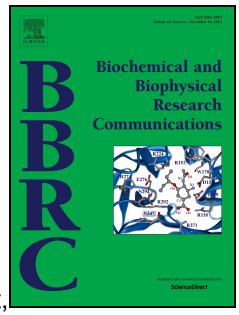
Aitken, J. F., Loomes, K. M., Riba-Garcia, I., Unwin, R. D., Prijic, G., Phillips, A. S., ... Cooper, G. J. S. (2017). Rutin suppresses human-amylin/hIAPP misfolding and oligomer formation in-vitro, and ameliorates diabetes and its impacts in human-amylin/hIAPP transgenic mice. *Biochemical and Biophysical Research Communications*, *482*(4), 625–631. https://doi.org/10.1016/j.bbrc.2016.11.083

Accepted Manuscript



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PII: S0006-291X(16)31946-5

DOI: 10.1016/j.bbrc.2016.11.083

Reference: YBBRC 36787

To appear in: *Biochemical and Biophysical Research Communications*

Received Date: 31 October 2016

Accepted Date: 15 November 2016

Please cite this article as: J.F. Aitken, K.M. Loomes, I. Riba-Garcia, R.D. Unwin, G. Prijic, A.S. Phillips, A.R.J. Phillips, D. Wu, S.D. Poppitt, K. Ding, P.E. Barran, A.W. Dowsey, G.J.S. Cooper, Rutin suppresses human-amylin/hIAPP misfolding and oligomer formation *in-vitro*, and ameliorates diabetes and its impacts in human-amylin/hIAPP transgenic mice, *Biochemical and Biophysical Research Communications* (2016), doi: 10.1016/j.bbrc.2016.11.083.

Rutin suppresses human-amylin/hIAPP misfolding and oligomer formation *in-vitro*, and ameliorates diabetes and its impacts in human-amylin/hIAPP transgenic mice

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ABSTRACT

Pancreatic islet β-cells secrete the hormones insulin and amylin, and defective β-cell function plays a central role in the pathogenesis of type-2 diabetes (T2D). Human amylin (hA, also termed hIAPP) misfolds and forms amyloid aggregates whereas orthologous mouse amylin does neither. Furthermore, hA elicits apoptosis in cultured β-cells and β-cell death in *ex-vivo* islets. In addition, hA-transgenic mice that selectively express hA in their β-cells, manifest β-cell apoptosis and progressive islet damage that leads to diabetes closely resembling that in patients with T2D. Aggregation of hA is thus linked to the causation of diabetes.

We employed time-dependent thioflavin-T spectroscopy and ion-mobility mass spectrometry to screen potential suppressors of hA misfolding for anti-diabetic activity. We identified the dietary flavonol rutin as an inhibitor of hA-misfolding and measured its anti-diabetic efficacy in hA-transgenic mice. *In vitro*, rutin bound hA, suppressed misfolding, disaggregated oligomers and reverted hA-conformation towards the physiological. In hA-transgenic mice, measurements of glucose, fluid-intake, and body-weight showed that rutin-treatment slowed diabetes-progression by lowering of rates of elevation in blood glucose (P=0.030), retarding deterioration from symptomatic diabetes to death (P=0.014) and stabilizing body-weight (P<0.0001).

In conclusion, rutin treatment suppressed hA-aggregation *in vitro* and doubled the lifespan of diabetic mice (P=0.011) by a median of 69 days compared with vehicle-treated control-diabetic hA-transgenic mice.

KEYWORDS: amylin, rutin, ion-mobility mass-spectrometry, transgenic mice, type-2 diabetes

1. Introduction

Diabetes exerts an enormous toll on patients, their families and caregivers, and health-care systems globally [1], and T2D accounts for >90% of all cases [1, 2]. Available therapies have not stemmed the alarming increase in numbers affected and there is agreed need for more effective approaches that can suppress pathogenic mechanisms (disease-modifying treatments) [3, 4].

