Common structural features determine the effectiveness of carvedilol, daunomycin and rolitetracycline as inhibitors of Alzheimer β-amyloid fibril formation

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One of the major pathological features of Alzheimer's disease is the deposition of β-amyloid peptide (Aβ). Cellular toxicity has been shown to be associated with fibrillar forms of Aβ; preventing this fibril formation is therefore viewed as a possible method of slowing disease progression in Alzheimer's disease. With the use of a series of tetracyclic and carbazole-type compounds as inhibitors of Aβ fibril formation, we here describe a number of common structural features that seem to be associated with the inhibitory properties of these agents. Compounds such as carvedilol, rolitetracycline and daunomycin, which are shown to inhibit Aβ fibril formation, also prevent the formation of species of peptide that demonstrate biological activity in a human neuroblastoma cell line. Molecular modelling data suggest that these compounds have in common the ability to adopt a specific three-dimensional pharmacophore conformation that might be essential for binding to Aβ and preventing it from forming fibrils. Understanding such drug–peptide interactions might aid the development of disease-modifying agents.

Key words: Alzheimer's disease, carbazoles, three-dimensional pharmacophore.

INTRODUCTION

Alzheimer's disease develops as a result of the progressive degeneration of specific neuronal pathways in the brain. The disease is characterized by the presence of large numbers of neuritic plaques, one of the main constituents of which is the 39–43-residue β-amyloid peptide (Aβ). The production and deposition of Aβ are believed to be fundamental to the pathology of the disease and to involve a process of direct neurotoxicity. Although there is a lack of consensus on the mechanism by which the neurotoxicity is brought about, there is general acceptance that the neuronal cell loss is associated with the aggregation or formation of fibrils of Aβ. Consequently, preventing this process might provide a method of slowing the development of the pathology of Alzheimer's disease.

Several series of compounds have been described that effectively inhibit Aβ fibril formation. These include β-cyclodextrin [1], rifamycins [2], sulphonate dyes [3], nicotine [4], haemin [5] and quaternary ammonium salts [6]. There have been no reports on the effects of any of these compounds on Aβ deposition *in vivo*, although peripheral amyloid formation has been attenuated by sulphonates [7]. Short peptide fragments, based primarily on the central hydrophobic region of Aβ, have been proposed as being capable of preventing β-sheet formation [8,9]. It has been claimed that peptides of this type inhibit fibrillogenesis in a rat brain model of amyloidosis in which both Aβ 1–42 and peptide inhibitor were injected directly into rat brain amygdala [10]. Although transgenic mouse models of Alzheimer's disease with Aβ deposits have now been described [11], there are no published reports describing the effects of the administration of inhibitors of fibril formation in these models. The anthracycline 4′-deoxy-4′-iododoxorubicin (IDOX) binds to amyloid fibrils from Alzheimer brain and also to amyloid fibrillar forms of immunoglobulin light chains, amyloid A, β2-microglobulin and transthyretin. It is effective in a model of murine peripheral amyloidosis *in vivo* [12]. Limited clinical data have also pointed to the therapeutic efficacy for IDOX in systemic amyloidosis [13].

In this paper, the ability of non-iodo derivatives of IDOX and other compounds to prevent the conversion of Aβ into a biologically active form of the peptide is shown to be associated with a number of common structural features in these molecules. In particular, the demonstration of the effectiveness of a number of tetracyclic compounds and substituted carbazoles as anti-fibrillar agents suggests that this inhibitory activity of the compounds is dependent on the possession of a basic amino group and two cyclic hydrophobic ring centroids.

MATERIALS AND METHODS

Materials

Aβ 1–40 (lot no. ZM605) was purchased from Bachem (UK) Ltd. (Saffron Walden, Essex, U.K.). Carbazoles and doxorubicin were obtained from the SmithKline Beecham compound collection. Other compounds were from commercial sources: daunomycin from Apin Chemicals Ltd. (Abingdon, Oxon., U.K.), and all tetracyclines and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (Poole, Dorset,

Abbreviations used: Aβ, β-amyloid; 3D, three-dimensional; IDOX, 4′-deoxy-4′-iododoxorubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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U.K.). Monoclonal antibody 2F12 was raised against the 1–16 fragment of Aβ 1–40.

