Identifying targets for bioreductive agents: using GRID to predict selective binding regions of proteins

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A computational procedure is described for investigating potential binding sites of a target macromolecule for their ability to bind both a reduced probe molecule and an oxidized probe molecule. The interaction energies are obtained using a molecular mechanics method and can be displayed as three-dimensional (3D) energy contours, indicating regions of the target molecule that may have favorable interactions with the probe molecule. Differences in the interaction energies of the oxidized and reduced probe with the target can also be plotted as contours, indicating regions that are selective for the reduced probe. These selectivity contours can be used to show whether the macromolecule is a potential target for bioreductive agents. The method has been applied to the chicken liver dihydrofolate reductase enzyme and has indicated new binding regions that may be suitable binding sites for bioreductive agents.

Keywords: bioreductive agents, dihydrofolate reductase, GRID, molecular modeling, selective binding

INTRODUCTION

The use of bioreductive agents offers one possible method of designing drugs to be selective against tumor cells.[1,2] We recently proposed a new type of bioreductive activity in which DHFR (dihydrofolate reductase) is the target and in which the reduced form of a drug would bind to its target, while the oxidized form would have a greatly reduced or repulsive interaction energy.[3,4] The proposals are based upon theoretical calculations of the binding energies of proposed quinone-based drug molecules with DHFR. Although there are disadvantages to DHFR as a target for anticancer agents,[5,6] antifolates are currently used successfully in cancer chemotherapy, and our proposals may result in the design of antifolates with greater selectivity and hence fewer problems.

The background to our proposals lies in the well-known binding site that is readily discernible from the X-ray crystal structure[7] of the *E. coli* enzme; this binding site is composed of residues asp 27, ile 5 and ile 94. However, other regions of the enzyme may be equally exploitable but not so readily detectable. Therefore, we have developed a computational technique for probing the macromolecule in order to highlight such regions. The method, described below, was tested on the chicken liver DHFR X-ray crystal structure,[8] and indeed was found to reveal other amino acid residues that could be involved in selective bioreductive inhibition, in addition to those previously described. Moreover, the general approach described in this paper could be applied to other and perhaps more appropriate targets, such as thymidylate synthetase.

The potential for the use of bioreductive agents arises because solid tumors are particularly deficient in oxygen due to their poor blood supply and the rapid metabolism of oxygen by active cells in the outer regions of the tumor.[9] The low oxygen tension at the center of the tumor may thus create a reducing environment that is necessary for the enzymic reduction of the bioreductive agents.[10-12] Examples of other types of bioreductive agents currently under investigation are the nitro-heterocyclic drugs, such as misonidazole and metronidazole;

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the more recent bifunctional bioreductive agents, such as RSU 1069; and the quinone alkylating agents, such as mitomycin C.[2,13,14] These drugs may act either upon DNA and involve covalent bond formation, or potentiate the effects of ionizing radiation. We believe that there may be advantages in developing the types of bioreductive agents described below.

METHODS

Our method is based on the computer program GRID[15,16] and because full details are given elsewhere,[15] we will present only an outline of the method here. GRID is essentially a program for highlighting interesting areas of a protein for which the three-dimensional (3D) crystal structure is known. For example, it may be used to predict regions within an enzyme where a water molecule or some other ligand will bind. This is done by defining a water probe to have appropriate charge, van der Waals parameters and hydrogen bonding capabilities. Similar parameters are also assigned to the atoms of the enzyme. The electrostatic interaction is calculated using the method of images,[15] which allows for the difference in the dielectric constant that occurs when the probe interacts with either an exposed group or a similar group the same distance away but buried within the protein. (This situation is illustrated by the interaction of a carboxy oxygen probe with arg 52 and arg 57 in DHFR from *L. casei*.[15]) The van der Waals term is described by a 12–6 repulsion-dispersion term. hydrogen bonding is described by an energy term of the general form

$$EHB = Er \times Et \times Ep \quad (1)$$

where Er is an 8–6 function of the interatomic distance between the hydrogen-bonding atoms and Et and Ep are functions of the angle made by the H-bond at the target atom and probe group, respectively.[16] At each point at which the interaction energy is calculated, the probe is rotated to optimize the hydrogen bond energy.

