and probably by most of the other pyrazolobenzothiazine derivatives, might be partially due to drug-induced steatosis. A plausible explanation might be the inhibition of mitochondrial β-oxidation by pyrazolobenzothiazines, although other mechanisms of drug-induced steatosis could be equally feasible.[41]

In depth analysis of the data obtained by different stainings performed on compound **2b** highlighted that this derivative also induced pospholipidosis/steatosis (i.e., pleiotropic effect) but with an altered effect compared to **2a** (see Supporting Information). Notably, compound **2b** did not show any significant alteration in cell morphology as a consequence of pleiotropic effect at all time points tested.

CONCLUSIONS

We have designed and synthesized novel pyrazolobenzothiazine derivatives with the aim of both tracing a clearer SAR for this class of NS5B NNIs and identifying compounds with improved anti-HCV profile. Biochemical studies and mutant counterscreen assay confirmed that our pyrazolobenzothiazines act as NS5B PSI inhibitors. Most derivatives exhibited EC_{50} values of $\leq$15 μM against HCV in the cell-based replicon assay. Notably, pyrazolobenzothiazine NS5B inibitors **2b** and **2h** proved to be true anti-HCV agents, as they did not display any antimetabolic and cell morphology alteration effects. Thus, these new derivatives represent an important improvement with respect to the previously reported parent compounds.

A combination of HCV replicons with the same genetic makeup but different cell culture adaptive mutations in several cell types was also employed to understand the effect of compound **2a**, as a representative analogue, on HCV RNA replication, host cell proliferation, and metabolism.

Finally, compound **2a** was also selected among the derivatives showing the undesired pleiotropic effect to assess possible perturbation of cellular lipid metabolism during pyrazolobenzothiazine treatment. Differential staining with dyes such as LipidTOX red and Nile Red revealed that the alteration of cell morphology induced by the pyrazolobenzothiazine **2a** might be partially due to drug-induced steatosis. Additional experimental studies highlighted physicochemical and ADME issues for the representative derivative **2a**.

These findings argue in favor of including phospholipidosis/drug-induced steatosis and pharmacokinetic profile as factors during further chemical optimization of the pyrazolobenzothiazine scaffold to obtain more selective and potent anti-HCV inhibitors and to prevent any side effect of the compounds on hepatic tissue.

EXPERIMENTAL SECTION

Chemistry. Reagents and solvents were purchased from common commercial suppliers and were used as such unless otherwise indicated. Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator at low pressure. All reactions were routinely checked by thin-layer chromatography (TLC) on silica gel 60 F254 (Merck) and visualized by using UV or iodine. Column chromatography separations were carried out with Merck silica gel 60 (mesh 70−230). Flash chromatography separations were carried out with Merck silica gel 60 (mesh 230−400). Melting points were determined in capillary tubes (Büchi Electrotermal model 9100) and are uncorrected. Yields were of purified products and were not optimized. ^{1}H NMR spectra were recorded at 200 or 400 MHz (Bruker Avance DRX-200 or -400, respectively), while ^{13}C NMR spectra were recorded at 100 MHz (Bruker Avance DRX-400). Chemical shifts (δ) are given in ppm relative to TMS and calibrated using residual undeuterated solvent as internal reference. Spectra were acquired at 298 K. Data processing was performed with standard Bruker software XwinNMR, and the spectral data are consistent with the assigned structures. The purity of the tested compounds was evaluated by combustion analysis using a Fisons elemental analyzer, model EA1108CHN, and data for C, H, and N are within 0.4% of the theoretical values (≥95% sample purity).

General Procedure for Condensation of Key Intermediate 6 with Hydrazine Hydrochlorides. Method A. A hot solution of the appropriate hydrazine hydrochloride (1.2 mmol) dissolved in hot MeOH (2 mL) was added at once to a refluxing solution of key intermediate **6**[19] (1 mmol) in MeOH (20 mL). The mixture was maintained at reflux for 2−20 h, then concentrated under reduced pressure to half volume and cooled to room temperature. The crystallized solid was filtered under vacuum to give the pyrazolobenzothiazine 3-carboxymethyl esters **7d−g**, **7i−k**, and **7m−u** used as is in the next reaction step.

Methyl 1-(3-Bromophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7d). Following the general procedure method A and using the 3-(bromophenyl)hydrazine hydrochloride, compound **7d** was obtained in 87% yield (reaction time, 15 h) as a whitish amorphous solid: mp 246−248 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.20 (s, 3H, NCH_3), 3.95 (s, 3H, OCH_3), 7.00 (dd, J = 1.6 and 7.6 Hz, 1H, H-9), 7.55−7.60 (m, 2H, H-5′ and H-6′), 7.70−7.80 (m, 2H, H-7 and H-8), 7.85−7.90 (m, 2H, H-2′ and H-4′), 8.00 (dd, J = 1.4 and 7.0 Hz, 1H, H-6).

Methyl 1-(4-Bromophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7e). Following the general procedure method A and using the 4-(bromophenyl)hydrazine hydrochloride, compound **7e** was obtained in 50% yield (reaction time, 3 h) as an orange amorphous solid: mp 196−199 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.15 (s, 3H, NCH_3), 3.95 (s, 3H, OCH_3), 7.05 (dd, J = 1.3 and 7.3 Hz, 1H, H-9), 7.55−7.60 (m, 2H, H-3′ and H-5′), 7.65−7.75 (m, 2H, H-7 and H-8), 7.80−7.90 (m, 2H, H-2′ and H-6′), 8.00 (dd, J = 1.5 and 7.6 Hz, 1H, H-6).

Methyl 1-(2-Bromophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7f). Following the general procedure method A and using the 2-(bromophenyl)hydrazine hydrochloride, compound **7f** was obtained in 90% yield (reaction time, 4 h) as an orange amorphous solid: mp 215−218 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.20 (s, 3H, NCH_3), 3.95 (s, 3H, OCH_3), 6.75 (d, J = 7.9 Hz, 1H, H-9), 7.60−7.75 (m, 4H, H-7, H-8, H-4′, and H-5′), 7.85 (d, J = 7.7 Hz, 1H, H-6′), 7.95 (d, J = 7.8 Hz, 1H, H-3′), 8.00 (d, J = 7.7 Hz, 1H, H-6).

Methyl 4-Methyl-1-(3-methylphenyl)-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7g). Following the general procedure method A and using the 3-(methylphenyl)hydrazine hydrochloride, compound **7g** was obtained in 80% yield (reaction time, 14 h) as a whitish amorphous solid: mp 199−200 °C. ^{1}H NMR (200 MHz, acetone-d_6): δ 2.50 (s, 3H, CH_3), 3.30 (s, 3H, NCH_3), 4.00 (s, 3H, OCH_3), 7.10−7.15 (m, 1H, H-9), 7.35−7.60 (m, 4H, H-2′, H-4′, H-5′, and H-6′), 7.65 (dt, J = 1.6 and 7.9 Hz, 1H, H-8), 7.70 (dt, J = 1.3 and 7.9 Hz, 1H, H-7), 8.00−8.10 (m, 1H, H-6).

Methyl 4-Methyl-1-(2-methylphenyl)-1,4-dihydropyrazolo-[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7i). Following the general procedure method A and using the 2-(methylphenyl)hydrazine hydrochloride, compound **7i** was obtained in 90% yield (reaction time, 10 h) as a whitish amorphous solid: mp 180−182 °C. ^{1}H NMR (400 MHz, acetone-d_6): δ 2.00 (s, 3H, CH_3), 3.30 (s, 3H, NCH_3), 4.00 (s, 3H, OCH_3), 6.85 (d, J = 8.0 Hz, 1H, H-9), 7.50−7.65 (m, 5H, H-8, H-3′, H-4, H-5′, and H-6′), 7.70 (dt, J = 1.1 and 7.8 Hz, 1H, H-7), 8.00 (dd, J = 0.9 and 7.8 Hz, 1H, H-6).

Methyl 1-(3-Methoxyphenyl)-4-methyl-1,4-dihydro-pyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7j). Following the general procedure method A and using the 3-(methoxyphenyl)hydrazine hydrochloride, compound **7j** was obtained in 60% yield (reaction time, 10 h) as yellow amorphous solid: mp 197−199 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.13 (s, 3H, NCH_3), 3.75 (s, 3H, OCH_3), 3.90 (s, 3H, CO_2CH_3), 6.95 (dd, J = 1.3 and 8.0 Hz, 1H, H-9), 6.95−7.05 (m, 1H, H-4′), 7.13 (t, J = 2.1 Hz, 1H, H-2′), 7.15−7.20 (m, 1H, H-6′), 7.50 (t, J = 8.1 Hz, 1H, H-5′), 7.65 (dt, J = 1.4 and 8.0 Hz, 1H, H-8), 7.70 (dt, J = 1.3 and 7.2 Hz, 1H, H-7), 7.90 (dd, J = 1.4 and 7.2 Hz, 1H, H-6).

Methyl 1-(4-Methoxyphenyl)-4-methyl-1,4-dihydro-pyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7k). Following the general procedure method A and using the 4-(methoxyphenyl)hydrazine hydrochloride, compound **7k** was obtained in 90% yield (reaction time, 15 h) as a white amorphous solid: mp 206−208 °C. ^{1}H NMR (400 MHz, acetone-d_6): δ 3.25 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 4.00 (s, 3H, CO_2CH_3), 7.15 (dd, J = 1.4 and 7.5 Hz, 1H, H-9), 7.20−7.25 (m, 2H, H-3′ and H-5′), 7.50−7.55 (m, 2H, H-2′ and H-6′), 7.60−7.80 (m, 2H, H-7 and H-8), 8.05 (m, 1H, H-6).