Human amylin is the main component of islet amyloid in T2DM patients [5, 6]. It is a small protein that can spontaneously misfold and aggregate to form structures that elicit death by apoptosis in islet β-cells, as well as β-cell/islet degeneration and failure of insulin secretion in animal models [7, 8]. Amylin-mediated cytotoxicity is identified as a potential target for developing new anti-diabetic molecules that act by suppressing β-cell death. One example, tetracycline shows anti-diabetic activity linked to its ability to suppress hA-misfolding and aggregation [9]. However, tetracyclines have side-effect profiles that are incompatible with chronic use for the treatment of diabetes, and orally-active molecules suitable for clinical investigation that suppress hA-aggregation have hitherto been lacking.

2. Materials and methods

2.1 Materials

General laboratory chemicals were of analytical grade from Gibco/BRL (Grand Island, NY) or Sigma-Aldrich (St Louis, MO).

2.2 Thioflavin-T (ThT) Assay

Actions of quercetin and rutin on amylin aggregation were measured by fluorescence spectroscopy with ThT, according to our established methods [10]. Each experiment was performed in triplicate and repeated at least twice on separate occasions.

2.3 Ion-mobility mass spectrometry

Human amylin (Bachem; H7905): 10 mg was dissolved in 500 µl hexafluoroisopropanol (HFIP) by vortex-mixing as necessary to fully solubilize, and incubated overnight at room temperature in the dark to disaggregate. Samples were then divided into 50 µl aliquots and vacuum-dried (Savant RVT4104; ThermoScientific, Waltham, MA) to yield 1 mg monomeric hA/tube: samples were stored at -20^{o}C until analysis.

For ion-mobility experiments, amylin (1 mg) was dissolved in 50 µl HFIP, and 15 µl aliquots were further diluted to 250 µl in HFIP; samples were then incubated for 1 h at room temperature, and 2- or 5-µl aliquots (according to required final concentration) were re-dried, and stored (-20^{o}C) until analysis.

Amylin was dissolved in 0.1% (w/v) formic acid to yield a final concentration of 50 µM. It was then injected into a Synapt G2 mass spectrometer (Waters, UK) using in-house-fabricated nanospray glass-emitter tips containing an internal-Pt wire to yield suitable ionization conditions using the manufacturer's recommended settings. The capillary voltage, cone voltage and source temperature were typically set at values of 1.9 kV, 40 V and 60°C respectively. The ion-mobility (IM) wave was operated at 1300 m/s with a constant wave-height of 40 V; data were acquired over a range of 500-4000 m/z. For ligand-binding experiments, rutin or raffinose were dissolved in water and added to amylin solutions at 1:1 ratios prior to mass spectral analysis, with no prior incubation period.

2.4 Human-amylin transgenic mice

Study protocols were approved by the University of Auckland Animal Ethics Committee and performed in accordance with the NZ Animal Welfare Act (1999), the UK Animals (Scientific Procedures) Act 1986, and associated guidelines. All animal studies complied with the ARRIVE guidelines [11]. Male FVB/N transgenic/control mice were housed from weaning (21 days old) in environmentally-controlled conditions with a 12-h light/day cycle. Animals were fed standard rodent chow (Teklad TB 2018; Harlan, Madison, WI) and water *ad libitum*.

The construction, characterisation, husbandry and measurement of glucose concentrations in tail-vein blood of hA-transgenic mice in such studies were performed as previously described [8, 9, 12].

2.5 Rutin treatment

Rutin (0.5 mg/ml, pH 7.6-8.0 in 18 MΩ/cm water) was administered to hemizygous hA-transgenic mice and non-transgenic littermate-controls, orally via the drinking water from weaning. Control groups comprised hemizygous male mice and their non-transgenic male littermates treated with pure water only (18 MΩ/cm).

3. Results and discussion

Rutin (Suppl. Fig. 1A) and quercetin (Suppl. Fig. 1B) are closely-related flavonols, and rutin comprises quercetin 3-O-rutinoside. Their molecular structures are shown with that of raffinose (Suppl. Fig. 1C), which was here used as an *in-vitro* control carbohydrate treatment for rutin, whose carbohydrate moiety it resembles.