Aβ 1–40 was dissolved in 0.1 % (v/v) acetic acid at 2 mg/ml. Inhibitors were dissolved in DMSO at 10 mg/ml.

Immunoassay

The ability of compounds to prevent Aβ fibril formation was assessed by a fibril-dependent immunoassay [5]. This assay employed a sandwich assay format based on the use of a single antibody. Specificity for polymerized Aβ was achieved by the use of 2F12 monoclonal antibody as the capture antibody and biotin-labelled 2F12 for detection. Only polymerized peptide, bound to the 2F12 immobilized on the capture plate, provided exposed epitope for the binding of the biotin-labelled detection antibody.

In brief, peptide and inhibitors were diluted in PBS, pH 7.4, containing 0.02 % (v/v) Tween 20 to prevent the binding of peptide to microtitre plates. Peptide at 11.6 μM (50 μg/ml) was incubated at 37 °C overnight in the presence or absence of competing compound. Peptide was subsequently captured on microtitre plates (Nunc Maxisorb; Life Technologies, Paisley, Scotland) precoated with the monoclonal antibody 2F12 (2.7 μg/ml in PBS). The detection antibody was a biotin-labelled form of 2F12 (0.8 μg/ml); binding of streptavidin–europium (Wallac, Milton Keynes, U.K.) allowed quantification by delayed enhanced lanthanide fluorescence immunoassay ('DELFIA'; Wallac). In a typical assay, 10 pmol per well of freshly dissolved Aβ or equivalent vehicle both gave background signals of approx. 20000 light units. After the overnight incubation of Aβ at 11.6 μM in the absence of competing compound, a signal of 600000–800000 light units was observed.

MTT assay for biological activity

Peptide plus inhibitors were dissolved as described above for the immunoassay and were incubated overnight at 37 °C. To assess the activity of Aβ after the incubation, IMR32 human neuroblastoma cells (ECACC; Porton Down, Salisbury, Wilts., U.K.) were plated at 6×10^4 cells/cm^2 in a 96-well microtitre plate. After attachment for 2 h, peptide solutions (containing pre-incubated Aβ, Aβ plus inhibitor or inhibitor alone) were added at concentrations of 0.1–200 pmol/ml Aβ. After incubation for a further 24 h, cell viability was assessed as the inhibition of the ability of cells to reduce the metabolic dye MTT to a blue formazan product [15].

Pharmacophore mapping

The DISCO pharmacophore mapping methodology [16], as implemented within the SYBYL software, was used to propose a plausible pharmacophore model and to overlay chemically common features of all the structures. Thirty different energy-minimized conformers (each within 42 kJ/mol and obtaining a root-mean-square deviation greater than 1.0 Å^2 between the representative conformations) were generated and used as representative binding motifs for the compounds. All combinations of conformers were considered and only those with a root-mean-square tolerance of less than 0.5 Å were considered as representative of the pharmacophore.

RESULTS

Inhibition of fibril formation

Aβ peptide, freshly dissolved in 0.1 % (v/v) acetic acid, was characterized by a lack of any fibrillar material discernible under the electron microscope or a lack of activity in the MTT assay or immunoassay (results not shown). Incubation of Aβ overnight (for 18 h) in the absence of competing compound resulted in a product that inhibited MTT reduction by up to 50 % and showed, by electron microscopy, the presence of long straight fibrils and a lack of amorphous deposits (Figure 1). The immunoassay signal for polymerized product was maximal after 18 h incubation, with a slightly less than maximum value being maintained for up to at least 96 h (Figure 1). Incubations for longer than 18 h did not have any significant further effects on either MTT reduction or the presence and nature of long straight fibrils discernible by electron microscopy (results not shown).