Methyl 4-Methyl-1-[4-(trifluoromethyl)phenyl]-1,4-dihydro-pyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7m). Following the general procedure method A and using the 4-(trifluoromethylphenyl)hydrazine hydrochloride, compound **7m** was obtained in 60% yield (reaction time, 3.5 h) as a white amorphous solid: mp 189−191 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.20 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 7.10 (dd, J = 2.1 and 7.0 Hz, 1H, H-9), 7.65−7.75 (m, 2H, H-7 and H-8), 7.80−7.85 (m, 2H, H-2′ and H-6′), 7.95−8.10 (m, 3H, H-6, H-3′, and H-5′).

Methyl 4-Methyl-1-[2-(trifluoromethyl)phenyl]-1,4-dihydro-pyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7n). Following the general procedure method A and using the 2-(trifluoromethylphenyl)hydrazine hydrochloride, compound **7n** was obtained in 70% yield (reaction time, 4 h) as an orange amorphous solid: mp 222−225 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.20 (s, 3H, NCH_3), 3.95 (s, 3H, OCH_3), 6.60 (d, J = 7.6 Hz, 1H, H-9), 7.60 (dt, J = 1.3 and 7.6 Hz, 1H, H-8), 7.70 (dt, J = 1.0 and 7.6 Hz, 1H, H-7), 7.80 (d, J = 7.2 Hz, 1H, H-3′), 7.95−8.05 (m, 3H, H-4′, H-5′, and H-6′), 8.10 (dd, J = 1.3 and 7.6 Hz, 1H, H-6).

Methyl 1-(3,4-Difluorophenyl)-4-methyl-1,4-dihydro-pyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7o). Following the general procedure method A and using the 3,4-(difluorophenyl)hydrazine hydrochloride, compound **7o** was obtained in 80% yield (reaction time, 4 h) as a white amorphous solid: mp 241−243 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.15 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 7.10 (dd, J = 1.0 and 8.2 Hz, 1H, H-9), 7.40−7.50 (m, 1H, H-6′), 7.65−7.75 (m, 3H, H-7, H-8, and H-5′), 7.85−7.95 (m, 1H, H-2′), 8.05 (dd, J = 1.3 and 7.7 Hz, 1H, H-6).

Methyl 1-(3,4-Dichlorophenyl)-4-methyl-1,4-dihydro-pyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7p). Following the general procedure method A and using the 3,4-(dichlorophenyl)hydrazine hydrochloride, compound **7p** was obtained in 80% yield (reaction time, 3 h) as a whitish amorphous solid: mp 300−302 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.15 (s, 3H, NCH_3), 3.95 (s, 3H, OCH_3), 7.15 (dd, J = 1.0 and 7.4 Hz, 1H, H-9), 7.60 (dd, J = 2.6 and 8.6 Hz, 1H, H-6′), 7.45−7.55 (m, 2H, H-7 and H-8), 7.85 (d, J = 8.6 Hz, 1H, H-5′), 8.00−8.05 (m, 2H, H-6 and H-2′).

Methyl 1-(3,4-Dimethylphenyl)-4-methyl-1,4-dihydro-pyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7q). Following the general procedure method A and using the 3,4-(dimethylphenyl)hydrazine hydrochloride, compound **7q** was obtained in 50% yield (reaction time, 4 h) as a whitish amorphous solid: mp 245−247 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.20 and 2.30 (s, each 3H, CH_3), 2.75 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 6.95 (dd, J = 1.0 and 7.7 Hz, 1H, H-9), 7.20 (dd, J = 2.3 and 7.9 Hz, 1H, H-6′), 7.30−7.40 (m, 2H, H-2′ and H-5′), 7.55−7.65 (m, 2H, H-7 and H-8), 7.90 (dd, J = 1.3 and 6.9 Hz, 1H, H-6).

Methyl 1-(3-Chloro-4-methylphenyl)-4-methyl-1,4-dihydro-pyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7r). Following the general procedure method A and using the 1-(3-chloro-4-methylphenyl)hydrazine hydrochloride, compound **7r** was obtained in 80% yield (reaction time, 4 h) as a whitish amorphous solid: mp 285−287 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.45 (s, 3H, CH_3), 3.10 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 7.00 (dd, J = 1.6 and 7.1 Hz, 1H, H-9), 7.40 (dd, J = 2.2 and 8.1 Hz, 1H, H-6′), 7.55 (d, J = 8.1 Hz, 1H, H-5′), 7.60−7.65 (m, 3H, H-7, H-8, and H-2′), 7.90 (dd, J = 1.6 and 6.8 Hz, 1H, H-6).

Methyl 1-[4-Chloro-3-(trifluoromethyl)phenyl]-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7s). Following the general procedure method A and using the 1-(4-chloro-3-trifluoromethylphenyl)hydrazine hydrochloride, compound **7s** was obtained in 50% yield (reaction time, 2 h) as a whitish amorphous solid: mp 291−293 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.10 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 7.10 (d, J = 7.7 Hz, 1H, H-9), 7.60−7.75 (m, 2H, H-7 and H-8), 7.85 (dd, J = 2.4 and 8.6 Hz, 1H, H-6′), 7.90 (d, J = 8.6 Hz, 1H, H-5′), 8.00 (dd, J = 1.0 and 7.3 Hz, 1H, H-6), 8.10 (d, J = 2.4 Hz, 1H, H-2′).

Methyl 1-Cyclohexyl-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylate 5,5-Dioxide (7t). Following the general procedure method A and using the cyclohexylhydrazine hydrochloride, compound **7t** was obtained in 50% yield (reaction time, 20 h) as a white solid: mp 198−200 °C. ^{1}H NMR (200 MHz, acetone-d_6): δ 1.25−1.85 (m, 4H, Cy-CH_2), 1.90−2.35 (m, 6H, Cy-CH_2), 3.20 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 4.70−5.00 (m, 1H, Cy-CH), 7.75−7.85 (m, 1H, H-8), 7.90−8.05 (m, 3H, H-6, H-7, and H-9).

Methyl 4-Methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzo-thiazine-3-carboxylate 5,5-Dioxide (7u). Following the general procedure method A and using the hydrazine hydrochloride, compound **7u** was obtained as a yellow solid in 70% yield (reaction time, 5 h) after purification by flash column chromatography, eluting with CH_2Cl_2/MeOH (95:5): mp 218−220 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.25 (s, 3H, NCH_3), 4.00 (s, 3H, OCH_3), 7.70 (t, J = 7.4 Hz, 1H, H-7), 7.80−8.00 (m, 2H, H-8 and H-9), 8.05 (d, J = 7.4 Hz, 1H, H-6).

General Procedure of Basic Hydrolysis. Method B. A stirred mixture of appropriate pyrazolobenzothiazine methyl esters **7a−c**,[19] **7d−g**, **7h**,[19] **7i−k**, and **7m−u** (1 mmol) in aqueous 10% NaOH (6 mL) and MeOH (6 mL) was refluxed for 1 h and then concentrated to one-third of the volume under reduced pressure. The mixture was poured into ice/water and acidified with 2 N HCl (pH 3). The precipitate formed was then filtered under vacuum, washed with Et_2O, and dried to give the pyrazolobenzothiazine-3-carboxylic acids **8a−k**, and **8m−u** used as is in the next reaction step.

1-(3-Chlorophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8a). Following the general procedure method B, compound **8a** was obtained from **7a**[19] in 85% yield as a white solid: mp 247−248 °C. ^{1}H NMR (200 MHz, acetone-d_6): δ 3.40 (s, 3H, NCH_3), 7.25 (dd, J = 0.8 and 6.8 Hz, 1H, H-9), 7.60−7.85 (m, 6H, H-7, H-8, H-2′, H-4′, H-5′, and H-6′), 8.05 (dd, J = 1.2 and 7.5 Hz, 1H, H-6).

1-(4-Chlorophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8b). Following the general procedure method B, compound **8b** was obtained from **7b**[19] in 80% yield as a white solid: mp 259−260 °C. ^{1}H NMR (200 MHz, acetone-d_6): δ 3.30 (s, 3H, NCH_3), 7.20−7.25 (m, 1H, H-9), 7.65−7.85 (m, 6H, H-7, H-8, H-2′, H-3′, H-5′, and H-6′), 8.05−8.10 (m, 1H, H-6).

1-(2-Chlorophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8c). Following the general procedure method B, compound **8c** was obtained from **7c**[19] in 75% yield as a white solid: mp 224−226 °C. ^{1}H NMR (200 MHz, acetone-d_6): δ 3.40 (s, 3H, NCH_3), 6.95 (dd, J = 1.1 and 6.9 Hz, 1H, H-9), 7.65 (dt, J = 1.5 and 6.9 Hz, 1H, H-8), 7.70−7.85 (m, 4H,

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H-7, H-3′, H-4′, and H-5′), 7.90−7.95 (m, 1H, H-6′), 8.05 (dd, J = 1.5 and 8.1 Hz, 1H, H-6).

1-(3-Bromophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8d). Following the general procedure method B, compound **8d** was obtained from **7d** in 90% yield as a white solid: mp 228−230 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.25 (s, 3H, NCH_3), 7.05 (dd, J = 1.3 and 7.4 Hz, 1H, H-9), 7.50−7.60 (m, 2H, H-5′ and H-6′), 7.65−7.75 (m, 2H, H-7 and H-8), 7.80−7.90 (m, 2H, H-2′ and H-4′), 8.00 (dd, J = 1.7 and 7.0 Hz, 1H, H-6).

1-(4-Bromophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8e). Following the general procedure method B, compound **8e** was obtained from **7e** in 80% yield as a white solid: mp 255−257 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.10 (s, 3H, NCH_3), 7.05 (d, J = 7.5 Hz, 1H, H-9), 7.50−7.60 (m, 2H, H-3′ and H-5′), 7.65−7.80 (m, 2H, H-7 and H-8), 7.80−7.85 (m, 2H, H-2′ and H-6′), 8.05 (d, J = 7.0 Hz, 1H, H-6).

