Bioorganic & Medicinal Chemistry Letters 23 (2013) 663–668

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Ligand-based discovery of *N*-(1,3-dioxo-1*H*,3*H*-benzo[*de*]isochromen-5-yl)-carboxamide and sulfonamide derivatives as thymidylate synthase A inhibitors

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ARTICLE INFO

Article history:
Received 16 October 2012
Revised 27 November 2012
Accepted 29 November 2012
Available online 7 December 2012

Keywords:
Deconstruction approach
Enzyme inhibition
Thymidylate synthase
Antibacterial compound
Anticancer compound

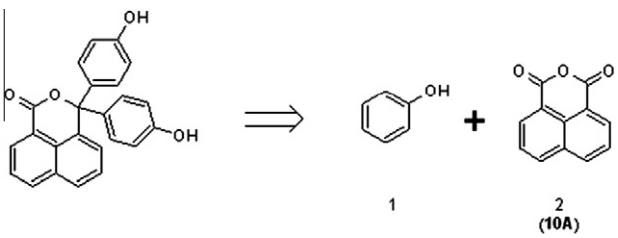
ABSTRACT

Phenolnaphthalein derivatives show potential for pharmacological activity as inhibitors of thymidylate synthase (TS) but difficulties in their synthesis and derivatization hinder their development. A deconstruction approach aimed at identifying a suitable new scaffold was proposed. A new scaffold was identified and two compound libraries based on this scaffold were designed. The carboxamide library (Library B) showed specific inhibition activity against *Escherichia coli* TS, whereas the sulfonamide library (Library C) showed a non-specific inhibition profile against hTS. *N*-(1,3-Dioxo-1*H*,3*H*-benzo[*de*]isochromen-5-yl)-sulfonamide derivatives, **1C** and **9C**, showed one order of magnitude improvement in inhibition constant against hTS with respect to the starting lead and represent potential compounds for further lead development.

Thymidylate synthase (TS) (EC: 2.1.1.45) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP) and is assisted by the cofactor N^5,N^{10}-methylene tetrahydrofolate (MTHF).[1] Because TS is essential for the only synthetic source of dTMP in human cells, it is a major target for the design of chemotherapeutic agents.[2] Among the drugs known to target TS, 5-fluorouracil, raltitrexed and pemetrexed are used therapeutically as anticancer agents.

Previous studies of phenolnaphthalein derivatives led to the discovery of compounds with species-specific inhibition profiles, including **α156** (3,3-bis(3-chloro-4-hydroxy-phenyl)-3*H*-benzo-[*de*]isochromen-1-one) and **GA9** (3,3-bis(3-bromo-4-hydroxy-phenyl)-7-chloro-3*H*-benzo[*de*]isochromen-1-one).[3–6] **α156** is fifty times more active toward *Escherichia coli* TS (EcTS) than human TS (hTS) (K_i = 0.6 and 30 μM, respectively), **GA9** shows a K_i versus EcTS of 4.1 μM with no inhibition of hTS at 50 μM concentration. In particular, **α156** has been proposed to be an antibacterial agent against vancomycin-resistant infections caused by Gram-positive bacteria, such as *Staphylococcus aureus*, because of its low toxicity to human cells. X-ray crystallographic studies of this class of compounds [**α156**, PDB ID: 1TSL; **MR20** (3,3-bis(4-hydroxy-phenyl)-6-nitro-3*H*-benzo[*de*]isochromen-1-one), PDB ID: 1TSM; **GA9**, PDB ID: 2A9W] have shown that their interactions with TS are characterized by multiple binding modes[3,6] (Supplementary Fig. SI-1). This observation has been corroborated by molecular docking studies.[7]

In general, phenolnaphthalein derivatives show appreciable pharmacological potential in terms of species specificity but exhibit low chemical tractability and require improvement to be considered as drug candidates.[4] To develop new inhibitors targeting TS, we applied a ligand-based approach to phenolnaphthalein derivatives, which were then deconstructed into fragments[8] (Scheme 1). A novel scaffold was sought and identified (Library A, Table 1), two libraries (Libraries B and C, Table 2) were synthesized and screened against hTS and EcTS.



Scheme 1. Deconstruction approach.