The primary structures of hA and its corresponding non-aggregating orthologue, mouse amylin (mA) are shown (Suppl. Fig. 1D) [6]. The sequence differences (*residues underlined in bold*, Suppl. Fig. 1D) between hA and mA in the segment corresponding to NNFGAILSS (*dashed line*) in large part generate the amyloidogenic nucleus in hA that is largely responsible for its propensity to misfold, aggregate, elicit death by apoptosis of islet β-cells, and cause islet degeneration and diabetes [13]. The intramolecular disulphide bond and carboxyl-terminal amide group are required for physiological hormonal activity of hA [6, 14].

We asked whether rutin or quercetin might inhibit hA-mediated aggregation by measuring their interactions with hA in solution by applying a ThT fluorescence assay (Fig. 1). When ThT bound to aggregating hA, there was a marked increase in fluorescence that was both time- and aggregation-state-dependent: both flavonols, at 10-fold molar excess, completely inhibited ThT-dependent fluorescence, providing evidence for the ability of each to suppress aggregation (Fig. 1A). Subsequently, we showed that rutin inhibits hA-mediated aggregation in a dose-dependent manner, at molar ratios of 1:1 and 1:0.1 hA:rutin (Suppl. Fig. 2).

Addition of rutin to hA at two different molar ratios, showed that it causes concentration-dependent disaggregation of hA that had previously been allowed to aggregate for 1440 min (Fig. 1B). Thus rutin can elicit two separate but related effects to suppress misfolding and aggregation of hA: these are *a priori* prevention of aggregate formation and *a posteriori* disaggregation of pre-formed aggregates. Either of these effects could contribute to the *in-vivo* anti-diabetic response to rutin treatment in hA-transgenic mice.

We analysed misfolding of hA by ion-mobility mass spectrometry in formic acid (0.1% w/v) in the presence or absence of rutin or raffinose (Fig. 2) according to established methods [15, 16]. Several charge-states of hA were observed in rutin's absence and their pattern was altered immediately following addition of rutin (at an hA-to-rutin ratio of 1:10 mol/mol; Fig. 2A *middle panel*), whereas the charge-state pattern was

not modified by an equivalent concentration of raffinose employed as a negative control (Fig. 2A *lower panel*): for example, the $[M+5H]^{5+}$ charge state of hA disappeared following rutin treatment but was not modified by raffinose treatment at the same concentration ratio.

Analysis of the peak-cluster at *m/z* = 1301.61 identified two overlapping species in the absence of rutin, corresponding to an hA-monomer with three charges, $[M+3H]^{3+}$ and a dimeric-hA species (hA-dimer) with 6 charges, $[2M+6H]^{6+}$ (Fig. 2B *upper panel*). This hA-dimer was not detected following addition of rutin (Fig. 2B *middle panel*) but persisted with raffinose (Fig. 2B *lower panel*). Arrival-time distributions in this *m/z* window confirmed the presence of these two distinct structures (Fig. 2C *upper panel*), the hA-dimeric species $[2M+6H]^{6+}$ and the hA-monomer, $[M+3H]^{3+}$, with suppression of dimeric-hA following addition of rutin (Fig. 2C *middle panel*), but not raffinose (Fig. 2C *lower panel*).

Analysis of the region spanning *m/z* = 1450-1600 showed there was no monomeric-hA in the absence of rutin (Fig. 2D *upper panel*), but instead dimeric-, $[2M+5H]^{5+}$ and trimeric-hA, $[3M+8H]^{8+}$ species were observed. While some of the dimeric-hA species remained in the rutin-treated sample (Fig. 2D *middle panel*), its levels were much reduced, whereas trimeric hA was undetectable (Fig. 2D *middle panel*). Instead, a species corresponding to monomeric-hA bound to rutin was observed, $[M+Rutin+3H]^{3+}$. Although raffinose bound to hA $[M+raffinose+3H]^{3+}$, it did not alter the hA-species profile shown by hA without addition (Fig. 2D *lower panel*).