1-(2-Bromophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8f). Following the general procedure method B, compound **8f** was obtained from **7f** in 85% yield as a white solid: mp 274−276 °C. ^{1}H NMR (400 MHz, acetone-d_6): δ 3.30 (s, 3H, NCH_3), 6.90 (d, J = 7.6 Hz, 1H, H-9), 7.60 (dt, J = 1.2 and 8.0 Hz, 1H, H-4′), 7.70−7.80 (m, 3H, H-7, H-8, and H-5′), 7.80 (dd, J = 1.2 and 6.0 Hz, 1H, H-6′), 7.95 (dd, J = 1.3 and 8.0 Hz, 1H, H-3′), 8.00 (dd, J = 0.7 and 7.8 Hz, 1H, H-6).

4-Methyl-1-(3-methylphenyl)-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8g). Following the general procedure method B, compound **8g** was obtained from **7g** in 80% yield as a white solid: mp 238−240 °C. ^{1}H NMR (400 MHz, acetone-d_6): δ 2.50 (s, 3H, CH_3), 3.25 (s, 3H, NCH_3), 7.10 (d, J = 7.9 Hz, 1H, H-9), 7.40−7.60 (m, 4H, H-2′, H-4′, H-5′, and H-6′), 7.65 (dt, J = 1.0 and 7.6 Hz, 1H, H-8), 7.75 (dt, J = 1.1 and 7.6 Hz, 1H, H-7), 8.00 (dd, J = 1.0 and 7.6 Hz, 1H, H-6).

4-Methyl-1-(4-methylphenyl)-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8h). Following the general procedure method B, compound **8h** was obtained from **7h**[19] in 80% yield as a white solid: mp 260−261 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 2.40 (s, 3H, CH_3), 3.15 (s, 3H, NCH_3), 7.10 (dd, J = 1.2 and 7.0 Hz, 1H, H-9), 7.40−7−80 (m, 4H, H-2′, H-3′, H-5′, and H-6′), 7.80−7.90 (m, 2H, H-7 and H-8), 8.00 (d, J = 1.2 and 7.1 Hz, 1H, H-6).

4-Methyl-1-(2-methylphenyl)-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8i). Following the general procedure method B, compound **8i** was obtained from **7i** in 70% yield as a white solid: mp 214−215 °C. ^{1}H NMR (400 MHz, acetone-d_6): δ 2.00 (s, 3H, CH_3), 3.25 (s, 3H, NCH_3), 6.80 (dd, J = 1.0 and 7.0 Hz, 1H, H-9), 7.50−7.60 (m, 5H, H-8, H-3′, H-4′, H-5′, and H-6′), 7.70 (dt, J = 1.0 and 7.7 Hz, 1H, H-7), 8.00 (dd, J = 0.8 and 7.7 Hz, 1H, H-6).

1-(3-Methoxyphenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8j). Following the general procedure method B, compound **8j** was obtained from **7j** in 90% yield as a whitish solid: mp 202−204 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.10 (s, 3H, NCH_3), 3.80 (s, 3H, OCH_3), 6.90 (d, J = 7.6 Hz, 1H, H-9), 7.05 (dd, J = 1.7 and 7.4 Hz, 1H, H-4′), 7.15 (t, J = 2.2 Hz, 1H, H-2′), 7.20 (dd, J = 2.2 and 8.2 Hz, 1H, H-6′), 7.50 (t, J = 8.2 Hz, 1H, H-5′), 7.55−7.65 (m, 2H, H-7 and H-8), 7.90 (dd, J = 1.0 and 7.8 Hz, 1H, H-6).

1-(4-Methoxyphenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8k). Following the general procedure method B, compound **8k** was obtained from **7k** in 90% yield as a whitish solid: mp 244−245 °C. ^{1}H NMR (200 MHz, acetone-d_6): δ 3.30 (s, 3H, NCH_3), 3.95 (s, 3H, OCH_3), 7.10 (dd, J = 1.3 and 7.7 Hz, 1H, H-9), 7.20−7.30 (m, 2H, H-3′ and H-5′), 7.55−7.60 (m, 2H, H-2′ and H-6′), 7.65 (dt, J = 1.4 and 7.7 Hz, 1H, H-8), 7.75 (dt, J = 1.3 and 7.6 Hz, 1H, H-7), 8.05 (dd, J = 1.4 and 7.6 Hz, H-6).

4-Methyl-1-[4-(trifluoromethyl)phenyl]-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8m). Following the general procedure method B, compound **8m** was obtained from **7m** in 90% yield as a whitish solid: mp 187−189 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.20 (s, 3H, NCH_3), 7.10 (d, J = 7.4 Hz, 1H, H-9), 7.65−7.75 (m, 2H, H-7 and H-8), 7.80−7.85 (m, 2H, H-2′ and H-6′), 8.00−8.05 (m, 3H, H-6, H-3′, and H-5′).

4-Methyl-1-[2-(trifluoromethyl)phenyl]-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8n). Following the general procedure method B, compound **8n** was obtained from **7n** in 70% yield as a whitish solid: mp 228−230 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.15 (s, 3H, NCH_3), 6.60 (d, J = 7.9 Hz, 1H, H-9), 7.60 (dt, J = 1.3 and 7.9 Hz, 1H, H-8), 7.65 (dt, J = 1.0 and 7.7 Hz, 1H, H-7), 7.85 (d, J = 7.2 Hz, 1H, H-3′), 7.90−8.00 (m, 3H, H-4′, H-5′, and H-6′), 8.05 (dd, J = 1.3 and 7.6 Hz, 1H, H-6).

1-(3,4-Difluorophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8o). Following the general procedure method B, compound **8o** was obtained from **7o** in 80% yield as a whitish solid: mp 244−246 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.10 (s, 3H, NCH_3), 6.95−7.05 (m, 1H, H-9), 7.35−7.45 (m, 1H, H-6′), 7.60−7.75 (m, 3H, H-7, H-8, and H-2′), 7.85−7.90 (m, 1H, H-5′), 7.95−8.00 (m, 1H, H-6), 13.50 (bs, 1H, COOH).

1-(3,4-Dichlorophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8p). Following the general procedure method B, compound **8p** was obtained from **7p** in 90% yield as a whitish solid: mp 245−247 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.15 (s, 3H, NCH_3), 7.15 (dd, J = 1.5 and 7.5 Hz, 1H, H-9), 7.55 (dd, J = 2.6 and 8.7 Hz, 1H, H-6′), 7.65−7.80 (m, 2H, H-7 and H-8), 7.85 (d, J = 8.7 Hz, 1H, H-5′), 7.90−8.05 (m, 2H, H-6 and H-2′).

1-(3,4-Dimethylphenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8q). Following the general procedure method B, compound **8q** was obtained from **7q** in 80% yield as a whitish solid: mp 258−260 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 and 2.30 (s, each 3H, CH_3), 3.15 (s, 3H, NCH_3), 6.90 (d, J = 7.6 Hz, 1H, H-9), 7.23 (dd, J = 2.0 and 7.9 Hz, 1H, H-6′), 7.30−7.35 (m, 2H, H-2′ and H-5′), 7.60−7.70 (m, 2H, H-7 and H-8), 7.90 (d, J = 7.6 Hz, 1H, H-6).

1-(3-Chloro-4-methylphenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8r). Following the general procedure method B, compound **8r** was obtained from **7r**, in 80% yield as a whitish solid: mp 244−246 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.40 (s, 3H, CH_3), 3.20 (s, 3H, NCH_3), 7.00 (d, J = 7.5 Hz, 1H, H-9), 7.35−7.40 (m, 1H, H-6′), 7.55 (d, J = 7.5 Hz, 1H, H-5′), 7.60−7.70 (m, 3H, H-7, H-8, and H-2′), 7.90−8.00 (m, 1H, H-6).

1-[4-Chloro-3-(trifluoromethyl)phenyl]-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8s). Following the general procedure method B, compound **8s** was obtained from **7s**, in 75% yield as a whitish solid: mp 242−244 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.15 (s, 3H, NCH_3), 7.10 (d, J = 7.7 Hz, 1H, H-9), 7.60−7.75 (m, 2H, H-7 and H-8), 7.84 (dd, J = 2.4 and 8.6 Hz, 1H, H-6′), 7.90 (d, J = 8.6 Hz, 1H, H-5′), 8.00 (dd, J = 1.0 and 7.3 Hz, 1H, H-6), 8.10 (d, J = 2.4 Hz, 1H, H-2′), 13.75 (bs, 1H, COOH).

1-Cyclohexyl-4-methyl-1,4-dihydropyrazolo[4,3-*c*][1,2]-benzothiazine-3-carboxylic Acid 5,5-Dioxide (8t). Following the general procedure method B, compound **8t** was obtained from **7t**, in 70% yield as a white solid: mp 229−231 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.30−1.70 (m, 4H, Cy-CH_2), 2.00−2.45 (m, 6H, Cy-CH_2), 3.30 (s, 3H, NCH_3), 4.50−4.75 (m, 1H, Cy-CH), 7.60−7.90 (m, 3H, H-7, H-8, and H-9), 8.10 (d, J = 7.7 Hz, 1H, H-6).

4-Methyl-1,4-dihydropyrazolo[4,3-c][1,2]benzothiazine-3-carboxylic Acid 5,5-Dioxide (8u). Following the general procedure method B, compound **8u** was obtained from **7u**, in 84% yield as yellow solid: mp 169−172 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.10 (s, 3H, NCH_3), 7.60−7.65 (m, 1H, H-8), 7.80−7.95 (m, 2H, H-7 and H-9), 8.05 (d, J = 7.7 Hz, 1H, H-6), 14.50 (s, 1H, pyrazolo-NH).

