Rifampicin Does Not Prevent Amyloid Fibril Formation by Human Islet Amyloid Polypeptide but Does Inhibit Fibril Thioflavin-T Interactions: Implications for Mechanistic Studies of β-Cell Death[†]

Fanling Meng,[‡] Peter Marek,[‡] Kathryn J. Potter,[§] C. Bruce Verchere,[§] and Daniel P. Raleigh*[,‡,||]

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-3400, Department of Pathology and Laboratory Medicine and Child and Family Research Institute, University of British Columbia, Vancouver, BC, Canada V5Z 4H4, and Graduate Program in Biochemistry and Structural Biology and Graduate Program in Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11794-3400

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ABSTRACT: Amyloid formation has been implicated in more than 20 different human diseases, including Alzheimer's disease, Parkinson's disease, and type 2 diabetes. The development of inhibitors of amyloid is a topic of considerable interest, both because of their potential therapeutic applications and because they are useful mechanistic probes. Recent studies have highlighted the potential use of rifampicin as an inhibitor of amyloid formation by a variety of polypeptides; however, there are conflicting reports on its ability to inhibit amyloid formation by islet amyloid polypeptide (IAPP). IAPP is the cause of islet amyloid in type 2 diabetes. We show that rifampicin does not prevent amyloid formation by IAPP and does not disaggregate preformed IAPP amyloid fibrils;, instead, it interferes with standard fluorescence-based assays of amyloid formation. Rifampicin is unstable in aqueous solution and is readily oxidized. However, the effects of oxidized and reduced rifampicin are similar, in that neither prevents amyloid formation by IAPP. Furthermore, use of a novel *p*-cyanoPhe analogue of IAPP shows that rifampicin does not significantly affect the kinetics of IAPP amyloid formation. The implications for the development of amyloid inhibitors are discussed as are the implications for studies of the toxicity of islet amyloid. The work also demonstrates the utility of *p*-cyanoPhe IAPP for the screening of inhibitors. The data indicate that rifampicin cannot be used to test the relative toxicity of IAPP fibrils and prefibril aggregates of IAPP.

Amyloid formation plays an important role in approximately 20 different diseases including Alzheimer's disease, Parkinson's disease, and type 2 diabetes (*1, 2*). Human islet amyloid polypeptide (IAPP,[1] also known as amylin) is the major protein component of the pancreatic islet amyloid associated with type 2 diabetes (*3*−*6*). IAPP is a 37-residue polypeptide hormone (Figure 1) which is synthesized in the pancreatic β-cells where it is processed in parallel with insulin, stored in the same secretory granules as insulin, and secreted in response to the same stimuli (*7*−*10*). Synthetic amyloid fibrils formed by IAPP are toxic to β-cells in culture,

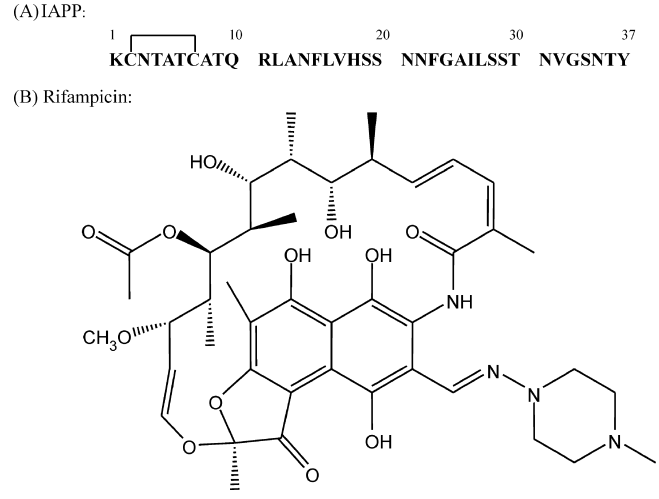

FIGURE 1: (A) The primary sequence of human IAPP. The peptide contains a disulfide bridge between Cys-2 and Cys-7 and has an amidated C-terminus. (B) The structure of the drug rifampicin. The naphthohydroquinone ring can be oxidized to the quinone form in aqueous buffer.

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* Corresponding author. Phone: (631) 632-9547. Fax: (631) 632-7960. E-mail: draleigh@notes.cc.sunysb.edu.

[‡] Department of Chemistry, State University of New York at Stony Brook.

[§] University of British Columbia.

[||] Graduate Programs in Biochemistry and Structural Biology and in Biophysics, State University of New York at Stony Brook.

[1] Abbreviations: DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HFIP, hexafluoro-2-propanol; HOBT, *N*-hydroxybenzotriazole monohydrate; HPLC, high-performance liquid chromatography; IAPP, islet amyloid polypeptide; IAPP $Y37F_{CN}$, human islet amyloid polypeptide with a *p*-cyanoPhe for Tyr-37 substitution; MALDI-TOF MS, matrix-assisted laser desorption ionization−time-of-flight mass spectrometry; PAL-PEG, 5-(4′-Fmoc-aminomethyl-3′,5-dimethoxyphenyl)valeric acid; TEM, transmission electron microscopy; TFA, trifluoroacetic acid.

