Articles

Design, Synthesis, Computational Prediction, and Biological Evaluation of Ester Soft Drugs as Inhibitors of Dihydrofolate Reductase from *Pneumocystis carinii*

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A series of lipophilic soft drugs structurally related to the nonclassical dihydrofolate reductase (DHFR) inhibitors trimetrexate and piritrexim have been designed, synthesized, and evaluated in DHFR assays, with special emphasis on the inhibition of *P. carinii* DHFR. The best inhibitors, encompassing an ester bond in the bridge connecting the two aromatic systems, were approximately 10 times less potent than trimetrexate and piritrexim. The metabolites were designed to be poor inhibitors. Furthermore, molecular dynamics simulations of three ligands in complex with DHFR from *Pneumocystis carinii* and from the human enzyme were conducted in order to better understand the factors determining the selectivity. A correct ranking of the relative inhibition of DHFR was achieved utilizing the linear interaction energy method. The soft drugs are intended for local administration. One representative ester was selected for a pharmacokinetic study in rats where it was found to undergo fast metabolic degradation to the predicted inactive metabolites.

Introduction

The AIDS epidemic, cancer chemotherapy, and organ transplantation have significantly increased the number of patients with impaired immune systems who are suffering from severe opportunistic infections including pneumonia caused by the fungus *Pneumocystis carinii.*[1−3] *P. carinii* pneumonia (PCP) is a serious disease with high prevalence and constitutes the major cause of death in patients with AIDS.[4−8] However, with the widespread use of highly active antiretroviral therapy (HAART) and prophylaxis in patients known to be at risk for developing PCP, the incidence of PCP in patients with AIDS has declined dramatically.[9−11] Current treatment with trimethoprim (TMP, Figure 1), a nonclassical inhibitor of dihydrofolate reductase (DHFR), in combination with a sulfonamide is still the standard therapy for PCP.[7,9,12,13] Severe side effects associated with sulfa drugs often lead to discontinuation of therapy.[5,14−16] Attempts have been made to use drugs locally, and inhaled aerosolized pentamidine[17] is currently used for prophylaxis,[18−21] but it is inadequate for treatment of active infection. When applied systematically, pentamidine exhibits considerable toxicity.[9,13,19,22] Trimetrexate (TMQ)[23,24] and piritrexim (PTX),[25] two lipophilic agents originally developed against cancer,[26−30] are now used in clinic as second-line therapy in moderate to severe PCP[31] (Figure 1). Although TMQ and PTX are both potent inhibitors of DHFR from *P. carinii*, they are not selective and inhibit the mammalian enzyme even more efficiently.[32,33] The clinical use of TMQ and PTX is therefore limited because of their systemic host toxicity and requirement of expensive cotherapy with the rescue agent leucovorin (5-formyl-tetrahydrofolate).[32,34−37] Leucovorin, a classical folate cofactor for one-carbon metabolism, is taken up via active transport only by mammalian cells and reverses toxicity associated with the lipophilic DHFR inhibitors.[32,36,38] Today, considerable research efforts are devoted to the identification of more selective and potent DHFR inhibitors

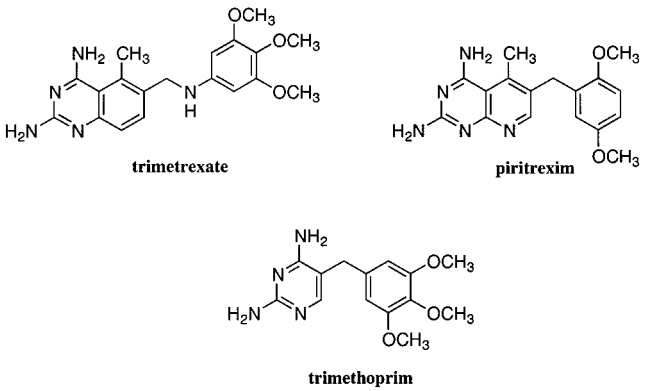

Figure 1.

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with the overall goal to improve therapy and to minimize the adverse effects.

The application of the soft drug concept could allow design of new agents for inhalation therapy and could make this route available to safer DHFR inhibitors. Soft drugs are active isosteric−isoelectronic analogues of lead compounds that are deactivated in a predictable and controllable way after exerting their biological effects.[39] These drugs are intended to undergo a fast metabolism, which minimizes toxicity to the host. Soft drugs are most often used for local treatment and are therefore, in general, administered near the site of action.[39] The concept has successfully been utilized, e.g., in the design of the recently launched inhaled antiasthma glucocorticosteroid budesonide,[40] which essentially lacks systemic side effects due to its rapid first-pass metabolism in the liver.[41]

We aim to test the hypothesis of whether local administration of soft drugs of lipophilic DHFR inhibitors might provide a new entry to efficient and safe treatment of PCP and, in a longer perspective, of other serious pulmonary diseases. We herein report the synthesis and the subsequent biological evaluation of a series of ester soft drugs structurally related to TMQ and PTX. Furthermore, molecular dynamics simulations of three ligands in complex with DHFR from *P. carinii* and the human enzyme were conducted to better understand the molecular basis for the observed selectivity. A pharmacokinetic study of one of the derivatives was performed in rat in vivo.

Results

Chemical Synthesis. The synthetic approach employed for the preparation of the target compounds **1a**−**10a**, encompassing an ester bond in the bridge between the aromatic ring systems, is outlined in Schemes 1 and 2. We have previously described[42] the synthesis of compounds **1a** and **2a**, as well as the metabolites **17a** and **18a**. Thus, protection of the aminogroups of **15a** with pivalic anhydride in anhydrous DMF afforded the dipivaloated aldehyde **16a**, which was further oxidized to the corresponding carboxylic acid with sodium chlorite, employing 2-methyl-2-butene as a scavenger.[43−45] After subsequent esterification, the two amino-protected 2,4-diaminoquinazoline esters were depivaloated using ammonia in dioxane,[46] finally yielding the desired esters **1a** and **2a**.

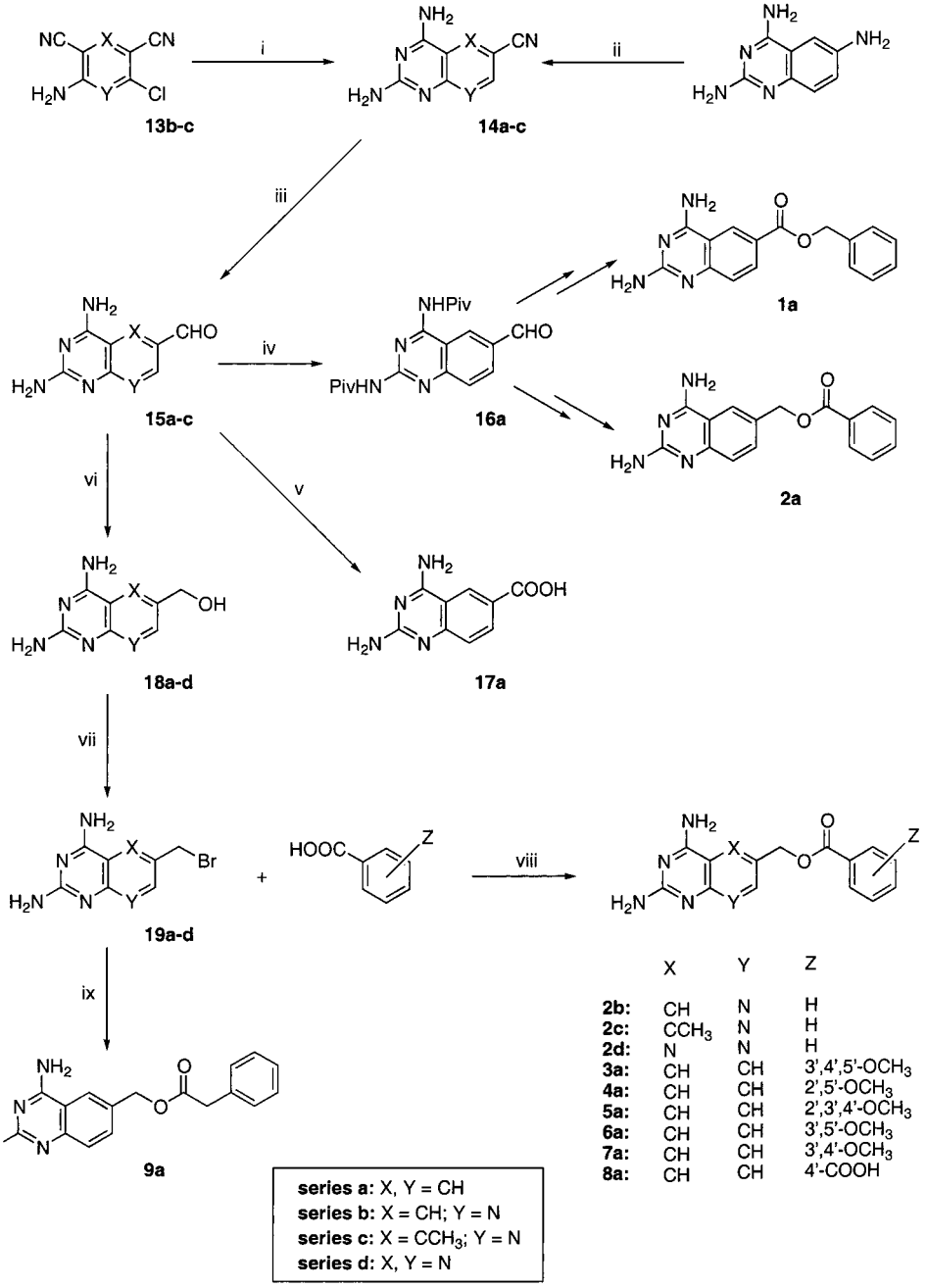
The synthesis of compound **14a** is previously described.[42,47] The intermediates **14b,c**, used for the synthesis of **2b,c**, were prepared from **13b,c** according to slightly modified literature procedures.[48,49] Thus, compounds **13b** and **13c** were synthesized by a HCl-mediated condensation of malononitrile with triethyl orthoformate and triethyl orthoacetate,[48,49] respectively. Dechlorination was followed by cyclization with guanidine in refluxing ethanol, affording the two substituted 2,4-diaminopyrido[2,3-*d*]pyrimidines **14b,c**. Treatment with a Ni−Al alloy in refluxing formic acid[42,50] converted the nitriles **14a,c** into the aldehydes **15a,c**, which were further reduced to the corresponding alcohols **18a,c** by sodium borohydride in methanol.[51,52] Prolonged reaction time leads to overreduction of the alcohol to the 6-methylanalogues.[53] For the synthesis of **2d**, commercially available **18d** was employed as starting material. The bromination of the alcohols **18a**−**d** was performed with methods employed in antifolate chemistry, including the use of dibromotriphenylphosphorane (Ph_3PBr_2) in DMAc,[54] HBr in dioxane,[52,55] PBr_3 in THF,[56] or HBr in AcOH.[57] The last procedure provided the best results in the present systems. Displacement of the bromide with the appropriate carboxylic acids using potassium carbonate or cesium carbonate as bases in DMF, DMSO, or DMAc, respectively, afforded the esters **2b**−**9a** in various yields. The synthesis of the ester **8a** is previously described.[58] Compound **20a**[59,60] was prepared by diazotation of 2,4,6-triaminoquinazoline, which was obtained by reduction of commercially available 2,4-diamino-6-nitroquinazoline. A standard Heck reaction[61−63] with phenyl acrylate provided the α,*β*-unsaturated ester **10a** in 40% yield (Scheme 2).

Finally, the two reference compounds **11a**[47] and **12a** were synthesized (Scheme 3). Diazotation of 2,4,6-triaminoquinazoline to the nitrile **14a**[42,47] with potassium cyanide and cuprous sulfate was followed by hydrogenation over Raney Ni at atmospheric pressure with aniline, providing **11a**. A subsequent reductive methylation using 37% formaldehyde and sodium cyanoborohydride at pH 2−3[49,64] provided the N10-methylated compound **12a** in 89% yield.

Inhibition of Dihydrofolate Reductase. Compounds **1a**−**12a** were tested for their ability to inhibit dihydrofolate reductase from *P. carinii* (pcDHFR) and rat liver (rlDHFR). The methodologies of the assay are described elsewhere.[33,65] All results are presented as IC_{50} values in Table 1 together with the reference compounds shown in Figure 1. For comparison, the IC_{50} values for inhibition of *Toxoplasma gondii* DHFR (tgDHFR) are shown. The IC_{50} values for the metabolites **17a** and **18a**−**d**, formed after hydrolysis of the esters, are also shown in the same table.