The probe is passed through the enzyme on a regular 3D grid, and the interaction energies obtained at each point are shown as energy contours using an appropriate graphics program such as FRODO.[17] Because our proposed bioreductive activity is based on the reduction of a quinone to a hydroquinone, the probes we used were those for a hydroxy group (as found in tyrosine) and a carbonyl group (as found in thymine). However, since we are more interested in the relative binding of these two groups, in order to predict bioreductive selectivity rather than their absolute values, it is more instructive to plot the differences ΔE in binding energies. These differences were obtained as follows:

$$\text{If } E_{OH}, E_{CO} < 0.0 \qquad \Delta E = -E_{CO} + E_{OH} \quad (2a)$$

$$\text{If } E_{OH} < 0.0, E_{CO} > 0.0 \qquad \Delta E = +E_{OH} \quad (2b)$$

$$\text{If } E_{OH} > 0.0, E_{CO} < 0.0 \qquad \Delta E = -E_{CO} \quad (2c)$$

$$\text{If } E_{OH} > 0.0, E_{CO} > 0.0 \qquad \Delta E = 0.0 \quad (2d)$$

where E_{OH} and E_{CO} are the probe interaction energies for the hydroxyl and carbonyl probes. Equation (2a) cannot be used throughout because points on the grid may fall within the van der Waals radius of a target atom; this could give rise to extremely large negative differences in binding energies for points at which binding could not occur. It is appropriate to calculate only the selectivity contours for points that are accessible to the probes, and these selectivity contours may be displayed with equations 2a, b, c and d.

To test the predictions from these selectivity contours, we designed a small number of prototype drug molecules to fit the selectivity contours and docked them into the enzyme using the AMBER suite of programs.[18–20] The geometries and charges of the drugs were obtained using the AM1 semi-empirical molecular orbital method.[21,22] Because AMBER uses a different molecular mechanics method from GRID, it is instructive to compare the results of the AMBER calculations with the GRID predictions. During the docking with AMBER, the enzyme was held rigid in order to facilitate direct comparison with GRID.

The available X-ray coordinates for chicken liver DHFR did not contain an inhibitor. Because we know that there are appreciable changes in conformation upon binding of the inhibitor methotrexate, especially in glu 30, we performed the GRID calculations on the DHFR structure after the cofactor, inhibitor (2,4-diamino-pteridine ring of methotrexate) and the residues ile 7, glu 30 and val 115 had been energy refined with AMBER. Since there is some doubt as to the ionization state of glu 30, we also performed GRID calculations on structures in which glu 30 was protonated. In addition, we observed that the conformation of the side chain of asn 21 could markedly affect the selectivity maps; GRID runs were therefore performed on alternative conformations of this residue.

RESULTS AND DISCUSSION

Contour maps

The GRID contour maps for hydroxyl and carbonyl probes (at $-6 kcal mol^{-1}$ and $-4.5 kcal mol^{-1}$, respectively) are shown in Color Plates 1 and 2. These maps show that the contours for hydroxyl are generally more extensive than those for carbonyl—there are virtually no carbonyl contours at $-6 kcal mol^{-1}$—and those shown occur almost exclusively in the vicinity of the cofactor (and hence away from the main active site region), although they do extend toward Oγ of ser 59. The hydroxyl contours, however, occur near glu 30, trp 24 (O and HNε1), ser 59 (Oγ and O), val 115 (O) and the cofactor.

Selectivity contour maps. The corresponding selectivity contours at $-3 kcal mol^{-1}$ are shown in Color Plate 3; they comprise six main regions in which there is a predicted selective preference for hydroxyl groups. These contours, therefore, show potential binding sites that can be exploited in the design of bioreductive molecules. Figure 1 shows a similar orientation of the active site

