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Bioactivity and Molecular Modelling of Diphenylsulfides and Diphenylselenides

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Abstract—Bis(2-bromo-4,5-dimethoxyphenyl)sulfide (**5**) and bis(2-bromo-4,5-dimethoxyphenyl) selenide (**7**) have been shown to block cells in the G2/M phase of the cell cycle, whereas the debromo (**4**, **6**) equivalents do not. The dibromoselenide (**7**) is cytotoxic to tumour cells *in vitro* and has been shown to increase the mitotic index of treated cells. These biological effects are consistent with disruption of the mitotic apparatus. This agent does not inhibit microtubule assembly *in vitro*, but does bind to tubulin. Molecular modelling of these structures indicates that their spatial and electronic structures may make an important contribution to the biological activity.

Introduction

The phenylthioindole amphethinile (**1**) and the *cis*-stilbene combretastatin A4 (**2**) have shown some promise as anti-tumour agents.[1] The biochemical target for these agents is the protein tubulin, the major constituent the microtubule, an organelle involved in a variety of cellular processes including chromosome separation at mitosis. Both amphethinile (**1**) and combretastatin A4 (**2**) have been shown to bind tightly to tubulin,[2] with affinity constants comparable to that of colchicine (**3**), a known tubulin-binding drug. Similarly, both drugs have been shown to be very potent inhibitors of the formation of microtubules from isolated tubulin *in vitro* and can compete with colchicine (**3**) for a binding site on the tubulin protein.[2] A structural study of colchicine (**3**), combretastatin A4 (**2**), and amphethinile (**1**) has shown that these agents share a common pharmacophore which is important for binding to tubulin. This is the existence of two aromatic/planar ring systems which are tilted with respect to each other.[2]

The diphenyl sulfides (**4**, **5**) and diphenyl selenides (**6**, **7**) combine the structural features of both amphethinile (**1**) (the phenylthio unit) and combretastatin A4 (**2**) (the oxygenated aromatic rings) (Figure 1). Selenium and its compounds have also previously been shown to exhibit toxicity.[3] These features attracted our laboratories to test these sulfides (**4**, **5**) and selenides (**6**, **7**) for their biological activities and to discover whether any such activity could be attributed to molecular shape and/or charge.

Results

Cytotoxicity

The dibromoselenide (**7**) gave an IC_{50} value (the drug concentration required to cause a 50 °C growth inhibition) of 24.1 μM in the parental P388 cell line and an IC_{50} value of 15.6 μM in the P388 R8/22 multi-drug resistant cell line. Against the A2780 human ovarian cell line and multidrug resistant counterpart A2780ADR ovarian carcinoma cell line, the dibromoselenide (**7**) afforded IC_{50} values of 2.1 μM and 5.2 μM, respectively. The dibromosulfide (**5**), and both debromo-derivatives (**4**, **6**) did not exhibit cytotoxicity at levels up to 50 μM. This concentration (50 μM) was the maximum level for these agents used due to solubility problems at higher concentrations.