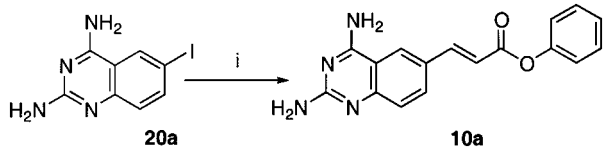
The impact of various linkers connecting the two ring systems has previously been studied thoroughly,[66] but to our knowledge the biological effects of antifolates with scissile ester bridges have not been reported. In general, two-atom bridges are preferred, although derivatives with three- and also four-atom bridges are frequently acceptable and retain some inhibitory potency.[67] A comparison of compounds, comprising different tethers, is presented in Table 1 (see **1a** and **2a**, **9a** and **10a**, and **11a** and **12a**). Two-atom bridge esters were not included in this investigation because of their chemical instability and their expected susceptibility to a too fast metabolism in vivo. The N10-methylated reference compound **12a** was approximately 10 times more potent in the three assay systems than the secondary amine **11a**, the latter possessing the same two-atom linker as TMQ. Incorporation of a three-atom ester delivered an inhibitor **1a**, which was almost as potent as **12a** against rlDHFR but with much poorer activity against pcDHFR. On the other hand, when the three-atom bridge was reversed to provide a methyleneoxycarbonyl linker in **2a**, a significantly more potent inhibitor of pcDHFR was achieved (cf. **1a** and **2a**). By elongation of the bridge affording compounds **9a** and **10a**, the activity declined vs pcDHFR. The methyleneoxycarbonyl unit was considered most suitable as a tether connecting the two ring systems and was therefore retained in further studies. Subsequently, the influence of (i) various substitution

Scheme 1[a]



[a] Reagents and conditions: (i) (a) H_2, 10% Pd/C, Et_3N, DMF, (b) guanidine hydrochloride, NaOMe, reflux; (ii) $NaNO_2$, 2 M HCl, KCN, $Cu(II)SO_4 \cdot 5H_2O$; (iii) Ni−Al alloy, 75% formic acid, reflux; (iv) pivalic anhydride, DMF, 80 °C; (v) $NaClO_2$, 2-methyl-2-butene, $NaH_2PO_4 \cdot 1H_2O$; (vi) $NaBH_4$, MeOH; (vii) 30% HBr in AcOH; (viii) K_2CO_3 or Cs_2CO_3, DMF or DMSO; (ix) phenylacetic acid, Cs_2CO_3, DMAc.

Scheme 2[a]

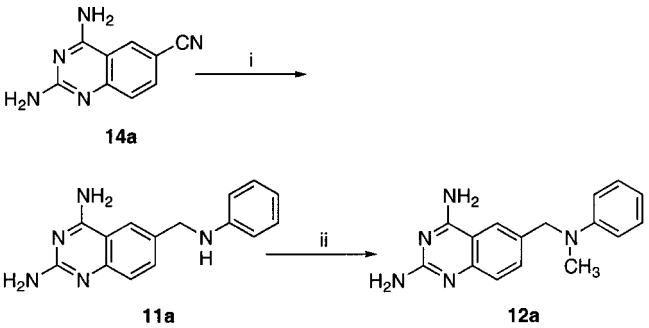


[a] Reagents and conditions: (i) phenyl acrylate, $Pd(OAc)_2$, P(*o*-tol)$_3$, Et_3N, 80 °C.

patterns of the phenyl ring with focus on methoxy groups found in TMQ and PTX and (ii) the effect of some different bicyclic heterocycles were examined.

Substitution of the ortho position with a methoxy group in the phenyl ring, as seen in compounds **4a** and **5a**, resulted in poor inhibition of pcDHFR. Compound **4a** exhibited in fact 70-fold less activity than the related PTX against pcDHFR, while the effect on the inhibitory capacity regarding tgDHFR was less pronounced. However, a migration of the ortho methoxy group in **4a** to the meta position provided an inhibitor **6a** with significantly better activity against pcDHFR. The three compounds **3a**, **6a**, and **7a** were all equipotent against pcDHFR, but their activity toward rlDHFR differed. Thus, **3a**, the ester most structurally similar to TMQ, was found to be a very potent inhibitor of rlDHFR, which accounts for the very low rlDHFR/pcDHFR ratio observed (0.05). Both **3a** and **7a** showed a good activity against tgDHFR. The benzoic acid derivative, compound

Scheme 3[a]



[a] Reagents and conditions: (i) H_2 (1 atm), Raney Ni, aniline; (ii) 30% formaldehyde, $NaCNBH_3$.

8a, exhibited the best selectivity against pcDHFR among the compounds tested, but the affinity was in the micromolar range.

Alterations of the inhibitors in the heterocyclic part of the molecule exerted a strong impact on the biological response, as obvious from a comparison of the series **a**−**d**. Thus, replacing the carbon in the 8-position of **2a** for a nitrogen delivered an almost 10 times less powerful inhibitor **2b**, and incorporation of a second nitrogen into the heterocycle, providing the pteridine analogue **2d**, rendered an IC_{50} value in the 10 μm range. A methyl group at the 5-position (**2c**) improved the activity considerably in accordance with what was observed in related systems.[48,68,69]

As shown in Table 1, the activity of the hydroxymethyl metabolites **18a**−**d** and that of the carboxylic acid **17a**, the latter also expected to be formed in vivo after metabolic oxidation of **18a**, are presented. All of the potential metabolites, with **18c** as the exception ($IC_{50} = 8.5$ μM), exhibited very low inhibitory potencies against rlDHFR.

Molecular Dynamics. The linear interaction energy (LIE) method has previously been used to predict the absolute binding free energies of molecules similar to methotrexate for human DHFR.[70] Herein, we employ the LIE method to predict the selectivity of inhibitors against DHFR from different species (*P. carinii* and human). The relative free energies of binding to these enzymes were calculated for compounds **1a**, **2a**, and **3a**. The results from the calculations are presented in Table 2. When the affinities of the three inhibitors of dihydrofolate reductase from the two species are compared, the relative binding free energies seem to agree well with the experimental data. The potencies of the inhibitors can be correctly ranked in both enzymes, with the reservation that the calculations are based on human DHFR and that rat liver DHFR is used in the experimental assay. Substantial homology has been shown at the N-terminal ends[71,72] of the enzyme, and inhibitors generally bind with similar affinities to both enzymes.[73] Recently completed studies, soon to be published, show substantial homology throughout.[74]

In the case of pcDHFR the simulations predict that compound **1a** is the least potent inhibitor, whereas **2a** and **3a** have approximately equal affinities, 20 times stronger than **1a**. For hDHFR compound **1a** is again ranked as the least potent inhibitor. For the mammalian enzyme compound **3a** is predicted to bind 60 times more strongly than **1a** and 5 times more strongly compared to **2a**. The calculated selectivity ratios hDHFR/pcDHFR agree nicely with the experimentally determined values of rlDHFR/pcDHFR. Compound **3a**, which was the most potent compound among the three simulated inhibitors, had the lowest selectivity ratio, i.e., higher selectivity against mammalian DHFR, which is in accordance with the experimental values.

Pharmacokinetics in Rat. We previously reported the pharmacokinetic profile in rat of the classic antifolate ester **21** (Figure 2), which was found to be relatively stable toward metabolism, both after oral and after intravenous administration.[58] After intravenous administration, compound **21** was not prone to undergo enzymatic hydrolysis and displayed a plasma half-life ($t_{1/2}$) of 1 h and a plasma clearance (CL) of 15 mL min^{-1} kg^{-1} compared to $t_{1/2}$ of 0.9 h and a CL of 11 mL min^{-1} kg^{-1} for methotrexate, the reference substance, evaluated in the same system. To be suitable as a soft drug, a significantly faster metabolism was required. We anticipated that the trimetrexate analogue, the lipophilic trimethoxy-substituted compound **3a**, because of its higher lipophilicity, should be better accommodated in esterases and display an improved tendency to metabolic degradation when subjected to the same animal model. The pharmacokinetics of the quinazoline ester **3a** was evaluated in rat after oral and intravenous administration. After oral administration, we were unable to detect **3a** in plasma after a period of 2 min while the alcohol metabolite **18a** appeared, suggesting a very low systemic bioavailability. After intravenous administration we found **3a**, as well as the alcohol metabolite **18a**, in plasma (Figure 3). In vitro studies in blood from human and rat showed that no metabolism, of neither **21** nor **3a**, had occurred after 45 min. Furthermore, small amounts of the carboxylic acid metabolite **17a** were detected. As obvious from the curve profile in Figure 3, compound **3a** exhibits a very short elimination half-life. Also, a very high plasma clearance was measured, even higher than the liver blood flow, indicating an extra hepatic elimination of compound **3a**. These results are consistent with the high activity of esterases in other organs. Thus, we argue that lipophilic esters related to **3a** could provide sufficiently good substrates to esterases to serve as soft drugs.

Discussion

Esterases are ubiquitous and are expressed in a variety of tissues, including liver, stomach, small intestine, colon, plasma, macrophage, and monocytes.[75] A diversity of structurally different, efficiently hydrolyzed ester prodrugs have successfully been developed.[76] Esterases also constitute suitable enzymatic systems for metabolic deactivation of soft drugs.[39] The rank of order among esters tends to be similar in different tissues,[42,77] but the stability of the ester bond shows in general a high variability in different animal species.[75,78] The stability of esters in human is often much higher than in rodent.[79,80] Thus, for local therapy, e.g., inhalation, we believe one should seek ester soft drugs that are metabolized rapidly in rodent to ensure a sufficient hydrolysis rate in human. In the design of soft drugs, the improvement of the therapeutic index is the main objective, and the avoidance of a long-term toxicity by

Table 1. Inhibition Concentrations (IC_{50}, μM) of DHFR from *P. carinii, T. gondii,* and Rat Liver and Selectivity Ratios

	IC_{50} (μM)			selectivity index[a]	
compd	pcDHFR	rlDHFR	tgDHFR	pcDHFR	tgDHFR
1a	2.5	0.42	0.36	0.17	1.2
2a	0.60	0.39	0.099	0.64	3.9
2b	5.45	3.9	1.9	0.72	2.1
2c	0.25	0.23	0.056	0.91	4.2
2d	9.8	7.0	5.8	0.72	1.2
3a	0.56	0.026	0.050	0.05	0.51
4a	2.4	0.42	0.22	0.17	1.9
5a	1.4	0.5	0.14	0.37	3.5
6a	0.46	0.35	0.15	0.76	2.4
7a	0.51	0.11	0.066	0.21	1.6
8a	1.0	1.1	1.0	1.1	1.1
9a	11.2	2.3	1.9	0.21	1.2
10a	2.1	1.29	0.189	0.61	6.8
11a	0.98	0.44	0.1	0.45	4.4
12a	0.096	0.069	0.011	0.72	6.0
17a	11%[b] @ 32 μM	3%[b] @ 32 μM	16%[b] @ 32 μM	ND	ND
18a	51.7	40.3	44.9	0.78	0.90
18b	17%[b] @ 104 μM	19%[b] @ 104 μM	9%[b] @ 104 μM	ND	ND
18c	10.5	8.5	3.2	0.81	2.6
18d	10%[b] @ 51 μM	2%[b] @ 51 μM	12%[b] @ 51 μM	ND	ND
PTX[c]	0.034	0.0044	0.017	0.13	0.26
TMQ[c]	0.042	0.008	0.01	0.19	0.80
TMP[c]	26.8	121	2.7	4.5	44.8

[a] Defined as the ratio IC_{50}(rlDHFR)/IC_{50}(pcDHFR) or IC_{50}(rlDHFR)/IC_{50}(tgDHFR). [b] Percent inhibition at highest concentration tested is given when IC_{50} cannot be calculated. [c] Data taken from refs 33 and 65.

Table 2. Experimental and Calculated IC_{50}, K_d, and Selectivity Index Values

	experimental values			calculated values[a]		
compd	relative IC_{50} pcDHFR[b]	relative IC_{50} rlDHFR	selectivity index	relative K_d pcDHFR	relative K_d hDHFR	selectivity index
1a	1	0.17	0.17	1	0.31	0.31
2a	0.24	0.16	0.64	0.049	0.025	0.51
3a	0.23	0.01	0.05	0.068	0.005	0.08

[a] The relative K_d values are obtained from the relation $K_d(B)/K_d(A) = \exp[(\Delta G_{bind}(B) - \Delta G_{bind}(A)]/(RT)$ where the errors in ΔG_{bind} are 0.6 kcal/mol. [b] The relative binding constants are normalized to 1 for the complex with lowest affinity (**1a** in complex with pcDHFR).