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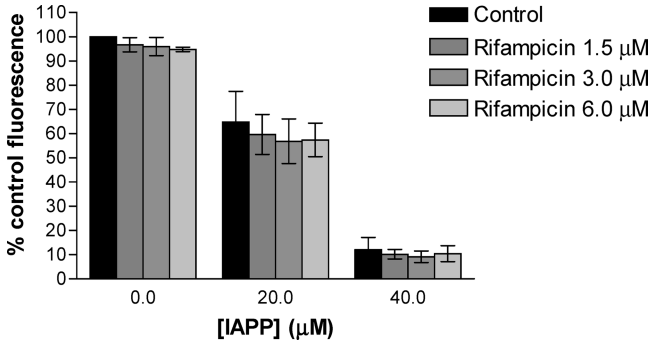

FIGURE 2: Rifampicin does not inhibit the cytotoxicity of human IAPP. Alamar Blue fluorescence assays monitored INS-1 cell viability. Cells were treated for 24 h with human IAPP alone or in the presence of varying concentrations of rifampicin. Bars indicate means ± standard error. There were no significant differences between control and rifampicin-treated cells at any concentration in the presence of 0, 20, and 40 μM human IAPP.

suggesting that amyloid could contribute to the loss of β-cell mass associated with type 2 diabetes (*11*−*15*). Furthermore, the level of amyloid correlates with the progression of the disease, suggesting a relationship between islet amyloid formation and β-cell loss (*15*).

There is considerable interest in developing inhibitors of amyloid formation, both because of their obvious therapeutic potential and also because they can provide powerful tools for mechanistic studies (*16*−*21*). In particular, there is a lively debate on what constitutes the toxic species in the amyloid diseases. Early work focused on the potential toxicity of the fibrils themselves, and fibril deposits certainly contribute to the progression of the systemic amyloidoses (*22*). In recent years, however, considerable attention has focused on oligomeric intermediate species and their potential toxicity, although there is also evidence that fibrils can be toxic (*23*−*31*). Molecules which inhibit fibril formation but not the formation of intermediates are attractive mechanistic probes since they will inhibit toxicity if fibrils are the only toxic entity but will not inhibit toxicity if intermediates are the key player (*23, 32*).

Epidemiological investigations have shown that leprosy patients have a statistically lower probability of senile dementia provided they have been treated with rifampicin or dapsone, suggesting that the drug might prevent Aβ amyloid formation (*33, 34*). *In vitro* studies showed that rifampicin did indeed inhibit fibril formation by Aβ_{1-40} and

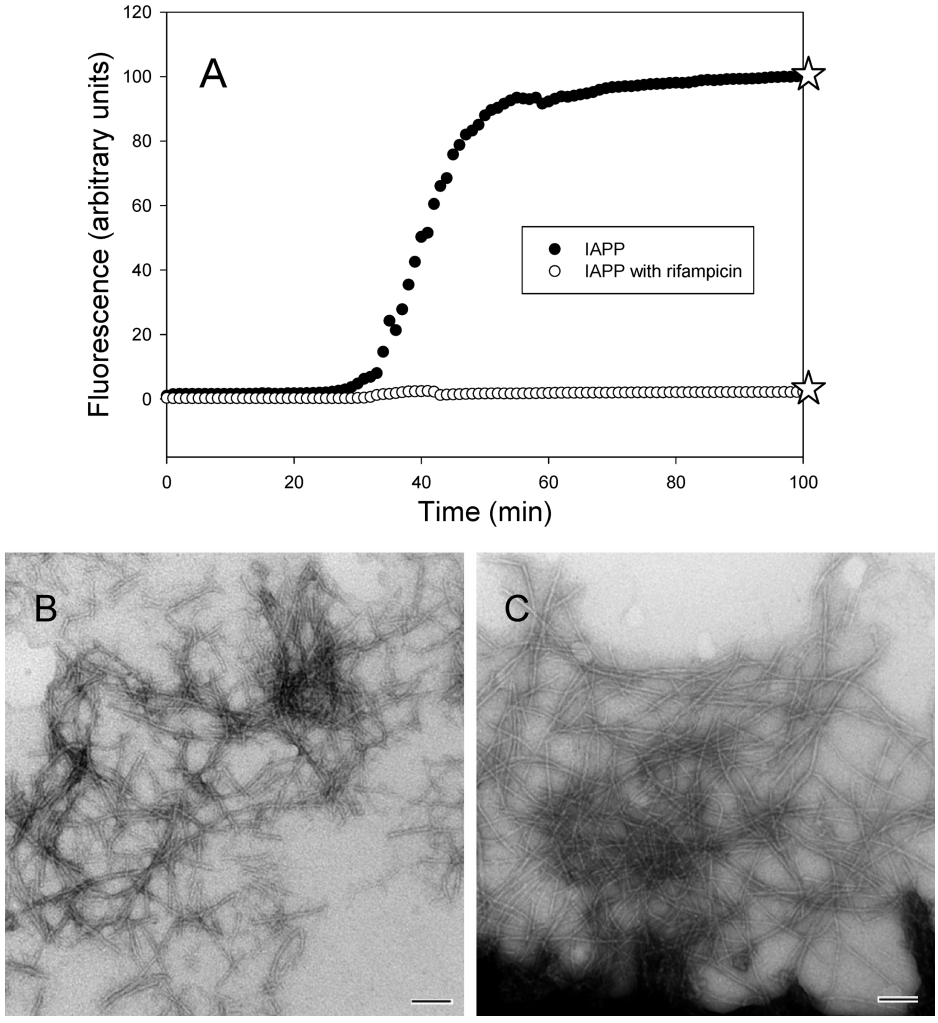

FIGURE 3: Rifampicin does not prevent amyloid formation by human IAPP. (A) Thioflavin-T fluorescence monitored assays of fibril formation. The closed circles (●) represent the experiment conducted in the absence of rifampicin. Open circles (○) correspond to an experiment conducted in the presence of 15 μM rifampicin. The stars (☆) indicate the time points at which aliquots were removed for TEM. (B) TEM images of the fibrillization reaction product for IAPP without rifampicin. (C) TEM image of a sample of 32 μM peptide and 15 μM rifampicin collected at 100 min after the start of the fibrillization reaction. Reactions were conducted at 25 °C, pH 7.4, 32 μM IAPP, 25 μM thioflavin-T in 2% HFIP. The scale bar is 100 nm.