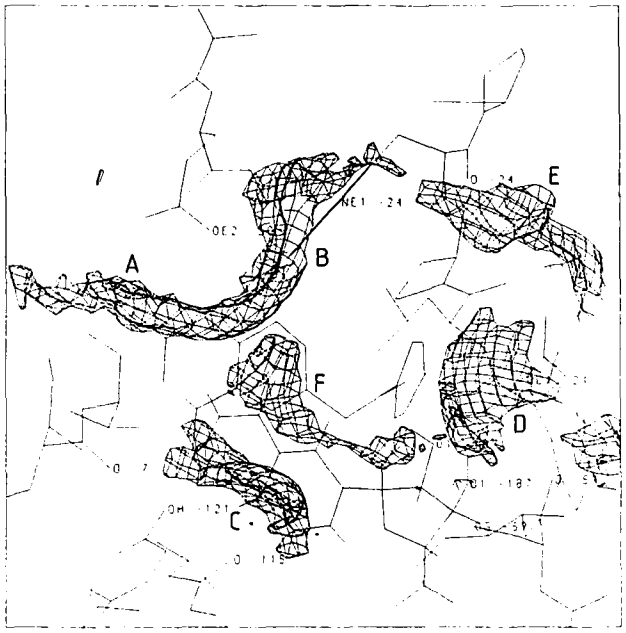

Figure 1. Grid selectivity contour map showing regions (A–F) selective for the hydroxyl probe — contours are shown at −3kcalmol⁻¹

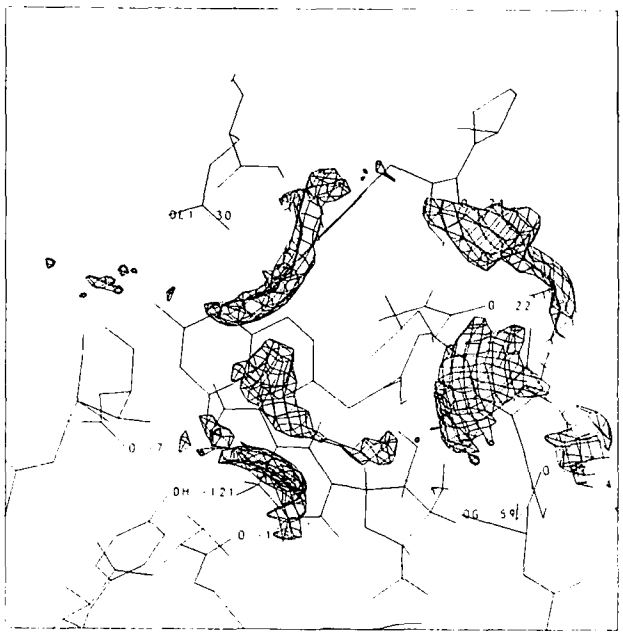

Figure 2. Grid selectivity contours at −3kcalmol⁻¹ with glu 30 protonated on Oδ1

in which the six regions are labeled A–F. There are virtually no regions that are selective for carbonyl, so the contour maps with reversed sign are not shown.

Region A is small and basically inaccessible to drug molecules because it delineates the site for a conserved water molecule (No. 335, c.f. 639 in *E. coli*). Region B is the most extensive site and runs into region A; it is due entirely to the carboxy oxygens of glu 30. Because there is some doubt about the state of ionization of glu 30, it is important to investigate whether DHFR would still be predicted to be a target for bioreductive activity if this residue were protonated. Clearly, it would not be such an attractive target in this latter case, but as Figures 2 and 3 show, extensive regions that are selective for reduced hydroxyl probe still remain, even when this residue is protonated (on either oxygen).

Together, regions B and C (Figure 1) are associated with the ideal binding site referred to in references 3 and 4; the ideal nature of this binding site results from the coplanarity of the four oxygens of ile 7, glu 30 and val 115. Region C results from the backbone carbonyl oxygens of ile 7 and val 115. There is also some contribution to the binding energy from tyr 121; fortunately, the hydroxyl group of this residue does not remove the selectivity in region C. (As shown in Color Plate 3, tyr 121 lies below residues ile 7 and val 115 and is consequently further from the inhibitor than these residues.)

Region D is coplanar with regions A, B and C; it results primarily from the backbone carbonyl oxygen of ser 59, although there is some contribution from the amide oxygen of asn 21. This is a complex region of the enzyme, since the side chain of asn 21 may rotate. When this side chain is optimized using AMBER, the amide group rotates through 180° and a different GRID map results; see Color Plate 4. In this new conformation, region D disappears, but again extensive selective areas exist, and region E is significantly enhanced. This suggests that it may not be easy to exploit the selectivity of region D.