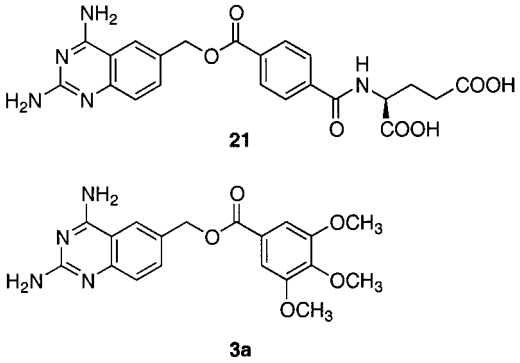


Figure 2.

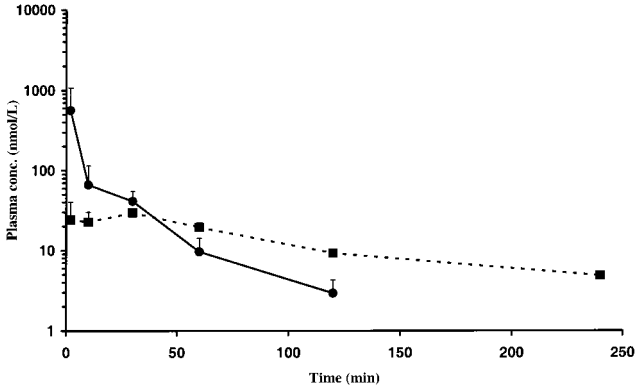


Figure 3. Plasma concentrations vs time curves of compound **3a** (●) and its metabolite **18a** (■) after intravenous administration of **3a** to male SD rats. The values are the means ± SD for three rats. The dose of **3a** is normalized to 1 μmol/kg.

providing an easily accessible metabolic pathway for degradation to inactive metabolites is a prerequisite.[39] Hence, to become an optimal inhaled soft drug against PCP, three requirements have to be fulfilled: (i) an efficient deactivation to inactive metabolites must occur, (ii) a high selectivity for pcDHFR to reduce the potential toxicity to the mammalian host cells at the site of administration must be achieved, and (iii) a sufficient efficacy against PCP must be obtained after local therapy alone. The recent reports that full protection against *P. carinii* was not achieved after inhalation of pentamidine, despite expected high local concentrations in the lung, constitute a major concern for the soft drug concept.[15,81,82]

Because we aimed to exploit structures related to TMQ and PTX as starting points, suitable positions in these molecules that can serve as handles for attacks from esterases first had to be identified. These ester bonds must be located in areas of the inhibitors not directly involved in the binding to pcDHFR. The inclusion of metabolically sensitive sites into (i) the lipophilic aromatic part, (ii) the bicyclic heterocycle, or (iii) the bridge connecting the two ring systems was initially considered. Aryl carboxylic acids, e.g., compound **8a**, exhibit pronounced and unacceptable inhibitory activity as a potential metabolite, and soft drugs based on the corresponding esters in the aromatic part were therefore excluded early as targets. Furthermore, because the incorporation of labile scissile bonds into the heterocyclic

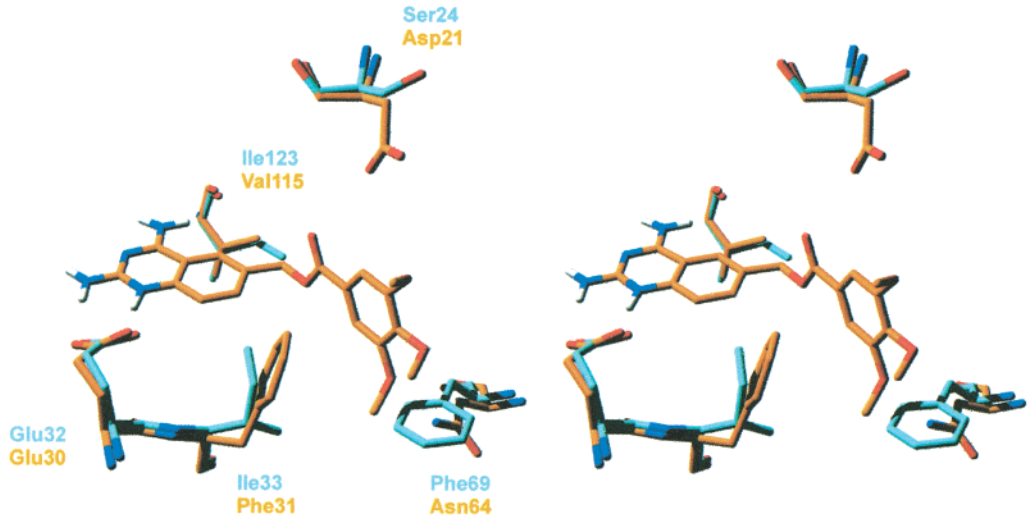


Figure 4. Stereoview showing the amino acid residues that are dissimilar in the binding site of pcDHFR (light blue) and hDHFR (orange). The glutamates interacting with the 2,4-diaminoquinazoline ring are also shown. The conformation of compound **3a** is the one that gives us the highest score when docked into the active site of hDHFR using FlexX.

system encompassing functionalities crucial for affinity to DHFR seemed difficult to pursue with the bioactivity retained, we focused our efforts on elaborations of the tether arm. The methyleneoxycarbonyl linker in **3a** resulted in superior properties among the four esters examined (cf. **3a** and **1a**, **9a**, and **10a**). Binding affinities similar to that of the methyleneamino linker, found in TMQ, were encountered, and **2a** exhibited in fact a better rlDHFR/pcDHFR ratio (0.64) than **11a** (0.45), which demonstrates that the three-atom chain is well tolerated. Although the quinazoline ester **2a** is about 15 times less potent than TMQ and PTX, it is notable that **2a** is slightly more selective. In summary, all compounds tested were less potent than TMQ and PTX, but in general, the ratio rlDHFR/pcDHFR was better. The best selectivity ratio, 0.91, was obtained with **2c**, the inhibitor with the 5-methylpyrido[2,3-*d*]pyrimidine structure also present in PTX. This has also previously been found in related systems.[83] Inhibitor **2c** was also the most active ester in the series against pcDHFR and almost 40-fold more potent than the corresponding pteridine analogue **2d**. A comparison of **2b** with **2c** reveals the remarkable impact of the 5-methyl group on the binding affinity, as expected from previous studies.[48,68,69,83] It is noteworthy that fairly high rlDHFR/tgDHFR ratios were achieved with **2c**, **3a**, **5a**, and the α,β-unsaturated four-atom bridge inhibitor **10a**, exhibiting ratios of 4.16, 3.92, 3.47, and 6.83, respectively. The corresponding values for TMQ and PTX are 0.80 and 0.26, respectively. Although **2c** is more selective than **2a** (0.91 vs 0.64) toward pcDHFR, the latter demonstrates a better and a more favorable metabolite (rlDHFR)/soft drug (pcDHFR) ratio because of the fact that the 5-methyl-substituted metabolite **18c** is more potent than metabolite **18a** against rlDHFR (Table 3).

The ability to predict the selectivity of inhibitors is of great advantage in the development of novel chemical entities aimed at interacting with proteins of pathogens where the homologous protein in human is functionally important. Sequence analysis and structural alignment of DHFR from *P. carinii* and human reveal that there are a few amino acid residues close to the active site

Table 3. Ratio of the IC_{50} Values for rlDHFR of the Metabolite and pcDHFR of the Soft Drug

compd	IC_{50} (μM) pcDHFR	IC_{50} (μM) rlDHFR	metabolite (rlDHFR)/soft drug (pcDHFR) ratio
2a	0.60		67
18a		40.3	
2c	0.25		33
18c		8.5	

that differ significantly between the two species.[84−88] Val115 in human corresponds to Ile123 in *P. carinii*, which results in a slightly larger binding pocket in the human enzyme. The change in selectivity ratio when adding substituents to the phenyl ring, e.g., compound **3a**, is anticipated to depend on the dissimilarity in the composition of amino acids in proximity to this part of the inhibitor. When the structures of the enzymes were compared, three amino acid residues are identified in this respect; Asp21, Phe31, and Asn64 in the human enzyme correspond to Ser24, Ile33, and Phe69 in *P. carinii*, respectively (Figure 4).

The greater selectivity against hDHFR for **3a** may partly be attributed to a hydrogen bond between one of the methoxy groups on the phenyl ring and the amide hydrogen of Asn64. To investigate the importance of Asn64, we also performed one simulation of compound **3a** in complex with the human Asn64Phe mutant. The calculated affinity to this "hybrid" protein was intermediate to that of the two wild-type proteins, indicating that this interaction is important, but may not account for the selectivity alone. In hDHFR, Phe31 may contribute to stronger binding by a ring stacking with the aromatic group of the inhibitor, an interaction that cannot be accomplished by the corresponding isoleucine in pcDHFR. The importance of Asp21 vs Ser24 is less obvious.

Docking these ester-linked inhibitors to DHFR is not trivial, although it seems that the diaminoquinazoline ring binds in the same manner as the diaminopteridine ring of methotrexate found in the hDHFR crystal structure.[89] The ester linkage has four rotatable bonds, and the orientation of the ester oxygens is not clear

because there are no polar groups in the proteins that seem particularly suitable for interactions. Different orientations of the carbonyl group were obtained among the highest ranked conformations generated by FlexX,[90] and therefore, simulations starting from a number of different orientations were performed. The position of the phenyl group and the torsional angles of the ester linkage of inhibitors **1a** and **2a** appeared to be rather flexible during the MD simulations, and hence, the starting conformation was not crucial for the final average energies.

Because of the relatively bulky methoxy groups of inhibitor **3a**, the aromatic ring does not appear to be as flexible as the phenyl group of compounds **1a** and **2a**. On the other hand, the ester linkage exhibits a high flexibility and several rotamers of this moiety are sampled during the simulations. However, the average orientation of the carbonyl oxygen appeared to vary depending on the surrounding enzyme, despite the large structural resemblance.

Conclusion

In summary, a series of ester soft drugs has been designed, synthesized, and evaluated in DHFR assays. Molecular dynamics simulations have been performed in order to determine the rank order of selectivity indexes using the linear interaction energy approach. These soft drugs, which are assumed to be rapidly metabolized to inactive species in vivo, are intended to be administered by inhalation to treat PCP. The best compounds were approximately 10 times less potent than trimetrexate and piritrexim in the DHFR assays but provided, in general, slightly better pcDHFR selectivity index. We believe that **2a** and **2c** should serve equally well as prototypes for further optimization. On the basis of the efficient elimination of **3a** in vivo, the compound was selected for a pharmacokinetic study because of its structural resemblance to trimetrexate, and also **2a** and **2c** can be anticipated to undergo a sufficiently fast metabolic degradation. Although **2c** is 2−3 times more potent against pcDHFR and also provides a better selectivity pcDHFR index than **2a**, the hydroxymethyl metabolite **18c**, which is generated from **2c**, unfortunately still retains a notable activity against rlDHFR, which makes compound **2c** somewhat less attractive.

Experimental Section

Chemistry. General Comments. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX270 spectrometer at 270 and 67.8 MHz, respectively, and a JEOL JNM-EX400 spectrometer at 400 and 100 MHz, respectively. Thin-layer chromatography (TLC) was performed by using aluminum sheets precoated with silica gel 60 F_{254} (0.2 mm) type E (Merck). Chromatographic spots were visualized by UV light or by an acidic ethanolic solution of 2,4-dinitrophenylhydrazine. Column chromatography was conducted on silica gel 60 (0.040−0.063 mm; Merck) unless otherwise noted. Centrifugal chromatography was carried out on a Harrison Research Chromatotron (model 7924T) with silica gel PF_{254} containing gypsum (E. Merck) as the solid phase. Melting points (uncorrected) were determined in open glass capillaries on an electrothermal apparatus. Infrared (IR) spectra were recorded on a Perkin-Elmer 1605 FT-IR spectrophotometer and are recorded in ν_{max} (cm^{-1}). The elemental analyses were performed by Mikro Kemi AB, Uppsala, Sweden, or Analytische Laboratorien, Gummersbach, Germany, and were within ±0.4% of the calculated values. All commercial chemicals were used without further purification.

