Suppression by polycyclic compounds of the conversion of human amylin into insoluble amyloid

Jacqueline F. AITKEN[1], Kerry M. LOOMES[1], Barbara KONARKOWSKA and Garth J. S. COOPER[2]
Biochemistry and Molecular Biology Group, School of Biological Sciences, University of Auckland, Auckland, New Zealand

There is a significant correlation between the occurrence of pancreatic islet amyloid and β-cell failure in advanced type II diabetes mellitus. Islet amyloid is composed primarily of the fibrillar form of the pancreatic hormone, amylin. Using thioflavin-T fluorescence binding and radioprecipitation assays, we investigated whether or not a series of small tricyclic compounds, tetracycline or Congo Red could interfere with the conversion of synthetic human amylin into its insoluble amyloid form. Of the compounds investigated, incubation of human amylin with a 20-fold molar excess of either Congo Red or Acridine Orange resulted in significant inhibition in the rate of amyloid formation. With Congo Red, maximal inhibition effectively occurred at a 1:1 molar ratio or greater over human amylin, whereas inhibition by Acridine Orange was dose-dependent. A 20-fold molar excess of the compound tetracycline also decreased insoluble amyloid content after extended incubation periods of approx. 20 h. Amyloid fibril morphology in the presence of tetracycline, as measured by transmission electron microscopy, was characterized by short fragmented fibrils compared with the longer and denser appearance of fibrils formed by amylin alone. These findings show that polycyclic compounds can suppress the formation of amyloid by human amylin, providing support for an alternative approach to peptide-based strategies by which islet amyloid formation could be modulated.

Key words: amylin, diabetes mellitus, islet amyloid, islet β-cell, pancreas, protein aggregation.

INTRODUCTION

Type II diabetes mellitus is a major disease [1] and is characterized by the onset of insulin resistance, the formation of islet amyloid and progressive pancreatic islet β-cell failure [2]. Although type II diabetes mellitus is a complex disorder, the occurrence of islet amyloid has been identified as a potentially significant factor contributing to late-stage islet β-cell failure [3]. Islet amyloid was first observed at the beginning of the last century and was assumed until more recent times to be insoluble deposits of insulin. Over the last fifteen years or so, molecular investigations revealed that these islet amyloid deposits are composed primarily of insoluble forms of the pancreatic hormone, amylin [4] {also known as insulinoma amyloid peptide (IAP), insulinoma amyloid polypeptide (IAPP) or islet amyloid polypeptide [5]}, which is normally co-secreted with insulin by pancreatic β-cells in response to glucose and other stimuli [6]. Amylin is a 37-amino-acid peptide characterized by a N-terminal cyclic ring structure and an amidated C-terminus that is essential for at least some biological activities [7].

Because amylin is co-regulated with insulin, the insoluble amyloid form of amylin most probably occurs as a consequence of excessive secretion during chronic hyperglycaemia, resulting in progressive extracellular islet deposition of amyloid [2]. Islet amyloid is present in approx. 95 % of type II diabetic patients and is strongly associated with islet β-cell loss [8]. Synthetic preparations of human amylin (hA) have been used to study mechanisms of amyloid formation *in vitro*, and have revealed the presence of structures that assemble spontaneously into higher-order polymorphic fibrils [9,10]. These preparations are cytotoxic to cultured RINm5F (rat insulinoma) islet β-cells in a time-dependent and concentration-dependent manner [11]. Transgenic models of hA support a more direct relationship between overexpression of amylin and induction of diabetes. Hemizygous transgenic animals that express hA, but do not exhibit any diabetic phenotype, develop diabetes-like syndromes when crossed into an insulin-resistant background [12,13]. In these cases, increased hA expression results in the occurrence of islet amyloid deposition in the pancreases of these animals, and is correlated with hyperglycaemia and β-cell loss.

Despite strong evidence linking the occurrence of islet amyloid to β-cell loss, no compounds have been identified that suppress islet amyloid formation *in vivo*. In the present study, we investigated the interactions of several polycyclic compounds with synthetic hA preparations. As some of these compounds were shown to suppress amyloid formation *in vitro*, these findings support an alternative approach to peptide-based strategies for modulation of islet amyloid formation.