The presence of the peaks at *m/z* = 1505 (Fig. 2D *middle panel*) indicates the existence of direct binding between rutin and monomeric-hA species, whose structure is modified by rutin-binding: thus, rutin acts as a suppressor of hA-misfolding and stabilises hA in a monomeric form. Moreover, the dimeric- and trimeric-hA species which represent hA-oligomers, are modified by rutin such that they are partially or entirely suppressed.

To detect potential *in-vivo* effects of rutin on hA-induced diabetes, we treated hemizygous hA-transgenic mice with oral rutin or water, and determined their individual dates of glucose deviation from baseline (diabetes-onset), rates of subsequent glucose increase (see Fig. 1A in Ref [17]), and rates of symptomatic deterioration as determined by the rates of increase in fluid consumption (see Fig. 1B in Ref [17]); we also measured time-dependent changes in body-mass (Suppl. Fig. 4A). The rutin dose employed replicated that we employed in a prior study of tetracycline, where we showed that this antibiotic can effectively

ameliorate severity of diabetes in the line of hA-transgenic mice [9] used for the current study: this rutin concentration was also consistent with concentrations that modified hA-aggregation in the *in-vitro* studies.

The time of diabetes-onset was first determined by a method validated for, and widely used in animal studies, which also reflects diagnostic criteria employed for diagnosis of the human disease: namely demonstration of consecutive twice-weekly measurements of blood glucose > 11 mM [9]. Following our previous study of tetracycline treatment of diabetes in hA-transgenic mice [9], we postulated that rutin might modify the rate of diabetes progression rather than risk of death *per se*: therefore, for survival analyses (Fig. 3) we employed the Wilcoxon test (which allows for a potential acceleration of failure time) rather than the log-rank test (which assumes proportional hazards).

The null hypothesis of 'no difference in time from diabetes-onset to death' (where 20% weight-loss was used as a surrogate for death) was rejected (P=0.044; see Table 1 in Ref [17]); rather, the rutin-treated mice survived a median of 91% longer (69 days) than the placebo-treated diabetic mice. Moreover, from the full glucose time-course data, it became evident that the conventional *a priori* method of diabetes diagnosis inaccurately estimates the precise time-point at which the blood-glucose trajectory changes from the pre- to post-diabetic state (Fig. 1A [17]). Hence the null hypothesis of 'no difference in time from inferred blood-glucose change-point to death' was rejected more strongly (P = 0.011; see Table 1 in Ref [17]). In future, a two-step process comprising the existing *a priori* method followed by the described *a posteriori* method [17], applied once complete data are available, should be employed for making optimized between-treatment comparisons.

To further investigate differences in time-course profiles between control- and rutin-treated groups, we employed parametric change-point regression analysis (*see* [17]) to extract, for each animal, baseline levels of blood glucose and fluid-intake, the change-point time at which blood glucose (diabetes-onset) and fluid-intake (onset of polydipsia) accelerated from baseline, and the rate of this acceleration (Figs. 1A, B and Table 1in Ref [17]). From the inferred change-point measurements, Kaplan-Meier curves were constructed (Fig. 3): these demonstrate that rutin-treatment significantly increased not only the time from blood-glucose change-point (CP) to death (P = 0.011), but also from fluid-intake change-point (CP) to death (P = 0.014), and from blood-glucose change-point to fluid-intake change-point (P = 0.036). Rutin-treatment thus prolonged the time interval between onset of diabetes and acceleration of fluid-intake, a marker for

worsening of diabetic symptoms, and prevented body-weight loss (Suppl. Fig. 4A) providing evidence for amelioration of the severity of the diabetic syndrome in treated mice.

Furthermore, the acceleration of blood glucose from baseline levels was slower in the rutin-treated group (P=0.030, two-tailed t-test after log-transformation to stabilise variance), and trending towards slower for the acceleration of fluid-intake from baseline levels (P = 0.059). In other words, the rate of disease progression was slowed by rutin treatment.