2-Amino-6-chloro-3,5-pyridinedicarbonitrile (13b).[48,91] Malononitrile (6.87 mg, 104 mmol) and triethyl orthoformate (8.65 mL, 52 mmol) were heated together under reflux in pyridine (4.2 mL, 52 mmol). After 20 min, concentrated HCl (80 mL) was added to the dark solid (foaming!). The reaction mixture was stirred at 80 °C for 1 h and cooled to room temperature. Water (100 mL) was added, and the solid was filtered off and washed with water, ethanol, and diethyl ether. Recrystallization from warm DMF (230 mL) and water (250 mL) gave **13b**, which was collected and washed as above, yielding 4.3 g (47%): mp 280 °C (sublimation) (lit.[48,91] 200 °C sublimation); IR (KBr) 2230 (nitrile) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.57 (s, 2H, *H*-4), 8.42 (br s, 2H, NH_2); ^{13}C NMR (DMSO-d_6) δ 160.13, 154.80, 149.10, 115.20, 114.60, 95.13, 89.20.

2-Amino-6-chloro-4-methyl-3,5-pyridinedicarbonitrile (13c).[48,91] A stirred solution of malononitrile (15.59 g, 236 mmol), triethyl orthoacetate (21.6 mL, 118 mmol), and pyridine (47.7 mL, 590 mmol) was heated under reflux for 40 min, cooled, and evaporated. The reaction mixture was treated with concentrated HCl (131 mL) with stirring at 80 °C. The mixture was cooled after 1 h, and water (220 mL) was added. The solid was collected and washed with water, ethanol, and diethyl ether. The crude product was recrystallized from warm DMF (110 mL) and water (220 mL), affording 11.6 g (51%) of **13c**: mp 275 °C (lit.[48] >270 °C); IR (KBr) 2231 (nitrile) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.30 (br s, 2H, NH_2) 2.50 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 160.06, 159.66, 154.74, 114.74, 114.12, 96.80, 89.99, 19.98.

[2,4-Diaminopyrido[2,3-*d*]pyrimidine-6-carbonitrile] (14b).[48] A solution of **13b** (3.9 g, 21.8 mmol) in DMF (60 mL) and triethylamine (3 mL) was hydrogenated at atmospheric pressure over 10% Pd/C at room temperature for 26 h until all of the starting material had been consumed according to TLC. The catalyst was filtered off through Celite, and the solution was evaporated to dryness in vacuo. The product was purified by flash chromatography [pentane/ethyl acetate (1:1)], yielding 1.0 g (32%) of 2-amino-3,5-pyridinedicarbonitrile:[48,92] ^{1}H NMR (DMSO-d_6) δ 8.58 (d, J = 2.3 Hz, 1H, *H*-4), 8.42 (d, J = 2.3 Hz, 1H, *H*-6), 7.97 (br s, 2H, NH_2); ^{13}C NMR (DMSO-d_6) δ 160.65, 156.79, 146.24, 117.14, 115.28, 95.49, 89.63.

Sodium methoxide (1.25 g, 23.2 mmol) and guanidine hydrochloride (2.22 g, 23.2 mmol) were stirred in ethanol (60 mL) for 20 min in a dry round-bottom flask. After addition of 2-amino-3,5-pyridinedicarbonitrile[48,92] (660 mg, 5.8 mmol) the mixture was refluxed for 60 h. After filtration and evaporation under reduced pressure the crude product was dissolved in concentrated acetic acid and precipitated with aqueous ammonia, finally yielding 725 mg (67%) of **14b**: IR (KBr) 2227 (nitrile) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.88 (d, J = 2.3 Hz, 1H, *H*-5), 8.83 (d, J = 2.3 Hz, 1H, *H*-7), 7.84 (br s, 2H, NH_2), 7.02 (br s, 2H, NH_2).

[2,4-Diamino-5-methylpyrido[2,3-*d*]pyrimidine-6-carbonitrile] (14c).[48] Compound **14c** was synthesized as described above for compound **14b** starting from **13c** (4.0 g, 20.8 mmol), DMF (60 mL), and triethylamine (2.9 mL, 20.7 mmol). Thin-layer chromatography showed the absence of the starting material after 24 h [pentane/ethyl acetate (3:1)]. The catalyst was removed by filtration through Celite, and the filtrate was evaporated to dryness under reduced pressure. Recrystallization from ethanol gave 2.6 g (80%) of 2-amino-4-methyl-3,5-pyridinedicarbonitrile[48] as yellow crystals: mp 220−223 °C (lit.[48] 222−223 °C); IR (KBr) 2223 (nitrile) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.49 (s, 1H, *H*-6), 7.87 (br s, 2H, NH_2), 2.47 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 161.10, 156.36 (2C), 116.60, 114.59, 97.07, 90.35, 19.21.

Guanidine hydrochloride (2.42 g, 25.3 mmol) was added to a suspension of sodium methoxide (1.37 g, 25.3 mmol) in absolute ethanol (90 mL). The mixture was allowed to stir at room temperature for 20 min before 2-amino-4-methyl-3,5-pyridinedicarbonitrile[48] (1.0 g, 6.3 mmol) was added. The reaction mixture was heated under reflux for 10 days. The

ethanol-insoluble product **14c** and sodium chloride were filtered off from the boiling mixture, and the solid cake was stirred with boiling ethanol to remove any existing starting material. The crude product was again collected and washed with water, ethanol, and diethyl ether before it was dissolved in boiling acetic acid (15%) and filtered. Addition of aqueous ammonia caused precipitation of pure product, providing 870 mg (69%): mp >360 °C; IR (KBr) 2228 (nitrile) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.74 (s, 1H, *H*-7), 7.36 (br s, 2H, NH_2), 6.92 (br s, 2H, NH_2), 2.83 (s, 3H, CH_3).

General Procedure for the Synthesis of Compounds 15a−c.[42,50,52] A combination of a method described by van Es et al.[93] and a modified version later described by Piper et al.[50] was adopted for the synthesis of the aldehydes **15a−c**. The nitrile (**14a−c**, 16.2 mmol), Ni−Al alloy (6 g), and 75% formic acid (70 mL) were refluxed for 75 min and then filtered while hot through Celite and washed with boiling 75% formic acid until the washings were colorless. The filtrate was concentrated in vacuo and washed with ethanol. The aldehyde was recrystallized from hot water (230 mL). Filtration while hot and precipitation of the final compound with 1 M NaOH gave, after storage in the refrigerator overnight with subsequent filtration under suction, the aldehyde as a solid.

General Procedure[51,52] for the Synthesis of Compounds 18a−c.[42,48,51,52] The aldehyde (**17a−c**, 0.53 mmol) was suspended in methanol (40 mL) with stirring. Small portions of sodium borohydride (20 mg, 0.53 mmol) were added. TLC showed full conversion to the alcohol after 45 min. The reaction mixture was filtered, a few milliliters of water was added, and the solution was evaporated under reduced pressure until about 4 mL was left. The mixture was put into the refrigerator, and the solid was later collected by filtration, yielding the alcohols **18a−c** as yellow powders.

General Procedure[57] for the Synthesis of Compounds 19a−d.[52,54,55,58] The appropriate alcohol (**18a−d**, 0.53 mmol) was dissolved in acetic acid (14 mL). In the synthesis of **19b−d**, warming was necessary to obtain dissolution of the alcohols **18b−d**. When the solution had reached room temperature, 30% hydrobromic acid in acetic acid (18 mL) was added to the reaction mixture. After being stirred at room temperature for 24−48 h in the absence of light, the mixture was poured with stirring into cold diethyl ether. The flask was put into the refrigerator, and the formed precipitation was collected under nitrogen and washed with cold diethyl ether. The bromomethyl compounds **19a−d**, generated as their hydrobromic salts, were all used immediately in the next step without further purification.

[2,4-Diaminopyrido[2,3-*d*]pyrimidine-6-methyl] Benzoate (2b). Freshly prepared **19b** (0.78 mmol) was added to a suspension of benzoic acid (286 mg, 2.34 mmol) and potassium carbonate (324 mg, 2.34 mmol) in dry DMF (18 mL). The reaction mixture was stirred under nitrogen at room temperature for 24 h before the crude product was evaporated under reduced pressure on a small portion of silica gel. The silica plug was loaded on the top of a silica column previously packed with dichloromethane, and the crude ester was purified by repeated flash chromatography [$CHCl_3$/(MeOH + NH_3) (19:1 and 39:1)], yielding 50 mg (22% over two steps): mp >360 °C; IR (KBr) 1721 (ester) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.75 (d, J = 2.14 Hz, 1H, *H*-5), 8.49 (d, J = 2.31 Hz, 1H, *H*-7), 8.00 (app d, 2H, Ar*H*), 7.67 (app t, 1H, Ar*H*), 7.56 (br s, 2H, NH_2), 7.53 (app t, 2H, Ar*H*), 6.40 (br s, 2H, NH_2), 5.35 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 165.64, 163.35 (2C), 161.14. 155.76, 133.47, 133.16, 129.52, 129.29 (2C), 128.79 (2C), 123.18, 104.01, 64.30. Anal. ($C_{15}H_{13}N_5O_2 \cdot 0.75H_2O$) C, H, N: calcd, 22.7; found, 22.2.

[2,4-Diamino-5-methyl-pyrido[2,3-*d*]pyrimidine-6-methyl] Benzoate (2c). Compound **2c** was prepared as described above in the synthesis of **2b** starting with **19c** (0.74 mmol), benzoic acid (267 mg, 2.19 mmol), and potassium carbonate (303 mg, 2.19 mmol) in dry DMF (20 mL). The mixture was stirred under nitrogen at room temperature for 48 h before it was worked up as for **2b**, yielding 36 mg (16% over two steps) of the final ester: mp >360 °C; ^1H NMR (DMSO-d_6) δ 8.59 (s, 1H, *H*-7), 7.97 (app d, 2H, Ar*H*), 7.65 (app t, 1H, Ar*H*), 7.51 (app t, 2H, Ar*H*), 7.07 (br s, 2H, NH_2), 6.35 (br s, 2H, NH_2), 5.40 (s, 2H, CH_2), 2.73 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 165.67, 164.24, 162.67, 162.21, 156.01, 146.76, 133.50, 129.62, 129.26 (2C), 128.87 (2C), 122.58, 105.45, 63.36, 17.82. Anal. ($C_{16}H_{15}N_5O_2 \cdot 0.70H_2O$) C, H, N: calcd, 21.75; found, 21.12.

[2,4-Diaminopteridine-6-methyl] Benzoate (2d). Compound **19d** (0.52 mmol) in anhydrous DMF (2 mL) was added dropwise to a mixture of benzoic acid (191 mg, 1.56 mmol) and potassium carbonate (216 mg, 1.56 mmol) in dry DMF (10 mL). The reaction mixture was stirred under nitrogen at room temperature for 2 days. Workup was performed as described above for **2b**, providing 52 mg (34% over two steps) of the ester as a yellow solid: mp >360 °C; IR (KBr) 1719 (ester) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.88 (s, 1H, *H*-7), 8.02 (app d, 2H, Ar*H*), 7.70−7.60 (m, 4H, NH_2 + Ar*H*), 7.53 (app t, 1H, Ar*H*), 6.73 (br s, 2H, NH_2), 5.45 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 165.58, 163.27, 162.83, 155.78, 150.32, 142.54, 133.61, 129.37 (2C), 129.25, 128.87 (2C), 121.61, 65.44. Anal. ($C_{14}H_{12}N_6O_2 \cdot 0.75H_2O$) C, H, N.