EXPERIMENTAL

Materials

Synthetic hA (Lot 0551805) and rat amylin (rA; Lot 0542554) were HPLC-purified products from Bachem (Torrance, CA, U.S.A.). Triprolyl hA [$(Pro_3^{25,28,29})$hA] was obtained from Auspep (Parkville, Vic., Australia; batch K31155). Peptides were freshly dissolved in sterile milliQ water (18 MΩ resistivity), and were then diluted to their final concentration in the appropriate buffer. Tritiated hA (145.3 MBq/mmol) and rA (22.6 GBq/mmol) were synthesized according to protocols described previously [14]. All incubations containing amylin peptides were carried out at 22 °C. All polycyclic compounds and thioflavin-T were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions were made fresh in sterile milliQ water for each experiment. Calcein-AM (acetoxymethyl ester) and ethidium homodimer-1 (EthD-1) were

Abbreviations used: AM, acetoxymethyl ester; EthD-1, ethidium homodimer-1; hA, human amylin; $(Pro_3^{25,28,29})$hA: triprolyl hA; rA, rat amylin.
[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed (e-mail g.cooper@auckland.ac.nz).

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obtained from Molecular Probes (Eugene, OR, U.S.A.). The rat insulinoma cell line RINm5F was obtained from the National Institutes of Health (Bethesda, MD, U.S.A.) and cultured at 37 °C in a humidified atmosphere containing 5 % CO_2. Cell culture medium and its supplements were purchased from Invitrogen (Auckland, New Zealand).

Amyloid-binding assays

Effects of different polycyclic compounds on amyloid formation were measured by fluorescence spectroscopy, using a SpectraMAX Gemini XS fluorescence spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.). Excitation and emission maxima were set to 450 nm and 510 nm respectively using a cut-off filter at 495 nm. The rate of amyloid formation was determined in 10 mM Tris, pH 7.4, by monitoring thioflavin-T fluorescence in the presence or absence of each of the polycyclic compounds. Tetracycline, Congo Red and Methylene Blue had no intrinsic fluorescence under the conditions used. Background fluorescence by acridine and Acridine Orange in the absence of amylin was subtracted from the experimental results. Fluorescence enhancement by a control preparation containing only hA and thioflavin-T was used as a comparison for amyloid formation in the presence of the polycyclic compounds. All other experimental conditions were identical. Each experiment was performed in triplicate and was repeated independently at least twice.

Electron microscopy

hA (60 μM) was incubated in 10 mM Tris, pH 7.4, in the presence or absence of tetracycline (1200 μM). Samples were removed at various time points and were prepared for electron microscopy. Aliquots of the amylin preparations were adsorbed to glow-discharged carbon-coated collodion film on 200-mesh copper grids for 1 min. Grids were blotted, washed twice in droplets of deionized water and stained with 2 % (w/v) uranyl acetate. Grids were examined in a Philips Technai transmission electron microscope operated at 120 kV.

Amyloid-precipitation assays

The time-dependent precipitation of insoluble amyloid was used as an independent method to monitor amyloid formation in the presence or absence of polycyclic compounds. Trace amounts of [^{3}H]hA were added (typically 10000 c.p.m./ml) to a 10 μM hA solution in 10 mM Tris, pH 7.4, which was incubated in the absence or presence of each of the polycyclic compounds (200 μM final concentration). Samples were removed from the incubation mixtures at the indicated time points and were centrifuged at 16000 $\boldsymbol{g}$ for 20 min. The amount of [^{3}H]hA remaining in the supernatant after centrifugation was determined (Beckman LSW 3801 β-counter; Beckman, Palo Alto, CA, U.S.A.) and precipitable amyloid content was expressed as a percentage of total radioactivity in the incubation mixture at that time point. All experiments were performed using the same amylin batch and tritiated amylin tracer in the presence of appropriate controls (hA and rA in the absence of polycyclic compound). Effects of polycyclic compounds on the precipitable amyloid content were reproducible within each set of experiments, performed at least twice independently in triplicate.