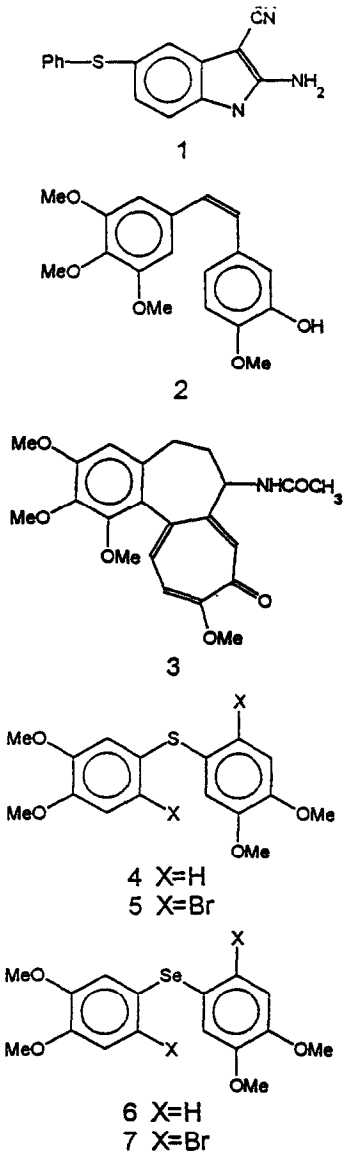

Figure 1. Structures of drugs

Cell cycle analysis

Treatment with the dibromoselenide (**7**) (50 μM, 1 cell cycle, 12 h) resulted in 75% of the P388 cells accumulating in the G2/M phase of the cell cycle. This compares with the control value of 13%. When the cells were incubated with the dibromoselenide (**7**) for longer than 12 h, cells with DNA contents greater than 4*n* (tetraploid) were observed resulting eventually in cells with 8*n* DNA contents. This indicates that disruption of the mitotic apparatus has occurred leading to the cells' failure to undergo cell division. This, however, has not affected the cells' ability to commence DNA synthesis, leading to cells with abnormally high DNA contents. The dibromosulfide (**5**) showed a similar but weaker effect, with 48% of cells blocked in the G2/M phase following treatment with the drug (50 μM, 12 h). The non-brominated compounds (**4**, **6**) showed no G2/M phase block at the limits of their solubilities at concentrations up to 50 μM.

After 4.5 h a rise in the mitotic indices (relative to controls) of the P388 cells treated with the dibromoselenide (**7**) (10.1% versus 3.6%) and the dibromosulfide (5.7% versus 3.6%) was observed. This is again indicative of disruption of the mitotic apparatus.

Tubulin assembly inhibition

None of these compounds (**4–7**) were capable of inhibiting tubulin assembly *in vitro*. However there was evidence of a drug–tubulin interaction between the dibromoselenide (**7**) and tubulin. This gave a consistently higher final absorbance value than that of the control (15% increase in absorbance compared to control). This may be the result of the formation of altered microtubules, or of an increased yield of microtubules. The microtubules, when examined by electron microscopy, were indistinguishable from those assembled in the absence of drug.

Effect of the dibromoselenide (7) on the binding of ^3H colchicine to tubulin

Combretastatin A4 (**2**) and amphethinile (**1**) have both been shown to compete for the colchicine (**3**) binding site on tubulin.[2] A competition assay between colchicine (**3**) and the dibromoselenide (**7**) was carried out. However at concentrations up to 200 μM (the limit of solubility of selenide **7**) no displacement of colchicine (**3**) was observed. This compares with combretastatin A4 (**2**) and amphethinile (**1**) which have been shown, under identical experimental conditions, to compete for colchicine (**3**) binding sites on tubulin at concentration of 6–7 μM.[2] The dibromoselenide (**7**) therefore does not compete for the colchicine binding site on the tubulin protein. This is evidence that the binding site on tubulin for the dibromoselenide (**7**) is not identical to that of colchicine (**3**).

Affinity of dibromoselenide (7) for tubulin

A fluorescence quenching assay was carried out to study the strength of the tubulin–dibromoselenide (**7**) interaction. The dibromoselenide was found to interact strongly with tubulin. After a 90 min incubation at 22 °C a dissociation constant of 4.6 μM and a free energy of binding of -7.7 kcal/mol was obtained. Increasing incubation time and temperature (37 °C, 150 min) did not significantly alter the dissociation constant (5.4 μM) or free energy of binding (-7.5 kcal/mol).

Molecular modelling

The minimum energies (obtained by the minimisation procedures described in the Experimental Section) for the diphenyl compounds (**4–7**) were 5.2, -4.3, 65.4, -7.0 kcal/mol respectively. Conformational analysis (see Experimental Section) of the four compounds (**4–7**) showed that they had varying degrees of conformational freedom. Tables 1–4 show some of the absolute torsion angles, the energy of the conformation, the difference in energy of the conformation to the lowest energy conformation and the Boltzmann populations (relative to the minimum energy population) of the conformations for the four diphenyl compounds (**4–7**). Table 5 shows the C–S(e) bond length, the C–S(e)–C bond angle, the angle between the planes of the aromatic and the angle between the C–S(e)–C plane and the plane of an aromatic ring for the minimum energy conformation of each of the sulfides (**4**, **5**) and selenides (**6**, **7**).

