Prediction of Selective Bioreductive Anti-tumour, Anti-folate Activity using a Modified *Ab initio* Method for calculating Enzyme–Inhibitor Interaction Energies

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Enzyme–ligand binding energies may be calculated using an *ab initio* molecular orbital method in which the ligand is treated fully and the enzyme is modelled by point charges. Modifications of the method are described whereby the two-electron integrals are re-used (thereby speeding up the calculations considerably) and the interaction energy is decomposed into an electrostatic energy and a polarization energy. The modified method has been used in connection with two different docking procedures, and is tested on a number of potential anti-cancer bioreductive anti-folates. The inhibitors of dihydrofolate reductase described here have been specifically designed to bind to the enzyme in their reduced form, but to have a repulsive interaction in their oxidized form. Since the reduced form would be favoured in the oxygen-deficient environment of solid tumours, the designed inhibitors should be tumour selective.

The determination of the *X*-ray crystal structure of dihydrofolate reductase (DHFR)[1] has led to great interest in the use of molecular graphics and molecular modelling in the design of inhibitors of this enzyme, because DHFR inhibitors (such as methotrexate) are potent anti-cancer agents.[2] Many of the molecular modelling methods use empirical energy functions, such as Lennard–Jones potentials. However, in an attempt to move away from the use of empirical functions we have developed an *ab initio* molecular orbital method in which the substrate is treated fully, and the enzyme is modelled by point charges. The method is based on work by Hayes and Kollman[3] and is able to reproduce binding energies remarkably well.[4–6] The approximation is based on the notion that the electrostatic field created by the point charges on the enzyme provides the environment which perturbs the ligand for which parameters suitable for molecular mechanics may not exist.

In order to calculate the binding energies, the position of the inhibitor must first be determined (if it has not been observed as part of the crystal structure). This has been achieved by using the method in conjunction with two docking procedures. Both the docking procedures were tested on the pteridine ring of the inhibitor methotrexate and the natural substrates folate and 7,8-dihydrofolate. It is known that despite their similarity, methotrexate and the natural substrates bind differently to DHFR. A reliable docking procedure should be able to predict this.

The use of currently available DHFR inhibitors in cancer chemotherapy has a number of drawbacks. One is the development of resistance which leads to an over-production of the enzyme;[7] another is the lack of selectivity for cancer cells.[8] While both of these drawbacks may be alleviated using an assortment of different therapeutic agents and a suitable dose regime, we have recently proposed a method for introducing selectivity into the inhibitors themselves.[9] The method depends on the observation that solid tumour cells may be deficient in oxygen due to a poor blood supply.[10] Using molecular graphics,[11,12] we have therefore designed inhibitors of DHFR which should bind tightly in their reduced form, but which should not bind so tightly in their oxidized form. It is possible that such inhibitors will be active only in oxygen-deficient cells. The importance of agents selective for oxygen-deficient (hypoxic) tumour cells has been pointed out a number of times.[13,14] This is because these cells are frequently resistant to chemotherapy and radiotherapy, and while they may lie dormant during hypoxia, they have the potential to start dividing if the oxygen tension increases again.[15] This observation forms the basis of the importance of our proposed bioreductive anti-folates.

It was suggested over a decade ago that there may be a therapeutically useable difference in oxygen tension between solid tumour cells and normal cells.[16] This approach has since been used with a number of quinoid-type molecules, such as mitomycin C, which are enzymatically reduced under hypoxic conditions to yield an alkylating agent which can cause strand breaks and cross-linking in DNA. The only other such agents of which we are aware are the nitroimidazole radiosensitizers.[17] It is appropriate to discuss mitomycin C here as its properties may well be relevant to our own proposed bioreductive agents. Mitomycin C can cause cell damage in several ways: as an alkylating agent, as an intercalating agent, and by redox cycling. Redox cycling involves first the production of oxygen radicals by the reaction of the one-electron reduction product—the semiquinone—with molecular oxygen, and subsequent regeneration of the quinone. Redox cycling can lead to undesirable side effects with quinone-type drugs;[18,19] it is linked with a preferential kill of oxic cells over hypoxic cells at high concentrations of the drug (10 μM), but at lower concentrations (1 μM) mitomycin C and its analogues are selective for hypoxic cells.[20]