Cell culture and cytotoxicity assays

RINm5F cells were cultured in RPMI 1640 medium containing 10 % foetal bovine serum, 290 μg/ml L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were plated in 24-well plates at a density of 15×10^4 cells per well, incubated for 48 h, rinsed with PBS and placed in fresh medium (200 μl/well) in the absence or presence of Congo Red (100 μM final concentration). Following a 30 min incubation, 12 μl of a freshly prepared aqueous solution of hA (500 μM) was added to the cell culture medium to give final amylin and Congo Red concentrations of 28 μM and 94 μM respectively. Following a 22 h incubation, cell viability was determined by double-staining with calcein-AM and EthD-1. Green fluorescence of live cells and red fluorescence marking nuclei of dead cells were simultaneously visualized using a Zeiss Axiovert S100 microscope equipped with a Zeiss filter set #09. Photographs were taken at 400× magnification using a Zeiss AxioCam digital camera.

RESULTS

Compounds were tested *in vitro* for their ability to interfere with the conversion of hA into its insoluble amyloid form. Interactions were measured using thioflavin-T fluorescence and radiolabelled amylin precipitation assays (Figure 1). When bound to hA

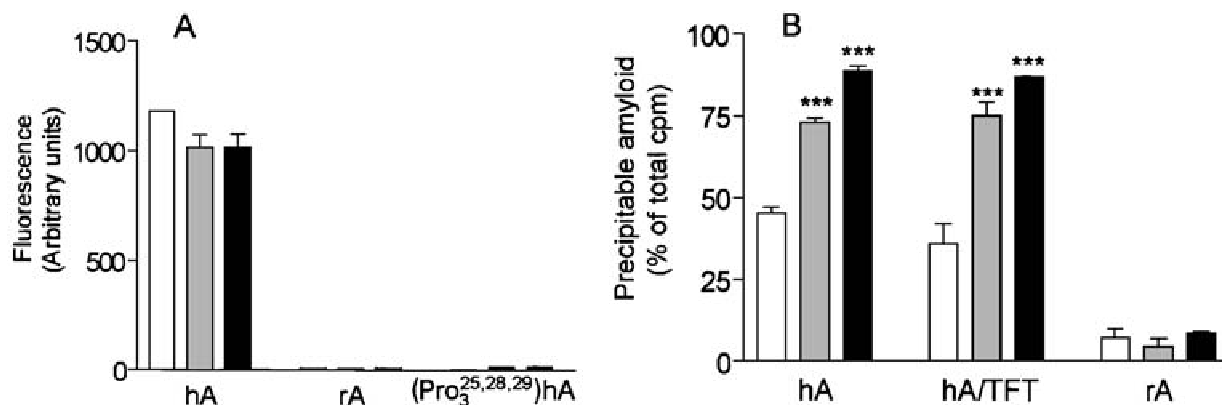

Figure 1 Amyloid formation by hA

(**A**) Thioflavin-T fluorescence following incubation of thioflavin-T (10 μM) with 60 μM hA, rA or (Pro$_3^{25,28,29}$)hA at 5 min (open bar), 5 h (shaded bar) and 23 h (solid bar). (**B**) Precipitable amyloid content following incubation of 10 μM hA in the absence or presence of 200 μM thioflavin-T (TFT) after 20 min (open bars), 5 h (shaded bars) and 24 h (solid bars). rA is shown for comparison as a non-amyloid-forming control. Results are means ± S.E.M. ($n = 3$). ***$P < 0.001$ compared with the corresponding control values at 20 min.

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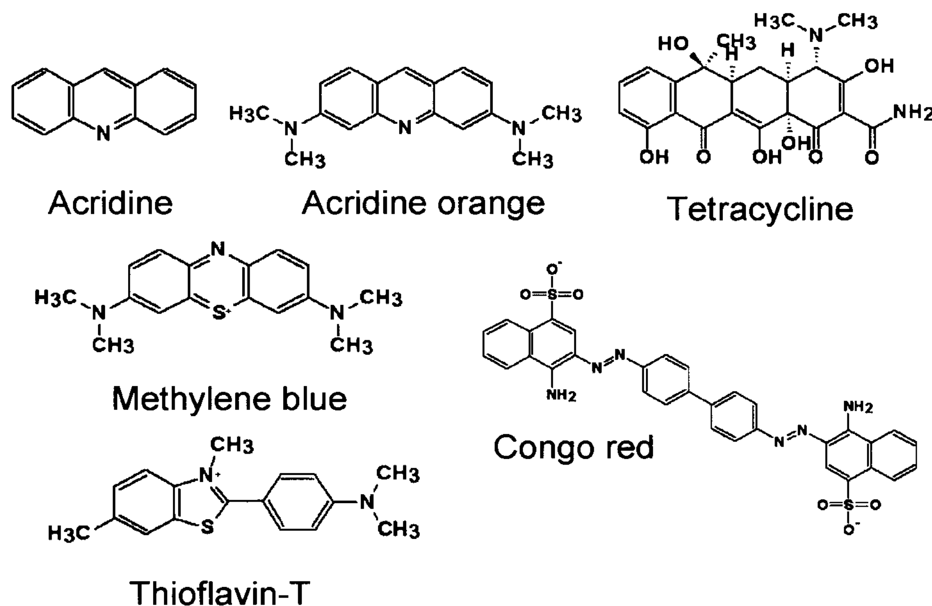