General Procedure for Amidation Reaction with *N*-(4-aminophenyl)methanesulfonamide. Method C. A mixture of the acid intermediates **8a−u** (1 mmol) in an excess of $SOCl_2$ was refluxed under stirring for 1 h. The solvent was distilled under reduced pressure, and the residues were washed three times with dry benzene. The corresponding pyrazolobenzothiazine-3-carbonyl chloride was

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immediately dissolved in dry DMF (2 mL) and added dropwise, under nitrogen flux, to a solution of *N*-(4-aminophenyl)-methanesulfonamide[20] (2 mmol) and Et_3N (2 mmol) in dry DMF (7 mL). The mixture was heated at 40 °C for 24 h. The solvent was then concentrated to half volume, and the mixture was poured into ice−water and acidified with 2 N HCl (pH 3). The precipitate formed was filtered under vacuum. The solid obtained was crystallized by EtOH/DMF (2:1) to give the target pyrazolobenzothiazine-3-carboxyamides **2a−s** or purified by flash column chromatography in the cases of compounds **2t** and **2u**.

1-(3-Chlorophenyl)-4-methyl-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2a). Following the general procedure method C, compound **2a** was obtained, from **8a**, in 40% yield as a white solid: mp 297−299 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.90 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 7.05 (dd, J = 1.3 and 7.9 Hz, 1H, H-9), 7.10−7.20 (m, 2H, H-3″ and H-5″), 7.50−7.55 (m, 1H, H-4′), 7.60 (t, J = 7.8 Hz, 1H, H-5′), 7.65−7.75 (m, 5H, H-7, H-8, H-6′, H-2″, and H-6″), 7.85 (t, J = 2.0 Hz, 1H, H-2′), 8.00 (dd, J = 1.5 and 7.0 Hz, 1H, H-6), 9.60 (s, 1H, SO_2NH), 10.40 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.12, 39.16, 121.26, 122.15, 123.33, 124.87, 124.95, 125.16, 126.18, 126.82, 130.48, 130.57, 130.76, 130.85, 132.04, 133.49, 134.65, 134.70, 135.02, 139.73, 140.37, 158.69. Anal. Calcd for $C_{24}H_{20}ClN_5O_5S_2$: C, 51.66; H, 3.61; N, 12.55. Found: C, 51.42; H, 3.80; N, 12.45.

1-(4-Chlorophenyl)-4-methyl-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2b). Following the general procedure method C, compound **2b** was obtained, from **8b**, in 30% yield as a white solid: mp 284−286 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.90 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 7.00 (d, J = 7.7 Hz, 1H, H-9), 7.20−7.25 (m, 2H, H-3″ and H-5″), 7.65−7.75 (m, 6H, H-7, H-8, H-2′, H-3′, H-5′, and H-6′), 7.75−7.80 (m, 2H, H-2″ and H-6″), 8.05 (dd, J = 1.5 and 7.8 Hz, 1H, H-6), 9.60 (s, 1H, SO_2NH), 10.50 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.15, 39.56, 121.30, 122.12, 123.39, 124.95, 125.16, 126.77, 127.97, 130.55, 130.59, 130.69, 130.87, 133.52, 134.68, 134.92, 135.06, 138.09, 139.79, 158.76. Anal. Calcd for $C_{24}H_{20}ClN_5O_5S_2$: C, 51.66; H, 3.61; N, 12.55. Found: C, 51.39; H, 3.86; N, 13.05.

1-(2-Chlorophenyl)-4-methyl-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2c). Following the general procedure method C, compound **2c** was obtained, from **8c**, in 30% yield as a white solid: mp 250−252 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.00 (s, 3H, SO_2CH_3), 3.50 (s, 3H, NCH_3), 6.55 (s, 1H, SO_2NH), 6.80 (dd, J = 7.9 Hz, 1H, H-9), 7.20−7.25 (m, 2H, H-3″ and H-5″), 7.45 (t, J = 7.9 Hz, 1H, H-8), 7.55−7.75 (m, 7H, H-7, H-3′, H-4′, H-5′, H-6′, H-2″, and H-6″), 8.05 (d, J = 7.8 Hz, 1H, H-6) 8.75 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): δ 39.12, 39.16, 121.18, 122.47, 122.91, 123.52, 125.05, 126.09, 128.45, 129.26, 129.76, 131.15, 131.48, 131.91, 132.04, 132.30, 132.40, 132.74, 135.23, 137.00, 139.10, 157.79. Anal. Calcd for $C_{24}H_{20}ClN_5O_5S_2$: C, 51.66; H, 3.61; N, 12.55. Found: C, 51.93; H, 3.89; N, 12.30.

1-(3-Bromophenyl)-4-methyl-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2d). Following the general procedure method C, compound **2d** was obtained, from **8d**, in 60% yield as a white solid: mp 297−300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 7.05 (dd, J = 1.3 and 7.4 Hz, 1H, H-9), 7.15−7.20 (m, 2H, H-3″ and H-5″), 7.55−7.65 (m, 2H, H-4′ and H-5′), 7.70−7.80 (m, 4H, H-7, H-8, H-2″, and H-6″), 7.85−7.90 (m, 1H, H-6′), 8.00−8.05 (m, 2H, H-6 and H-2′), 9.65 (s, 1H, SO_2NH), 10.50 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.13, 39.32, 121.25, 122.16, 122.85, 123.33, 124.95, 125.16, 125.21, 126.81, 128.93, 130.55, 130.76, 130.83, 132.23, 133.36, 133.48, 134.71, 135.01, 139.73, 140.42, 158.68. Anal. Calcd for $C_{24}H_{20}BrN_5O_5S_2$: C, 47.85; H, 3.35; N, 11.62. Found: C, 48.08; H, 3.62; N, 11.30.

1-(4-Bromophenyl)-4-methyl-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2e). Following the general procedure method C, compound **2e** was obtained, from **8e**, in 30% yield as a white solid: mp 243−245 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 7.05 (dd, J = 1.6 and 7.6 Hz, 1H, H-9), 7.15−7.25 (m, 2H, H-3″ and H-5″), 7.60−7.65 (m, 2H, H-3′ and H-5′), 7.70−7.80 (m, 4H, H-7, H-8, H-2″, and H-6″), 7.85−7.90 (m, 2H, H-2′ and H-6′), 8.00−8.05 (m, 1H, H-6), 9.65 (s, 1H, SO_2NH), 10.45 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.15, 39.36, 121.28, 122.11, 123.39, 123.41, 124.98, 125.16, 126.81, 128.15, 130.53, 130.70, 130.85, 133.48, 133.54, 134.70, 135.05, 138.51, 139.83, 158.75. Anal. Calcd for $C_{24}H_{20}BrN_5O_5S_2$: C, 47.85; H, 3.35; N, 11.62. Found: C, 47.73; H, 3.26; N, 11.80.

1-(2-Bromophenyl)-4-methyl-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2f). Following the general procedure method C, compound **2f** was obtained, from **8f**, in 50% yield as a white solid: mp 240−242 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.95 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 6.80 (d, J = 7.6 Hz, 1H, H-9), 7.15−7.20 (m, 2H, H-3″ and H-5″), 7.65−7.80 (m, 6H, H-7, H-8, H-4′, H-5′, H-2″, and H-6″), 7.95 (dd, J = 1.0 and 7.3 Hz, 1H, H-3′), 8.00 (dd, J = 1.1 and 7.2 Hz, 1H, H-6′), 8.05 (dd, J = 1.0 and 7.8 Hz, 1H, H-6), 9.65 (s, 1H, SO_2NH), 10.45 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.37, 39.39, 121.24, 121.34, 122.30, 123.22, 123.50, 125.22, 125.96, 130.15, 130.63, 130.87, 130.90, 131.88, 133.28, 133.68, 134.38, 134.67, 135.03, 138.52, 139.71, 158.67. Anal. Calcd for $C_{24}H_{20}BrN_5O_5S_2$: C, 47.85; H, 3.35; N, 11.62. Found: C, 47.90; H, 3.40; N, 11.50.

4-Methyl-1-(3-methylphenyl)-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2g). Following the general procedure method C, compound **2g** was obtained, from **8g**, in 60% yield as yellowish solid: mp 283−285 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.40 (s, 3H, CH_3), 3.00 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 7.05 (d, J = 7.6 Hz, 1H, H-9), 7.20−7.25 (m, 2H, H-3″ and H-5″), 7.45 (d, J = 7.7 Hz, 1H, H-4′), 7.50−7.60 (m, 3H, H-2, H-5′, and H-6′), 7.70 (dt, J = 1.4 and 7.8 Hz, 1H, H-8), 7.75 (dt, J = 1.2 and 7.8 Hz, 1H, H-7), 7.80−7.85 (m, 2H, H-2″ and H-6″), 8.05 (dd, J = 1.4 and 7.8 Hz, 1H, H-6), 9.75 (s, 1H, SO_2NH), 10.60 (s, 1H, NHCO). ^{13}C NMR (DMSO-d_6): δ 21.21, 39.22, 39.33, 121.28, 122.12, 123.34, 123.61, 124.71, 125.14, 126.52, 126.62, 130.25, 130.46, 130.57, 130.81, 131.16, 133.35, 134.62, 135.11, 139.27, 139.34, 140.46, 158.86. Anal. Calcd for $C_{25}H_{23}N_5O_5S_2$: C, 55.85; H, 4.31; N, 13.03. Found: C, 56.01; H, 4.50; N, 12.85.