Abbreviations: DHFR, dihydrofolate reductase; DMSO, dimethylsulfoxide; dTMP, 2′-deoxythymidine-5′-monophosphate; dUMP, 2′-deoxyuridine-5′-monophosphate; EcTS, *Escherichia coli* TS; hTS, human TS; IC_{50}, concentration of inhibitor required for inhibition of 50% of target enzyme activity; K_i, inhibition constant; LcTS, *Lactobacillus casei* TS; MTHF, N^5,N^{10}-methylene tetrahydrofolate; Py, pyridine; TS, thymidylate synthase.

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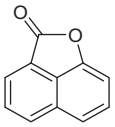
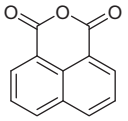
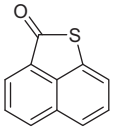
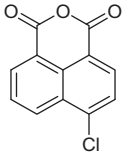
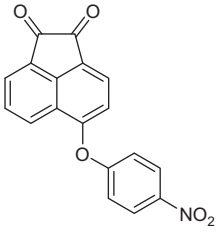
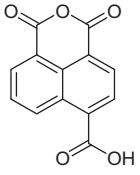
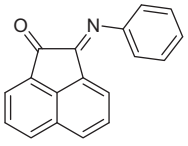
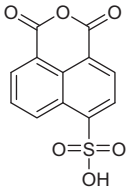
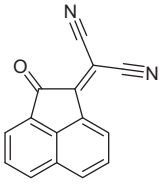
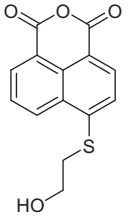
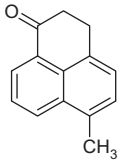
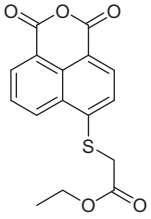
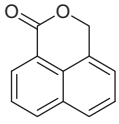
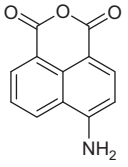
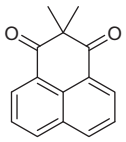
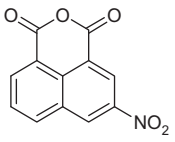
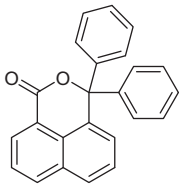
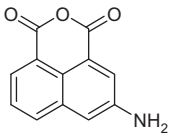
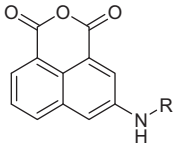
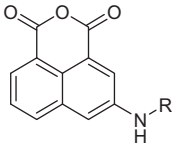
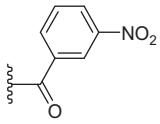
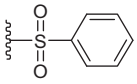
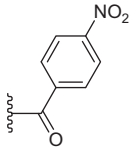
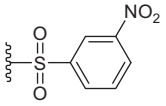
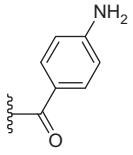
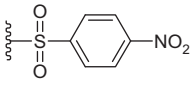
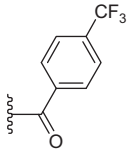
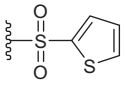
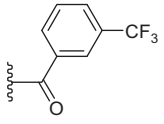
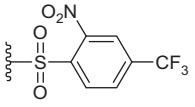
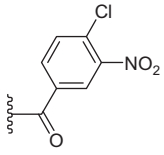
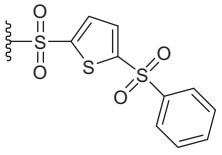
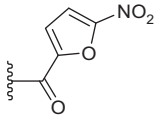
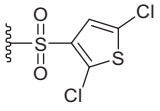
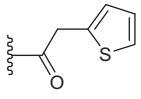
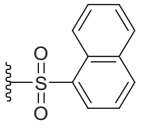
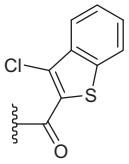
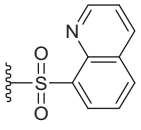
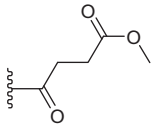
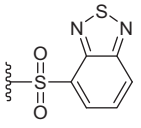
http://dx.doi.org/10.1016/j.bmcl.2012.11.117

Table 1
Inhibition activity profiles of compounds from Library A

Code	Structure	K_i EcTS (μM)[a]	K_i hTS (μM)[a]	Code	Structure	K_i EcTS (μM)[a]	K_i hTS (μM)[a]
1A		>>265 NI (54)	4.2	**10A**		>>294 NI (60)	2.0
2A		>>196 NI (40)	3.1	**11A**		105	7.3
3A		>>103 NI (20)	>>98 NI (20)	**12A**		26.8	2.0
4A		>>294 NI (60)	>>309 NI (60)	**13A**		>>784 NI (160)	>>824 NI (160)
5A		>>294 NI (60)	>>309 NI (60)	**14A**		>>451 NI (92)	>>474 NI (92)
6A		>>294 NI (60)	3.1	**15A**		>>588 NI (120)	12
7A		>>304 NI (62)	47	**16A**		>>152 NI (31)	>>160 NI (31)
8A		>>588 NI (120)	33	**17A**		>>196 NI (40)	>>206 NI (40)
9A		>>294 NI (60)	>>309 NI (60)	**18A**		6.8	>>412 NI (80)