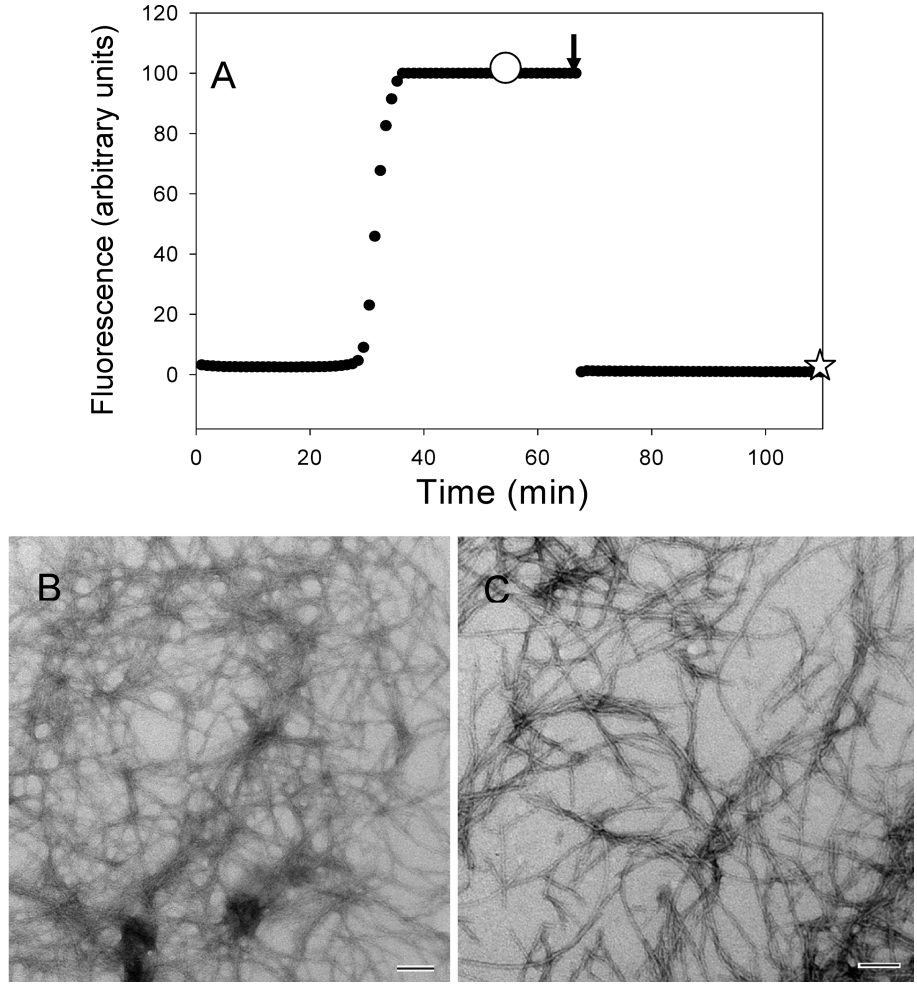

FIGURE 4: Effects of adding rifampicin to human IAPP fibrils. (A) Thioflavin-T fluorescence monitored time course of IAPP fibril formation. Rifampicin was added at the point indicated by the arrow (↓). (B) TEM image recorded before the drug was added at a time indicated by the (◯). (C) TEM image recorded 35 min after addition of the drug, indicated by the (☆). All samples were 32 μM IAPP, 25 μM thioflavin-T in 2% HFIP, 25 °C, pH 7.4. The scale bar is 100 nm.

reduced its toxicity to cultured rat PCL2 cells (*35*). Rifampicin has been reported to inhibit *in vitro* amyloid formation by a number of proteins (*32, 35*−*41*). More recently, rifampicin has been used to probe the mechanism of IAPP-induced cell death (*32, 42*). However, the IAPP literature is confusing since some reports argue that rifampicin is an inhibitor of fibril formation and does not inhibit toxicity (*32*) while others claim that it does not inhibit amyloid formation but inhibits the toxicity of preaggregated IAPP fibrils (*41*). Rifampicin has also been reported to inhibit the membrane disrupting activity of IAPP but not its ability to form amyloid (*43*). Further complicating the issue, Fink and colleagues, in a series of careful studies, showed that rifampicin inhibits fibrillization of α-synuclein but found that an oxidation product of the drug is actually the most potent inhibitory compound (*39*). The conflicting reports on the effects of rifampicin on IAPP toxicity and fibril formation and the importance of these studies for understanding the origin of cellular toxicity prompted us to reexamine the effects of the drug on the *in vitro* fibrillization of IAPP using transmission electron microscopy (TEM) and fluorescence-detected thioflavin-T binding assays as well as newly developed fluorescent analogues of IAPP. We show that rifampicin does not prevent amyloid fibril formation by IAPP and does not disaggregate preformed IAPP amyloid but does interfere with thioflavin-T assays.

EXPERIMENTAL PROCEDURES

Reagents. Rifampicin was purchased from Sigma (lot no. R3501), ascorbic acid from Fisher Scientific, and thioflavin-T from Aldrich Chemical Co.