4-Methyl-1-(4-methylphenyl)-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2h). Following the general procedure method C, compound **2h** was obtained, from **8h**, in 40% yield as a white solid: mp 279−281 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.50 (s, 3H, CH_3), 3.00 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 6.95 (dd, J = 1.0 and 7.7 Hz, 1H, H-9), 7.20−7.25 (m, 2H, H-3″ and H-5″), 7.40−7.45 (m, 2H, H-3′ and H-5′), 7.50−7.55 (m, 2H, H-2′ and H-6′), 7.65 (dt, J = 1.4 and 7.7 Hz, 1H, H-8), 7.70 (dt, J = 1.0 and 7.7 Hz, 1H, H-7), 7.75−7.80 (m, 2H, H-2″ and H-6″), 8.05 (dd, J = 1.4 and 7.7 Hz, 1H, H-6), 9.60 (s, 1H, SO_2NH), 10.45 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 21.28, 39.33, 39.51, 121.29, 122.11, 123.65, 124.68, 125.14, 126.09, 126.46, 130.47, 130.53, 130.83, 130.90, 133.36, 134.62, 135.12, 136.92, 139.32, 140.33, 158.88. Anal. Calcd for $C_{25}H_{23}N_5O_5S_2$: C, 55.85; H, 4.31; N, 13.03. Found: C, 55.71; H 4.25; N 13.18.

4-Methyl-1-(2-methylphenyl)-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2i). Following the general procedure method C, compound **2i** was obtained, from **8i**, in 60% yield as a white solid: mp 251−253 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.00 (s, 3H, CH_3), 3.00 (s, 3H, SO_2CH_3), 3.40 (s, 3H, NCH_3), 6.75 (d, J = 7.9 Hz, 1H, H-9), 7.25−7.30 (m, 2H, H-3″ and H-5″), 7.45−7.75 (m, 6H, H-7, H-8, H-3′, H-4′, H-5′,and H-6′), 7.75−7.80 (m, 2H, H-2″ and H-6″), 8.05 (d, J = 7.7 Hz, 1H, H-6), 9.65 (s, 1H, SO_2NH), 10.50 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 17.08, 39.27, 39.32, 121.25, 122.19, 123.44, 123.57, 125.14, 125.81, 128.12, 128.20, 130.74, 130.94, 131.31, 131.48, 132.07, 133.63, 134.60, 135.10, 135.42, 138.58, 139.34, 158.84. Anal. Calcd for $C_{25}H_{23}N_5O_5S_2$: C, 55.85; H, 4.31; N, 13.03. Found: C, 56.14; H, 4.60; N, 12.75.

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1-(3-Methoxyphenyl)-4-methyl-*N*-{4-[(methylsulfonyl)-amino]phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2j). Following the general procedure method C, compound **2j** was obtained, from **8j**, in 30% yield as a white solid: mp 286−288 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.25 (s, 3H, NCH_3), 3.80 (s, 3H, OCH_3), 7.05 (dd, J = 1.1 and 7.0 Hz, 1H, H-9), 7.15 (dd, J = 2.0 and 8.0 Hz, 1H, H-4′), 7.15−7.20 (m, 2H, H-3″ and H-5″), 7.25 (dd, J = 2.0 and 8.2 Hz, 1H, H-6′), 7.30 (t, J = 2.0 Hz, 1H, H-2′), 7.55 (t, J = 8.2 Hz, 1H, H-5′), 7.65−7.75 (m, 2H, H-7 and H-8), 7.75−7.80 (m, 2H, H-2″ and H-6″), 8.05 (dd, J = 1.4 and 7.5 Hz, 1H, H-6), 9.60 (s, 1H, SO_2NH), 10.50 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.22, 39.35, 56.21, 111.87, 116.50, 118.35, 121.28, 122.14, 123.53, 124.81, 125.12, 126.52, 130.52, 130.61, 130.81, 131.32, 133.38, 134.65, 135.09, 139.35, 140.33, 158.84, 160.70. Anal. Calcd for $C_{25}H_{23}N_5O_6S_2$: C, 54.24; H, 4.19; N, 12.65. Found: C, 54.48; H, 4.32; N, 12.38.

1-(4-Methoxyphenyl)-4-methyl-*N*-{4-[(methylsulfonyl)-amino]phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2k). Following the general procedure method C, compound **2k** was obtained, from **8k**, in 40% yield as a white solid: mp 269−271 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.25 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 6.95 (dd, J = 1.2 and 7.5 Hz, 1H, H-9), 7.15−7.25 (m, 4H, H-3′, H-5′, H-3″, and H-5″), 7.55−7.60 (m, 2H, H-2′ and H-6′), 7.65−7.75 (m, 2H, H-7 and H-8), 7.75−7.80 (m, 2H, H-2″ and H-6″), 8.00 (dd, J = 1.4 and 7.6 Hz, 1H, H-6), 9.60 (s, 1H, SO_2NH), 10.40 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.23, 39.34, 56.13, 115.53, 121.29, 122.12, 123.69, 124.58, 125.15, 126.24, 127.90, 130.49, 130.64, 130.88, 132.21, 133.40, 134.60, 135.15, 139.14, 158.91, 160.65. Anal. Calcd for $C_{25}H_{23}N_5O_6S_2$: C, 54.24; H, 4.19; N, 12.65. Found: C, 53.04; H, 4.06; N, 12.78.

4-Methyl-*N*-{4-[(methylsulfonyl)amino]phenyl}-1-[3-(trifluoromethyl)phenyl]-1,4-dihydropyrazolo[4,3-*c*][1,2]-benzothiazine-3-carboxamide 5,5-Dioxide (2l). Following the general procedure method C, compound **2l** was obtained, from **8l**,[19] in 60% yield as a white solid: mp 200−202 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 7.00 (d, J = 7.6 Hz, 1H, H-9), 7.15−7.20 (m, 2H, H-3″ and H-5″), 7.65−7.75 (m, 4H, H-7, H-8, H-2″, and H-6″), 7.80−7.90 (m, 2H, H-5′ and H-6′), 7.95−8.00 (m, 2H, H-6 and H-4′), 8.15−8.20 (bs, 1H, H-2′), 9.70 (s, 1H, SO_2NH), 10.60 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.15, 39.33, 121.27, 122.19, 123.09−123.13 (m, C-2′), 123.28, 123.90 (q, J_{C-F} = 270 Hz, CF_3), 124.92, 125.22, 126.96, 127.07−127.11 (m, C-4′), 130.20, 130.68, 130.82, 131.14 (q, J_{C-F} = 32 Hz, C-3′), 131.82, 133.45, 134.73, 134.99, 139.81, 140.00, 158.69. Anal. Calcd for $C_{25}H_{20}F_3N_5O_5S_2$: C, 50.76; H, 3.41; N, 11.84. Found: C, 50.80; H, 3.46; N, 11.74.

4-Methyl-*N*-{4-[(methylsulfonyl)amino]phenyl}-1-[4-(trifluoromethyl)phenyl]-1,4-dihydropyrazolo[4,3-*c*][1,2]-benzothiazine-3-carboxamide 5,5-Dioxide (2m). Following the general procedure method C, compound **2m** was obtained, from **8m**, in 60% yield as a white solid: mp 285−286 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.95 (s, 3H, SO_2CH_3), 3.25 (s, 3H, NCH_3), 7.10 (d, J = 7.4 Hz, 1H, H-9), 7.15−7.25 (m, 2H, H-3″ and H-5″), 7.70−7.80 (m, 4H, H-7, H-8, H-2″, and H-6″), 7.90−7.95 (m, 2H, H-2′ and H-6′), 8.00−8.10 (m, 3H, H-6, H-3′, and H-5′), 9.60 (s, 1H, SO_2NH), 10.50 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.00, 39.11, 121.27, 122.09, 123.29, 125.17−125.22 (m, C-2′ and C-6′), 126.10 (q, J_{C-F} = 300 Hz, CF_3), 126.69, 127.16, 127.70−127.75 (m, C-3′ and C-5′), 130.49 (q, J = 32 Hz, C-4′), 130.57, 130.80, 130.83, 133.59, 134.75, 134.98, 140.24, 142.36, 158.67. Anal. Calcd for $C_{25}H_{20}F_3N_5O_5S_2$: C, 50.76; H, 3.41; N, 11.84. Found: C, 50.96; H, 3.58; N, 11.66.

4-Methyl-*N*-{4-[(methylsulfonyl)amino]phenyl}-1-[2-(trifluoromethyl)phenyl]-1,4-dihydropyrazolo[4,3-*c*][1,2]-benzothiazine-3-carboxamide 5,5-Dioxide (2n). Following the general procedure method C, compound **2n** was obtained, from **8n**, in 30% yield as a white solid: mp 255−257 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 6.70 (d, J = 8.0 Hz, 1H, H-9), 7.15−7.20 (m, 2H, H-3″ and H-5″), 7.65 (dt, J = 1.2 and 7.9 Hz, 1H, H-8), 7.70 (dt, J = 1.2 and 7.9 Hz, 1H, H-7), 7.75−7.80 (m, 2H, H-2″, and H-6″), 7.95−8.05 (m, 4H, H-3′, H-4′, H-5′, and H-6′), 8.10−8.15 (m, 1H, H-6), 9.70 (s, 1H, SO_2NH), 10.50 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.33, 39.37, 121.21, 122.31, 123.05 (q, J_{C-F} = 270 Hz, CF_3), 123.28, 123.68, 125.26, 126.03, 126.68 (q, J_{C-F} = 30 Hz, C-2′), 128.63−128.68 (m, C-6′), 130.84, 130.98, 131.28, 132.54, 132.57, 133.62, 134.69, 134.96, 135.24, 136.62, 139.63, 158.61. Anal. Calcd for $C_{25}H_{20}F_3N_5O_5S_2$: C, 50.76; H, 3.41; N, 11.84. Found: C, 50.67; H, 3.36; N, 12.00.