We have also sought to employ the ready reduction of quinones to hydroquinones, as hydroquinones may donate hydrogen bonds to the carboxylate and carbonyl groups of the active site of DHFR—see Figures 1 and 2. A non-enzymic reduction is likely to give at most a 10-fold change in the ratio of oxidized to reduced forms—based on oxygen tensions of about 32 mmHg in a normal cell and 0.25 mmHg in an hypoxic cell. A standard redox potential of about 0.5—0.7 V would be required. The redox potentials of quinones may be adjusted to fall in this range. 1,4-Benzoquinone, for example, has a standard redox potential of +0.699 V; the redox potential is decreased by electron-donating groups and by increasing the size of the fused ring system; it is raised by electron-accepting substituents.[21]

The work on the bioreductive alkylating agents suggests that the reduction may be carried out enzymatically. The bioreductive step is dependent on the presence of non-specific enzymes in the cell. It appears that mitomycin C preferentially

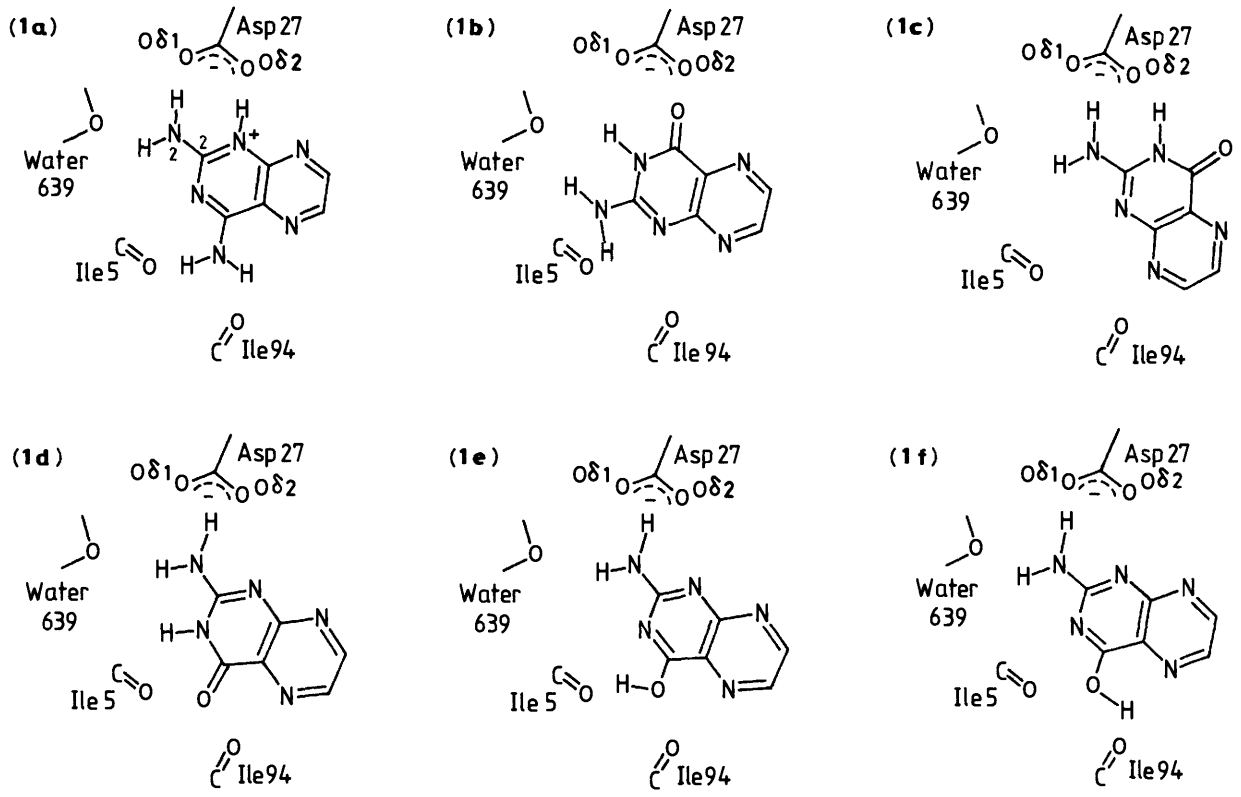


Figure 1. DHFR Active site containing substrate tautomers: (a) methotrexate; (b) folate binding in methotrexate conformation; (c) and (d) folate binding in conformation opposite to that of methotrexate; (e) and (f) enol forms of folate