In the Aβ-fibril-specific immunoassay, the anthracyclines daunomycin and doxorubicin were found to be effective inhibitors of Aβ 1–40 fibril formation, with IC_{50} values of 30 μM for both

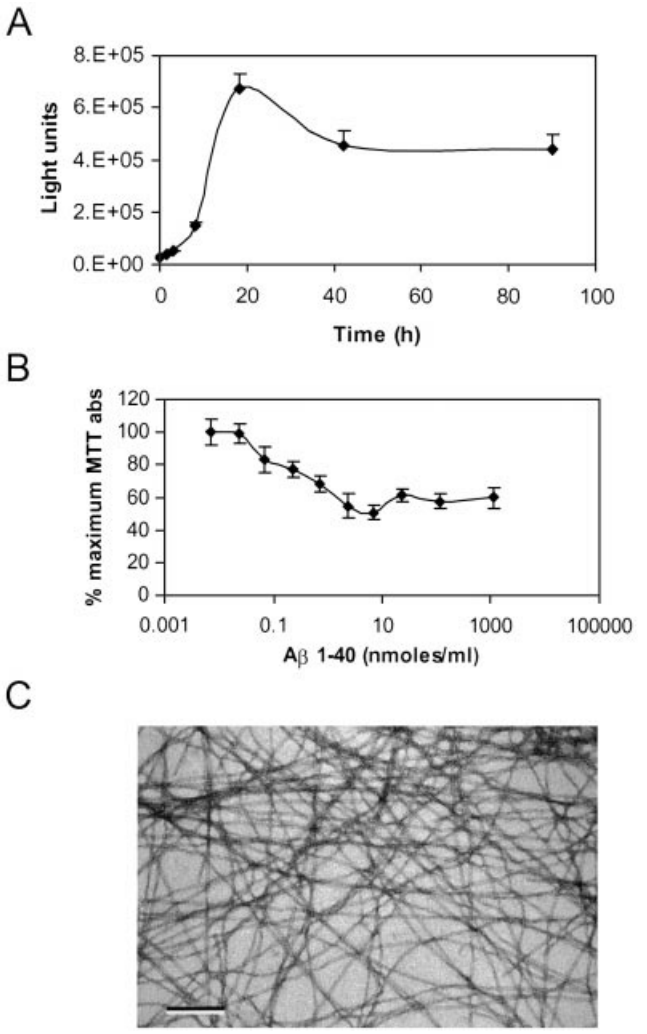
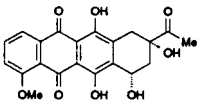
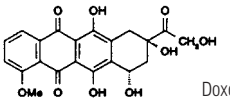
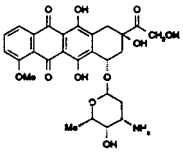
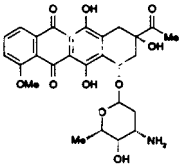
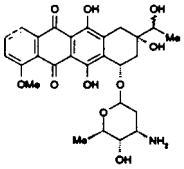
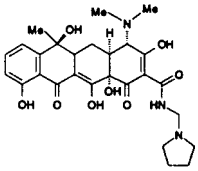
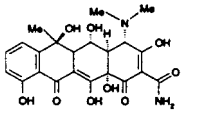
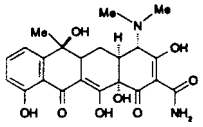


Figure 1 Polymerization of Aβ 1–40 in the absence of competing compound

(**A**) Time course of development for immunoassay product. Aβ 1–40 was dissolved and incubated for up to 96 h; assessment was via Aβ polymer-specific immunoassay (see the Materials and methods section for details). On the y-axis, 2.E + 5 is equivalent to 2×10^5, etc. (**B**) Aβ 1–40 (11.6 μM) that had been incubated for 18 h was added to IMR 32 neuroblastoma cells at the indicated concentrations. Biological activity was determined by MTT assay and is expressed as a percentage of the MTT reduction in the absence of Aβ. Results for the immunoassay and the MTT assay are means ± S.E.M. for three determinations. (**C**) Electron micrograph image of Aβ preparation after 18 h of incubation. Scale bar, 200 nm.