One objective of this study was to identify natural compounds which suppress human amylin misfolding, and to test their efficacy *in vivo* in an hA-transgenic mouse line. We show here by ThT fluorescence and CD spectroscopy (Suppl. Fig. 3) that treatment with rutin and quercetin suppressed misfolding and aggregation of hA *in vitro*. These data provide direct evidence for the first time that rutin binds to hA monomers and disaggregates hA oligomers. These findings indicate that the pharmacophore responsible for these effects resides solely in the quercetin structure. These data are consistent with a rutin interaction with soluble hA oligomers that prevents or restricts their conversion into cytotoxic structures and thus shields islet β-cells from Fas-evoked destruction [8-10].

Oral treatment with rutin from the time of weaning, at mg/kg doses similar to those used in humans for dietary supplementation [18], delayed the onset and progression of diabetes in a transgenic model of hA-evoked diabetes wherein the phenotype closely resembles that of T2D in patients. Oral rutin treatment ~doubled the long-term survival of affected individuals.

The parametric change-point regression approach applied here to determine the modal change-point allows a much more accurate determination of the exact time of diagnosis of diabetes than does the standard approach that we have used hitherto and is expected to enable more precise comparisons between aspects of the efficacy of different treatments. This and the above observations are pertinent to the ‘real-life’ treatment of diabetes, and support further investigation of the potential use of rutin or related flavonols with similar biological activities, for the treatment of diabetes.

The ORAC assay (Suppl. Results) did not provide convincing evidence that the observed effects of rutin were mediated via an oxidative mechanism. These data are insufficient to establish the existence of such a mechanism as the cause of the anti-diabetic effects observed in rutin-treated hA-transgenic mice. Oral rutin effectively increased glucose tolerance in hA-transgenic animals after 60 days’ treatment (Suppl. Fig.

4B). This improvement in glucose metabolism is a likely result of the rutin-hA interaction and probably contributes to its anti-diabetic effects.

Quercetin and quercetin-containing compounds comprise the most prevalent flavonoids in the diet of many humans [19]. Dietary quercetin is plant-derived and comprises a mixture of free quercetin and quercetin glycosides, including rutin (quercetin-3-O-rutinoside) and quercetin-4'-O-glucoside. Rutin is probably the main glycosidic form of quercetin in the human diet [19]. There is evidence that the glycoside moiety is a major determinant of the absorption of dietary flavonoids in man [20] but ongoing uncertainty persists concerning the *in-vivo* physiology in mammals of quercetin-containing flavonoids including rutin [21]. Rutin and quercetin are polycyclic compounds, whose structures imply that they could interact with the amyloidogenic region in hA to suppress β-sheet formation: they evidently promote the formation of α-helix by hA, either by allowing its spontaneous formation or by promoting its formation from random coil. Alternatively, it has been suggested that the rutinoside group of rutin might be cleaved during its uptake from the gut, meaning that the polycyclic quercetin group and its metabolites could be the (potentially) bioactive compounds: therefore, the possibility remains that the *in-vivo* effects observed in this study may have been caused by interactions between quercetin and/or its metabolites and hA. However, a recent pharmacokinetic study found no evidence of free quercetin in the blood following dosing with rutin or quercetin-4'-O-glucoside [21], so further investigation concerning the *in-vivo* active moiety is clearly called for. In addition, the exact molecular interaction between rutin-derived metabolites and hA requires further investigation.

Accruing evidence from other hA-transgenic mouse lines also points to a role for soluble hA oligomers in the increased frequency of β-cell apoptosis in late-stage diabetes [22-24]. There is also evidence that cytotoxic effects of pre-fibrillar aggregates of amyloidogenic proteins such as β-amyloid, α-synuclein and transthyretin can elicit cell death via equivalent mechanisms [25-27]. Therefore, mechanistic studies of rutin may be indicated in suitable animal models of these other syndromes.