1-(3,4-Difluorophenyl)-4-methyl-*N*-{4-[(methylsulfonyl)-amino]phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2o). Following the general procedure method C, compound **2o** was obtained, from **8o**, in 70% yield as a white solid: mp 290−292 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.95 (s, 3H, SO_2CH_3), 3.30 (s, 3H, NCH_3), 7.10 (d, J = 7.5 Hz, 1H, H-9), 7.15−7.25 (m, 2H, H-3″ and H-5″), 7.50−7.55 (m, 1H, H-6′), 7.65−7.85 (m, 5H, H-7, H-8, H-2′, H-2″, and H-6″), 7.95−8.05 (m, 2H, H-6 and H-5′), 9.65 (s, 1H, SO_2NH), 10.50 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.20, 39.30, 116.62 (d, J_{C-F} = 20 Hz, C-2′), 119.27 (d, J_{C-F} = 18.6 Hz, C-5′), 121.26, 122.13, 123.23, 123.65−123.80 (m, C-6′), 125.00, 125.18, 126.62, 130.77, 130.83, 130.85, 133.62, 134.69, 135.02, 135.75 (dd, J_{C-F} = 3.4 and 8.3 Hz, C-1′), 139.69, 150.10 (dd, J_{C-F} = 13.1 and 247.5 Hz, C-4′), 150.80 (dd, J_{C-F} = 12.2 and 248 Hz, C-3′), 158.67. Anal. Calcd for $C_{24}H_{19}F_2N_5O_5S_2$: C, 51.51; H, 3.42; N, 12.52. Found: C, 51.76; H, 3.67; N, 12.22.

1-(3,4-Dichlorophenyl)-4-methyl-*N*-{4-[(methylsulfonyl)-amino]phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2p). Following the general procedure method C, compound **2p** was obtained, from **8p**, in 40% yield as a white solid: mp 259−261 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.25 (s, 3H, NCH_3), 7.20−7.25 (m, 3H, H-9, H-3″, and H-5″), 7.60 (dd, J = 2.3 and 8.6 Hz, 1H, H-6′), 7.70−7.80 (m, 4H, H-7, H-8, H-2″, and H-6″), 7.85 (d, J = 8.6 Hz, 1H, H-5′), 8.00 (dd, J = 1.1 and 7.2 Hz, 1H, H-6), 8.10 (d, J = 2.3 Hz, 1H, H-2′), 9.60 (s, 1H, SO_2NH), 10.45 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.09, 39.33, 121.25, 122.15, 123.20, 125.15, 125.21, 126.14, 126.95, 128.07, 130.61, 130.82, 130.83, 132.21, 132.93, 133.09, 133.63, 134.74, 134.97, 138.80, 139.90, 158.61. Anal. Calcd for $C_{24}H_{19}Cl_2N_5O_5S_2$: C, 48.65; H, 3.23; N, 11.82. Found: C, 48.33; H, 2.29; N, 12.06.

1-(3,4-Dimethylphenyl)-4-methyl-*N*-{4-[(methylsulfonyl)-amino]phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2q). Following the general procedure method C, compound **2q** was obtained, from **8q**, in 40% yield as a white solid: mp 301−303 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 and 2.30 (s, each 3H, CH_3), 2.95 (s, 3H, SO_2CH_3), 3.20 (s, 3H, NCH_3), 7.00 (d, J = 8.0 Hz, 1H, H-9), 7.15−7.20 (m, 2H, H-3″ and H-5″), 7.30 (dd, J = 2.0 and 8.0 Hz, 1H, H-6′), 7.40 (d, J = 8.0 Hz, 1H, H-5′), 7.45 (d, J = 2.0 Hz, 1H, H-2′), 7.65−7.75 (m, 2H, H-7 and H-8), 7.75−7.80 (m, 2H, H-2″ and H-6″), 8.00 (dd, J = 1.3 and 7.8 Hz, 1H, H-6), 9.50 (s, 1H, SO_2NH), 10.35 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 19.66, 19.70, 39.21, 39.36, 121.29, 122.11, 123.46, 123.70, 124.66, 125.11, 126.40, 126.89, 130.39, 130.51, 130.81, 131.12, 133.36, 134.61, 135.12, 137.11, 138.99, 139.08, 139.18, 158.89. Anal. Calcd for $C_{26}H_{25}N_5O_5S_2$: C, 56.61; H, 4.57; N, 12.70. Found: C, 56.59; H, 4.53; N, 12.75.

1-(3-Chloro-4-methylphenyl)-4-methyl-*N*-{4-[(methylsulfonyl)amino]phenyl}-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2r). Following the general procedure method C, compound **2r** was obtained, from **8r**, in 40% yield as a white solid: mp 287−289 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.45 (s, 3H, CH_3), 2.95 (s, 3H, SO_2CH_3), 3.20 (s, 3H, NCH_3), 7.10 (dd, J = 1.4 and 7.5 Hz, 1H, H-9), 7.15−7.20 (m, 2H, H-3″ and H-5″), 7.50 (dd, J = 2.1 and 8.0 Hz, 1H, H-6′), 7.60 (d, J = 8.0 Hz, 1H, H-5′), 7.65−7.80 (m, 4H, H-7, H-8, H-2″, and H-6″), 7.85 (d, J = 2.1 Hz, 1H, H-2′), 8.05 (dd, J = 1.6 and 7.3 Hz, 1H, H-6), 9.60 (s, 1H, SO_2NH), 10.40 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 19.92, 39.12, 39.33, 121.26, 122.14, 123.41, 124.72, 124.90, 125.14, 126.41, 126.69, 130.49, 130.70, 130.82, 132.68, 133.48, 134.63, 134.68, 135.03, 138.10, 138.14, 139.55, 158.72. Anal. Calcd for $C_{25}H_{22}ClN_5O_5S_2$: C, 52.49; H, 3.88; N, 12.24. Found: C, 52.89; H, 4.93; N, 12.15.

1-[4-Chloro-3-(trifluoromethyl)phenyl]-4-methyl-*N*-{4-[(methylsulfonyl)amino]phenyl}-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2s). Following

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the general procedure method C, compound **2s** was obtained, from **8s**, in 50% yield as a white solid: mp 288−290 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.95 (s, 3H, SO_2CH_3), 3.20 (s, 3H, NCH_3), 7.10−7.20 (m, 3H, H-9, H-3″, and H-5″), 7.65−7.80 (m, 4H, H-7, H-8, H-2″, and H-6″), 7.85 (dd, J = 2.5 and 8.6 Hz, 1H, H-6′), 7.95 (d, J = 8.6 Hz, 1H, H-5′), 8.00 (dd, J = 1.2 and 7.4 Hz, 1H, H-9), 8.25 (d, J = 2.5 Hz, 1H, H-2′), 9.60 (s, 1H, SO_2NH), 10.50 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.08, 39.34, 121.25, 122.22, 122.68 (q, J_{C-F} = 272 Hz, CF_3), 123.14, 125.17, 125.28, 125.60−125.70 (m, C-2′), 127.11, 128.63 (q, J_{C-F} = 31.4 Hz, C-3′), 130.70, 130.86, 130.89, 131.28 (bs, C-5′), 132.35 (bs, C-4′), 133.60, 133.74, 134.78, 134.93, 138.25, 140.15, 158.58. Anal. Calcd for $C_{25}H_{19}ClF_3N_5O_5S_2$: C, 47.96; H, 3.06; N, 11.19. Found: C, 47.76; H, 3.18; N, 11.02.

1-Cyclohexyl-4-methyl-*N*-{4-[(methylsulfonyl)amino]-phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxyamide 5,5-Dioxide (2t). Following the general procedure method C, compound **2t** was obtained, from **8t**, as a white solid in 40% yield after purification by flash column chromatography eluting with $CHCl_3$/MeOH (95:5): mp 254−256 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.25−1.35 (m, 2H, Cy-CH_2), 1.50−1.65 (m, 2H, Cy-CH_2), 1.70−1.80 (m, 2H, Cy-CH_2), 1.85−1.95 (m, 4H, Cy-CH_2), 2.95 (s, 3H, SO_2CH_3), 3.25 (s, 3H, NCH_3), 4.70−4.80 (m, 1H, Cy-CH), 7.15−7.25 (m, 2H, H-3″, and H-5″), 7.70−7.85 (m, 3H, H-8, H-2″, and H-6″), 7.90−8.05 (m, 3H, H-6, H-7, and H-9), 9.60 (s, 1H, SO_2NH), 10.10 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 25.05, 25.15, 32.93, 38.99, 39.35, 60.63, 121.22, 122.29, 123.77, 124.90, 125.28, 125.55, 130.20, 130.25, 131.17, 134.15, 134.61, 135.02, 137.59, 159.07. Anal. Calcd for $C_{24}H_{27}N_5O_5S_2$: C, 54.43; H, 5.14; N, 13.22. Found: C, 54.63; H, 5.34; N, 13.01.

4-Methyl-*N*-{4-[(Methylsulfonyl)amino]phenyl}-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (2u). Following the general procedure method C, compound **2u** was obtained from **8u** as yellow solid in 60% yield after purification by flash column chromatography, eluting with $CHCl_3$/acetone (6:4): mp 298−299 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.40 (s, 3H, NCH_3), 7.15−7.20 (m, 2H, H-3″ and H-5″), 7.70−7.80 (m, 3H, H-8, H-2″, and H-6″), 7.85−7.95 (m, 2H, H-7 and H-9), 8.05 (d, J = 7.7 Hz, 1H, H-6), 9.50 (bs, 1H, SO_2NH), 10.40 (s, 1H, CONH), 14.50 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 39.22, 39.35, 114.70, 115.50, 121.41, 121.90, 124.30, 124.56, 124.78, 127.13, 130.34, 130.52, 133.78, 134.45, 135.34, 159.10. Anal. Calcd for $C_{18}H_{17}N_5O_4S_2$: C, 50.10; H, 3.97; N, 16.23. Found: C, 49.90; H, 3.76; N, 16.52.