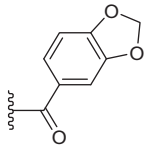
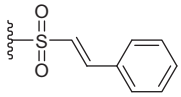
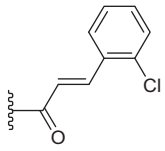
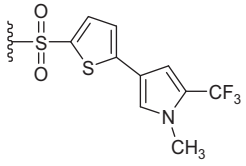
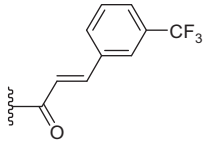
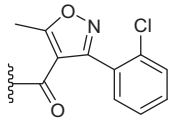
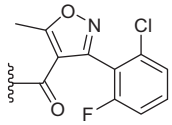
[a] NI indicates no detectable inhibition at the concentration reported in brackets ([*I*]). In these cases K_i was estimated considering an inhibition percentage (*I*%) of 2% at this concentration and applying the formulas $IC_{50} = (100 - I\%) \times [I]/I\%$ and $K_i = IC_{50}/(1 + [S]/K_m)$[21] where [*S*] is the concentration of folate used in the assay and K_m is the affinity constant of the enzyme for that substrate.[5]

Table 2
Inhibition activity profiles of compounds from Libraries B and C

Code	 R chain	K_i EcTS (μM)[a]	K_i hTS (μM)[a]	Code	 R chain	K_i EcTS (μM)[a]	K_i hTS (μM)[a]
1B		7.3	2.3	**1C**		4.2	0.3
2B		17	17.3	**2C**		2.1	1.1
3B		21.6	>>823 NI (160)	**3C**		1.2	1.3
4B		6.4	>>206 NI (40)	**4C**		2.2	0.5
5B		4.4	44.6	**5C**		1.3	0.9
6B		4.0	25.4	**6C**		1.2	1.3
7B		6.7	12.3	**7C**		4.6	1.2
8B		5.3	13.8	**8C**		1.1	0.8
9B		6.5	>>411 NI (80)	**9C**		2.9	0.3
10B		7.1	16.2	**10C**		0.9	5.0

(*continued on next page*)

Table 2 (*continued*)

Code	R chain	K_i EcTS (μM)[a]	K_i hTS (μM)[a]	Code	R chain	K_i EcTS (μM)[a]	K_i hTS (μM)[a]
11B		7.4	57.2	**11C**		0.3	0.6
12B		32.3	>>616 NI (120)	**12C**		1.9	0.8
13B		10.6	>>206 NI (40)				
14B		44.7	>>826 NI (160)				
15B		6.1	25.9				

[a] NI indicates no detectable inhibition at the concentration reported in brackets ([*I*]). In these cases K_i was estimated considering an inhibition percentage (*I*%) of 2% at this concentration and applying the formulas $IC_{50} = (100 - I\%) \times [I]/I\%$ and $K_i = IC_{50}/(1 + [S]/K_m)$[21] where [*S*] is the concentration of folate used in the assay and K_m is the affinity constant of the enzyme for that substrate.[5]

A deconstruction approach was applied to three known phenolnaphthalein derivatives: **α156**, **MR20** and **GA9**. Two simple fragments were identified: phenol (**1**) and benzo[*de*]isochromene-1,3-dione (known also as 1,8-naphthalic anhydride) (**2**) (Scheme 1). We focused on fragment **2** and an initial library of commercially available compounds (Library A, **1A**–**18A**) (Table 1) that represented potentially new scaffolds was compiled and screened against hTS and EcTS. The library was designed using ChemFinder from the ChemOffice program suite[9]; the ACXProd database and ChemFinder were searched using the substructure search (see Supplementary Fig. SI-2 for queries used). The 306 retrieved results were filtered based on each candidate's suitability for further derivatization, commercial availability and cost. All of the compounds were commercially available, with the exception of compound **18A**, which was obtained by the reduction of compound **17A**.[10,11]

With the exception of three compounds (**11A**, **12A** and **18A**; K_i = 105, 26.8 and 6.8 μM, respectively) (Table 1), none of the compounds inhibited EcTS.[12] Almost half of the compounds in Library A (**3A**–**5A**, **9A**, **13A**–**14A** and **16A**–**18A**) did not show inhibition against hTS. The most active compounds against hTS were **10A** and **12A**, with K_i values of 2.0 μM (Table 1, Fig. 1).