The orientation and indeed the nature of the side chain of residue 21 clearly have a marked effect on the GRID maps. It is therefore interesting to note the results of homology studies that show conclusively that while this residue is an asn in the vertebrate chicken liver enzyme, it is an asp in the mammalian enzymes[23] (mouse 11210

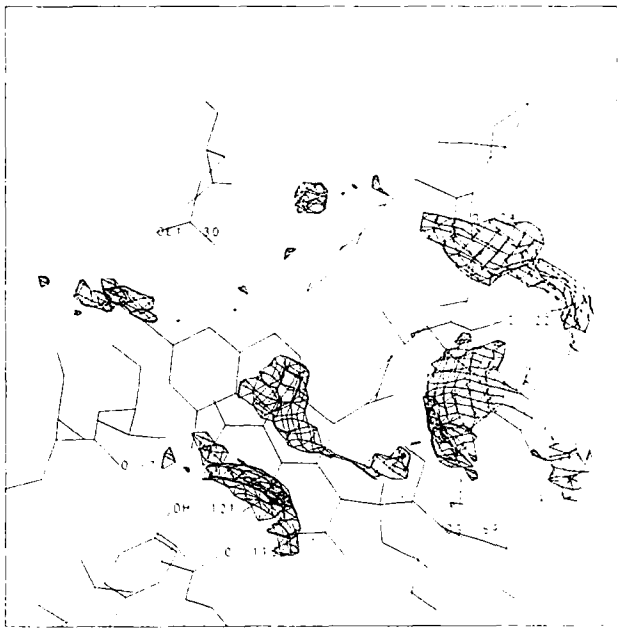

Figure 3. Grid selectivity contours at −3kcalmol⁻¹ with glu 30 protonated on Oδ2

and human enzyme). This mutation is accompanied by a corresponding mutation of asp 19 to asn 19. (The human enzyme was sequenced using cDNA techniques;[24] the human, mouse and chicken liver enzymes have also been sequenced using traditional Edmond degradation,[25-27] combined with thin layer chromatography to confirm the presence of asp or asn.) The GRID selectivity map for asn 21 mutated into asp is shown in Color Plate 5. In Color Plate 5, region D is greatly enlarged and runs into region E, suggesting that region D may indeed be exploitable. Asp 19 is 8 Å further from the active site than asn 21, and its side chain points away from the active site into the solvent; the GRID selectivity maps for the doubly mutated enzyme are very similar to that shown in Color Plate 5 and are therefore not shown.

The potential problems in exploiting region D in either the vertebrate or mammalian enzymes lie with the hydroxyl groups on the side chain of ser 59 on C2′ and C3′ of the cofactor. These hydroxyl groups give rise to one of the main carbonyl binding regions; see Color Plate 2. Region D, however, may still be exploited by the use of blocking groups on the drug to prevent these hydroxyl groups from interacting with the carbonyl groups in the oxidized form of the drug.

Region E results from the backbone carbonyls of trp 24 and pro 22. However, it is some 10 Å to 15 Å from the main active site region, and it has the disadvantage of not being coplanar with the other regions; it is also in the proximity of other nonselective groups, such as the amide hydrogen of the backbone of trp 24. Region E is therefore unlikely to be a useful selective binding region.

Region F is in the vicinity of the cofactor and will not be discussed further.

Exploitation of the selective regions

In order to design bioreductive agents selective for dihydrofolate reductase in tumor cells, it is essential to design ligands that are predicted by calculations to have a significantly greater binding energy in their reduced form than in their oxidized form. The selectivity contours should enable such ligands to be designed more effectively. While the purpose of this article is to present the method as an aid to designing bioreductive agents rather than to present predictions of drugs with potential bioreductive activity, calculations have been performed on four prototype molecules that were designed using the GRID contours. These molecules are shown in Figure 4. They contain a fragment similar to the pteridine ring fragment of methotrexate, a well-known inhibitor of DHFR; this is primarily to hold the molecule in position.

Regions B and C have been exploited in the earlier works on this subject.[3,4] However, the extent of region B is greater than was initially realized, and inhibitors 1 and 2 (Figure 4) were designed to exploit the upper part of region B. The binding of methotrexate to val 115 in the chicken liver enzyme is weaker than that to the corresponding ile 94 in the *E. coli* enzyme.[8] This is due to the greater distance of val 115 from the inhibitor. Consequently, the part of region C due to val 115 lies lower in the plane of the binding site than it would in *E. coli*. Inhibitor 3 is designed to exploit this lower region. Inhibitor 4 was designed to exploit region D. The binding energies of these reduced inhibitors and their oxidized forms, together with individual contributions to the binding energy from key residues of the enzyme, are shown in Table 1. These results show that the GRID selectivity contours can indeed be used to design molecules that should have an increased binding energy in their reduced forms. A clear selectivity is predicted for the reduced forms of molecules 1–4. The selectivity for inhibitor 4, however, is not so marked, and some of the reasons for this are described above. One potential problem is that if the enzyme was allowed to relax fully, the hydroxyl groups near to region D could interact with the oxidized form, and this could result in improved binding for the oxidized form. Under normal circumstances where an inhibitor, designed by molecular graphics, makes extra interactions with the enzyme, this is an advantage. However, this is clearly not advantageous when one wants one form of the drug to have a reduced interaction.