The results of the Probe Interaction (see Discussion) are depicted in Figure 2. The contours are set at 27.6 kcal/mol with the red contour depicting the Van der Waals plus electrostatic interaction for the bromoselenide (**7**) and the green contour depicting the same for selenide (**6**). (Other energy contours were calculated but have been omitted from Figure 2 for the sake of clarity.)

Discussion

The dibromosulfide (**5**) and dibromoselenide (**7**) share structural features with amphethinile (**1**) and combretastatin A4 (**2**). A comparison of biological activity shows that the dibromo compounds (**5**, **7**) have the ability to disrupt the cell cycle by causing cells to block in the G2/M phase. DNA content greater than 2*n* (normal diploid) was observed which indicated that DNA synthesis was continuing in the cells which were unable to divide. The increase in mitotic index of P388 cells treated with these compounds (**5**, **7**) suggested that cells were being transiently blocked in metaphase. The decrease in the number of mitoses with increasing time is consistent with cells decondensing their chromosomes in preparation for a second cycle of DNA synthesis without cell division. These cellular events are consistent with the action of agents which disrupt the mitotic spindle.[6] Disruption of this organelle can lead to the appearance of cells with excessive DNA contents. These agents show considerably reduced cytotoxicity compared with combretastatin A4 (**2**) (>10,000-fold) or ampethinile (**1**) (>50-fold). The relatively low cytotoxicity, together with poor solubility would indicate that these compounds are not themselves good candidates for anti-tumour agents but may be useful lead structures. However *in vitro* activity is a poor predictor of *in vivo* effects.

Table 1. Molecular modelling parameters associated with the two C–S torsion angles of sulfide (4)

Absolute torsion angles (°)	Potential Energy (kcal/mol)	ΔE from minimum energy conformation (kcal/mol)	Boltzmann probability*
330, 270	6.00	0.83	19.93
330, 300	5.31	0.14	44.16
330, 330	5.88	0.71	23.33
300, 240	7.12	1.95	3.67
300, 270	5.84	0.63	25.81
300, 300	5.17	-	-
300, 330	5.30	0.13	44.57
270, 210	6.57	1.40	8.74
270, 240	6.19	1.02	15.32
270, 270	6.02	0.85	19.39
270, 300	5.82	0.65	25.17
270, 330	5.91	0.74	22.44
240, 210	6.29	1.12	13.27
240, 240	5.89	0.72	23.02
240, 270	6.17	1.00	15.76

* = % probability of structure occupying this conformation relative to the lowest energy conformation.

Table 2. Molecular modelling parameters associated with the two C–S torsion angles of dibromosulfide (5)

Absolute torsion angles (°)	Potential Energy (kcal/mol)	ΔE from minimum energy conformation (kcal/mol)	Boltzmann probability*
330, 270	-1.90	2.25	2.25
330, 300	-1.78	2.37	1.85
300, 270	-2.85	1.3	10.2
300, 300	-4.15	-	-
300, 330	-1.78	2.37	1.85
270, 270	-3.8	0.35	35.74
270, 300	-2.85	1.3	10.2
270, 330	-1.9	2.25	2.25

* = % probability of structure occupying this conformation relative to the lowest energy conformation.

Table 3. Molecular modelling parameters associated with the two C–Se torsion angles of selenide (**6**)

Absolute torsion angles (°)	Potential Energy (kcal/mol)	ΔE from minimum energy conformation (kcal/mol)	Boltzmann probability*
270, 240	67.4	2.0	3.38
270, 270	65.4	-	-
240, 240	67.1	1.7	5.47
240, 270	67.5	2.1	2.88
300, 300	69.0	3.6	0.24
300, 270	68.9	3.5	0.28

* = % probability of structure occupying this conformation relative to the lowest energy conformation.