Table 1 Inhibition of Aβ fibril formation by anthracycline and tetracyclic compounds

Aβ 1–40 (11.6 μmole/litre) was incubated (18h) in the presence or absence of competing compound. Fibrillar product was determined by either immunoassay or MTT cell viability assay, as described in "Methods". Immunoassay data represents mean ± S.E.M. (where given) of three experiments. Cell toxicity is expressed as the percentage inhibition of the MTT reduction produced by 1 pmole/ml of fibrillar Aβ 1–40 at the stated concentration of compound and is the mean of ≥3 experiments carried out in triplicate. (n.t. = not tested).

	Immunoassay IC_{50} (μM)	MTT assay (% Inhibition)
 Daunomycinone	Inactive at 250 μM	Inactive at 250 μM
 Doxorubicinone	Inactive at 250 μM	n.t.
 Doxorubicin	30 ± 4 μM	n.t.
 Daunomycin	30 ± 5 μM	80 % at 20 μM
 Daunomycinol	100 ± 14 μM	10 % at 20 μM
 Rolitetracycline	59 ± 5 μM	100 % at 20 μM
 Oxytetracycline	34% inhibition at 250 μM	10–20 % at 20 μM
 Tetracycline	20% inhibition at 250 μM	n.t.
Chlortetracycline, demeclocycline, doxycyline, meclocycline, minocycline, penimepicycline	Inactive at 250 μM	n.t.

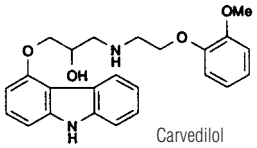
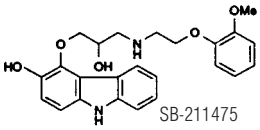
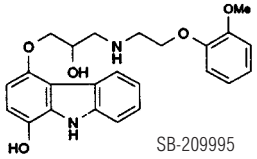
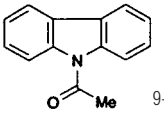
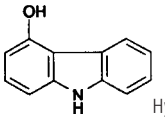
compounds (Table 1). This activity was partly retained by daunomycinol but was lost in the corresponding aglycones. The biological activity of the fibrillar Aβ incubation product was assessed by MTT assay. A similar disparity between daunomycin and daunomycinol was observed in this assay (Table 1). Of the tetracycline analogues, only rolitetracycline, the derivative with an extra basic amine group, showed activity comparable to that of the anthracyclines in both the immunoassay and the MTT assay. The substituted carboline carvedilol and two hydroxylated analogues were also active when assessed by either assay, whereas the non-substituted hydroxycarbazole and 9-acetyl derivative were less than 10% as active (Table 2). At the concentrations described, the compounds alone had no effect on cell viability and did not directly inhibit the effects of prefibrillized Aβ on MTT reduction (results not shown). The aglycone derivatives of daunomycin and doxorubicin, and tetracyclines other than rolitetracycline, were ineffective in preventing Aβ fibril formation.

Pharmacophore mapping

Three of the compounds showing the ability to inhibit the formation of fibrillar Aβ (carvedilol, rolitetracycline and

Table 2 Inhibition of Aβ fibril formation by carbazole compounds

Aβ 1–40 (11.6 μmole/litre) was incubated (18 h) in the presence or absence of competing compound. Fibrillar product was determined by either immunoassay or MTT cell viability assay, as described in "Methods". Immunoassay data represents mean ± S.E.M. (where given) of three experiments. Cell toxicity is expressed as the percentage inhibition of the MTT reduction produced by 1 pmole/ml of fibrillar Aβ 1–40 at the stated concentration of compound and is the mean of ≥3 experiments carried out in triplicate. (n.t. = not tested).