Peptide Synthesis and Purification. Peptides were synthesized on a 0.25 mmol scale using an Applied Biosystems 433A peptide synthesizer, using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry as described (*44*). Pseudoprolines were incorporated to facilitate the synthesis. The 5-(4′-Fmoc-aminomethyl-3′,5-dimethoxyphenol)valeric acid (PAL-PEG) resin was used to afford an amidated C-terminal. Standard Fmoc reaction cycles were used. The first residue attached to the resin, β-branched residues, residues directly following β-branched residues, and pseudoprolines were double coupled. Crude peptides were oxidized by dimethyl sulfoxide (DMSO) for 24 h at room temperature (*45*). The peptides were purified by reverse-phase HPLC using a Vydac C18 preparative column. Analytical HPLC was used to check the purity of the peptides before each experiment. The identity of the pure

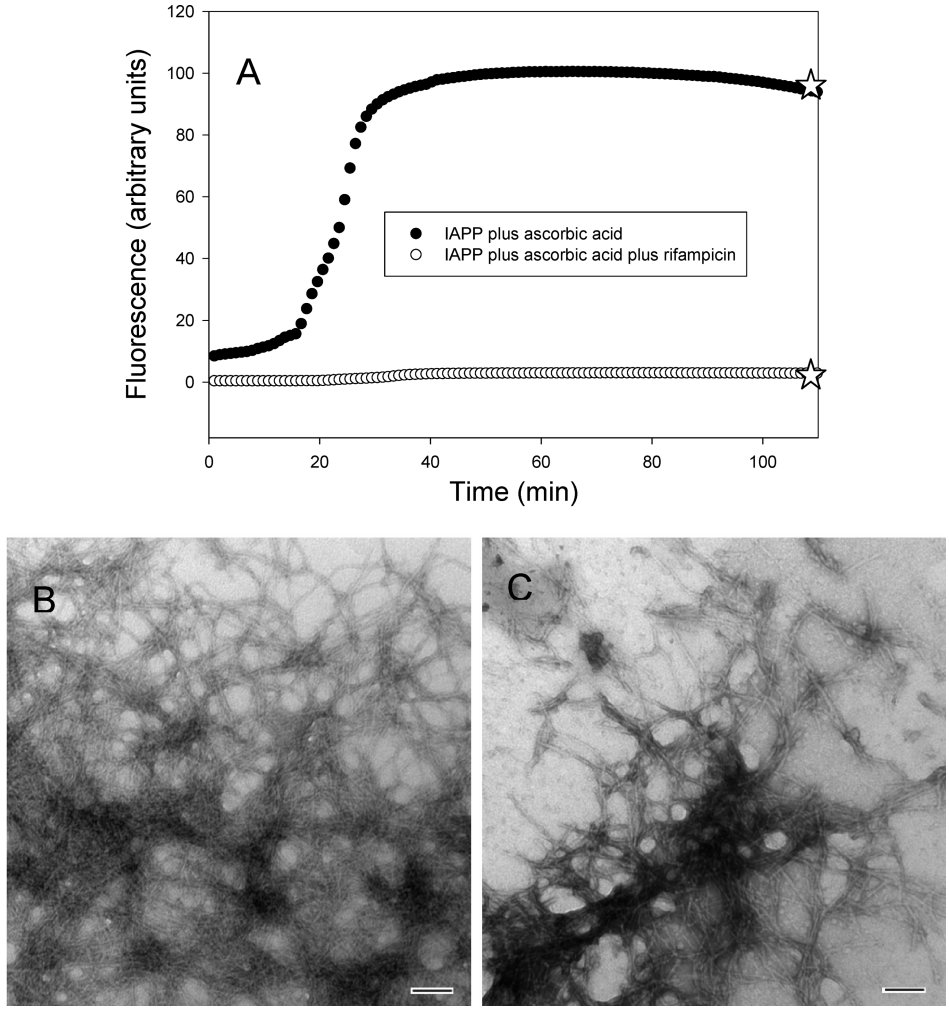

FIGURE 5: Rifampicin fails to prevent amyloid formation by human IAPP in the presence of antioxidants. (A) Thioflavin-T fluorescence monitored assays of fibril formation. The closed circles (●) represent the experiment conducted in the absence of rifampicin. Open circles (○) correspond to an experiment conducted in the presence of 15 μM rifampicin. The stars (☆) indicated the time points at which aliquots were removed for TEM. (B) TEM images of the product of the fibrillization reaction for IAPP without rifampicin. (C) TEM image of a sample of 32 μM peptide and 15 μM rifampicin collected at 110 min after the start of the fibrillization reaction. Reactions were conducted at 25 °C, pH 7.4, 32 μM IAPP, 83 mM ascorbic acid, 25 μM thioflavin-T in 2% HFIP. The scale bar is 100 nm.

peptides was confirmed by mass spectrometry using a Bruker MALDI-TOF MS.

Sample Preparation. A 1.58 mM peptide solution was prepared in 100% hexafluoro-2-propanol (HFIP) or DMSO and stored at −20 °C. A 20 mM rifampicin stock solution was prepared by dissolving rifampicin in DMSO. For the antioxidant experiments, ascorbic acid was dissolved in Tris-HCl buffer and adjusted to pH 7.4 to give a 100 mM ascorbic acid stock solution. The stability of rifampicin in Tris-HCl buffer and ascorbic acid solutions was tested by UV−vis absorbance at 483 nm (*46, 47*).