Table 4. Molecular modelling parameters associated with the two C–Se torsion angles of dibromoselenide (**7**)

Absolute torsion angles (°)	Potential Energy (kcal/mol)	E (kcal/mol)	Boltzmann probability*
330, 270	-5.34	1.65	5.92
330, 300	-5.78	1.21	11.63
330, 330	-4.23	2.76	0.97
300, 270	-6.53	0.46	31.63
300, 300	-6.99	-	-
300,330	-5.78	1.21	11.63
270, 240	-4.21	2.78	0.94
270, 270	-6.46	0.53	29.15
270, 300	-6.53	0.46	31.63
270, 330	-5.34	1.65	5.92

* = % probability of structure occupying this conformation relative to the lowest energy conformation.

Table 5. Geometries of compounds (**4**–**7**) derived from their minimum energy conformations

Compound	C-S(e) (Å) bond length	C-S(e)-C (°) bond angle	Angle between plane of aromatic rings	Angle between plane of aromatic ring and the C-S(e)-C plane
4	1.77	103.5	94.2	59.8
5	1.78	102.6	95.6	119.3
6	1.96	60.7	76.0	86.1
7	1.91	96.6	82.6	116.6

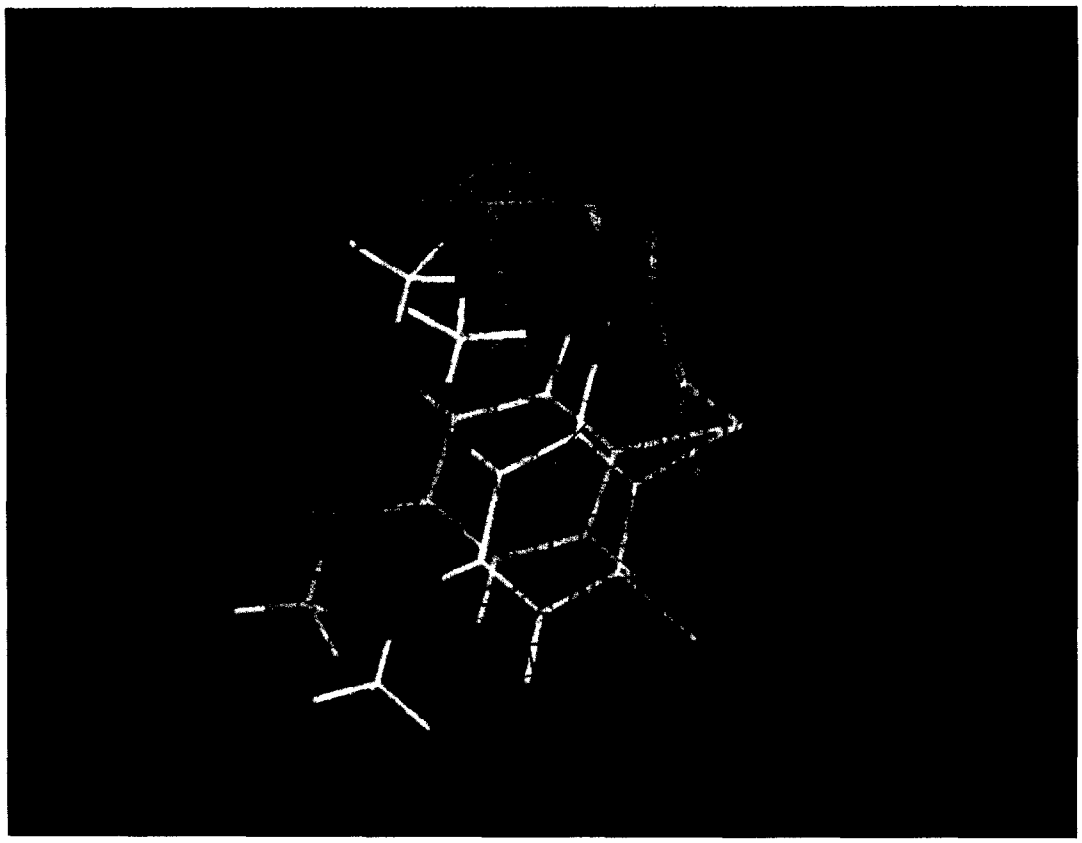

Figure 2. Probe interaction map. The green contour (selenide, **6**) and the red contour (dibromoselenide, **7**) depict their combined Van der Waals and electrostatic interaction contours at 27.6 kcal/mol