Figure 2 Compound structures

Acridine, Acridine Orange and Methylene Blue are selected examples of fused tricyclic ring compounds. Tetracycline is a fused four-ring compound, whereas Congo Red comprises a combination of two paired fused rings with intervening biphenyl structures. Thioflavin-T is widely used in fluorescent assays to measure amylin fibril formation.

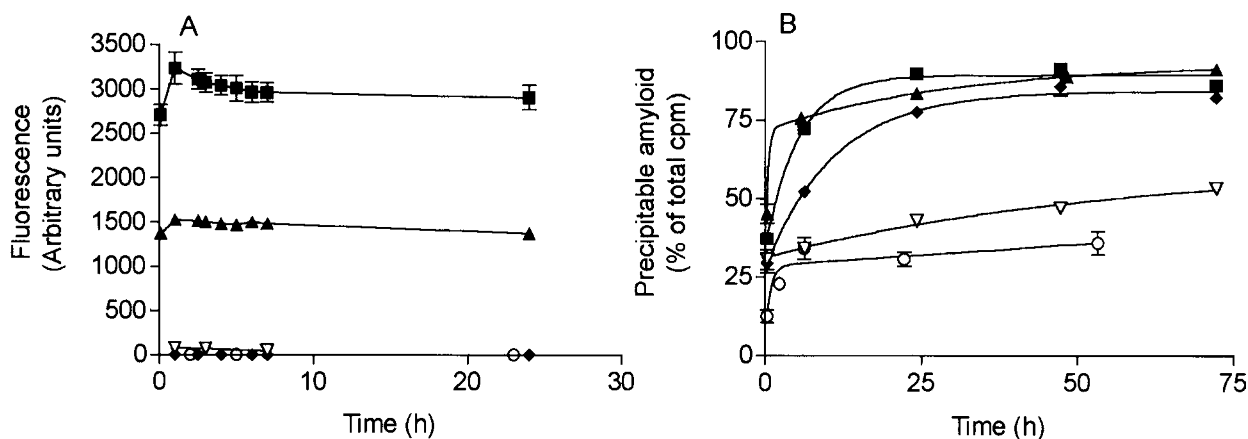

Figure 3 Effects of polycyclic compounds on amyloid formation

(**A**) Thioflavin-T fluorescence following incubation of hA (60 μM) and thioflavin-T (10 μM) in the absence (■) or presence of 1200 μM of either acridine (▲), Acridine Orange (▽), Congo Red (○) or Methylene Blue (◆). Results are means ± S.E.M. ($n = 3$) (**B**) Time-dependent incorporation of radioactivity into the insoluble pellet of an incubation mixture containing 10 μM hA with added [^{3}H]hA in the absence (■) or presence of 200 μM acridine (▲), Acridine Orange (▽), Congo Red (○), or Methylene Blue (◆). Results are shown as the percentage of precipitable radioactive amyloid, relative to total radioactivity in the supernatant at each time point, and are means ± S.E.M. ($n = 3$).

fibrils, thioflavin-T showed a marked increase in fluorescence that could be quantified. However, with either rA or the three-proline-substituted hA structure [($Pro_3^{25,28,29}$)hA], both of which are non-fibrillogenic, there were no increases in fluorescence, showing that the effects are amyloid-specific (Figure 1A). For the radioprecipitation assays (Figure 1B), insoluble amyloid was removed from the solution by centrifugation, and the amount of radioactivity in the precipitable amyloid was used as a measure of amyloid content. rA solutions containing tritiated rA as the tracer molecule, which is not amyloid-forming, showed no significant precipitable radioactivity over the time course of the experiments. Furthermore, a 20-fold molar excess of thioflavin-T had no effect on the rate of amyloid formation by hA. Tritiated hA (10 μM) retained the ability to form amyloid structures with associated cytotoxicity to RINm5F cells, showing that the tritiated structure behaved identically with non-modified hA (results not shown). Both radioprecipitation and thioflavin-T fluorescence enhancement assays were validated further on separate commercial batches of hA that exhibited varying rates of fibril formation (results not shown).