Thioflavin-T Fluorescence. All fluorescence experiments were performed with a Jobin Yvon Horiba fluorescence spectrophotometer or with an Applied Phototechnology fluorescence spectrophotometer. An excitation wavelength of 450 nm and emission wavelength of 485 nm were used for the thioflavin-T studies. The excitation and emission slits were set at 5 nm. A 1.0 cm cuvette was used, and each point was averaged for 1 min. All solutions for these studies were prepared by diluting filtered stock solution (0.45 μm filter) into a Tris-HCl-buffered (20 mM, pH 7.4) thioflavin-T solution immediately before the measurement. The final concentration was 32 μM peptide and 25 μM thioflavin-T with or without rifampicin in 2% HFIP. Some experiments made use of DMSO stock solutions. For these studies, the final conditions were 32 μM peptide and 25 μM thioflavin-T in 20 mM Tris-HCl buffer (pH 7.4) and 2% DMSO. The final concentration of ascorbic acid used in antioxidant experiments was 83 μM. All solutions were stirred during the fluorescence experiments. *p*-CyanoPhe fluorescence was excited at 240 nm and detected at 296 nm, with both excitation and emission slits of 10 nm.

Transmission Electron Microscopy (TEM). TEM was performed at the Life Science Microscopy Center at the State University of New York at Stony Brook. The same solutions that were used for the fluorescence measurements were used so that samples could be compared under as similar conditions as possible. Fifteen microliters of peptide solution was placed on a carbon-coated Formvar 300 mesh copper grid for 1 min and then negatively stained with saturated uranyl acetate for 1 min.

Alamar Blue Viability Assay. Alamar Blue (Biosource, Camarillo, CA) reduction was used as a measure of cell viability. *p* values were determined using one-way ANOVA, and significant differences ($p < 0.05$) were determined using Tukey's multiple comparison post hoc test. Rat (INS-1)

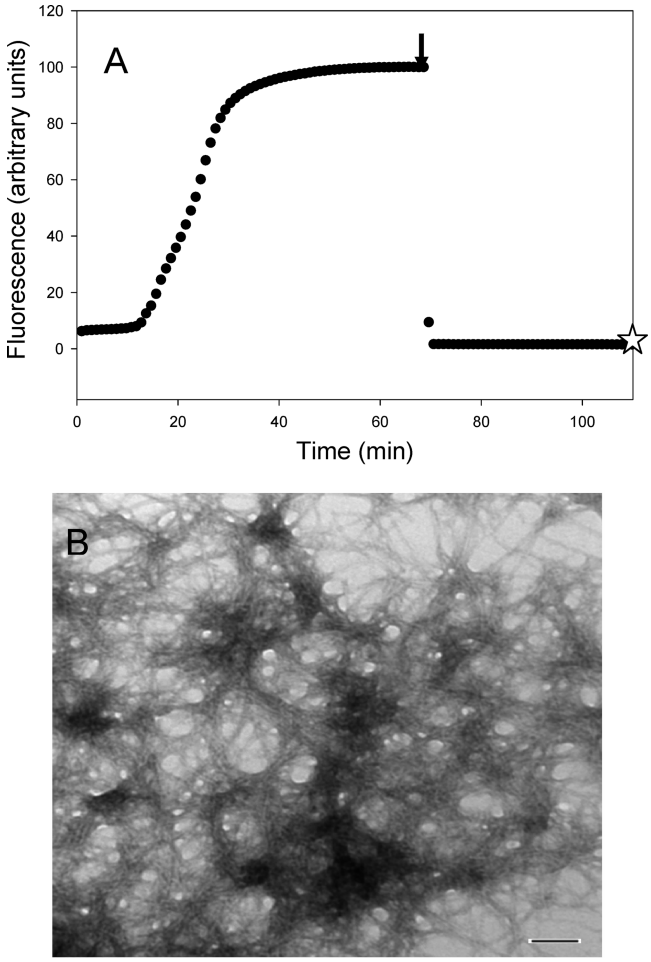

FIGURE 6: Effects of adding rifampicin to human IAPP fibrils in the presence of antioxidant. (A) Thioflavin-T fluorescence monitored time course of human IAPP fibril formation. Rifampicin was added at the point indicated by the arrow (↓). (B) TEM image recorded 35 min after addition of the drug, indicated by the (☆). All samples were 32 μM IAPP, 83 mM ascorbic acid, 25 μM thioflavin-T in 2% HFIP, 25 °C, pH 7.4. The scale bar is 100 nm.

insulinoma cells were seeded at a density of 20000 cells per well. After 24 h culture in RPMI (11 mM glucose) plus 10% fetal bovine serum, HEPES (0.5 M), L-glutamine (102 mM), sodium pyruvate (50 mM), β-mercaptoethanol (50 μM), penicillin (50 units/mL), and streptomycin (50 μg/mL), culture media were removed and replaced with fresh media and the appropriate amount of rifampicin, freshly dissolved human IAPP (Bachem, Torrance, CA), and/or DMSO vehicle. The final DMSO concentration did not exceed 0.1%, and viability was not reduced in control cells treated with 0.1% DMSO. After 24 h incubation, media were replaced with fresh media containing 10% Alamar Blue and incubated for 3 h. Fluorescence was measured using a Fluoroskan Ascent microplate reader (Labsystems; Fischer Scientific, Pittsburgh, PA) using excitation and emission wavelengths of 530 and 590 nm, respectively.

RESULTS AND DISCUSSION

Rifampicin Does Not Inhibit the Cytotoxicity of IAPP. There are apparently conflicting reports on the effects of rifampicin on IAPP-induced toxicity; however, the discrepancy may be due to the different nature of the studies. Tomiyama and colleagues (*41*) showed that rifampicin inhibited the toxicity of preformed fibril aggregates of IAPP while Meier and co-workers (*32*) examined the effects of treating cells with initially soluble IAPP and rifampicin. The second study demonstrated that rifampicin had no effects on toxicity with this protocol. Thus the two studies, while seemingly at variance, need not be contradictory since different conditions were examined. Given the importance of the toxicity results, we reexamined the effect of added rifampicin on IAPP-induced cytotoxicity.