The dibromoselenide (**7**) has been shown to bind strongly to tubulin. The dissociation constant (4.6 μM) for binding is comparable to those of the known anti-mitotic agents (the dissociation constants of amphethinile (**1**), combretastatin A4 (**2**) and colchicine (**3**) are 0.77, 1.02 and 0.71 μM respectively).[1] However whereas these agents share a common binding site the dibromoselenide (**7**) appears to bind at a separate, distinct site and does not displace colchicine (**3**) from the protein.

The dibromoselenide (**7**) is cytotoxic in the cell lines tested. Interestingly the P388 R8/22 multi-drug resistant cells showed collateral sensitivity (the processes whereby resistance to one agent is accompanied by increased sensitivity to another agent) towards this agent (**7**). The mechanism of resistance operating in these resistant cell lines is increased expression of the multi-drug resistance pump (gP170), which exports drug from within the cell. However, a human ovarian multidrug resistant A2780/ADR cell line which also overexpresses the gP170 protein did not demonstrate this phenomenon.

Although the dibromo compounds (**5**, **7**), especially the dibromoselenide (**7**), showed biochemical effects similar to those of known anti-mitotic agents (binding to tubulin, increased number of cells in G2/M phase of the cell cycle), they failed to exhibit other properties expected of this type of agent. The dibromoselenide (**7**) failed to displace colchicine (**3**) from its binding site on tubulin at a concentration of 200 μM. This is the equivalent of 1 tubulin heterodimer per 200 drug molecules. Also neither of the thio (**4**, **5**) or seleno (**6**, **7**) ethers showed an ability to inhibit the polymerisation of tubulin. However, with the dibromoselenide (**7**), the observed final absorbance value was consistently higher than that of the control. The tubulin polymers appeared normal when viewed in an electron microscope. No abnormal sheet or ribbon structures were observed and the average microtubule length was similar to those in control samples. It may be, however, that these drugs, upon binding to tubulin alter the monomer–polymer equilibrium in favour of the polymer. This would result in increased production of microtubules, which would lead to an increased absorbance. This effect has been observed for taxol.[10] Therefore this agent may be a lead compound for other simple agents which can interact with tubulin to alter microtubule dynamics. The dibromoselenide (**7**) is less potent than taxol (15% increase in microtubule assembly compared to 50% for taxol).

Molecular modelling studies show a large variation in the flexibility of these agents (Tables 1–4). The dihydrosulfide (**4**) is conformationally flexible (Table 1), whereas the dihydroselenide (**6**) is predicted to have a rigid structure (Table 3). The dibromoselenide (**7**) and the dibromosulfide (**5**), agents which show biological activity, have similar torsion angles in their minimum energy conformations (Tables 2 and 4). These compounds (**5**, **7**) also show other

structural similarities (Table 5) including the C–S(e)–C bond angle, and the angle between the aromatic ring and the C–S(e)–C plane. A comparison of the minimised structures shows excellent structural homology between the dibromoselenide (**7**) and the dibromosulfide (**6**) (Figure 3). This structural similarity is not evident when the dibromoselenide (**7**) and the dihydroselenide (**6**) are compared (Figure 4). This is reflected by the good overlap achieved by the two dibromo-compounds (RMS deviation of 0.08 Å over a seven atom overlap). Overlap of the dibromoselenide (**7**) with the dihydroselenide (**6**) (RMS = 0.56 Å), showed much poorer structural homology.