undergoes one-electron reductions, involving NADPH cytochrome P450 reductase[22] and NADPH cytochrome c reductase,[23] along with a two-electron reducing enzyme.[24] For our proposed bioreductive agents it may be desirable to stimulate two-electron reductions, possibly by DT-diaphorase.[25,26] It may also be advantageous to restrict the availability of oxygen to the tissues of the body; this can be achieved using compounds such as 5-(2-formyl-3-hydroxyphenoxy)pentanoic acid which directly changes the oxygen affinity of haemoglobin.[27] This compound might therefore play a role in cancer chemotherapy, as it could be used to favour the bioreduction step.

Methods

The *X*-ray crystal structure of DHFR from *E. coli* was used.[1] It is known that the vertebrate enzyme is very similar in the region considered in this article.[28] Where necessary, hydrogen atoms were added using standard geometries. The structures of the substrates and inhibitors were fully optimized using the semiempirical AM1 Hamiltonian[29] within the AMPAC program.[30] (The AM1 method is considered an improvement upon its predecessor MNDO.[29])

In order to locate the position of the substrates and inhibitors, two docking procedures were used. The first used the AMI method. The enzyme was modelled by 28 atoms, primarily from residues Ile 5, Asp 27, Ile 94, and water 639—the main residues involved in the pteridine binding site. The docking was then carried out by optimizing only the intermolecular internal co-ordinates. For this step it is not necessary to optimize more than six internal co-ordinates. A translation and rotation was then performed to superimpose the optimized co-ordinates on to the entire binding site of the enzyme.[31] The binding site involved residues Ile 5, Ala 6, Ala 7, Trp 22, Asp 27, Leu 28, Phe 31, Ile 94, Thr 113, and water 639 of the B subunit of DHFR. (Water 639 was orientated to give a favourable interaction with the bound methotrexate).

The second method utilized the ECEPP,[32] parameters of Némethy[33] which were developed to describe polypeptide interactions. The method uses an electrostatic term, a 12–6 Lennard–Jones repulsion–attraction term and a corresponding 12–10 term to replace the 12–6 term in hydrogen-bonding situations. In this work the charges for the electrostatic term were taken from the AM1 Mulliken charges. The interaction energy was minimized using a Newton-like method by allowing free translation and rotation about the centroid of the drug molecule or substrate. In both cases the initial position was found by quaternion-fitting[31] the inhibitor to the methotrexate moiety in the crystal structure. In both cases the position of the oxidized inhibitor was found by quaternion-fitting it to the reduced form.

Once the position of the inhibitors had been predicted, the interaction energy was calculated using the *ab initio* point charge method in which the inhibitor is treated fully and the interaction with the enzyme is modelled by a modified Hamiltonian.[4] The method was incorporated[34] into Gaussian 82. The modifications allowed the re-use of the two-electron integrals; the interaction energy can then be determined at very little expense above that of a single-point calculation on the inhibitor alone. (The increase in CPU time is about 30%.) Moreover, by using the wavefunction from the first part of the calculation as the starting vectors for the second part, it was possible to decompose the interaction energy into an electrostatic part and a part representing the polarization energy of the substrate—in a manner similar to that of Kitaura and Morokuma.[35] Although an attempt is being made to move away from the use of empirical parameters, the method clearly