From Library A, compound **18A** was selected because it has a chemical handle which allows easy structural variation. This compound was active at low micromolar levels against EcTS and showed no inhibition of the human enzyme. Two libraries, *N*-(1,3-dioxo-1*H*,3*H*-benzo[*de*]isochromen-5-yl)-carboxamide derivatives (Library B, **1B**–**15B**) (Table 2) and *N*-(1,3-dioxo-1*H*,3*H*-benzo[*de*]isochromen-5-yl)-sulfonamide derivatives (Library C, **1C**–**12C**) (Table 2), were designed, synthesized and screened. Fragments considered for addition at the amino group at position 5 were selected for their drug-like properties and their adherence to Lipinski's rules,[13] with the aim of exploring molecular diversity while focusing on aromatic and hetero-aromatic compounds. Substituents included small aliphatic chains (**10B**); aromatic systems with zero, one, or two substituents (**1B**–**7B**, **9B**, **11B**, **1C**–**5C** and **7C**–**10C**); longer chains with a variable-size aliphatic bridge (**8B**, **12B**, **13B** and **11C**); two aromatic systems with variable substituents (**14B**, **15B**, **6C** and **12C**). MW values ranged from 327 to 506 Da; Log*D* values at pH 7.4 ranged from 0.37 to 4.76. Except for compound **3B**, which had three H-bond donor atoms, all of the other derivatives had only one H-bond donor center. The number of H-bond acceptor atoms varied from 4 to 7.

Compounds **1B**–**15B** were obtained by reaction of **18A** with the corresponding acyl chloride as depicted in Scheme 2.[10] After reaction termination, ice was added to the solution and a precipitate was formed. In the case of **1B**, **2B**, **4B**–**7B**, **9B** and **12B**–**14B**, the reaction mixture was first filtered to separate a precipitate consisting of the starting material and then treated with ice. The filtered precipitate was suspended in water and treated with sodium bicarbonate (pH to 8 and 9); the reaction product was then separated out by filtration (Scheme 2).

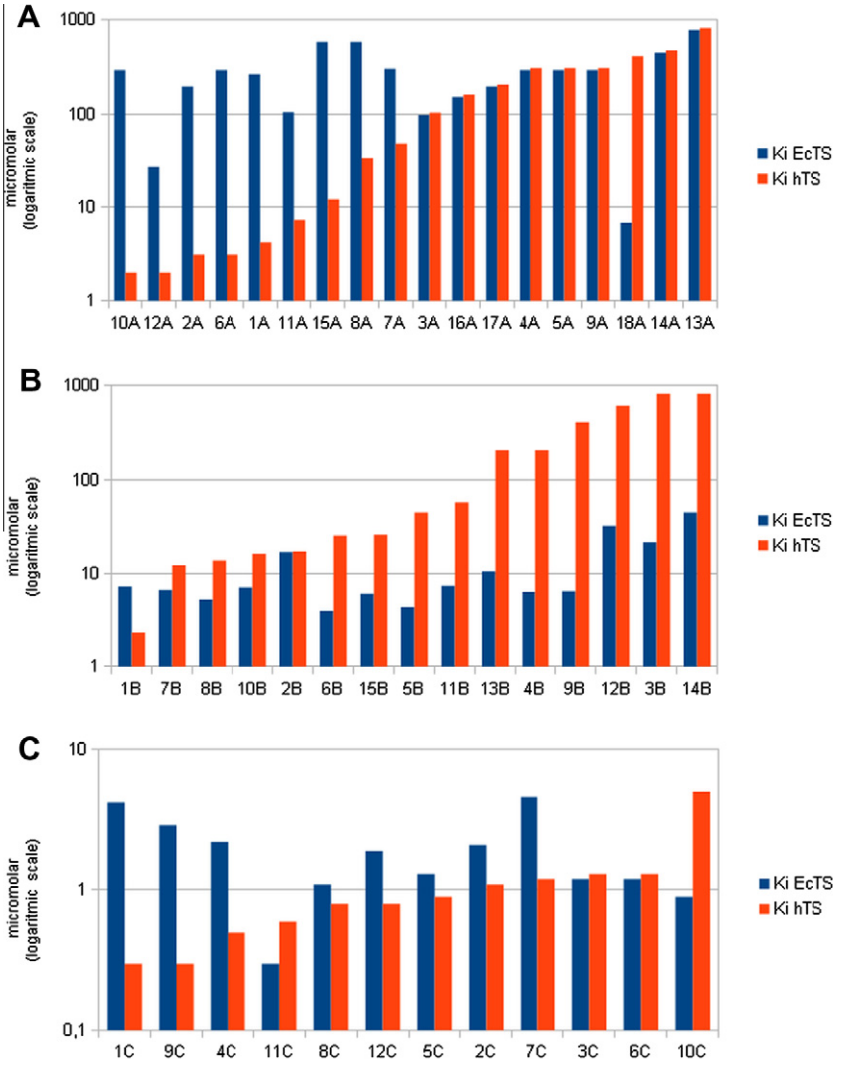
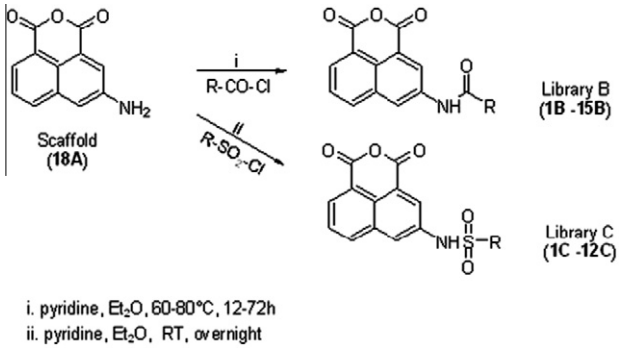