The probe interaction calculations on selenides (**6**, **7**) were performed on the conformations of their overlaid low energy structures by overlapping the Se atoms and just three aromatic carbons from each equivalent ring (i.e. four atoms from each structure). This means that two of the rings are separated by 35.9° (the difference between the two C–Se–C bond angles in the low energy structures of **6** and **7**). The Van der Waals and electrostatic repulsion at an energy of 27.6 kcal/mol of the dibromo compound (**7**) envelops a larger spatial volume around selenide (**7**) than the corresponding energy contour of the non-brominated selenide (**6**) (Figure 2). This extended repulsive volume manifests itself around the bromine atoms. The electronegative bromines attract the probe (a proton). This repulsion therefore arises due to the Van der Waals volume of the bromine atoms.

The dibromoselenide (**7**) is the most active of these agents. The dibromosulfide (**5**) however also shows some biological effects, particularly the arrest of cells in the G2/M phase of the cell cycle, the appearance of cells with greater than 4*n* DNA content, and an increase in mitotic index. All these biological effects are seen when the mitotic apparatus has been disrupted, resulting in inhibition of cell division. Agents which are known to cause similar effects include the anti-mitotic drugs vincristine, vinblastine, and vindesine, as well as colchicine (**3**), combretastatin A4 (**2**), and amphethinile (**1**). These agents bind to tubulin and disrupt the tubulin–microtubule equilibrium.

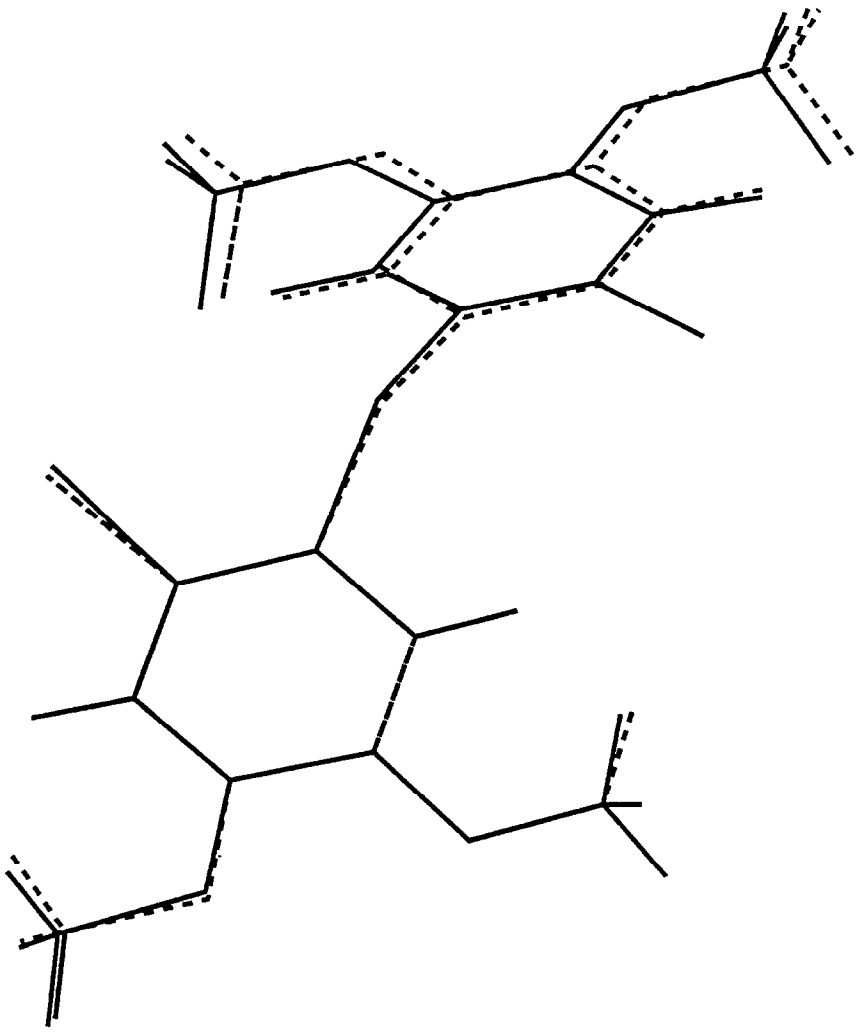

Figure 3. Overlap of minimum energy conformations of dibromoselenide (**7**) (solid lines) and dibromosulfide (**5**) (broken lines)