(2,4-Diaminoquinazoline-6-yl)methyl 3′,4′,5′-Trimethoxybenzoate (3a). Freshly prepared **19a** (1.58 mmol) in DMSO (15 mL) was added dropwise to a mixture of 3,4,5-trimethoxybenzoic acid (368 mg, 1.7 mmol) and powdered potassium carbonate (523 mg, 3.8 mmol) in anhydrous DMSO (5 mL). The reaction mixture was allowed to stir at room temperature under an atmosphere of nitrogen for 48 h until water (200 mL) was added, and the flask was put into the refrigerator. The precipitate was collected by filtration and washed with water. A solution of the crude product in methanol containing suspended silica gel was evaporated in vacuo. Further purification was performed as in the case of **2b**, using $CHCl_3$/MeOH (19:1) as eluent, finally affording 129 mg (21% over two steps) of a white powder: mp 223−227 °C; IR (KBr) 1702 (ester) cm^{-1}; ^1H NMR (DMF-d_6) δ 8.23 (d, J = 1.70 Hz, 1H, *H*-5), 7.62 (dd, J = 8.55, 1.92 Hz, 1H, *H*-7), 7.37 (br s, 2H, NH_2), 7.36 (s, 2H, Ar*H*), 7.28 (d, J = 8.55 Hz, 1H, *H*-8), 6.02 (br s, 2H, NH_2), 5.39 (s, 2H, CH_2), 3.96 (s, 6H), 3.82 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 165.28, 162.45, 161.06, 152.76, 152.56, 141.82, 132.90, 127.21, 124.76, 124.52 (2C), 123.97 (2C), 109.89, 106.55, 66.67, 60.16, 56.00 (2C). Anal. ($C_{19}H_{20}N_4O_5$) C, H, N.

(2,4-Diaminoquinazoline-6-yl)methyl 2′,5′-Dimethoxybenzoate (4a). Compound **4a** was prepared as in the case of **2d** starting with **19a** (1.58 mmol) in anhydrous DMF (15 mL), 2,5-dimethoxybenzoic acid (862 mg, 4.73 mmol), and potassium carbonate (654 mg, 4.73 mmol) in dry DMF (5 mL). After 48 h at room temperature under an atmosphere of nitrogen the mixture was filtered and worked up as for **2b**, using $CHCl_3$/MeOH (39:1) as eluent, to provide 160 mg (29% over two steps) of the pure ester: mp 195−197 °C; IR (KBr) 1703 (ester) cm^{-1}; ^1H NMR (DMF-d_6) δ 8.22 (app s, 1H, *H*-5), 7.69 (dd, J = 8.57, 1.98 Hz, 1H, *H*-7), 7.48 (br s, 2H, NH_2), 7.31 (d, J = 8.58 Hz, 1H, *H*-8), 7.29−7.17 (m, 3H, Ar*H*), 6.15 (br s, 2H, NH_2), 5.34 (s, 2H, CH_2), 3.83 (s, 3H), 3.80 (s, 3H); ^{13}C NMR (DMF-d_6) δ 165.89, 163.50, 161.67, 153.39, 153.28, 152.60, 133.39, 128.52, 124.84, 124.21, 121.62, 118.91, 116.14, 114.66, 110.51, 66.85, 56.59, 55.73. Anal. ($C_{18}H_{18}N_4O_4 \cdot 0.25H_2O$) C, H, N.

(2,4-Diaminoquinazoline-6-yl)methyl 2′,3′,4′-Trimethoxybenzoate (5a). Compound **5a** was prepared as described above for **2d** starting with **19a** (0.53 mmol), 2,3,4-trimethoxybenzoic acid (335 mg, 1.58 mmol), and potassium carbonate (218 mg, 1.58 mmol) in DMF (total amount: 5 mL). The reaction was allowed to proceed for 2 days before it was filtered and worked up as for **2b**, yielding 34 mg (17% over two steps): mp 216−218 °C; ^1H NMR (DMF-d_6) δ 8.19 (app s, 1H, *H*-5), 7.65 (dd, J = 8.57, 1.98 Hz, 1H, *H*-7), 7.53 (d, J = 8.91 Hz, 1H, Ar*H*), 7.51 (br s, 2H, NH_2), 7.28 (d, J = 8.58 Hz, 1H, *H*-8), 6.91 (d, J = 8.91 Hz, 1H, Ar*H*), 6.24 (br s, 2H, NH_2), 5.28 (s, 2H, CH_2), 3.88 (s, 3H), 3.78 (s, 3H), 3.76 (s, 3H); ^{13}C NMR (DMF-d_6) δ 165.71, 163.84, 161.98, 158.02, 154.90, 152.85, 143.63, 133.91, 128.97, 127.13, 125.12, 124.74, 118.69, 110.87, 108.34, 67.08, 62.05, 60.95, 56.50. Anal. ($C_{19}H_{20}N_4O_5 \cdot 0.25H_2O$) C, H, N.

(2,4-Diaminoquinazoline-6-yl)methyl 3′,5′-Dimethoxybenzoate (6a). Compound **6a** was prepared as described above for **2d** starting with **19a** (0.53 mmol), 3,5-dimethoxybenzoic acid (287 mg, 1.58 mmol), and potassium carbonate (218 mg, 1.58 mmol) in DMF (total amount: 5 mL). The reaction was allowed to proceed for 2 days before it was filtered and worked up as for **2b**, yielding 42 mg (23% over two steps): mp 219−221 °C; ^{1}H NMR (DMF-d_6) δ 8.19 (d, J = 1.65 Hz, 1H, *H*-5), 7.63 (dd, J = 8.58, 1.65 Hz, 1H, *H*-7), 7.42 (br s, 2H, NH_2), 7.25 (d, J = 8.58 Hz, 1H, *H*-8), 7.11 (d, J = 2.31 Hz, 2H, Ar*H*), 6.77 (t, J = 2.31 Hz, 1H, Ar*H*), 6.10 (br s, 2H, NH_2), 5.33 (s, 2H, CH_2), 3.81 (s, 6H); ^{13}C NMR (DMF-d_6) δ 166.32, 163.80, 162.92, 161.66, 153.90, 133.62, 132.79, 128.37, 125.77, 124.69, 110.98, 107.76 (2C), 105.57, 67.62, 55.98 (2C). Anal. ($C_{18}H_{18}N_4O_4$) C, H, N.

(2,4-Diaminoquinazoline-6-yl)methyl 3′,4′-Dimethoxybenzoate (7a). Compound **7a** was prepared as described above for **2d** starting with **19a** (0.53 mmol), 3,4-dimethoxybenzoic acid (287 mg, 1.58 mmol), and potassium carbonate (218 mg, 1.58 mmol) in DMF (total amount: 5 mL). The reaction was allowed to proceed for 2 days before it was filtered and worked up as for **2b**, yielding 56 mg (30% over two steps) of the pure ester: mp 212−215 °C; ^{1}H NMR (DMF-d_6) δ 8.19 (d, J = 1.65 Hz, 1H, *H*-5), 7.63 (dd, J = 8.25, 1.98 Hz, 1H, *H*-7), 7.62 (dd, J = 8.58, 1.98 Hz, 1H, *H*-7), 7.52 (d, J = 1.98 Hz 2H, Ar*H*), 7.44 (br s, 2H, NH_2), 7.25 (d, J = 8.58 Hz, 1H, *H*-8), 7.08 (d, J = 8.25 Hz, 1H, Ar*H*), 6.11 (br s, 2H, NH_2), 5.31 (s, 2H, CH_2), 3.87 (s, 3H, CH_3), 3.84 (s, 3H, CH_3); ^{13}C NMR (DMF-d_6) δ 166.41, 163.79, 162.95, 154.19, 153.82, 149.61, 133.58, 128.68, 125.71, 124.55, 124.06, 122.98, 112.60, 111.68, 110.96, 67.14, 56.22, 56.06. Anal. ($C_{18}H_{18}N_4O_4$) C, H, N.

(2,4-Diaminoquinazoline-6-yl)methyl Phenylacetate (9a). Freshly prepared **19a** (0.53 mmol) in dry DMAc (5 mL) was added dropwise to a suspension of phenylacetic acid (287 mg, 1.05 mmol) and cesium carbonate (514 mg, 1.58 mmol) in dry DMAc (5 mL). The reaction mixture was allowed to stir for 24 h at room temperature under an atmosphere of nitrogen. The workup was performed as for **2b**, providing 79 mg (49% over two steps) of pure compound: mp 183−185 °C; IR (KBr) 1712 (ester) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.97 (d, J = 1.65 Hz, 1H, *H*-5), 7.45 (dd, J = 8.58, 1.65 Hz, 1H, *H*-7), 7.35−7.21 (m, 5 + 2H, Ar*H* + NH_2), 7.16 (d, J = 8.58 Hz, 1H, *H*-8), 5.99 (br s, 2H, NH_2), 5.08 (s, 2H), 3.73 (s, 2H); ^{13}C NMR (CD_3OD) δ 173.46, 164.85, 162.23, 152.61, 135.70, 135.04, 130.80, 130.50 (2C), 129.68 (2C), 128.22, 125.11, 124.78, 111.32, 67.67, 42.13. Anal. ($C_{17}H_{16}N_4O_2\cdot0.25H_2O$) C, H, N.

2,4-Diamino-6-iodoquinazoline (20a).[59,60] Sodium nitrite (0.207 g, 3.00 mmol) in 1 mL of water (0 °C) was added to a cold solution of 2,4,6-triaminoquinazoline (0.5 g, 2.9 mmol) in 2 M hydrochloric acid (8 mL). The mixture was allowed to stir on an ice bath for 15 min. Potassium iodide (0.498 g, 3.00 mmol) in water (4 mL) was added to the mixture, and the reaction was allowed to proceed for 2 h before it was heated to 70 °C for only a short while. The reaction mixture was cooled to room temperature, and 7 M aqueous ammonia (4 mL) was added. The formed precipitate was filtered off and dissolved in DMF and filtered. After precipitation with water and subsequent filtration, the crude mixture was purified by flash chromatography [CH_2Cl_2/(MeOH + $NH_{3(aq)}$) (9:1)]. Concentration of the pooled fractions afforded a powder, which was recrystallized from DMF and water, finally yielding 166 mg (20%) of pure product: ^{1}H NMR (DMSO-d_6) δ 8.37 (d, J = 1.65 Hz, 1H, *H*-5), 7.72 (dd, J = 8.58, 1.98 Hz, 1H, *H*-7), 7.45 (br s, 2H, NH_2), 6.99 (d, J = 8.91 Hz, 1H, *H*-8), 6.24 (br s, 2H, NH_2).

Phenyl (2,4-Diaminoquinazoline-6-yl)acrylate (10a). Phenyl acrylate (150 mg, 1.01 mmol) and triethylamine (0.14 mL, 1.01 mmol) dissolved in dry DMF (2 mL) were added to a Pyrex flask containing **20a** (58 mg, 0.20 mmol), palladium acetate (2.5 mol %), and tri(*o*-tolyl)phosphine (5 mol %) in dry DMF (2 mL). The reaction mixture was heated to 80 °C and stirred overnight before it was evaporated under reduced pressure on a small portion of silica gel. Workup was performed as described above for **2b**, yielding 25 mg (40%) of pure compound: mp >360 °C; ^{1}H NMR (DMF-d_6) δ 8.54 (d, J = 1.98 Hz, 1H, *H*-5), 7.98 (dd, J = 8.58, 1.98 Hz, 1H, *H*-7), 7.87 (d, J = 15.84 Hz, 1H), 7.62 (br s, 2H, NH_2), 7.49 (app t, 2H, Ar*H*), 7.31−7.26 (m, 4H, *H*-8 + Ar*H*), 6.80 (d, J = 15.84 Hz, 1H), 6.41 (br s, 2H, NH_2); ^{13}C NMR (DMF-d_6) δ 165.75, 163.71, 162.57, 155.57, 151.54, 146.80, 132.07, 129.78 (2C), 126.17, 126.03, 125.97, 125.72, 122.29 (2C), 114.42, 110.85. Anal. ($C_{17}H_{14}N_4O_2\cdot1H_2O$) C, H, N.

2,4-Diamino-6-anilinomethylquinazoline (11a).[47] A heated solution of 2,4-diaminoquinazoline-6-carbonitrile (**14a**, 100 mg, 0.54 mmol) in 50% acetic acid (5 mL) was cooled, treated with aniline (60 μL, 0.64 mmol), and hydrogenated over Raney Ni (300 mg) at atmospheric pressure for 20 h. After filtration and evaporation under reduced pressure the product was purified on an Al_2O_3 column [$CHCl_3$/(MeOH + NH_3) (9:1)], affording 75 mg (52%) of the pure compound: mp 196−198 °C (lit.[47] 190−197 °C); ^{1}H NMR (DMSO-d_6) δ 7.97 (d, J = 1.65 Hz, 1H, *H*-5), 7.49 (dd, J = 8.58, 1.98 Hz, 1H, *H*-7), 7.18 (br s, 2H, NH_2), 7.15 (d, J = 8.58 Hz, 1H, *H*-8), 7.04 (app t, 2H, Ar*H*), 6.61 (app d, 2H, Ar*H*), 6.51 (app t, 1H, Ar*H*), 6.06 (t, J = 5.61 Hz, 1H, N*H*), 5.87 (br s, 2H, NH_2), 4.22 (d, J = 5.61 Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 162.32, 160.60, 151.73, 148.71, 132.29, 131.42, 128.77 (2C), 124.36, 122.29, 115.76, 112.31 (2C), 109.90, 46.88.