Figure 1. Inhibition activity profile of Libraries A, B and C.



Scheme 2. Synthesis of Libraries B and C.

Compounds **1C**–**12C** were synthesized by reacting **18A** with the corresponding sulfonyl chloride as depicted in Scheme 2.[10] After reaction termination, the reaction solution was diluted with water and the resulting precipitate was separated by filtration, dissolved in a mixture of acetone and ethanol (50/50 v/v) and subsequently dried under vacuum. This last step was repeated two or three times to eliminate the residual Py (Scheme 2).

Compounds **1B**–**15B** are active as inhibitors of EcTS, with K_i values in the range of 4.0–44.7 μM.[12] Six compounds (**3B**–**4B**, **9B** and **12B**–**14B**) are species-specific, that is, they showed inhibitory activity toward the bacterial enzyme without inhibiting the human enzyme. The other compounds were also active against hTS, with K_i values in the range of 2.3–58 μM (Table 2, Fig. 1).

Compounds **1C**–**12C** are better inhibitors of TS and exhibit K_i values in the narrower range of 0.3–5.0 μM, but they are not specific inhibitors of the bacterial TS, as they show the same level of inhibition against both bacterial and human enzymes[12] (Table 2, Fig. 1).

It is worth considering the change in the inhibition activity profile resulting from the derivatization of the scaffold at position 5 with a carboxamidic bridge or a sulfonamidic bridge. The carboxamidic bridge does not modify the activity level or the species specificity profile of the scaffold compound. In contrast, the sulfonamidic bridge leads to increased activity with respect to the scaffold compound (**18A**) over non-specific compounds and enhances the activity against hTS. Docking studies have been performed to explain the difference in activity profiles between Library B and Library C (see Supplementary data). The results obtained show that these molecules (**4B**, **1C**, **4C** and **9C**) are predicted to bind in the same region, that is, in the folate binding site where other folate-analog TS inhibitors bind. The sulfonamidic derivatives show a different overall shape compared to the carboxamide compounds and bind deeper in the active site (see Supplementary Figs. SI-2 and SI-3). The interaction pattern is different: the sulfonamidic

derivatives interact with the non conserved residue, Trp 83 in EcTS that corresponds to Asn 112 in hTS; the carboxamidic compound **4B** is predicted to interact only with the bulky Trp. These results can partly explain the observed biological activity profiles.

Some of the compounds in Library B were also tested against human dihydrofolate reductase (hDHFR), which is an enzyme of the thymidylate cycle, to identify potential off-target effects. All of the tested compounds were inactive toward hDHFR (see Supplementary Table SI-4), which demonstrates that they are selective inhibitors of the TS enzyme.