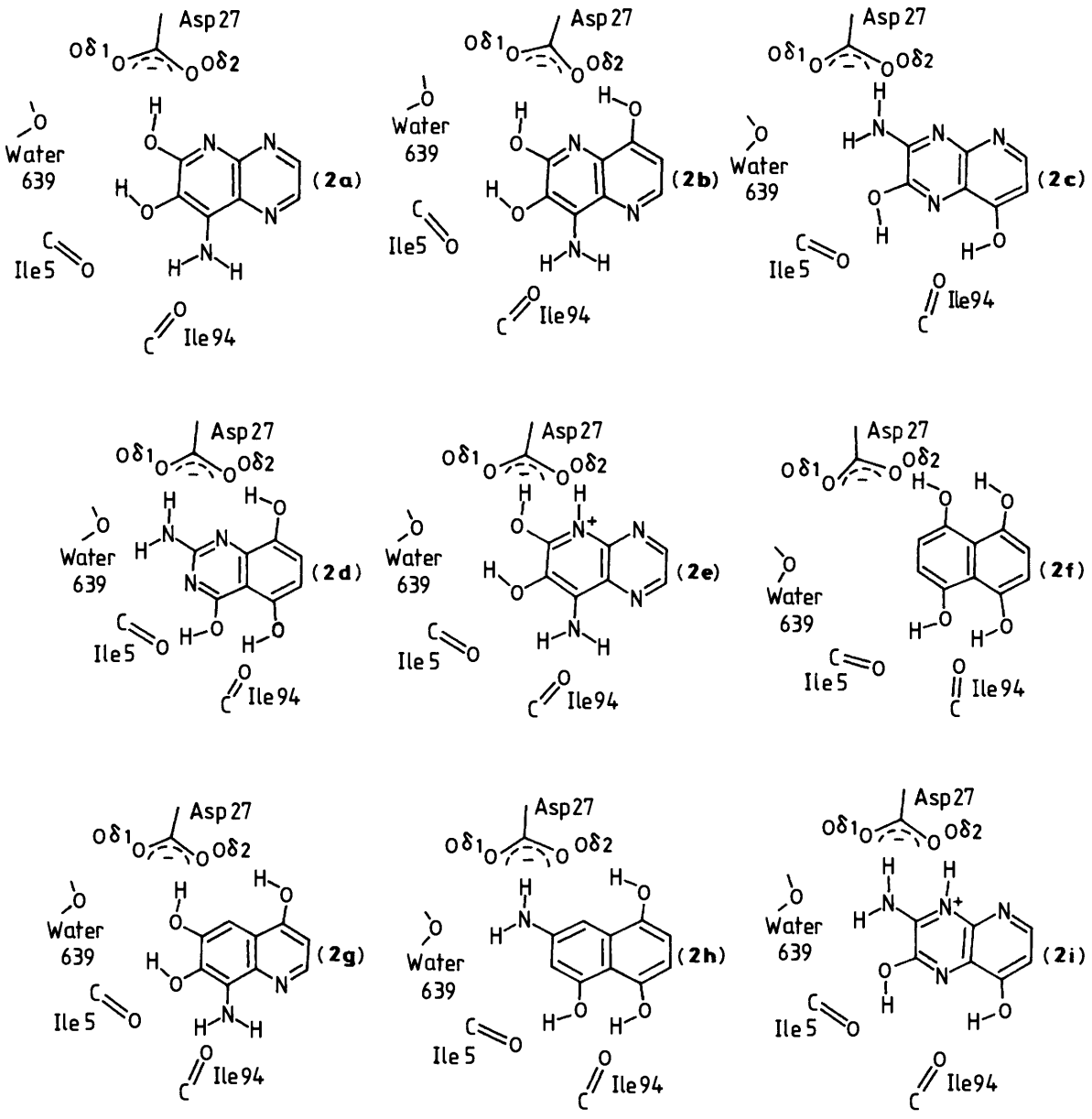


Figure 2. Binding of reduced bioreductive anti-folates

depends on the choice of point charges. Owing to the small expense involved in calculating the interaction energies, it was possible to determine the interaction energies for some molecules using two sets of different charges in the same calculation. In this work the charges of Kollman were used, both the Mulliken charges[36] and the electrostatic-potential-derived charges[37,38] within the united-atom framework. The STO-3G basis set was used throughout.[39] As this part of the method does not contain an explicit repulsion term, it is essential that the two molecules do not approach closer than the sum of their van der Waals' radii[4,40]—this constraint is largely satisfied by the use of the docking procedure.

Results

Table 1 shows the relative heats of formation of the various isomers of folate, as determined by the AM1 method. It also shows the binding energies of folate and dihydrofolate, determined by the AM1 method, by the *ab initio* method at the AM1 docked position, by the molecular mechanics method, and also by the *ab initio* method at the molecular mechanics docked position. The corresponding information for the proposed inhibitors is shown in Table 2. The various orientations of the substrates docked using molecular mechanics are shown in Figure 1; the positions of the inhibitors docked using molecular mechanics are shown in Figure 2. (The discussion below relates primarily to the molecular mechanics docked substrates and inhibitors.)