Several polycyclic compounds were investigated for potential effects on amyloid formation (Figure 2). Congo Red is a conjugated biphenyl structure that is used routinely as a diagnostic non-specific amyloid stain in histopathology [15]. It was reported to inhibit neurotoxicity of two fibrillar β-amyloid peptides in primary rat hippocampal cultures, either by inhibiting fibril formation and/or binding to preformed fibrils [16]. There is also a report that 5 days of *in vitro* incubation of β-amyloid-(1–42) with

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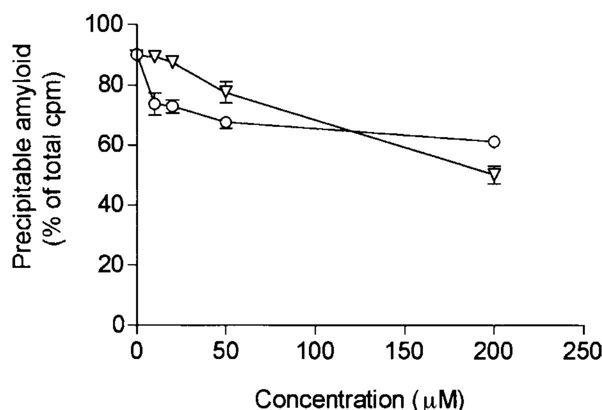

Figure 4 Concentration-dependent effects of Congo Red and Acridine Orange on precipitable amyloid content

Incorporation of radioactivity after 24 h into the insoluble pellets of incubation mixtures comprising 10 μM hA with added [^{3}H]hA in the presence of various concentrations of Congo Red (○) or Acridine Orange (▽) is shown. Results are means ± S.E.M. from at least 4–6 separate determinations from two independent experiments.

tetracycline inhibited fibril formation by this Alzheimer's peptide, as determined by electron microscopy [17]. Acridine and Acridine Orange are examples of core and derivatized tricyclic structures respectively, whereas Methylene Blue contains a phenothiazine core structure.

Of these compounds, incubation of hA with a 20-fold molar excess of Acridine Orange, Methylene Blue or Congo Red completely inhibited thioflavin-T-enhanced fluorescence, compared with an immediate and sustained increase in fluorescence when hA was incubated with thioflavin-T alone (Figure 3A). Acridine, which contains only the parental tricyclic structure, by contrast showed a moderate reduction in thioflavin-T-enhanced fluorescence. Further measurements showed that these relative reductions in fluorescence effects were not due to shifts in the emission spectrum of thioflavin-T, demonstrating direct interactions with hA amyloid structures.

Precipitation experiments showed clearly that the inhibitory effects on thioflavin-T fluorescence by acridine and Methylene Blue were not due to inhibition of amyloid formation (Figure 3B). Conversely, a 20-fold molar excess of Congo Red resulted in a significant and rapid 3-fold reduction in amyloid content after 5 h that was sustained over the incubation period. Comparable reductions in amyloid content after 24 h also occurred with lower concentrations of Congo Red, down to 1:1 molar ratios (Figure 4). In addition to Congo Red, we found that incubation of hA with a 20-fold molar excess of the tricyclic compound, Acridine Orange, resulted in an immediate and sustained reduction in amyloid content by approx. 25 % after 72 h (Figure 3B). However, unlike Congo Red, inhibition in the rate of amyloid formation was concentration dependent (Figure 4) with little effect at 1:1 molar ratios.