INS-1 Cell Viability Assays. Treatment of INS-1 cells for 24 h with 20 or 40 μM human IAPP reduced cell viability by 35.2% and 89.7%, respectively ($p < 0.05$ and $p < 0.001$, respectively; Figure 2). Rifampicin alone (1.5−6.0 μM) had no impact on cell viability (Figure 2), nor did treatment of control cells with the maximal concentration of 0.1% DMSO vehicle (data not shown). Addition of rifampicin to media containing human IAPP failed to protect INS-1 cells from human IAPP-induced death. Even at the maximal concentration of rifampicin used (6.0 μM), cell viability in the presence of human IAPP was not significantly different between control cells and cells treated with rifampicin (for 20 μM human IAPP, 64.8 ± 12.6% versus 57.4 ± 7.0%, p = NS; for 40 μM human IAPP, 12.1 ± 5.0% versus 10.4 ± 3.3%, p = NS). These results agree with the study of Meier and co-workers (*32*).

Rifampicin Interferes with Thioflavin-T Assays but Does Not Prevent Amyloid Formation by IAPP. We monitored the apparent time course of fibril formation of IAPP in the presence and in the absence of rifampicin using thioflavin-T assays. Thioflavin-T is a small dye molecule which has proven enormously useful in studies of amyloid formation. Thioflavin-T has a low fluorescence quantum yield in solution which increases significantly when bound to fibrils (*48*). There is no structure of thioflavin-T bound to any amyloid fibrils, but the dye is believed to bind to grooves on the surface of amyloid fibrils. Amyloid is made up of a cross-β structure in which individual β-strands are aligned perpendicular to the fibril axis. In such a structure, side chains in consecutive strands will form a ridge and a set of side chains at positions n and $n + 2$ will lead to two ridges separated by a groove. These grooves are the likely binding sites.

Samples were 32 μM in IAPP and contained either no rifampicin or 15 μM rifampicin. The ratio of drug to IAPP is higher than that reported to inhibit IAPP fibrillization (*32*). The data collected in the absence of rifampicin is typical of IAPP fibrillization experiments. A lag phase is observed followed by a growth phase with a rapid change in thioflavin-T fluorescence leading to a final plateau where the bound thioflavin-T fluorescence reaches a steady-state value (Figure 3). TEM images collected of samples corresponding to the end point of the reaction display the classic features of amyloid (Figure 3). The results of the experiment in the presence of rifampicin are strikingly different. No significant change in thioflavin-T fluorescence is observed over the entire time course of the reaction. Taken alone, the thioflavin-T fluorescence experiment would argue that rifampicin is a potent inhibitor of fibrillization. However, the drug could instead be a competitive inhibitor of thioflavin-T binding to IAPP fibrils, or it might quench the fluorescence of the bound thioflavin-T without preventing amyloid formation. Conse-