Several studies with rutin or quercetin have shown improvement in aspects of glucose homeostasis in diabetic rodent models, which has been attributed to antioxidant effects. Both rutin and quercetin reportedly improved enzymatic markers of oxidative stress, decreased lipid peroxidation, and lowered plasma glucose and glycated haemoglobin and nitric oxide production in streptozotocin-diabetic rats; they also increased insulin secretion and ameliorated β-cell function [28]. It is uncertain how data from rats with streptozotocin-induced

diabetes, an animal model of severe, type-1 diabetes, pertain to the mechanisms of T2D in patients, or to the results we report here from our studies in rutin-treated hA-transgenic mice.

In conclusion, these findings show that rutin evidently acts to stabilize soluble, nascent pre-fibrillar aggregates of hA and thereby delay the *in-vivo* progression of diabetes in hA-transgenic mice whose diabetes closely resembles T2D in patients. The observed effects of rutin support further investigation concerning its mechanism of action. Identification of classes of compounds that suppress the misfolding and aggregation processes whereby hA forms cytotoxic oligomers could provide a means for finding new anti-diabetic treatments that act by preserving β-cell function.

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Acknowledgements

We thank Cynthia Tse for her management and administrative support and for editing the manuscript, and Professor Sir P John Scott for his helpful discussions with us.

Figure Legends

Fig. 1. Rutin and quercetin inhibit fibril formation by human amylin *in vitro.* (A) Shows thioflavin-T fluorescence following incubation of hA (20 μM) with thioflavin-T (10 μM) in the absence or presence of either rutin (200 μM) or quercetin (200 μM). Results are means ± S.E.M. (*n* = 3/point). (B) Rutin causes concentration-dependent disaggregation of preformed hA-aggregates. Normalized time-dependent ThT fluorescence following incubation with hA alone (*red lines*), or following later addition of rutin (*at t = 1440 min*) at molar hA-to-rutin ratios indicated. Human amylin (20 μM) was pre-incubated with thioflavin-T (10 μM) for 1440 mins at room temperature prior to addition of rutin. Each experiment was performed in triplicate, and each line represents a replicate experiment.

Fig. 2. Study of misfolding and aggregation of hA and the effects of rutin-treatment by ion-mobility mass spectrometry. (A) Effects of treatment with rutin or raffinose on aggregation by hA. (B) Effects of treatment with rutin or raffinose on oligomerization of hA. (C) Ion-mobility analysis contrasting the effects of treatment with rutin or raffinose on aggregation by hA. (D) Higher-resolution analysis of the effects of rutin or raffinose on aggregation and oligomerization of hA. In each figure: upper panel shows hA with no addition; middle panel shows hA in the presence of rutin and lower panel shows hA in the presence of raffinose.

Fig. 3. Kaplan-Meier survival curves for control- (*red*) and rutin-treated (*blue*) groups of diabetic hemizygous hA-transgenic mice constructed from the results of the blood-glucose and fluid-intake parametric change-point modelling described in [17] (shown in Fig. 1A, 1B, Table 1[17]).