2,4-Diamino-6-(*N*-methylanilino)methylquinazoline (12a). Sodium cyanoborohydride (106 mg, 1.69 mmol) was added to a suspension of **11a** (150 mg, 0.57 mmol) and 37% formaldehyde (0.1 mL) in acetonitrile (24 mL). The reaction mixture was stirred at room temperature under an atmosphere of nitrogen. After 10 min, concentrated hydrochloric acid was added dropwise until a clear solution was obtained (pH 2−3). The reaction mixture was allowed to stir for an additional hour before thin-layer chromatography showed total consumption of the starting material. After evaporation in vacuo, a small volume of water was added together with aqueous ammonia until the pH reached 8−9. The resulting suspension was refrigerated before the product was collected by filtration. Drying in a vacuum oven at 70 °C yielded 141 mg (89%) of pure compound: mp 203−205 °C; ^{1}H NMR (DMF-d_6) δ 8.02 (s, 1H, *H*-5), 7.42 (dd, J = 8.58, 1.98 Hz, 1H, *H*-7), 7.38 (br s, 2H, NH_2), 7.22 (d, J = 8.58 Hz, 1H, *H*-8), 7.17 (app t, 2H, Ar*H*), 6.83 (app d, 2H, Ar*H*), 6.65 (app t, 1H, Ar*H*), 5.99 (br s, 2H, NH_2), 4.57 (s, 2H, CH_2), 3.05 (s, 3H, N−CH_3); ^{13}C NMR (DMF-d_6) δ 163.26, 161.58, 152.68, 150.18, 132.02, 131.46, 129.20 (2C), 125.32, 121.96, 116.42, 112.90 (2C), 110.80, 56.48, 38.32. Anal. ($C_{16}H_{17}N_5\cdot0.75H_2O$) C, H, N.

Computational Method. The linear interaction energy (LIE) method can be used for estimation of the binding free energy for protein−ligand complexes and is described in detail elsewhere.[94,95] A general version of the LIE formula can be written as[96]

$$\Delta G_{bind} = \alpha(\langle V_{l-s}^{vdW}\rangle_p - \langle V_{l-s}^{vdW}\rangle_w) + \beta(\langle V_{l-s}^{el}\rangle_p - \langle V_{l-s}^{el}\rangle_w) + \gamma \quad (1)$$

where the van der Waals and electrostatic components of the ligand-surrounding interaction energy V_{l-s} are denoted by the superscripts vdW and el, respectively. The terms $\langle\ \rangle_p$ and $\langle\ \rangle_w$ denote average energies from simulations of the solvated protein complex and of the free ligand in water. Here, we use the values α = 0.181 and β = 0.5 previously determined for a set of protein−ligand complexes with similar polar binding sites.[95] While no constant term (γ) was needed for that set of complexes, a recent study showed that a γ = −3 kcal/mol was required to reproduce binding affinities of a set of human thrombin inhibitors.[96] Our earlier results from LIE calculations of methotrexate analogue affinities for human DHFR also give some indication that $\gamma \neq 0$ may be needed to reproduce the absolute binding free energies in this case.[58] However, here we are only interested in examining the relative binding free energies, for which a constant term in eq 1 will cancel, and no attempt to recalibrate eq 1 is made. It may be noted in this respect that the earlier parametrization of eq 1[96] did yield accurate relative binding energies for the thrombin complexes, although the additional constant term was needed to reproduce the absolute affinities. Furthermore, it should be pointed out

that the experimental binding results herein are given in terms of IC_{50} and not K_d values, but for relative affinities a direct comparison of the two quantities should nevertheless be meaningful. All energy averages were calculated from MD simulations performed using the software package Q[97] and the GROMOS87 force field.[98] Nonstandard charges of NADPH and the inhibitors were derived as in earlier work.[58,99] The starting coordinates for the simulations of dihydrofolate reductase from *P. carinii* were taken from the crystal structure of the ternary complex with the enzyme in complex with NADPH and trimethoprim[100] (PDB entry 1DYR). The structure of human DHFR was the L22Y mutant enzyme in complex with NADPH and methotrexate[101] (PDB entry 1DLS) from which the wild-type enzyme was modeled prior to simulation. The protein modeling and the building of the inhibitors were done using InsightII (Biosym/MSI, San Diego). Docking of the inhibitors into the active sites was performed using FlexX.[90] The protein−inhibitor complex was soaked in an 18 Å spherical grid of SPC[98] water molecules. Water molecules generated closer than 2.4 Å to protein or inhibitor atoms were removed. Ionic groups on the protein within about 10 Å from the inhibitors were included, whereas those far from the active site were modeled as neutral dipolar groups. The net charge of the protein and the cofactor was zero. Simulations of the free inhibitors were carried out in water spheres of the same size as the protein simulations. The water molecules were subjected to radial and polarization surface restraints,[98] and the local reaction field (LRF)[102] was used for handling long-range electrostatics. The system was heated to 300 K by increasing the temperature incrementally during a 30 ps equilibration phase, keeping the protein atoms restrained in their crystallographic positions. The system was then equilibrated without restraining the protein in the simulation sphere for 50 ps. Data collection was performed at 300 K using 500 000 time steps of 1.5 fs.

Pharmacokinetics in Rat. The pharmacokinetics of **3a** was evaluated in male SD rats, weighing 233−264 g, after intravenous (1 μmol/kg) and oral (10 μmol/kg) administration, three animals for each route of administration. The test compound was dissolved in a DMA/PEG400/citrate buffer (40:40:20) vehicle to the concentration of 2 μmol/mL. The intravenous dose (100 μL) was given as a bolus into a tail vein. The oral dose (1 mL) was given by gavage. Blood was collected by repeated sampling from the tail vein opposite the intravenous administration site at 2, 10, 30, 60, 120, 240, and 360 min. No anesthetics were used during the experiment. Plasma, prepared by centrifugation of the blood for 5 min at 3000 *g* in 4 °C, was precipitated with ethanol, and the supernatant was analyzed for the presence of the compound using a LC−MS/MS technique. The formed metabolites **17a** and **18a**, respectively, were also quantified. As standards for the bioanalysis, the test formulations were added to blank plasma and diluted stepwise, giving rise to standard curves ranging from 2000 to 6000 nM. The concentration of compound in the test formulations was determined before and after each experiment by LC−MS. For calculation of the pharmacokinetic parameters, the noncompartmental analysis model in WinNonlin Professional (version 2.1), Scientific Consulting, was used.

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References

(1) Masur, H. Problems in the Management of Opportunistic Infections in Patients Infected with Human Immunodeficiency Virus. *J. Infect. Dis.* **1990**, *161*, 858−864.
(2) Kontoyannis, D. P.; Rubin, R. H. Infection in the Organ Transplant Recipient. An Overview. *Infect. Dis. Clin. North Am.* **1995**, *9*, 811−822.
(3) Sparano, J. A.; Sara, C. Infection Prophylaxis and Antiretroviral Therapy in Patients with HIV Infection and Malignancy. *Curr. Opin. Oncol.* **1996**, *8*, 392−399.
(4) Kovacs, J. A.; Hiemenz, J. W.; Macher, A. M.; Stover, D.; Murray, H. W.; Shelhamer, J.; Lane, H. C.; Urmacher, C.; Honig, C.; Longo, D. L.; Parker, M. M.; Natanson, C.; Parillo, J. E.; Fauci, A. S.; Pizzo, P. A.; Masur, H. *Pneumocystis carinii* Pneumonia: A Comparison Between Patients with the Acquired Immunodeficiency Syndrome and Patients with Other Immunodeficiencies. *Ann. Intern. Med.* **1984**, *100*, 663−671.
(5) Glatt, A. E.; Chirgwin, K. *Pneumocystis carinii* Pneumonia in Human Immunodeficiency Virus-Infected Patients. *Arch. Intern. Med.* **1990**, *150*, 271−279.
(6) McKenzie, R.; Travis, W. D.; Dolan, S. A. The Causes of Death in Patients with Human Immunodeficiency Virus Infections: A Clinical and Pathological Study with Emphasis on the Role of Pulmonary Diseases. *Medicine (Baltimore)* **1991**, *70*, 326−343.
(7) Miller, R. F.; Mitchell, D. M. AIDS and the Lung. Update 1995: 1 *Pneumocystis carinii* Pneumonia. *Thorax* **1995**, *50*, 191−200.
(8) Mills, J. *Pneumocystis carinii* and *Toxoplasma gondii* Infections in Patients with AIDS. *Rev. Infect. Dis.* **1986**, *8*, 1001−1011.
(9) Schliep, T. C.; Yarrish, R. L. *Pneumocystis carinii* Pneumonia. *Semin. Respir. Infect.* **1999**, *14*, 333−343.
(10) Kaplan, J. E.; Hanson, D.; Dworkin, M. S.; Frederick, T.; Bertolli, J.; Lindegren, M. L.; Holmberg, S.; Jones, J. L. Epidemiology of Human Immunodeficiency Virus-Associated Opportunistic Infections in the United States in the Era of Highly Active Antiretroviral Therapy. *Clin. Infect. Dis.* **2000**, *30* (Suppl. 1), S5−S14.
(11) Arozullah, A. M.; Yarnold, P. R.; Weinstein, R. A.; Nwadiaro, N.; McIlraith, T. B.; Chmiel, J. S.; Sipler, A. M.; Chan, C.; Goetz, M. B.; Schwartz, D. N.; Bennett, C. L. A New Preadmission Staging System for Predicting Inpatient Mortality From HIV-Associated *Pneumocystis Carinii* Pneumonia in the Early Highly Active Antiretroviral Therapy (HAART) Era. *Am. J. Respir. Crit. Care Med.* **2000**, *161*, 1081−1086.
(12) Fischl, M. A.; Dickinson, G. M.; La Voie, L. Safety and Efficiency of Sulfamethoxazole and Trimethoprim Chemoprophylaxis for *Pneumocystis carinii* Pneumonia in AIDS. *J. Am. Med. Assoc.* **1988**, *105*, 45−48.
(13) Walker, R. E.; Masur, H. In *Pneumocystis carinii Pneumonia*; Walzer, P. D., Ed.; Marcel Dekker: New York, 1994; pp 439−466.
(14) Gordin, F. M.; Simon, G. L.; Wofsy, C. B.; Mills, J. Adverse Reactions to Trimethoprim−Sulfamethoxazole in Patients with the Aquired Immunodeficiency Syndrome. *Ann. Intern. Med.* **1984**, *100*, 495−499.
(15) Masur, H. Prevention and Treatment of *Pneumocystis* Pneumonia. *N. Engl. J. Med.* **1992**, *327*, 1853−1860.
(16) Roudier, C.; Caumes, E.; Rogeauz, O.; Bricaire, F.; Gentilini, M. Adverse Cutanous Reactions to Trimethoprim−Sulfamethoxazole in Patients with the Acquired Immunodeficiency Syndrome and *Pneumocystis carinii* Pneumonia. *Arch. Dermatol.* **1994**, *30*, 1383−1386.
(17) Montgomery, A. B.; Debs, R. J.; Luce, J. M.; Corkery, K. J.; Turner, J.; Brunette, E. N.; Lin, E. T.; Hopewell, P. C. Aerosolised Pentamidine as Sole Therapy for *Pneumocystis carinii* Pneumonia in Patients with Acquired Immunodeficiency Syndrome. *Lancet* **1987**, 480−483.
(18) Golden, J. A.; Chernoff, D.; Hollander, H.; Feigal, D.; Conte, J. E. Prevention of *Pneumocystis carinii* by Inhaled Pentamidine. *Lancet* **1989**, *1*, 654−657.
(19) Monk, J. P.; Benfield, P. Inhaled Pentamidine. An Overview of Its Pharmacological Properties and a Review of Its Therapeutic Use in *Pneumocystis carinii* Pneumonia. *Drugs* **1990**, *39*, 741−756.
(20) Leoung, G. S.; Feigal, D. W.; Montgomery, A. B.; Corkery, K.; Wardlaw, L.; Adams, M.; Busch, D.; Cordon, S.; Jacobson, M. A.; Volberding, P. A.; Abrams, D. Aerosolized Pentamidine for Prophylaxis against *Pneumocystis carinii* Pneumonia. *N. Engl. J. Med.* **1990**, *323*, 769−775.
(21) Hirschel, B.; Lazzarin, A.; Chopard, P. A Controlled Study of Inhaled Pentamidine for Primary Prevention of *Pneumocystis carinii* Pneumonia. *N. Engl. J. Med.* **1991**, *324*, 1079−1083.
(22) Pearson, R. D.; Hewlett, E. L. Pentamidine for the Treatment of *Pneumocystis carinii* Pneumonia and Other Protozoal Diseases. *Ann. Intern. Med.* **1985**, *103*, 782−786.
(23) Elslager, E. F.; Davoll, J. In *Lectures in Heterocyclic Chemistry 2*; Castle, R. N., Townsend, L. B., Eds.; Hetero Corp. Orem: UT, 1974.
(24) Elslager, E. F.; Johnson, J. L.; Werbel, L. M. Folate Antagonists. 20. Synthesis, Antitumor, and Antimalarial Properties of Trimetrexate and Related 6-[(Phenylamino)methyl]-2,4-quinazolindiamines. *J. Med. Chem.* **1983**, *26*, 1753−1760.
(25) Grivsky, E. M.; Lee, S.; Sigel, C. W.; Duch, D. S.; Nichol, C. A. Synthesis and Antitumor Activity of 2,4-Diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-*d*]pyrimidine. *J. Med. Chem.* **1980**, *23*, 327−329.