By applying a ligand-based approach to the phenolnaphthalein class of TS inhibitors, we identified a new scaffold (**18A**) that could be easily modified through synthetic chemistry, thus allowing the development of two compound libraries stemming from an initial scaffold. Screening against hTS and EcTS revealed differences in the species-specific inhibition activity of the two libraries. Most of the carboxamide derivatives showed specificity toward the bacterial TS. In particular, compounds **4B** and **9B** will be further developed. Their levels of activity and specificity are similar to that of **GA9** but the chemical structure of this series of compounds allows further exploration of the neighbouring chemical space to obtain promising antibacterial lead compounds. In contrast, the sulfonamide derivatives are promising as anticancer compounds, as they inhibit hTS with K_i values in the range of 300 nM–5 μM. In particular, three compounds (**1C**, **4C** and **9C**) with K_i values from 300 to 500 nM will be proposed for the development of drug leads that are useful against human tumor cells.

Acknowledgments

This study was supported by MIUR-PRIN 2009 to Costi M.P. 200925BPZ5_004. We wish to thank the Cassa di Risparmio di Modena (CDRM) Foundation. We thank CIGS (Centro Interdipartimentale Grandi Strumenti) of the University of Modena and Reggio Emilia for the use of their instrumentation facilities. R.C.W. gratefully acknowledges the support of the Klaus Tschira Foundation. We also thank Professor M. Rinaldi for assistance with chemical synthesis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.11.117.

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9. ChemOffice Ultra 7.0, Cambridge Soft Corporation, http://www.cambridgesoft.com.
10. *Chemistry:* Reagents were purchased from Sigma–Aldrich. Reaction progress was monitored by TLC on pre-coated silica gel 60 F_{254} plates (Merck) and visualization was accomplished using UV light (254 nm). The purity of all materials was determined to be at least 95% by TLC, ^{1}H NMR and elemental analyses. Compounds **1A**–**17A** were purchased from different vendors and their purity was evaluated to be higher than 95% by elemental analyses (Supplementary Table SI-2). Reaction yields refer to purified products and are not optimized (Supplementary Table SI-3). The purity of all synthesized compounds was determined by elemental analyses performed on a Perkin–Elmer 240C instrument and the results for C, H and N microanalysis were within ±0.4% of theoretical values (Supplementary Table SI-3). All synthesized compounds were characterized by ^{1}H NMR on a Bruker FT-NMR AVANCE 200. Spectra were recorded in hexadeuterodimethylsulfoxide (DMSO-d_6). Chemical shifts are reported as δ values (ppm) and referenced to tetramethylsilane as an internal standard. When peak multiplicities are given, the following abbreviations are used: s, singlet; d, doublet; t, triplet; dd, double doublet; m, multiplet (see Supplementary data).
11. *Synthesis of 5-amino-benzo[de]isochromene-1,3-dione* ***(18A):*** The scaffold of Libraries B and C was obtained through the catalytic reduction of **17A** (5-nitro-benzo[*de*]isochromene-1,3-dione). The nitro compound, **17A** (5 g, 20 mmol), was dissolved in dimethylformamide (40 mL) and 10% Pd/C (0.5 g) was added. The mixture was stirred under H_2 (P = 2 atm, T = 40 °C), for 18 h. The catalyzer was then filtered off and the product **18A** was precipitated by addition of water.
12. *Inhibition activity profile evaluation:* Proteins were purified as described previously.[14–18] Folate cofactors and substrates were a gift from Merck & Co (Switzerland); all other substrates, cofactors and reagents were purchased from different companies at the highest purity grade possible. Kinetic experiments with TS were conducted under standard conditions.[19] Inhibition experiments were conducted by measuring the effects of different inhibitor concentrations on the initial rate of the enzymatic reaction, in the presence of a limited concentration of the folate substrate. Reactions were initiated by addition of the enzyme. IC_{50} (concentration of inhibitor required to inhibit 50% of enzymatic activity) values were determined and K_i values were detected, showing competitive inhibition with respect to MTHF for all of the compounds.[20,21] Kinetic measurements using human DHFR were performed at 25 °C in standard enzyme buffer.[22,23] For the determination of reductase activity, NADPH oxidation was followed at 340 nm. The kinetic experiments were performed in triplicate and no individual measurements differed by >20% from the mean. Stock solutions of each inhibitor were freshly prepared in dimethylsulfoxide (DMSO). The DMSO concentration was kept below the concentration known to affect enzymatic activity (5% for TS and human DHFR).
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