In contrast with these interactions seen with Congo Red and Acridine Orange, the behaviour of tetracycline was different in that its effects were relatively long-acting (Figure 5). A 20-fold molar excess of tetracycline resulted in only a gradual decrease in thioflavin-T fluorescence with a half-life of 3.4 h (Figure 5A). Parallel effects on amyloid content did not match this decrease. Instead, a significant reduction in amyloid content of approx. 25 % only occurred after a sustained 50 h incubation period (Figure 5B). Transmission electron microscopy of hA incubated with tetracycline for 24 h also revealed a marked change in the morphology of the resulting amyloid fibrils (Figure 6), characterized by short fragmented structures (Figure 6A), compared with the longer, more dense and characteristic amylin fibril appearance of the respective control (Figure 6B). At higher magnification, and in the presence of tetracycline, small globular lightly stained structures were observed together with short fragments of fibrils (Figure 6C). These globular structures were not observed at the higher magnification in the amylin control (Figure 6D). Consistent with the observed interactions in the other two assay systems, these globular structures indicate disruption of existing amylin fibrils after incubation with tetracycline. Interestingly, these globular structures were not observed when hA was incubated with Congo Red or Acridine Orange (results not shown).

We next investigated the effects of suppression of amyloid formation on amyloid-induced cytotoxicity in cultured RINm5F β-cells (Figure 7). These experiments were confined to Congo Red, which displayed no intrinsic cytotoxic effects under these experimental conditions. Results showed that incubation of

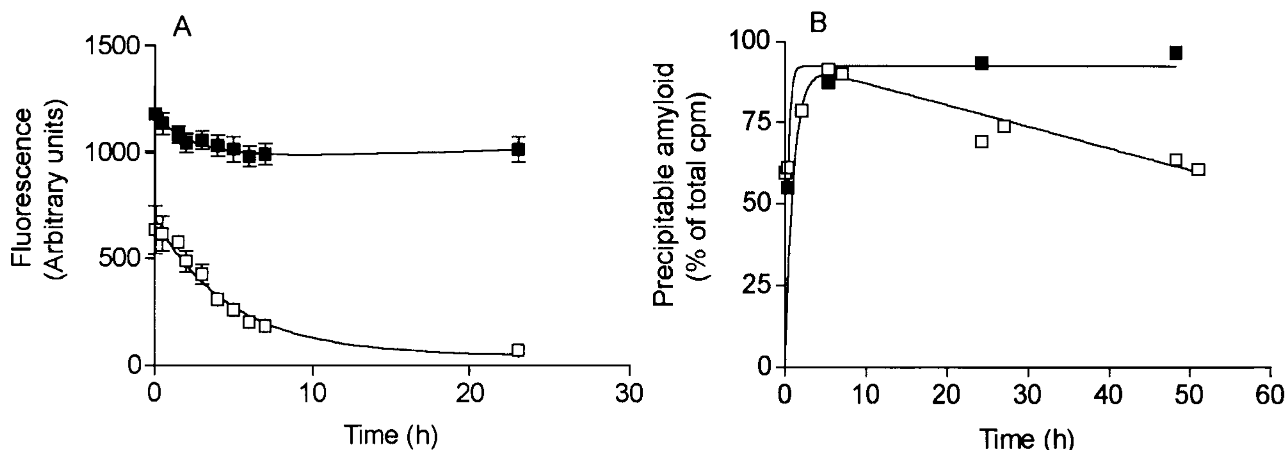

Figure 5 Amyloid interactions with tetracycline

(**A**) Thioflavin-T fluorescence following incubation of hA (60 μM) and thioflavin-T (10 μM) in the absence (■) or presence of 1200 μM tetracycline (□). Results are means ± S.E.M. ($n = 3$). (**B**) Time course of incorporation of radioactivity into the pellet of hA (10 μM) with added [^{3}H]hA incubated in the absence (■) or presence of 200 μM tetracycline (□). Results are means ± S.E.M. ($n = 3$).