Discussion

Folate Binding.—The *X*-ray structure of *E. coli* DHFR was published[41] in 1977 and demonstrated the mode of binding of methotrexate. Great excitement was caused two years later when it was shown that the natural substrate could not bind in a similar manner,[42] but rather that the heterocyclic ring was

Table 1. AM1 Relative heats of formation (dHf) and calculated binding energies (in kJ mol^{-1}) for DHFR substrates and methotrexate

			AM1 Docked position			MM Docked position		
Compound		dHf	AM1	Qmul[a]	Qmep[b]	MM	Qmul	Qmep
Methotrexate	(**1a**)			−156	−189	−81	−103	−131
Folate (keto)	(**1b**)	0				−27	−70	−60
	(**1c**)	0	−140	−151	−156	−111	−127	−129
Folate (enol)	(**1e**)	22	−88	−99	−109	−98	−69	−73
Folate (enol)	(**1f**)	26	−87	−76	−85	−91	−27	−29
Dihydrofolate	(**1b**)		−119	−156	−164	−98	−58	−53
	(**1c**)		−123	−142	−149	−99	−108	−107

[a] Qmul refers to the *ab initio* results calculated using Mulliken charges. [b] Qmep refers to the *ab initio* results calculated using Kollman's electrostatic potential-derived charges.

rotated through 180°. Two alternative conformations have been proposed.[43,44] These are shown in Figure 1. The first of these, folate (**1b**), is in the opposite orientation to methotrexate (**1a**). The second, folate (**1c**), is formed by rotation about the C-2–N-2 bond. Figure 1 shows not only the keto form of folate, (**1c**), as this is the form observed experimentally,[45,46] but also the enol forms (**1e**, **f**). The form (**1d**) has the same orientation as methotrexate.

We were not able to observe a minimum for the first proposed conformation (**1b**), as both of the alternative starting geometries (**1b**—**c**) gave the second, (**1c**), as the minimum—using both docking procedures. The reason for this—as pointed out previously [1]—lies primarily in the hydrogen bonds donated to both Oδ1 and Oδ2 of Asp 27.

While the hydroxy form is not observed experimentally, we have considered its binding due to the alternative hydrogen bonding arrangement of the enol form—particularly with Ile 5 or Ile 94—if the folate were to bind in the same orientation as methotrexate—see Figure 1 (**1e**) and (**1f**). It may be that a greater binding energy for the enol form could overcome the lack of stability of this form when free. However, as shown in Table 1, the mode of binding in (**1c**) is preferred over both the enol forms of the folate (**1e**, **f**) and the alternative arrangements for the keto form (**1b**, **d**)—for both the folate and the 7,8-dihydrofolate, in agreement with experiment. The only doubt about this conclusion lies in the small difference in binding energies, as calculated using molecular mechanics, between arrangements (**1b**) and (**1c**) for 7,8-dihydrofolate. The molecular orbital methods give a larger difference. We are not, however, aware of any reports on the binding enthalpies of folate.

The AM1 method and the molecular mechanics method give similar positions for some of the substrates. The main exception is for form (**1b**) of both the folate and the dihydrofolate. For the AM1 method the two protons on the amino group bind to the two oxygens of the aspartate carboxylate group (final position not shown.). This orientation would be impossible for the full folate molecule and this misleading finding is partly due to the lack of sufficient residues in the binding site (necessitated by the computational cost of the AM1 method).

The conclusion is that both methods may give similar positions for the docked inhibitors. On the other hand it would therefore seem reasonable to determine the position of the inhibitors using the cheaper molecular mechanics method which has the advantage of being able to include many more atoms in the binding site. It would, however, seem preferable to determine the interaction energy using the molecular orbital methods. Having thus tested the method on the known properties of folate, and having reproduced its known properties, we can use the method with a little more confidence in the prediction of new bioreductive anti-folates.