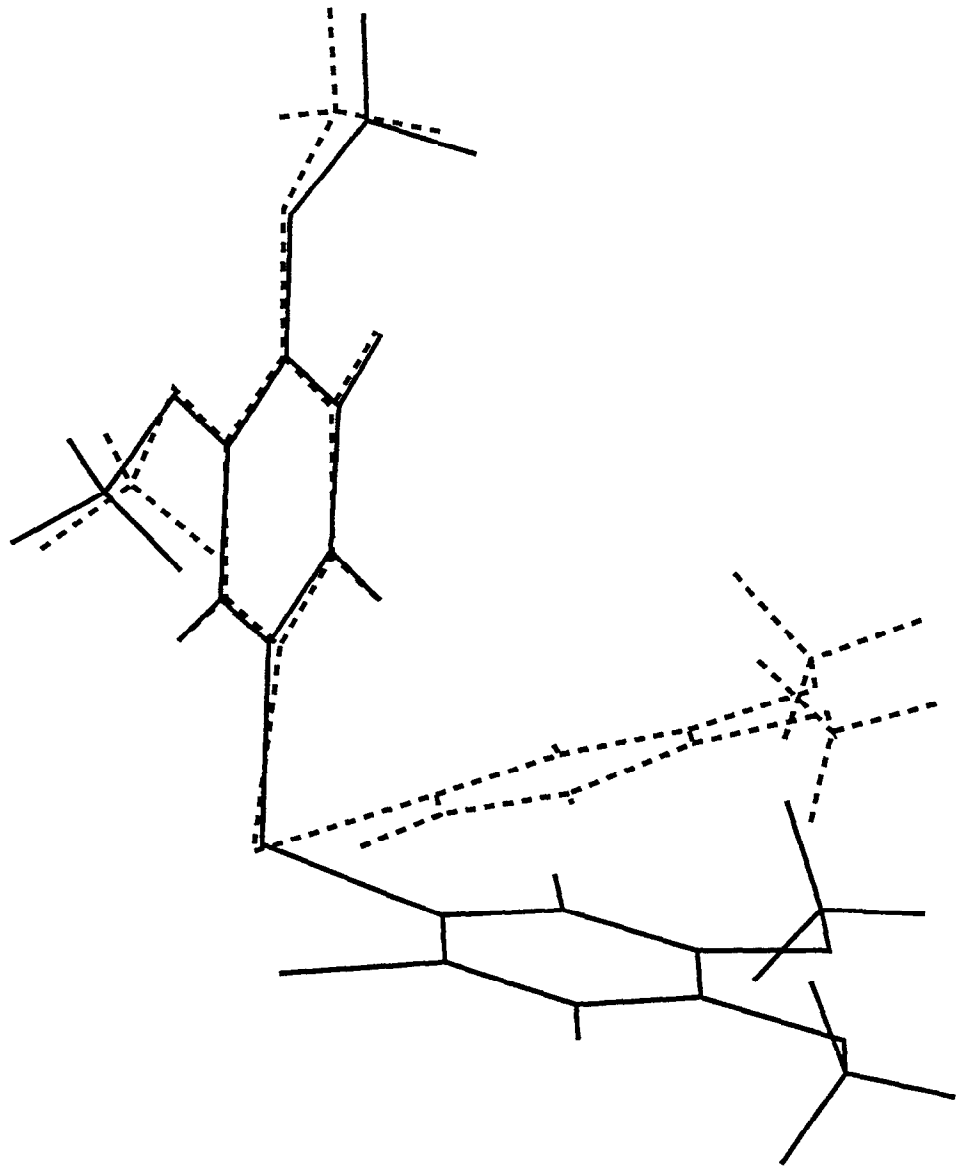

Figure 4. Overlap of minimum energy conformations of dibromoselenide (7) (solid lines) and selenide (6) (broken lines)