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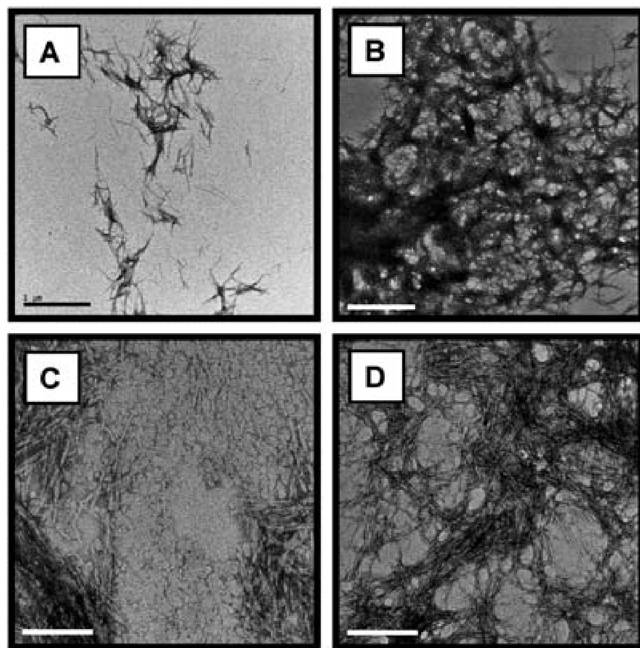

Figure 6 Effects of tetracycline on fibril morphology

hA (60 μM) was incubated in 10 mM Tris, pH 7.4, for 24 h in the presence (**A**) (20 500$\times$ magnification; scale bar, 1 μM) or absence (**B**) (20 500$\times$ magnification; scale bar, 1 μM) of 1200 μM tetracycline. Panels (**C**) and (**D**) are the respective incubations at 105 000$\times$ magnification (scale bar, 200 nm). All experiments were performed in triplicate and the images shown are representative of at least six photomicrographs taken at each magnification.

RINm5F cells with 28 μM hA for 22 h resulted in a significant increase in cell death compared with the vehicle control (Figure 7). In contrast, rA preparations under identical conditions were not cytotoxic. Co-incubation of hA with a 3.4-fold molar excess of Congo Red inhibited the cytotoxic effects of amylin (Figure 7).

The results shown are representative of a series of independent experiments that were performed using different commercial hA batches. Across these preparations, we noted significant variability in associated cytotoxicities that ranged from 7 to 45 % cell death. There is evidence that mature amylin fibril preparations are less cytotoxic than less mature intermediate-sized amylin aggregates [18]. Although the hA preparations used in the present study were freshly dissolved before addition to cultured β-cells, these observed variations in cytotoxicity may reflect different relative contents of these amylin aggregates.

DISCUSSION

Islet amyloidosis is regarded as one of a class of otherwise unrelated amyloid-associated states that occur in several diseases such as Alzheimer's disease [19], the prion encephalopathies [20] and other amyloidoses [21]. There are no primary structure similarities between the respective amyloid-forming proteins implicated in these states; however, they all include extended β-sheet fibrillar structures. In the case of islet amyloid, the positive correlation between amyloid deposition and the onset of diabetic symptoms makes targeting diabetes by the use of compounds that inhibit onset of amyloid formation an intriguing possibility. To date, studies have been confined to investigation of short amylin peptides from within the amyloid-forming region encompassing residues 20–29 [22]. In a further report, synthetic double-N-methylated five-, six-, eight- and ten-amino-acid-long

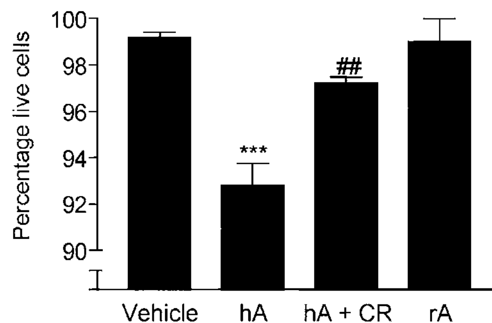

Figure 7 Protective effects of Congo Red against hA-mediated cytotoxicity in cultured RINm5F cells

The percentage of live cells was determined for cells treated for 22 h with vehicle, 28 μM hA, 28 μM hA in the presence of 94 μM Congo Red (hA + CR) or 30 μM rA. Experiments were repeated independently four times. Results are means $\pm$ S.E.M. of live and dead cell counts from at least six fields per condition. Statistical significance was tested by one-way ANOVA followed by post-hoc analysis using Tukey's Test. $^{***}P < 0.001$ compared with vehicle, $^{\#\#}P < 0.01$ compared with hA.

peptide analogues within this region were said to be devoid of β-sheet structure, amyloidogenicity and cytotoxicity, and to inhibit amyloid formation of their non-methylated peptide counterparts *in vitro* [23]. Two peptides, Ser-Asn-Asn-Phe-Gly-Ala and Gly-Ala-Ile-Leu-Ser-Ser-Thr, were reported to inhibit amyloid aggregation of the full-length molecule with associated decrements in cytotoxicity to cultured RIN-1056 cells [24].