	Immunoassay IC_{50} (μM)	Cell toxicity (% inhibition)
 Carvedilol	30 ± 6 μM	80–90 % at 20 μM
 SB-211475	42 ± 14 μM	80–90 % at 20 μM
 SB-209995	79 ± 23 μM	80–90 % at 20 μM
 9-Acetylcarbazole	Inactive at 250 μM	n.t.
 Hydroxycarbazole	450 ± 75 μM	Inactive at 250 μM

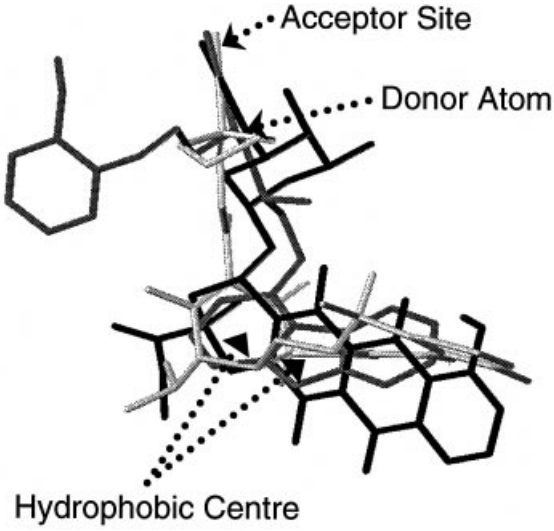


Figure 2 Pharmacophore mapping of carvedilol, rolitetracycline and daunomycin showing common features

The colours used are as follows: dark grey, carvedilol; black, daunomycin; pale grey, rolitetracycline. See the Results section for details.

daunomycin) were subjected to pharmacophore mapping (Figure 2). A three-dimensional (3D) conformation was identified that could be accessed by all three compounds. Thirty discrete energy-minimized conformers (each within 42 kJ/mol) were generated for each structure and were used to overlay and compare several chemical features. Four common features, comprising two aromatic centres, one donor atom and an acceptor site, were identified that could be overlaid to within a 0.5 Å tolerance. Compounds lacking activity in the fibril formation assays, such as oxytetracycline and tetracycline, possessed the required motifs but did not have the conformational flexibility to access the required 3D phamacophore (results not shown). Interestingly, the metabolites of carvedilol (SB-209995, SB-211475) that were effective inhibitors of Aβ fibril formation retained these structural motifs and had the flexibility to achieve the desired conformation (results not shown).

DISCUSSION

The polymerization of the Aβ peptide into a form that initiates the neurodegenerative process characteristic of Alzheimer's disease has been studied extensively [17]. However, the precise nature of the toxic form is unknown; small oligomers [18–21], protofibrils [22,23] and fibrils [24] have been proposed as the toxic species. Inhibition of Aβ polymerization has been assessed by monitoring the ability of Aβ preparations (1) to decrease MTT reduction and (2) to form long straight fibrils [2,25,26]. This inhibition has also been described by immunoassay for haemin [5] and benzofurans [25]. We have recently demonstrated that non-toxic low-molecular-mass oligomers of Aβ are not immunoassay-positive [27]. However, in the presence of daunomycin at concentrations that inhibit bioactive polymer production, no Aβ other than 4 kDa material is observed [27] and the preparation is free from distinct structural features (D. R. Howlett, unpublished work), thus resembling freshly dissolved material. This suggests that compounds such as daunomycin might inhibit the fibril formation cascade by binding to a 4 kDa form. Conversely, results with haemin [27] and benzofurans [25] point to interactions between the inhibitor and non-monomeric forms. Moreover, although incubation with benzofurans resulted in the prevention of formation of long fibrils, small, non-bioactive, immunoassay-negative forms were observed. The DISCO methodology can be extended to derive a functional 3D pharmacophore that can be used as a search criterion to screen compounds virtually. This pharmacophore was applied to flexible conformations of two other Aβ fibril-formation inhibitors, SKF-74652 ([5-chloro-2-(4-methoxyphenyl)-3-benzofuranyl]-{4-[3-(diethylamino)propoxy]phenyl}methanone hydrochloride), a representative of the benzofuran type [25], and haemin [5], to investigate their potential role (A. R. George, unpublished work). These two compounds did not fulfil the criteria, within reasonable distance tolerances; it can therefore be postulated that they act via a different mechanism or at a different binding site and suggests that there might be no common structural form that inhibitors bind to in preventing fibril formation.