Author Information

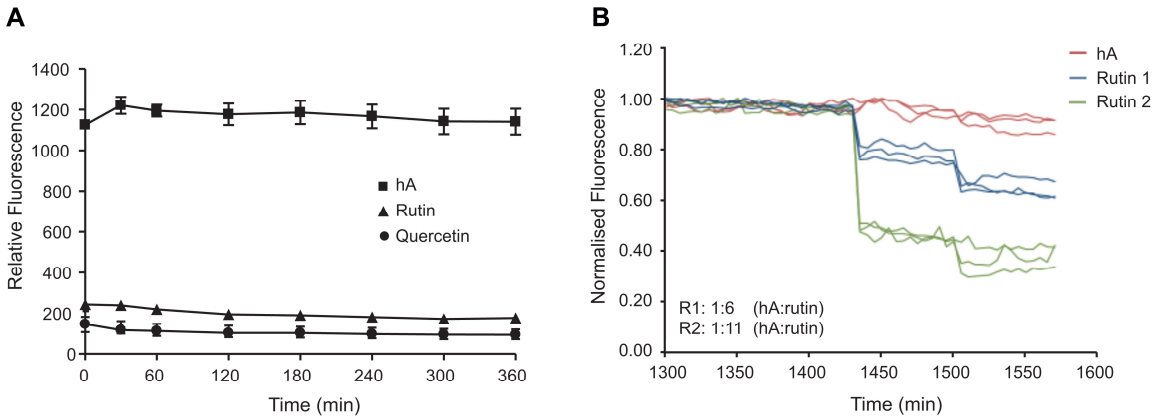
JA conceived, designed and performed research, analysed data, interpreted results, and wrote and revised the manuscript. KL designed research, interpreted results, and revised the manuscript. IR-G and RU designed, performed, and interpreted research, and wrote and revised the manuscript. GP and ASP performed research. ARJP designed and supervised research, and revised the manuscript. DW, SDP and KD reviewed and revised the manuscript. PB designed, supervised and interpreted research, and wrote and revised the manuscript.AD designed research, developed the Bayesian change-point regression methodology applied here, analysed and interpreted data, wrote and revised the manuscript, and is the co-senior author of this study. GC conceived, designed and supervised research, analysed data, interpreted results, and wrote and revised the manuscript, and is co-senior author: he is the guarantor of this work and, as such, had full access to all the study data, and takes responsibility for the integrity of the data and accuracy of the data analysis, and of the final manuscript.