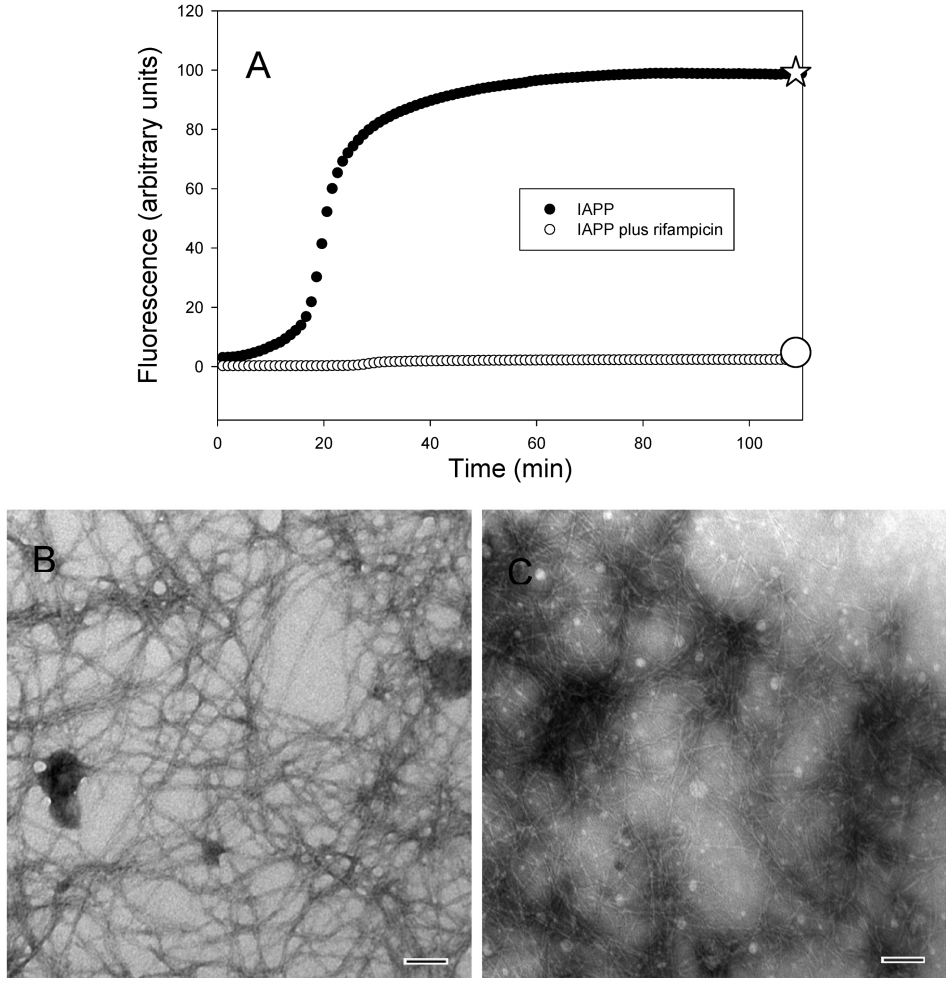

FIGURE 7: (A) Thioflavin-T fluorescence monitored time course of human IAPP fibril formation with or without rifampicin. The closed circles (●) represent the experiment conducted in the absence of rifampicin. Open circles (○) correspond to an experiment conducted in the presence of 15 μM rifampicin. Rifampicin in Tris-HCl buffer, pH 7.4, was incubated for 35 days at room temperature before the start of the experiment. All samples were 32 μM IAPP, 25 μM thioflavin-T in 2% HFIP, 25 °C, pH 7.4. (B) TEM image of a sample collected at the indicated time point (☆) for a sample of IAPP without rifampicin. (C) TEM image of a sample collected at the indicated time point (○) for a sample of IAPP plus rifampicin. The scale bar is 100 nm.

quently, we recorded TEM images of aliquots of each reaction mixture collected at a time point corresponding to 100 min after initiation of the reaction. This is much longer than the time required for IAPP to form amyloid fibrils. The images reveal numerous fibrils with the classic morphology associated with *in vitro* IAPP fibrils for both samples, indicating that rifampicin did not prevent amyloid formation by IAPP. Not all of the grids displayed fibrils, and qualitatively they appeared somewhat less abundant for the samples which contain rifampicin than for the samples of IAPP in the absence of rifampicin. Nonetheless, the key conclusion is that rifampicin, when present at the start of the fibrillization reaction, leads to false negatives in the thioflavin-T assay. We repeated the experiments at lower rifampicin concentration, examining solutions which were 5 or 2.5 μM rifampicin. The final fluorescence intensity is higher than observed in the 15 μM rifampicin experiment but is considerably lower than the value observed in the absence of rifampicin (Supporting Information). The final thioflavin-T fluorescence intensity is often used as a measurement of the amount of amyloid formation, and by this measure the drug would still be classified as a fibrillization inhibitor. However, TEM images recorded at the end of the reaction show that extensive fibrils are formed (Supporting Information). Thus the conclusion that rifampicin inhibits thioflavin-T fluorescence but not amyloid formation is independent of the range of rifampicin tested.

All of these experiments were conducted using stock solutions prepared in HFIP which is the standard protocol for biophysical studies of IAPP fibril formation. We tested the result of initially solubilizing IAPP in DMSO since DMSO stock solutions are sometimes used in studies of IAPP. An IAPP stock solution was prepared in 100% DMSO and fibrilization initiated by dilution into aqueous buffer (final DMSO concentration 2% by volume). The choice of cosolvent does not affect the conclusions. Rifampicin inhibits thioflavin-T fluorescence but not amyloid formation (Supporting Information). Thus our results are not an artifact of the choice of cosolvent.

We next investigated the effect of adding rifampicin to preformed fibrils. Such an experiment is often performed to test a compound's ability to disaggregate fibrils. In this case, a compound which eliminated the fluorescence of bound thioflavin-T or displaced bound thioflavin-T but did not dissociate fibrils would be incorrectly scored as having the ability to disaggregate fibrils; i.e., it would generate a false

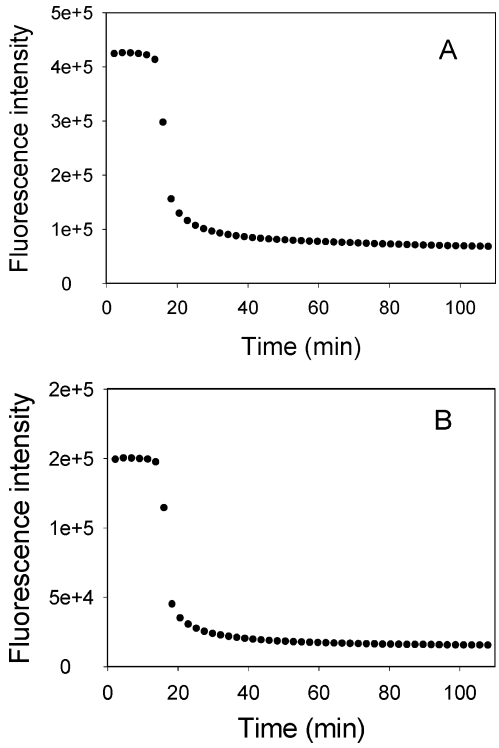

FIGURE 8: *p*-CyanoPhe fluorescence detected kinetics of IAPP $Y37F_{CN}$ in the absence of rifampicin (A) and in the presence of rifampicin (15 μM) (B). All samples were 32 μM peptide, 25 μM thioflavin-T in 2% HFIP, 25 °C, pH 7.4. Excitation was at 240 nm, and the emission was monitored at 296 nm. The difference in the maximum fluorescence intensity is likely due to inner filter effects.

positive. It is possible that rifampicin only interferes with thioflavin-T binding to IAPP if it is present in the initial reaction mixture. This seems unlikely since the dye generally does not bind to species populated in the lag phase (*44*), but it is important to test. Figure 4 displays the result of an experiment in which rifampicin is added to the plateau region of the reaction (indicated by the arrow). Addition of the drug leads to a rapid loss of thioflavin-T fluorescence. TEM images of a sample collected before the drug is added (Figure 4) confirm that abundant fibrils had formed. Strikingly, TEM images collected after the drug was added also display numerous amyloid fibrils (Figure 4). Once again, the drug interferes with the thioflavin-T fluorescence response but does not disaggregate IAPP fibrils.