1-(3-Fluorophenyl)-4-methyl-*N*-phenyl-1,4-dihydropyrazolo[4,3-c][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (3). The title compound was prepared following the general procedure method C. Starting from compound **9**[17] and replacing the *N*-(4-aminophenyl)methanesulfonamide with the aniline, compound **3** was obtained as a white solid in 70% yield after purification by flash chromatography, eluting with $CHCl_3$/MeOH (95:5): mp 202−204 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.25 (s, 3H, NCH_3), 7.05 (dd, J = 1.2 and 7.6 Hz, 1H, H-9), 7.10 (t, J = 7.4 Hz, 1H, H-4″), 7.35 (t, J = 7.5 Hz, 2H, H-3″ and H-5″), 7.45 (dd, J = 1.0 and 7.9 Hz, 1H, H-6′), 7.55 (dt, J = 1.8 and 8.5 Hz, 1H, H-4′), 7.65−7.75 (m, 4H, H-7, H-8, H-2″, and H-6″), 7.80−7.85 (m, 2H, H-2′ and H-6′), 8.05 (dd, J = 1.8 and 7.8 Hz, 1H, H-6), 10.30 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 39.15, 113.88 (d, J_{C-F} = 25 Hz, C-2′), 117.55 (d, J_{C-F} = 21 Hz, C-4′), 121.12, 122.44 (d, J_{C-F} = 3 Hz, C-6′), 123.36, 124.55, 124.91, 125.17, 126.79, 129.08, 130.61, 130.74, 130.86, 132.28 (d, J_{C-F} = 9.1 Hz, C-5′), 133.47, 138.63, 139.76, 140.51 (d, J_{C-F} = 10.2 Hz, C-1′), 158.88, 162.74 (d, J_{C-F} = 245.3 Hz, C-3′). Anal. Calcd for $C_{23}H_{17}FN_4O_3S$: C, 61.60; H, 3.82; N, 12.49. Found: C, 61.72; H, 3.93; N, 12.36.

***N*-[4-(Amino-2-oxoethoxy)phenyl]-1-(3-fluorophenyl)-4-methyl-1,4-dihydropyrazolo[4,3*c*][1,2]benzothiazine-3-carboxamide 5,5-Dioxide (4).** The title compound was prepared following the general procedure method C. Starting from **9**[17] and replacing the *N*-(4-aminophenyl)methanesulfonamide with the 2-(4-aminophenoxy)acetamide,[21] compound **4** was obtained as a white solid in 60% yield after purification by flash column chromatography, eluting with CH_2Cl_2/MeOH (98:2): mp 223−225 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.35 (s, 3H, NCH_3), 4.50 (s, 2H, OCH_2), 6.90−7.00 (m, 2H, H-3″ and H-5″), 7.05 (d, J = 7.7 Hz, 1H, H-9), 7.40 (bs, 1H, NH), 7.45 (dd, J = 1.8 and 7.3 Hz, 1H, H-4′), 7.50−7.55 (m, 2H, H-8 and NH), 7.65−7.75 (m, 6H, H-7, H-2′, H-5′, H-6′, H-2″, and H-6″), 8.00 (dd, J = 1.4 and 7.7 Hz, 1H, H-6), 10.40 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 39.16, 67.41, 113.88 (d, J_{C-F} = 25 Hz, C-2′), 115.10, 117.53 (d, J_{C-F} = 21 Hz, C-4′), 122.47 (bs, C-6′), 122.63, 123.37, 124.90, 125.16, 126.70, 130.57, 130.72, 130.84, 132.19, 132.28 (d, J_{C-F} = 9 Hz, C-5′), 133.47, 139.86, 140.53 (d, J_{C-F} = 10.3 Hz, C-1′), 154.76, 158.56, 162.73 (d, J_{C-F} = 245.4 Hz, C-3′), 170.42. Anal. Calcd for $C_{25}H_{20}FN_5O_5S$: C, 57.58; H, 3.87; N, 13.43. Found: C, 57.72; H, 4.02; N, 13.15.

1-(3-Fluorophenyl)-4-methyl-1,4-dihydropyrazolo[4,3-*c*]-[1,2]benzothiazin-3-amine 5,5-Dioxide (10). A mixture of the acid intermediate **9**[17] (1.48 g, 4.0 mmol) in an excess of $SOCl_2$ (4 mL) was refluxed under stirring for 1 h. The solvent was distilled off, and the residue was washed three times with dry benzene. The corresponding pyrazolobenzothiazine-3-carbonyl chloride was immediately dissolved in acetone (4 mL) and added dropwise to aqueous NaN_3 (1 M, 5.6 mL) at 0 °C. After the mixture was stirred at room temperature for 5 min, water was added and the precipitated pinkish solid was filtered. The crude product was immediately suspended into a mixture of toluene/37% HCl (1:1) (20 mL) and heated at 100 °C for 1.5 h. The mixture was then cooled and the solid formed was filtered to give compound **10** (0.95 g, 80%) as a white solid, used as is in the next reaction step: mp 185−187 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, NCH_3), 6.25 (s, 2H, NH_2), 7.15−7.20 (m, 2H, H-9 and H-4′), 7.25−7.30 (m, 2H, H-2′ and H-5′), 7.50−7.55 (m, 1H, H-6′), 7.65−7.70 (m, 2H, H-7 and H-8), 7.95−8.00 (m, 1H, H-6).

***N*-[1-(3-Fluorophenyl)-4-methyl-5,5-dioxido-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazin-3-yl]-4-nitrobenzamide (11).** To a stirred solution of amine derivative **10** (0.76 g, 2.3 mmol) in dry pyridine (10 mL), under nitrogen flux, a solution of 4-nitrobenzoyl chloride (0.85 g, 4.6 mmol) in dry pyridine (10 mL) was added dropwise. The mixture was heated at 40 °C for 18 h and then was concentrated to half volume and poured into ice−water, acidified with 2 N HCl (pH 3), and the precipitate formed was filtered under vacuum. The solid was suspended in 20% $NaHCO_3$, filtered, and purified by flash column chromatography, eluting with CH_2Cl_2/MeOH (95:5) to give compound **11** (0.43 g, 45%) as a white solid: mp 273−275 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.00 (s, 3H, NCH_3), 7.15 (d, J = 6.5 Hz, 1H, H-9), 7.25−7.30 (m, 1H, H-4′), 7.40−7.50 (m, 2H, H-2′ and H-5′), 7.55−7.70 (m, 3H, H-7, H-8, and H-6′), 7.90−8.00 (m, 1H, H-6), 8.15−8.25 (m, 2H, H-2″ and H-6″), 8.30−8.40 (m, 2H, H-3″ and H-5″), 11.05 (s, 1H, CONH).

4-Amino-*N*-[1-(3-fluorophenyl)-4-methyl-5,5-dioxido-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazin-3-yl]benzamide (12). A stirred solution of nitro derivative **11** (0.40 g, 0.8 mmol) in DMF (50 mL) was hydrogenated over a catalytic amount of Raney nickel at room temperature and atmospheric pressure for 2 h. The mixture was then filtered over Celite. The filtrate was evaporated to dryness and triturated with ethanol to give the amino derivative **12** (0.26 g, 66%) as a white solid: mp 218−220 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, NCH_3), 5.80 (s, 2H, NH_2), 6.55−6.65 (m, 2H, H-3″ and H-5″), 7.10−7.15 (m, 1H, H-9),7.30−7.35 (m, 1H, H-4′), 7−40−7.50 (m, 2H, H-2′ and H-5′), 7.55−7.70 (m, 3H, H-7, H-8, and H-6′), 7.75−7.80 (m, 2H, H-2″ and H-6″), 7.95−8.00 (m, 1H, H-6), 10.15 (s, 1H, CONH).

***N*-[1-(3-Fluorophenyl)-4-methyl-5,5-dioxido-1,4-dihydropyrazolo[4,3-*c*][1,2]benzothiazin-3-yl]-4-(methylsulfonyl)-aminobenzamide (5).** Methanesulfonyl choride (0.2 mL, 3 mmol) was added dropwise to a solution of the amino derivative **12** (0.28 g, 0.6 mmol) in dry CH_2Cl_2 (20 mL) and dry pyridine (2 mL) at 0 °C. The mixture was left overnight at room temperature and then poured into ice/water, acidified with 2 N HCl (pH 3), and extracted with CH_2Cl_2 (4 times). The combined organic layers were washed with brine (2 times), dried, filtered, and evaporated to dryness. The residue was purified by column chromatography, eluting with CH_2Cl_2/MeOH (95:5), affording the target compound **5** (0.16 g, 50%) as a white solid: mp 261−263 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H, SO_2CH_3), 3.20 (s, 3H, NCH_3), 7.15 (dd, J = 2.0 and 7.5 Hz, 1H, H-9),

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7.30−7.40 (m, 3H, H-4′, H-3″, and H-5″), 7.45−7.50 (m, 2H, H-2′ and H-5′), 7.60−7.75 (m, 3H, H-7, H-8, and H-6′), 7.95−8.10 (m, 3H, H-6, H-2″, and H-6″), 10.25 (bs, 1H, SO_2NH), 10.70 (s, 1H, CONH). ^{13}C NMR (DMSO-d_6): δ 36.65, 36.75, 112.84 (d, J_{C-F} = 24.1 Hz, C-2′), 116.65 (d, J_{C-F} = 21.1, C-4′), 118.29, 121.65 (bs, C-6′), 122.20, 123.79, 124.82, 124.96, 127.86, 129.22, 129.92, 130.32, 130.89, 131.24 (d, J_{C-F} = 10 Hz, C-5′), 133.33, 140.48, 140.77 (d, J_{C-F} = 10 Hz, C-1′), 142.61, 162.72 (d, J_{C-F} = 245.1 Hz, C-3′), 166.35. Anal. Calcd for $C_{24}H_{20}FN_5O_5S_2$: C, 53.23; H, 3.72; N, 12.93. Found: C, 51.43; H, 4.05; N, 12.66.