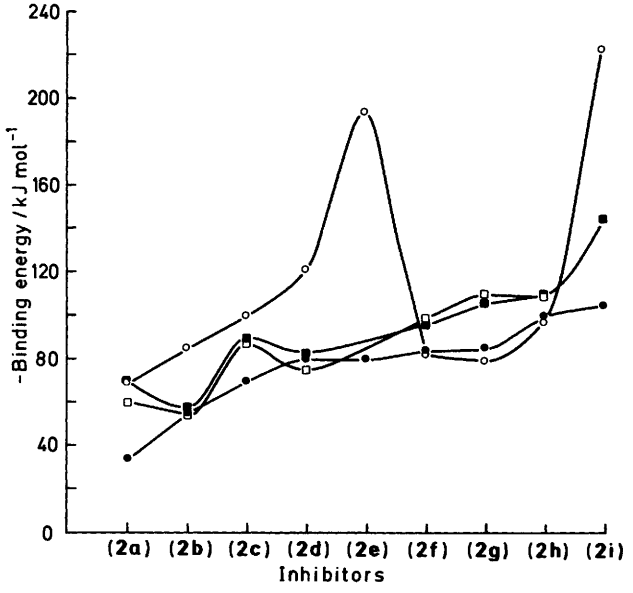


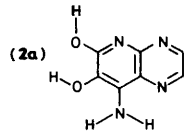
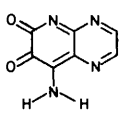
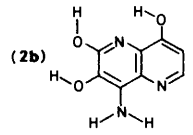
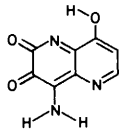
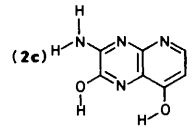
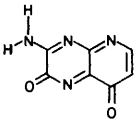
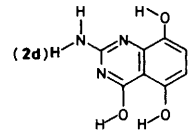
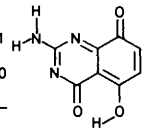
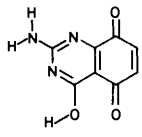
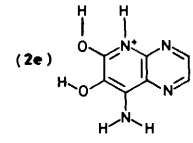
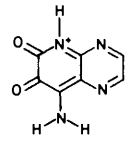
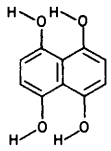
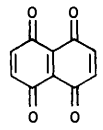
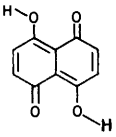
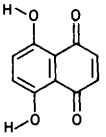
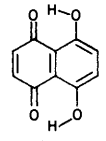
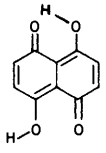
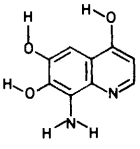
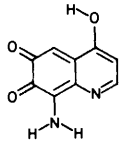
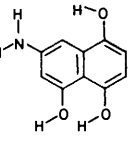
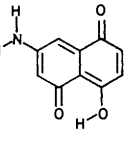
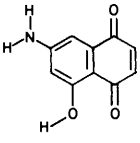
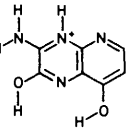
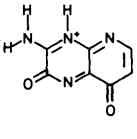
Figure 3. Calculated binding energy trends of inhibitors. ■ AM1 docked, *ab initio*; ● MM docked, *ab initio*; □ AM1 docked, AM1 energy; ○ MM docked, MM energy

Bioreductive Anti-folates.—The initial aim of this section is to obtain improved binding energies for the hypoxic-tumour-selective anti-folates previously proposed,[9] and to suggest some new bioreductive agents.

The predicted binding energies are shown in Table 2. Two sets of *ab initio* binding energies are given—at both the AM1 and molecular mechanics docked position. It can be seen that the two values are fairly similar. In some cases these are significantly more positive than in reference 9 in which the inhibitors were located using a quaternion-fitting procedure.[31] There are two reasons for this. First, the method employed is only valid if the interacting molecules do not come closer than the sum of their van der Waals radii [4,40]—if they do, excessively negative energies may result for close approaches due to the lack of a repulsion term. This effect shows the desirability of combining the approach with a docking procedure. Secondly, for some of the inhibitors there was a definite preference for moving away from water 639, and therefore not forming as many hydrogen bonds as was initially desired. This is clearly shown in Figure 2 inhibitor (**2f**), where the hydroxy protons were intended to bind to Oδ1 and Oδ2 of Asp 27.