Rifampicin Does Not Prevent IAPP Amyloid Formation in the Presence of Antioxidants. The naphthohydroquinone ring in rifampicin is easily oxidized to the quinone form, and aqueous solutions of rifampicin are not stable. Under basic conditions they break down to the rifampicin quinone form, and under acidic conditions 3-formylrifampicin is produced (*39, 46, 47*). Fink and co-workers have shown that the oxidation product is a more potent inhibitor of α-synuclein fibril formation (*39*). The experiments described in the previous subsection were conducted in the absence of antioxidants; thus rifampicin will be present as a mixture of the oxidized and reduced form. We conducted our first set of studies under those conditions because they were used in all reported studies of rifampicin−IAPP interactions. We repeated the studies in the presence of the same antioxidant used by Fink and co-workers, ascorbic acid, to test if our results were dependent on the oxidation state of the drug. The breakdown of rifampicin can be easily monitored by following changes in its absorption spectrum. In particular, the intensity at 483 nm is significantly decreased if a sample of rifampicin is incubated in aqueous buffer at physiological pH due to oxidation (*46, 47*). Control experiments showed no significant change in the absorbance of rifampicin at 483 nm for at least 1000 min in the presence of ascorbic acid. In contrast, a steady decrease is observed in the absence of ascorbic acid.

The results are not affected by the presence of the antioxidant; rifampicin still inhibits thioflavin-T fluorescence but does not prevent amyloid formation by IAPP. A sample of IAPP in the presence of 83 μM ascorbic acid yields a typical kinetic curve as monitored by thioflavin-T fluorescence (Figure 5), and TEM confirms that fibrils were formed. Addition of rifampicin to the reaction mixture at time zero leads to a flat curve with no significant change in the thioflavin-T fluorescence. A sample was removed from this reaction mixture after 100 min (Figure 5), and TEM images were recorded. They revealed abundant amyloid fibrils. We also repeated the experiment in which rifampicin was added to preformed fibrils. Again, we observed the same results in the presence and absence of ascorbic acid. Addition of rifampicin eliminated the thioflavin-T fluorescence but did not dissociate fibrils (Figure 6). We also examined the effect of oxidized rifampicin by testing samples which had been preincubated in aqueous solutions under conditions which promote oxidation of the drug. A sample of rifampicin was incubated in aqueous solution for 35 days prior to testing its inhibitory potential. This is, of course, a highly unlikely experimental protocol for any therapeutic studies, but it is useful for testing the properties of breakdown products of the drugs. The material interfered with thioflavin-T fluorescence measurements but failed to prevent amyloid formation although the resulting fibrils appeared to be somewhat thinner than those formed with unoxidized rifampicin (Figure 7).

Use of a Novel IAPP Fluorescent Analogue Allows the Kinetics of Amyloid Formation To Be Monitored in the Presence of Rifampicin. We have recently shown that the intrinsic fluorescence of *p*-cyanophenylalanine (*p*-cyanoPhe) can be used to follow the time course of amyloid formation by IAPP (*49*). *p*-CyanoPhe fluorescence is large in aqueous solution, but the fluorescence is significantly reduced in a hydrophobic environment (*50, 51*). An analogue of IAPP in which the C-terminal tyrosine is replaced by *p*-cyanoPhe (IAPP $Y37F_{CN}$) forms amyloid at the same rate as wild type, and the morphology of the resulting fibrils is identical as judged by TEM. Furthermore, the time course of the change in *p*-cyanoPhe fluorescence describes the same kinetic trace as the thioflavin-T fluorescence; thus *p*-cyanoPhe fluorescence can be used to follow fibrillization of IAPP. Figure 8 compares the time course of the fluorescence of IAPP $Y37F_{CN}$ in the presence and absence of rifampicin. The time courses of fibril formation are very similar in the presence and in the absence of the drug. Quantitative analysis of the data shows that the t_{50} time (the time for the reaction to reach 50% of the maximum fluorescence intensity) is 19 min when rifampicin is present and 18 min when it is absent. The respective growth phases, here defined as the time for the

reaction to go from 10% to 80% completion, are also very similar: 7 min in the presence of the drug and 6 min in its absence.

CONCLUSIONS

The studies described here demonstrate that rifampicin does not prevent amyloid formation by human IAPP; furthermore, the results are not an artifact of the choice of cosolvent or of failing to control the oxidation state of the drug. One important lesson from these studies is that the thioflavin-T based assay can lead to false positives in tests of fibrillization inhibitors. It is impossible to say how general this effect may be, but there are undoubtedly other small molecules which inhibit thioflavin-T binding or compromise its fluorescence response. This is very important since thioflavin-T binding is widely used, particularly for high-throughput screens of fibril inhibitors. The *p*-cyanoPhe analogue of IAPP used here overcomes these difficulties and should be a generally useful tool for the screening of inhibitors. The data presented here have important implications for studies which seek to define the toxic species in IAPP fibrillization by studying the effects of fibril inhibitors on cell death (*42*). In particular, studies which have used rifampicin will need to be reexamined, since the data presented here clearly demonstrate that rifampicin does not prevent amyloid formation by IAPP. Thus the observation of toxicity in the presence of rifampicin does not prove that IAPP fibrils are nontoxic.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

A figure showing the effects of rifampicin on the formation of IAPP fibrils for a reaction in 2% DMSO and two figures showing effects of 5 and 2.5 μM rifampicin on IAPP fibril formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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