Computational Methods. Pyrazolobenzothiazine derivative **2a** was built using the Schrödinger Maestro Interface[42] and then submitted to Polak−Ribiere conjugate gradient minimization [0.0005 kJ/(Å mol) convergence]. The docking of **2a** into the PSI of 3SKE was then performed with the same methodology previously described.[28]

Molecular dynamics (MD) simulation of **2a**/NS5B complex was run in explicit solvent, using the TIP4P water model[43] in a periodic boundary conditions orthorhombic box. Desmond[31] was used to set up and run the MD simulations. The simulated environment was built using the system builder utility, with the structures being neutralized by Na^+ and Cl^- ions, which were added until a concentration of 0.15 M was reached. The buried regions were solvated using the "solvate pocket" utility. Before the simulations were performed, a series of minimizations and short MD simulations were carried out to relax the model system by means of a relaxation protocol consisting of six stages: (i) minimization with the solute restrained; (ii) minimization without restraints; (iii) simulation (12 ps) in the NVT ensemble using a Berendsen thermostat (10 K) with non-hydrogen solute atoms restrained; (iv) simulation (12 ps) in the NPT ensemble using a Berendsen thermostat (10 K) and a Berendsen barostat (1 atm) with non-hydrogen solute atoms restrained; (v) simulation (24 ps) in the NPT ensemble using a Berendsen thermostat (300 K) and a Berendsen barostat (1 atm) with non-hydrogen solute atoms restrained; (vi) unrestrained simulation (24 ps) in the NPT ensemble using a Berendsen thermostat (300 K) and a Berendsen barostat (1 atm). At this point, a 10 ns long MD simulation was carried out at a temperature of 300 K in the NPT ensemble using a Nose−Hoover chain thermostat and a Martyna−Tobias−Klein barostat (1.01325 bar). The simulation analysis was carried out by means of the Simulation Interaction Diagram utility[44] embedded in the Schrödinger Suite 2013 update 2.

Vital Stains. Nile Red was obtained from Sigma. Monodansylcadaverine and LipidTOX Red Neutral lipid stain was obtained from life technologies. Huh-9-13 cells were seeded in glass bottom 35 mm culture dishes (Mattek) at a density of 25.000 cells/dish in complete DMEM without G418 at 37 °C and 5% CO_2. The next day medium was replaced with complete DMEM containing either the proper dilution of the compound to be tested or DMSO. The cells were further incubated for 3 days at 37 °C and 5% CO_2, after which the medium was replaced with complete medium without neutral red and either Nile Red (2 μg/mL), monodansylcadaverine (50 μM), or LipidTOX Red Neutral lipid stain (diluted 1/1000). Next, the cells were incubated for an additional 30 min at 37 °C and 5% CO_2 after which the cells were imaged using a laser-scanning SP5 confocal microscope (Leica Microsystems) equipped with a DMI 6000 microscope and an Acousto optical beam splitter. For Nile Red the yellow-gold fluorescence was monitored at an excitation wavelength (λ_{ex}) of 552 nm and an emission wavelength (λ_{em}) of 636 nm. The red fluorescence was monitored at λ_{ex} of 485 nm and λ_{em} of 525 nm. Monodansylcadaverine was imaged at λ_{ex} of 340 nm and λ_{em} of 530 nm, and LipidTOX Red Neutral lipid stain was assessed at λ_{ex} of 577 nm and λ_{em} of 609 nm.

Physicochemical and ADME Properties. Solubility. For thermodynamic and kinetic solubility determination, solutions were shaken in a shaker bath at room temperature for 48 h before the filtration. Determinations were performed in triplicate by LC−UV−MS assay. Quantification of the compound was made by comparison with corresponding calibration curves realized with standard solutions in methanol.

Shake Flask Method for Lipophilicity. The octanol/water partitioning experiment was performed in a test tube. The test tube was filled with 2.5 mL of water and 2.5 mL of octanol to form the shake flask setup. The tested compound was dissolved directly in octanol.

The sample was sealed and then agitated to produce good mixing between the phases. Time required to reach the equilibrium was 10 h. An aliquot was obtained from each of the phases and is analyzed, typically by HPLC, to determine the concentration of selected compound in each phase.

The experimental log P was calculate using the following equation:

$$\log P_{oct/wat} = \log\left(\frac{[\text{solute}]_{octanol}}{[\text{solute}]_{water}}\right)$$

LC analysis was performed with a Perkin-Elmer (series 200) instrument equipped with an UV detector (Perkin-Elmer 785A, UV/vis detector). Chromatographic analysis was conducted using a Polaris C18-A column (150 mm × 4.6 mm, 5 μm particle size) at a flow rate of 0.8 mL min^{-1} with a mobile phase composed of 50% ACN/50% H_2O.

PAMPA Assay. Donor solution (0.5 mM) was prepared by diluting 1 mM dimethylsulfoxide (DMSO) compound stock solution using phosphate buffer (pH 7.4, 0.025 M). Filters were coated with 5 μL of a 1% (w/v) dodecane solution of phosphatidylcholine or 4 μL of brain polar lipid solution (20 mg/mL 16% $CHCl_3$, 84% dodecane) prepared from $CHCl_3$ solution, 10% w/v, for intestinal permeability and BBB permeability, respectively. Donor solution (150 μL) was added to each well of the filter plate. To each well of the acceptor plate was added 300 μL of solution (50% DMSO in phosphate buffer). The compound was tested in three different plates on different days. The sandwich was incubated for 5 or 22 h at room temperature under gentle shaking. After the incubation time, the plates were separated and samples were taken from both receiver and donor sides and analyzed using LC with UV detection at 254 nm. LC analyses were performed with a Perkin-Elmer (series 200) instrument equipped with an UV detector (Perkin-Elmer 785A, UV/vis detector). Chromatographic separations were conducted using a Polaris C18-A column (150 mm × 4.6 mm, 5 μm particle size) at a flow rate of 0.8 mL min^{-1} with a mobile phase composed of 50% ACN/50% H_2O.

Permeability (P_{app}) for PAMPA was calculated according to the following equation, obtained from the Wohnsland and Faller[45] and Sugano et al.[46] equation with some modification in order to obtain permeability values in cm/s,

$$P_{app} = \frac{V_D V_A}{(V_D + V_A)At} - \ln(1 - r)$$

where V_A is the volume in the acceptor well, V_D is the volume in the donor well (cm^3), A is the "effective area" of the membrane (cm^2), t is the incubation time (s), and r is the ratio between drug concentration in the acceptor and equilibrium concentration of the drug in the total volume (V_D + V_A). Drug concentration was estimated by measuring the peak area.

Membrane retention (%) was calculated according to the following equation:

$$\%\,\text{MR} = \frac{[r - (D + A)]100}{\text{Eq}}$$

where r is the ratio between drug concentration in the acceptor and equilibrium concentration and where D, A, and Eq represent the drug concentration in the donor, acceptor, and equilibrium solution, respectively.

Metabolic Stability. The compound in DMSO solution was incubated at 37 °C for 60 min in 125 mM phosphate buffer (pH 7.4), 5 μL of human liver microsomal protein (0.2 mg/mL), in the presence of a NADPH-generating system at a final volume of 0.5 mL (compound final concentration, 50 μM); DMSO did not exceed 2% (final solution). The reaction was stopped by cooling in ice and adding 1.0 mL of acetonitrile. The reaction mixtures were then centrifuged,

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and the parent drug and metabolites were subsequently determined by LC–UV–MS. Chromatographic analyses were performed with an Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies, Palo Alto, CA) consisting of a vacuum solvent degassing unit, a binary high-pressure gradient pump, an 1100 series UV detector, and an 1100 MSD model VL benchtop mass spectrometer. Chromatographic separation was obtained using a Varian Polaris C18-A column (150 mm × 4.6 mm, 5 μm particle size) and gradient elution, eluent A being ACN and eluent B consisting of water. The analysis started with 2% of eluent A, which was rapidly increased to 70% in 12 min, then slowly increased to 98% in 20 min. The flow rate was 0.8 mL min^{-1}, and injection volume was 20 μL. The Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument was equipped with the orthogonal spray API-ES (Agilent Technologies, Palo Alto, CA). Nitrogen was used as nebulizing and drying gas. The pressure of the nebulizing gas, the flow of the drying gas, the capillary voltage, the fragmentor voltage, and the vaporization temperature were set at 40 psi, 9 L/min, 3000 V, 70 V, and 350 °C, respectively. UV detection was monitored at 280 nm. The LC–ESI-MS determination was performed by operating the MSD in the negative ion mode. Spectra were acquired over the scan range m/z 100–1500 using a step size of 0.1 μm. The percentage of compound not metabolized was calculated by comparison with reference solutions.

■ ASSOCIATED CONTENT

Supporting Information

Biochemical and biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

RBV, ribavirin; pegIFN-α, pegylated interferon α; SVR, sustained virological response; DAA, direct-aging antiviral; NS5B, nonstructural 5B; NI, nucleoside inhibitor; NNI, non-nucleoside inhibitor; TSI, thumb site I; TSII, thumb site II; PSI, palm site I; PSII, palm site II; PSIII, palm site III; SI, selectivity index; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; neo, neomycin phosphotransferase; MDC, monodansylcadaverine; PAMPA, parallel artificial membrane permeability; MR, membrane retention; HLM, human liver microsome

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