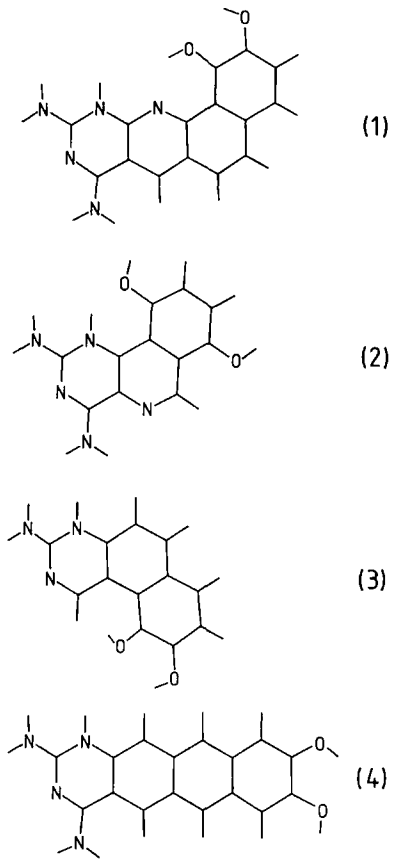

Figure 4. Reduced forms of prototype bioreductive agents

CONCLUSIONS

A method for identifying targets for bioreductive agents has been presented and applied to chicken liver dihydrofolate reductase. The resulting GRID selectivity con-

Table 1. Total calculated binding energies and certain residue contributions to the binding energies of prototype inhibitors. The difference between the binding energy of oxidized and reduced forms is a measure of the selectivity. These calculations were carried out using AMBER and employed a distance-dependent dielectric constant

		Binding energy/kcalmol^{-1}					
Drug	Form	Total	ile 7	asn 21	glu 30	ser 59	vall 15
1	Reduced	−86.7	−8.4	−0.5	−46.7	−0.3	−1.3
1	Oxidized	−75.4	−8.2	−1.5	−40.9	−0.7	−1.8
2	Reduced	−77.9	−8.2	−0.3	−52.1	−0.2	−1.4
2	Oxidized	−65.5	−2.4	−0.2	−41.7	−0.1	−1.4
3	Reduced	−76.5	−3.8	−0.2	−44.5	−0.2	−10.6
3	Oxidized	−68.2	−3.3	−0.2	−46.7	−0.1	−2.8
4	Reduced	−78.1	−8.1	−5.3	−40.9	−3.7	−1.7
4	Oxidized	−71.4	−8.1	−1.7	−42.3	−0.5	−1.7

tours predict that the chicken liver enzyme, like the *E. coli* enzyme, is a potential target for bioreductive agents. This potential for bioreductive activity lies in the specific arrangement of ile 7, glu 30 and val 115, which is comparable to the homologous residues in the *E. coli* enzyme. In addition, the method suggests that it may be possible to involve trp 24 and ser 59. We proposed that the human enzyme may be an even better target due to the mutation of asn 21 to asp.

A number of bioreductive inhibitors were designed to exploit the features highlighted by the selectivity contours and were indeed predicted to have increased binding energies in their reduced forms. A number of potential problems that may arise in exploiting all the selectivity regions suggested by the contours have been discussed and will be addressed more fully elsewhere. The results from the initial application of the method suggest that it may be worthwhile to apply the method to other key macromolecular targets with a view to identifying other targets for bioreductive agents.

In order to design effective bioreductive agents, it is necessary for the drug molecules to have an appropriate redox potential; we have recently shown that it may also be possible to treat this aspect of the design process theoretically, in a quantitative manner.[28]

The calculations involved in this research were carried out using the AMBER and GRID programs; these programs exemplify different approaches to the study of macromolecular systems, employing different functions and different parameterizations. It is therefore interesting that both methods, which were useful at different stages of the research, arrive at mutually compatible conclusions.

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