The nature of the biological activity assessed by the MTT assay is unknown, although it is generally described as an inhibition of MTT reduction or exocytosis [28,29]. However, the activity has been shown to be coupled with an increase in the release of lactate dehydrogenase from the challenged cell [30]. Nevertheless, MTT reduction itself does not necessarily measure cell death but is an index of the cell's metabolic integrity, which, as shown in the present study, can be compromised at concentrations of Aβ as low as 1 pmol/ml, well below those usually associated with causing cell death [8,9,30,31]. The use of the

MTT assay also has the advantage that it permits analyses of biological activity of Aβ after a relatively short exposure time (in comparison with cell-death assays), thus minimizing the time during which Aβ could undergo further conformational changes. At the concentrations used in the MTT assay, however, this is highly unlikely, given the high-order kinetics that are characteristic of Aβ polymerization [32].

We have demonstrated previously that compounds of structurally diverse types such as cyclodextrins [33], haemin [5] and benzofurans [25] are able to prevent the formation of a biologically active form of Aβ, as assessed by MTT assay. The means by which inhibitors of the fibril formation process are effective is not understood, although a number of mechanisms have been proposed for various compounds [34]. It has been suggested that compounds might interact with fibrillar Aβ and decrease MTT reduction either (1) by binding directly to polymerized Aβ and blocking interactions between the peptide and the cell [35,36] or (2) by facilitating the dissassembly of polymerized Aβ [37]. The lack of effect of the present compounds on the inhibition of MTT reduction resulting from a challenge of cells with prefibrillized Aβ peptide suggests that neither of these possibilities can account for the results reported.

The present results show that a range of compounds such as anthracyclines and carbazoles can assume similar 3D conformations that are correlated with their ability to inhibit Aβ fibril formation. The ability of the anthracycline IDOX to bind to various amyloids has been described previously [12]. We have recently shown that in a computational model of the interaction of IDOX with Aβ, the iodine of IDOX orientates out from the fibril surface, presumably decreasing the ability of additional Aβ molecules to bind to the fibril [38]. As an inhibitor of Aβ fibrillization, IDOX seems to exhibit 1:1 stoichiometry with peptide, as assessed with the Aβ 25–35 fragment [39]. With the use of the fibril-specific immunoassay, the anthracyclines doxorubicin and daunomycin at 30 μM are able to inhibit the fibril formation of 11.6 μM Aβ 1–40 peptide. Hence these compounds might inhibit fibril formation by binding to Aβ in a similar manner to IDOX.

Our results describe the substituted carbazole carvedilol as a novel anti-fibrillar agent. The comparable activities of carvedilol and its hydroxylated metabolites as inhibitors of fibril formation suggest that the effects are unrelated to the anti-oxidant properties specific to the metabolites [40,41]. Possession of the side chain is obviously necessary for binding because carbazoles with simple acetyl or hydroxy substitutions were inactive.

Although the modelling results suggest that the active inhibitors of fibril formation share an ability to assume a specific 3D pharmacophore conformation, we are unable to conclude whether these compounds inhibit the process by binding to Aβ monomers or small oligomers. Nevertheless, the effectiveness of compounds in the immunoassay seems to be reflected in the ability of the compounds, where assessed, to inhibit the formation of biologically active forms of the peptide. Further studies will be directed towards generating a more precise picture of the Aβ form and conformation to which compounds, such as those described above, will bind.

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