This work has shown that the dibromoselenide (7) interacts with tubulin, and this results in a G2/M phase accumulation of cells. Microtubules are in equilibrium with free tubulin. This equilibrium can be disturbed by the interaction of a drug with tubulin, leading to either increased (e.g. taxol) or decreased (e.g. combretastatin A4) microtubule production. It may be that binding of the dibromoselenide (7) leads to the adoption of a tubulin structure which more favours microtubule assembly. This would be reflected by the increased optical absorbance seen following microtubule assembly in the presence of the dibromoselenide (7). The cytotoxicity seen for this agent may be a result of this stabilisation. The greater biological activity of the dibromoselenide (7) to that of the virtually identically shaped dibromosulfide (5) cannot readily be explained but may be simply a result of altered cellular accumulation or distribution.

In conclusion the dibromoselenide (7) is a novel agent which possesses cytotoxic effects against tumour cells *in vitro*. This has been shown to bind to tubulin. Cells treated with this agent accumulate in the G2/M phase of the cell cycle before restarting DNA synthesis without cell division. This probably results from disruption or inhibition of the mitotic apparatus. However this agent does not inhibit the formation of microtubules *in vitro*. Therefore, although an interaction with tubulin has been demonstrated, the possibility that the dibromoselenide (7) is acting on another microtubular protein cannot be excluded. The biological activity of the dibromoselenide (7), and to a lesser degree the dibromosulfide (5), may be attributable to the molecular shape. These two agents show structural homology, whereas the dihydro-derivatives (4, 6) possess either a different geometry (6) or are very flexible (4). The shape of the dibromoselenide (7) may be a useful lead in the search for novel agents which inhibit mitosis.

Experimental Section

The four compounds (**4–7**) were kindly provided by G Klar.[4]

Biochemical studies

The sulfides (**4, 5**) and selenides (**6, 7**) were tested for cytotoxicity by the previously published MTT assay.[5] The cell lines used were P388 mouse leukaemia, a multi-drug resistant sub-line P388R8/22, A2780 human ovarian carcinoma and its multi-drug resistance sub-line A2780ADR. Cell cycle analysis,[6] measurement of mitotic indices[7] and the measurement of the inhibition of tubulin assembly[2] were performed as previously described. The dissociation constant[8] and the effect on binding of ^{3}H colchicine to tubulin[9] for the dibromoselenide (**7**) were determined as published, except that porcine, rather than bovine tubulin was used.

Molecular modelling

The molecular modelling was performed using the Quanta (3.3) and Charmm programs of Molecular Simulations Inc. (Burlington, MA, U.S.A.). The crystal structures of the sulfide (**4**) and the two selenides (**6, 7**) were imported into Quanta from the Cambridge Crystallographic Database via the Chemical Information Service at Daresbury Laboratories, Warrington. No crystal structure for sulfide (**5**) was available so this structure was built in Quanta. These molecular structures were assigned to the correct Quanta atom types, minimised by the steepest descents program (50 iterations) followed by Newton–Raphson minimisation (50 iterations). Conformational studies on the sulfides (**4, 5**) and selenides (**6, 7**) were carried out using the conformational search program from Quanta. For each structure the torsion angles of the two C–S(e) bonds were altered stepwise by 30° over 360° and minimised in these fixed positions by the Adopted Basis Newton–Raphson procedure following each step. This process afforded a two dimensional contour plot which was analysed to discover the minimum energy conformations of the compounds (**4–7**) (Tables 1–4).

Calculations of overlap of one conformation of one of the compounds (**4–7**) with one conformation of another of the four compounds were performed using the Molecular Similarity program from Quanta. Unless otherwise stated seven equivalent atoms were overlaid (3 atoms from each aromatic ring and the sulfur or selenium atom for each structure). The probe interaction contour maps (Figure 2) of the minimum energy conformations of the selenide (**6**) and of the dibromoselenide (**7**) were calculated using the Probe Interaction program from Quanta. The probe chosen was a proton, the grid size selected at 0.5 Å which was centred on the S(e) atom for each case. The contours shown (Figure 2) show a combined Van der Waals and electrostatic repulsion at a potential energy of 27.6 kcal/mol.

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