(26) Bertino, J. R.; Sawacki, W. L.; Moroson, B. A.; Cashmore, A. R.; Elslager, E. F. 2,4-Diamino-5-methyl-6-[(2,4,5-trimethoxyanilino)methyl]quinazoline (TMQ), A Potent Non-Classical Folate Antagonist Inhibitor-I. *Biochem. Pharmacol.* **1979**, *28*, 1983−1987.

(27) Weir, E. C.; Cashmore, A. R.; Dreyer, R. N.; Graham, M. L.; Hsiao, N.; Moroson, B. A.; Sawacki, W. L.; Bertino, J. R. Pharmacology and Toxicity of a Potent "Nonclassical" 2,4-Diamino Quinazoline Folate Antagonist, Trimetrexate, in Normal Dogs. *Cancer Res.* **1982**, *42*, 1696−1702.

(28) Duch, D. S.; Edelstein, M. P.; Bowers, S. W.; Nichol, C. A. Biochemical and Chemotherapeutic Studies on 2,4-Diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-*d*]pyrimidine (BW301U), A Novel Lipid-Soluble Inhibitor of Dihydrofolate Reductase. *Cancer Res.* **1982**, *42*, 3987−3994.

(29) Lin, J. T.; Cashmore, A. R.; Baker, M.; Dreyer, R. N.; Ernstoff, M.; Marsh, J. C.; Bertino, J. R.; Whitfield, L. R.; Delap, R.; Grillo-Lopez, A. Phase I Studies with Trimetrexate: Clinical Pharmacology, Analytical Methodology, and Pharmacokinetics. *Cancer Res.* **1987**, *47*, 609−616.

(30) Kovacs, J. A.; Allegra, C. A.; Swan, J. C.; Drake, J. C.; Parillo, J. E.; Chabner, B. A.; Masur, H. Potent Antipneumocystis and Antitoxoplasma Activities of Piritrexim, a Lipid-Soluble Antifolate. *Antimicrob. Agents Chemother.* **1988**, *32*, 430−433.

(31) FDA Approves Trimetrexate As A Second Line Therapy For *Pneumocystis carinii* Pneumonia. *Am. J. Hosp. Pharm.* **1994**, *51*, 591−592.

(32) Allegra, C. J.; Kovacs, J. A.; Drake, J. C.; Swan, J. C.; Chabner, B. A.; Masur, H. Activity of Antifolates Against *Pneumocystis carinii* Dihydrofolate Reductase and Identification of a Potent New Agent. *J. Exp. Med.* **1987**, *165*, 926−931.

(33) Broughton, M. C.; Queener, S. F. *Pneumocystis carinii* Dihydrofolate Reductase Used to Screen Potential Antipneumocystis Drugs. *Antimicrob. Agents Chemother.* **1991**, *35*, 1348−1355.

(34) Allegra, C. J.; Chabner, B. A.; Tuazon, C. U.; Ogata-Agakaki, D.; Baird, B.; Drake, J. C.; Simmons, J. T.; Lack, E. E.; Shelhamer, J. H.; Balis, F.; Walker, R.; Kovacs, J. A.; Lane, H. C.; Masur, H. Trimetrexate for the Treatment of *Pneumocystis carinii* Pneumonia in Patients with the Acquired Immunodeficeincy Syndrome. *N. Engl. J. Med.* **1987**, *317*, 978−985.

(35) Sattler, F. R.; Allegra, C. J.; Verdegem, T. D. Trimetrexate−Leucoverin Dosage Evaluation Study for the Treatment of *Pneumocystis carinii* Pneumonia. *J. Infect. Dis.* **1990**, *161*, 91−96.

(36) Masur, H.; Polis, M. A.; Tuazon, C. V.; Ogota, A. D.; Kovacs, J. A.; Katz, D.; Hilt, D.; Simmons, T.; Feuerstein, I.; Lundgren, B.; Lane, H. C.; Chabner, B. A.; Allegra, C. J. Salvage Trial of Trimetrexate−Leucovorin for the Treatment of Cerebral Toxoplasmosis. *J. Infect. Dis.* **1993**, *167*, 1422−1426.

(37) Falloon, J.; Allegra, C. J.; Kovacs, J.; O'Neill, D.; Ogata-Arakaki, D.; Feuerstein, I.; Polis, M.; Davey, R.; Lane, H. C.; LaFon, S.; Rogers, M.; Zunich, K.; Turlo, J.; Tuazon, C.; Parenti, D.; Simon, G.; Masur, H. Piritrexim with Leucovorin for the Treatment of *Pneumocystis* Pneumonia (PCP) in AIDS Patients. *Clin. Res.* **1990**, *38*, 361A.

(38) Chabner, B. A. In *Pharmacologic Principles of Cancer Treatment*; Chabner, B. A., Ed.; W. B. Saunders: Philadelphia, 1982; pp 229−238.

(39) For a review, see the following. Bodor, N.; Buchwald, P. Soft Drug Design: General Principles and Recent Applications. *Med. Res. Rev.* **2000**, *20*, 58−101.

(40) Clissold, S. P.; Heel, R. C. Budesonide. A Preliminary Review of Its Pharmacodynamic Properties and Therapeutic Efficacy in Asthma and Rhinitis. *Drugs* **1984**, *28*, 485−518.

(41) Johansson, S.-Å.; Andersson, K.-E.; Brattsand, R.; Gruvstad, E.; Hedner, P. Topical and Systemic Glucocorticoid Potencies of Budesonide and Beclomethasone Dipropionate in Man. *Eur. J. Clin. Pharmacol.* **1982**, *22*, 523−529.

(42) Graffner-Nordberg, M.; Sjödin, K.; Tunek, A.; Hallberg, A. Synthesis and Enzymatic Hydrolysis of Esters, Constituting Simple Models of Soft Drugs. *Chem. Pharm. Bull. (Tokyo)* **1998**, *46*, 591−601.

(43) Lindgren, B. O.; Nilsson, T. Preparation of Carboxylic Acids from Aldehydes (Including Hydroxylated Benzaldehydes) by Oxidation with Chlorite. *Acta Chem. Scand.* **1973**, *27*, 888−890.

(44) Kraus, G. A.; Roth, B. Synthetic Studies toward Verrucarol. 2. Synthesis of the AB Ring System. *J. Org. Chem.* **1980**, *45*, 4825−4830.

(45) Kraus, G. A.; Taschner, M. J. Model Studies for the Synthesis of Quassinoids. 1. Construction of the BCE Ring System. *J. Org. Chem.* **1980**, *45*, 1175−1176.

(46) Taylor, E. C.; Otiv, S. R.; Durucasu, I. Protection and Deprotection of Fused 2-Amino-4(3H)-pyrimidinones. Conversion of Pterins and 5-Deazapterins to 2,4-Diamino Derivatives. *Heterocycles* **1993**, *36*, 1883−1895.

(47) Davoll, J.; Johnson, A. M. Quinazoline Analogues of Folic Acid. *J. Chem. Soc.* **1970**, 997−1002.

(48) Piper, J. R.; McCaleb, G. S.; Montgomery, J. A.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. Synthesis and Antifolate Activity of 5-Methyl-5-Deaza-Analogues of Aminopterin. Methotrexate, Folic Acid, and N-10 Methyl Folic Acid. *J. Med. Chem.* **1986**, *29*, 1080−1086.

(49) Gangjee, A.; Vasudevan, A.; Queener, S. F.; Kisliuk, R. L. 6-Substituted 2,4-Diamino-5-methylpyrido[2,3-*d*]pyrimidines as Inhibitors of Dihydrofolate Reductases from *Pneumocystis carinii* and *Toxoplasma gondii* and as Antitumor Agents. *J. Med. Chem.* **1995**, *38*, 1778−1785.

(50) Piper, J. R.; McCaleb, G. S.; Montgomery, J. A.; Kisliuk, R. L.; Gaumont, Y.; Thorndike, J.; Sirotnak, F. M. Synthesis and Antifolate Activity of 5-Methyl-5,10-dideaza Analogues of Aminopterin and Folic Acid and an Alternative Synthesis of 5,10-Dideazatetrahydrofolic Acid, a Potent Inhibitor of Glycinamide Ribonucleotide Formyltransferase. *J. Med. Chem.* **1988**, *31*, 2164−2169.

(51) De Graw, J. I.; Tagawa, H.; Christie, P. H.; Lawson, J. A.; Grown, E. G.; Kisliuk, R. L.; Gaumont, Y. Synthesis of 5,10-Dideazaaminopterin. *J. Heterocycl. Chem.* **1986**, *23*, 1−4.

(52) Piper, J. R.; Malik, N. D.; Rhee, M. S.; Galivan, J.; Sirotnak, F. M. Synthesis and Antifolate Evaluation of the 10-Propargyl Derivatives of 5-Deazafolic Acid, 5-Deazaaminopterin, and 5-Methyl-5-deazaaminopterin. *J. Med. Chem.* **1992**, *35*, 332−337.

(53) Taylor, E. C. New Pathways from Pteridines. Design and Synthesis of a New Class of Potent and Selective Antitumor Agents. *J. Heterocycl. Chem.* **1990**, *27*, 1−12.

(54) Piper, J. R.; Montgomery, J. A. Preparation of 6-(Bromomethyl)-2,4-pteridinediamine Hydrobromide and Its Use in Improved Syntheses of Methotrexate and Related Compounds. *J. Org. Chem.* **1977**, *42*, 208−211.

(55) Su, T.-L.; Huang, J.-T.; Burchenal, J. H.; Watanabe, K. A.; Fox, J. J. Synthesis and Biological Activites of 5-Deaza Analogues of Aminopterin and Folic Acid. *J. Med. Chem.* **1986**, *29*, 709−715.

(56) Srinivasan, A.; Broom, A. D. Pyridopyrimidines. 12. Synthesis of 8-Deaza Analogues of Aminopterin and Folic Acid. *J. Org. Chem.* **1981**, *46*, 1777−1781.

(57) Piper, J. R.; Johnson, C. A.; Maddry, J. A.; Malik, D. N.; McGuire, J. J.; Otter, G. M.; Sirotnak, F. M. Studies on Analogues of Classical Antifolates Bearing the Naphthoyl Group in Place of the Benzoyl in the Side Chain. *J. Med. Chem.* **1993**, *36*, 4161−4171.

(58) Graffner-Nordberg, M.; Marelius, J.; Ohlsson, S.; Persson, Å.; Swedberg, G.; Andersson, P.; Andersson, S. E.; Åqvist, J.; Hallberg, A. Computational Predictions of Binding Affinities to Dihydrofolate Reductase: Synthesis and Biological Evaluation of Methotrexate Analogues. *J. Med. Chem.* **2000**, *43*, 3852−3861.

(59) Harris, N. V.; Smith, C.; Bowden, K. A Simple Synthesis of 5,8,10-Trideazaminopterin Analogues. *Synlett* **1990**, 577−578.