In the present study, we show that polycyclic compounds can also modulate amyloid formation. Some of these were not only able to bind to amylin oligomeric forms, but were also able to inhibit the rate of amyloid formation. Thus incubation with 1:1 or greater molar excesses of Congo Red led to immediate and sustained significant reductions in amyloid content. These findings are in contrast with those of Lorenzo and Yankner [16], who found no effects of Congo Red on hA fibril formation after a 24 h incubation in PBS. This observation was based on a single time-point measurement comprising centrifugation and analysis of insoluble and soluble peptide fractions by SDS/PAGE [16]. The reason(s) for the discrepancy between these results [16] and those of the present study is not clear, but could be related to the different experimental conditions used by Lorenzo and Yankner [16]; for example, the buffer system used for hA incubations. Also, the inability to detect soluble hA peptide in the presence of Congo Red by SDS/PAGE may reflect a limitation of this detection method to quantitatively discriminate between any relative reductions in insoluble amyloid content.

In addition to Congo Red, similar decreases in the rate of amyloid formation were observed with Acridine Orange, although these effects were dose-dependent with little inhibition at 1:1 molar ratios. In contrast, acridine and Methylene Blue were able to bind to hA oligomeric forms, but with no effects on amyloid content even at 20-fold molar excesses. Incubation of hA preparations with a 20-fold molar excess of tetracycline also resulted in an eventual reduction in amyloid content and associated morphological changes in fibril structure. Although the precise molecular mechanisms underlying these interactions await confirmation, the marked differences between these compounds point towards the existence of distinct structural factors that enable binding to amylin oligomeric structures and subsequent suppression of insoluble amyloid formation. For example, an aromatic ring structure of a polycyclic compound could confer binding through aromatic π–π interactions [25], whereas the chemical nature of the ring structure and the stereochemistry of

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side-chain groups could enable interactions that destabilize the formation of mature insoluble amyloid.

Also, unlike other amyloidoses, including Alzheimer's β-amyloid and the prion protein, PrP^c, where α-helix/β-strand-discordant stretches appear to be associated with amyloid formation [26], amyloid formation in the case of amylin may instead proceed via a pathway involving the aggregation of relatively unfolded amyloid-forming regions [10]. Although there is uncertainty over the precise identities of the folding assemblies involved, these aggregates lead to the formation of protofibrils composed of extended β-sheet structures with β-strand orientations perpendicular to the longitudinal axes. Of interest is the amyloidogenic region defined by hA residues 20–29, which includes the sequence Asn-Phe-Gly-Ala-Ile-Leu [22]. Substitutions within this region with proline residues at positions 25, 28 and 29 are sufficient to substantially decrease amyloid formation by the intact molecule. It is possible that the decrease in amyloid content evoked by Congo Red, Acridine Orange and tetracycline is attributable to disruptive interactions either within or between protofibrils at such amyloid-forming regions. Further studies of these and other compounds with intact amylin and amyloid-forming fragments of amylin will be of value.

The identification of polycyclic compounds that can affect fibril morphology and/or amyloid content provides an alternative strategy to peptide-based approaches [22–24] to decrease the impact of islet amyloid on pancreatic β-cell function. At the present time, experiments on the *in vivo* modulation of *de novo* amyloid formation or disruption of existing islet amyloid deposits are being planned. Although the molecular identities of the cytotoxic species have not been established beyond doubt, they are believed to comprise precursor amyloid conformers as opposed to mature amyloid fibrils [18]. With these considerations in mind, the present study nevertheless shows that hA preparations incubated with 1:1 or greater molar excesses of Congo Red contained less precipitable amyloid content and were less cytotoxic to cultured islet β-cells compared with incubation with hA alone. Consequently, modulation of amyloid formation may not necessarily be cytotoxic and may even be cytoprotective. Also, even subtle inhibitory effects on islet amyloid formation, *in vivo*, may be sufficient for endogenous clearance mechanisms to predominate and facilitate amyloid removal. The existence of such clearance mechanisms could account for the absence of islet amyloid and diabetic symptoms observed in some hA transgenic mouse lines [27].

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