The final positions determined by both AM1 and molecular

Table 2. Interaction energies of inhibitors (**2a—i**) and their oxidized forms at the AM1 and molecular mechanics docked position[a]

	Reduced	Binding energies	Oxidized	Interaction energies	Difference in interaction energies
(2a)		-60 -69 -70 -34 -80 -36		+48 +64 +42 +60	118 98 144 96
(2b)		-54 -85 -58 -55 — —		+58 +57 — —	116 112 — —
(2c)		-87 -100 -90 -70 -105 -82		-77 -56 -78 -54	13 14 27 28
(2d)		-75 -121 -83 -80 — —		-31 -26 — —	52 54 — —
				-34 -29 — —	49 51 — —
(2e)		— -194 — -80 — -101		— +38 — +14	— 42 — 87
(2f)		-99 -82 -96 -84 — —		— -5 — —	— 79 — —
				— -73 — —	— 11 — —
				— -64 — —	— 20 — —
				— -20 — —	— 64 — —
				— -13 — —	— 71 — —
(2g)		-110 -79 -106 -85 — —		-3 +2 — —	103 87 — —
(2h)		-109 -97 -110 -100 — —		-80 -79 — —	30 21 — —
				-81 -75 — —	29 25 — —
(2i)		— -223 -145 -105 -168 -127		-144 -106 -152 -114	1 -1 16 13

[a] Energies at the AM1 position are on the left for each ligand. The binding energies for the docking method are in the first row, those for the *ab initio* method using the Mulliken charges in the second row, and those using the Kollman charges in the third row.

mechanics are similar. However, a closer examination showed that the AM1 method tended to predict the final position to be closer to Asp 27. Individual hydrogen bonds differed by up to 0.3 Å in length. This disparity seems undesirable, because it can lead to differences in the *ab initio*-predicted binding energies of up to 30 kJ mol^{-1}. However, a plot of the binding energies for all the inhibitors, using the four different methods (Figure 3), shows that the predicted *ab initio* binding energy trends are very similar for the two docking methods, and are similar to the AM1 binding energies. The molecular mechanics binding energies, however, follow a different trend. This again suggests that while the position of the inhibitor may be located using molecular mechanics, it may be desirable to determine the energy quantum mechanically. (A different molecular mechanics scheme may of course give superior results).

In several instances, the predicted binding energies are

appreciably different from our earlier predictions,[9] in particular the specificity intended was not realized by either docking procedure, except for Figure 2 inhibitors (**2d**) and (**2i**). This now suggests that the most specific bioreductive anti-folates, of those considered here, should possess an amino group in position 2. (This constraint may not be necessary if the docking is carried out while allowing for conformational flexibility of the enzyme.)

The repulsive interaction energy given for the oxidized inhibitors in Table 2 is calculated at essentially the same position as for the reduced inhibitors, and is therefore a rather arbitrary value; the oxidized forms are unlikely to align themselves in that position as they do not possess the necessary complimentarity. However, it does give a measure of the possible selectivity for hypoxic cells. On this basis, while Figure 2 inhibitor (**2d**) is still predicted to be selective for hypoxic cells, inhibitor (**2i**) is not. Moreover, inhibitors (**2a**), (**2b**), (**2g**), and (**2h**) all have large differences in binding energy between the oxidized and reduced forms, despite not forming an explicit hydrogen-bond pattern to the five key oxygens in the binding site.

As in our previous article,[9] we conclude from our calculations that it is possible to design inhibitors of DHFR which should bind in their reduced form, but which in their oxidized forms would have a greatly diminished binding energy or even a repulsive interaction.

Summary

We have reported modifications to our program for calculating enzyme–substrate binding energies. Comparison of this work with our earlier work [9] shows that it is desirable to use the method in conjunction with a docking algorithm. A molecular mechanics method seems adequate for determining the position of the inhibitor, but it seems more desirable to determine the interaction energy using a quantum mechanical method. This scheme is able to describe adequately the binding of folate (or at least the binding of the pteridine ring fragment of folate) and the trends in the binding energies of a series of inhibitors.

The method has been used to determine the binding energies of DHFR inhibitors which have the property of binding in their reduced forms, but not in their oxidized forms. The calculations have borne out the hypothesis that these inhibitors may bind strongly in their reduced form, but not in their oxidized form. They may therefore be selective anti-tumour agents against the therapeutically important hypoxic cell population of solid tumours.

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