(60) Harris, N. V.; Smith, C.; Bowden, K. Antifolate and Antibacterial Activities of 6-Substituted 2,4-Diaminoquinazolines. *Eur. J. Med. Chem.* **1992**, *27*, 7−18.

(61) For a review, see the following. Heck, R. F. Palladium-Catalyzed Vinylation of Organic Halides. *Org. React.* **1982**, *27*, 345−390.

(62) Bräse, S.; de Meijere, A. In *Metal-Catalyzed Cross-coupling Reactions*; Diedrich, F., Stang, P. J., Eds.; Wiley-VCH: Weinheim, 1998.

(63) For a review, see the following. Beletskaya, I. P.; Cheprakov, A. V. The Heck Reaction as a Sharpening Stone of Palladium Catalysis. *Chem. Rev.* **2000**, *100*, 3009−3066.

(64) Temple, C., Jr.; Elliott, R. D.; Montgomery, J. A. Pyrido[2,3-*d*]-pyrimidines. Synthesis of the 5-Deaza Analogues of Aminopterin, Methotrexate, Folic Acid, and N^{10}-Methylfolic Acid. *J. Org. Chem.* **1982**, *47*, 761−764.

(65) Chio, L.-C.; Queener, S. F. Identification of Highly Potent and Selective Inhibitors of *Toxoplasma gondii* Dihydrofolate Reductase. *Antimicrob. Agents Chemother.* **1993**, *37*, 1914−1923.

(66) For a review, see the following. Blaney, J. M.; Hansch, C.; Silipo, C.; Vittoria, A. Structure−Activity Relationships of Dihydrofolate Reductase Inhibitors. *Chem. Rev.* **1984**, *84*, 333−407.

(67) Gangjee, A.; Vasudevan, A.; Queener, S. F.; Kisliuk, R. L. 2,4-Diamino-5-deaza-6-Substituted Pyrido[2,3-*d*]pyrimidine Antifolates as Potent and Selective Nonclassical Inhibitors of Dihydrofolate Reductases. *J. Med. Chem.* **1996**, *39*, 1438−1446.

(68) Schornagel, J. H.; Chang, P. K.; Sciarini, L. J.; Moroson, B. A.; Mini, E.; Cashmore, A. R.; Bertino, J. Synthesis and Evaluation of 2,4-Diamino Quinazoline Antifolates with Activity against Methotrexate Resistan Human Tumor Cells. *Biochem. Pharmacol.* **1984**, *33*, 3251−3255.

(69) Hynes, J. B.; Harmon, S. J.; Floyd, G. G.; Farrington, M.; Hart, L. D.; Gale, G. R.; Washtein, W. L.; Susten, S. S.; Freisheim, J. H. Chemistry and Antitumor Evaluation of Selected Classical 2,4-Diaminoquinazoline Analogues of Folic Acid. *J. Med. Chem.* **1985**, *28*, 209−215.

(70) Hansson, T.; Marelius, J.; Åqvist, J. Ligand Binding Affinity Prediction by Linear Binding Free Energies. *J. Comput. Aided Mol. Des.* **1998**, *12*, 27−35.

(71) Johnson, S. J.; Gupta, S. V.; Stevenson, K. J.; Freisheim, J. H. Purification and Characterization of Dihydrofolate Reductase form Walker 256 Carcinosarcoma. *Can. J. Biochem.* **1982**, *60*, 1132−1142.
(72) Delcamp, T. J.; Susten, S. S.; Blankenship, D. T.; Freisheim, J. H. Purification and Characterization of Dihydrofolate Reductase from Methotrexate-Resistant Human Lymphoblastoid Cells. *Biochemistry* **1983**, *22*, 633−639.
(73) Burchall, J. J.; Hitchings, G. H. Inhibitors Binding Analysis of Dihydrofolate Reductases from Various Species. *Mol. Pharmacol.* **1965**, *1*, 126−136.
(74) Cody, V. Personal communication.
(75) Satoh, T.; Hosokawa, M. The Mammalian Carboxylesterases: from Molecules to Functions. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 257−288.
(76) *Design of Prodrugs*; Bundgaard, H., Ed.; Elsevier: Amsterdam, 1985.
(77) Kam, S.-T.; Matier, W. L.; Mai, K. X.; Barcelon-Yang, C.; Borgman, R. J.; O'Donnell, J. P.; Stampfli, H. F.; Sum, C. Y.; Anderson, W. G.; Gorczynski, R. J.; Lee, R. J. ((Arylcarbonyl)-oxy)propanolamines. 1. Novel *β*-Blockers with Ultrashort Duration of Action. *J. Med. Chem.* **1984**, *27*, 1007−1016.
(78) Augustinsson, K. B. Multiple Forms of Esterase in Vertebrate Blood Plasma. *Ann. N.Y. Acad. Sci.* **1961**, *94*, 844−860.
(79) Quon, C. Y.; Mai, K.; Patil, G.; Stampfli, H. F. Species Differences in the Stereoselective Hydrolysis of Esmolol by Blood Esterases. *Drug Metab. Dispos. Biol. Fate Chem.* **1988**, *16*, 425−428.
(80) Minagawa, T.; Kohno, Y.; Suwa, T.; Tsuji, A. Species Differences in Hydrolysis of Isocarbacyclin Methyl Ester (TEI-9090) by Blood Esterases. *Biochem. Pharmacol.* **1995**, *49*, 1361−1365.
(81) Schneider, M. M. E.; Hoepelman, A. I. M. E.; Schattenkerk, J. K. M.; Nielsen, T. L.; van der Graaf, Y.; Frissen, J. P. H. J.; van der Ende, I. M. E.; Kolsters, A. F. P.; Borleffs, J. C. C. A Controlled Trial of Aerosolized Pentamidine or Trimethoprim−Sulfamethoxazole as Primary Prophylaxis Against *Pneumocystis carinii* Pneumonia in Patients with Human Immunodeficiency Virus Infection. *N. Engl. J. Med.* **1992**, *327*, 1836−1841.
(82) Hardy, W. D.; Feinberg, J.; Finkelstein, D. M.; Power, M. E.; He, W.; Kaczka, C.; Frame, P. T.; Holmes, M.; Waskin, H.; Fass, R. J.; Powderly, W. G.; Steigbigel, R. T.; Zuger, A.; Holzman, R. S. A Controlled Trial of Trimethoprim−Sulfamethoxazole or Aerosolized Pentamidine for Secondary Prophylaxis of *Pneumocystis carinii* Pneumonia in Patients with the Aquired Immunodeficiency Syndrome. *N. Engl. J. Med.* **1992**, *327*, 1842−1848.
(83) Piper, J. R.; Johnson, C. A.; Krauth, C. A.; Carter, R. L.; Hosmer, C. A.; Queener, S. F.; Borotz, S. E.; Pfefferkorn, E. R. Lipophlic Antifolates as Agents against Opportunistic Infections. 1. Agents Superior to Trimetrexate and Piritrexim against *Toxoplasma gondii* and *Pneumocystis carinii* in *in Vitro* Evaluations. *J. Med. Chem.* **1996**, *39*, 1271−1280.
(84) Edman, J. C.; Edman, U.; Cao, M.; Lundgren, B.; Kovacs, J. A.; Santi, D. V. Isolation and Expression of the *Pneumocystis carinii* Dihydrofolate Reductase Gene. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 8625−8629.
(85) Queener, S. F. New Drug Developments for Opportunistic Infections in Immunosuppressed Patients: *Pneumocystis carinii*. *J. Med. Chem.* **1995**, *38*, 4739−4759.
(86) Gschwend, D. A.; Sirawaraporn, W.; Santi, D. V.; Kuntz, I. D. Specificity in Structure-Based Drug Design: Identification of a Novel, Selective Inhibitor of *Pneumocystis carinii* Dihydrofolate Reductase. *Proteins* **1997**, *29*, 59−67.
(87) Rosowsky, A.; Cody, V.; Galitsky, N.; Fu, H.; Papoulis, A. T.; Queener, S. F. Structure-Based Deisgn of Selective Inhibitors of Dihydrofolate Reductase: Synthesis and Antiparasitic Activity of 2,4-Diaminopteridine Analogues with a Bridged Diarylamine Side Chain. *J. Med. Chem.* **1999**, *42*, 4853−4860.
(88) Cody, V.; Chan, D.; Galitsky, N.; Rak, D.; Luft, J. R.; Pangborn, W.; Queener, S. F.; Laughton, C.; Stevens, M. F. G. Structural Studies on Bioactive Compounds. 30. Crystal Structure and Molecular Modeling Studies on the *Pneumocystis carinii* Dihydrofolate Reductase Cofactor Complex with TAB, a Highly Selective Antifolate. *Biochemistry* **2000**, *39*, 3556−3564.
(89) Queener, S. F.; Bartlett, M. S.; Jay, M. A.; Durkin, M. M.; Smith, J. W. Activity of Lipid-Soluble Inhibitors of Dihydrofolate Reductase Against *Pneumocystis carinii* in Culture and in a Rat Model of Infection. *Antimicrob. Agents Chemother.* **1987**, *31*, 1323−1327.
(90) Rarey, M.; Kramer, B.; Lengauer, T.; Klebe, G. A Fast Flexible Docking Method Using an Incremental Construction Algorithm. *J. Mol. Biol.* **1996**, *261*, 470−489.
(91) Schmidt, H.; Junec, H. Syntheses with Nitriles. Part 46. Synthesis of Alkoxymethylenemalononitriles, Tetracyanopropenides, and Highly Substituted α-Aminopyridines. *Monatsh. Chem.* **1977**, *108*, 895−900.
(92) Mulvey, D. M.; Cottis, S. G.; Tieckelmann, H. The Synthesis of 2,4,6-Trisubstituted Pyrido[2,3-*d*]pyrimidines from 2-Amino-3,5-Dicyanopyridine. *J. Org. Chem.* **1964**, *29*, 2903−2907.
(93) van Es, T.; Staskun, B. Reductions with Raney Alloy in Acid Solution. *J. Chem. Soc.* **1965**, 5775−5777.
(94) Åqvist, J.; Medina, C.; Samuelsson, J. E. A New Method for Predicting Binding Affinity in Computer-Aided Drug Design. *Protein Eng.* **1994**, *7*, 385−391.
(95) Hansson, T.; Marelius, J.; Åqvist, J. Ligand Binding Affinity Prediction by Linear Interaction Energy Methods. *J. Comput. Aided Mol. Des.* **1998**, *12*, 27−35.
(96) Ljungberg, K. B.; Marelius, J.; Musil, D.; Svensson, P.; Nordén, B.; Åqvist, J. Computational Modelling of Inhibitor Binding to Human Thrombin. *Eur. J. Pharm. Sci.* **2001**, *12*, 441−446.
(97) Marelius, J.; Kolmodin, K.; Feierberg, I.; Åqvist, J. Q: An MD Program for Free Energy Calculations and Empirical Valence Bond Simulations in Biomolecular Systems. *J. Mol. Graph. Modell.* **1998**, *16*, 213−225.
(98) van Gunsteren, W. F.; Berendsen, H. J. C. *Groningen Molecular Simulation (GROMOS) Library Manual*; Biomos, B. V.: Groningen, The Netherlands, 1987.
(99) Marelius, J.; Graffner-Nordberg, M.; Hansson, T.; Hallberg, A.; Åqvist, J. Computation of Affinity and Selectivity: Binding of 2,4-Diaminopteridine and 2,4-Diaminoquinazoline Inhibitors to Dihydrofolate Reductases. *J. Comput. Aided Mol. Des.* **1998**, *12*, 119−131.
(100) Champness, J. N.; Achari, A.; Ballantine, S. P.; Bryant, P. K.; Delves, C. J.; Stammers, D. K. The Structure of *Pneumocystis Carinii* Dihydrofolate Reductase to 1.9 Å Resolution. *Structure* **1994**, *2*, 915−924.
(101) Lewis, W. S.; Cody, V.; Galitsky, N.; Luft, J. R.; Pangborn, W.; Chunduru, S. K.; Spencer, H. T.; Appleman, J. R.; Blakley, R. L. Methotrexate-Resistant Variants of Human Dihydrofolate Reductase with Substitutions of Leucine 22: Kinetics, Crystallography, and Potential as Selectable Markers. *J. Biol. Chem.* **1995**, *270*, 5057−5064.
(102) Lee, F. S.; Warshel, A. A Local Reaction Field Method for Fast Evaluation of Long-Range Electrostatic Interactions in Molecular Simulations. *J. Chem. Phys.* **1992**, *97*, 3100−3107.

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