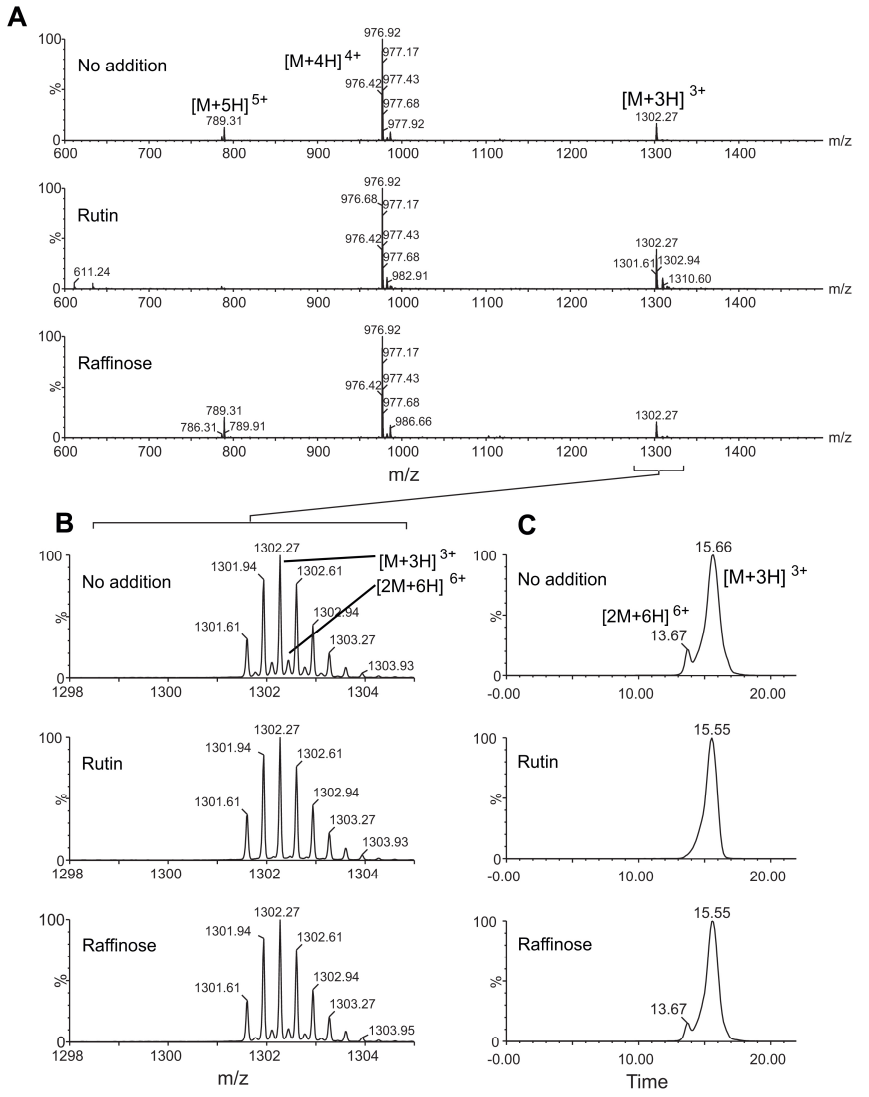
Funding

This research was supported by grants from the Endocore Research Trust (60147); the Maurice and Phyllis Paykel Trust (various equipment grants; 3601069; co-funding of CD-Spectrometer); the Health Research Council of New Zealand (HRC 03/190); the Ministry of Business, Innovation & Employment, New Zealand (MBIE; UOAX0815); the Maurice Wilkins Centre for Molecular Biodiscovery (Tertiary Education Commission 9431-48507); Lottery Health New Zealand (3354520; co-funding of CD-Spectrometer); the Medical Research Council (UK, MR/L010445/1 and MR/L011093/1); the Guangdong High-end Foreign Expert Fund; a Key International Collaborative Fund from the Chinese Academy of Sciences (154144KYSB20150019, DW); The University of Manchester, the Central Manchester NHS Foundation Trust, and the Northwest Regional Development Agency through a combined programme grant to CADET; and was facilitated by the Manchester Biomedical Research Centre and the Greater Manchester Comprehensive Local Research Network.

Conflict of Interest Statement

All the authors have declared that they have no conflict of interest with respect to this work. Sponsors had no role in the study design; the collection, analysis and interpretation of the data; the writing of the manuscript; or the decision to submit the article for publication.

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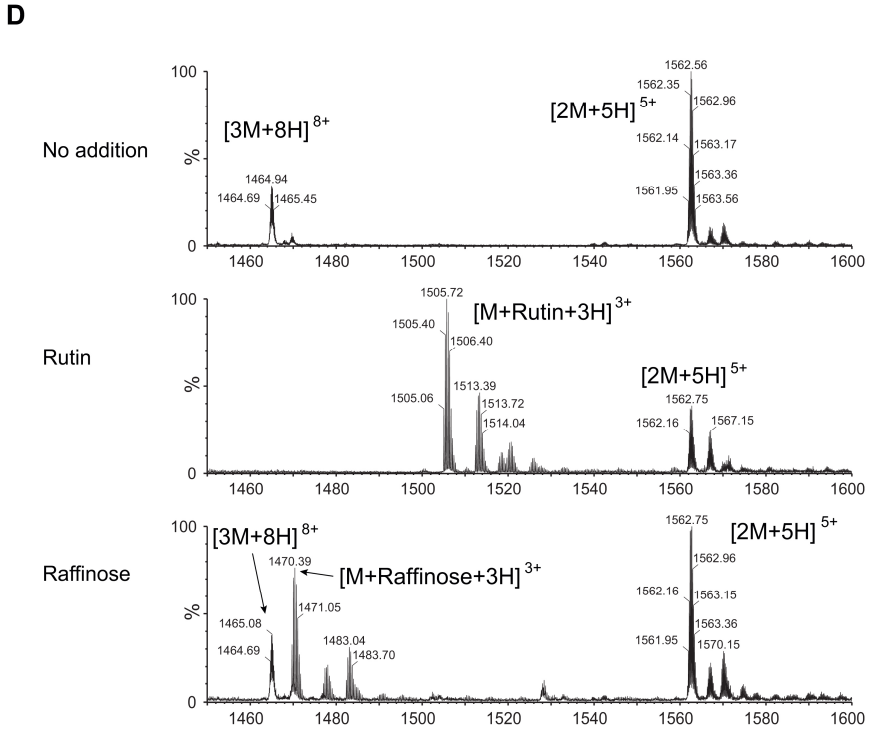
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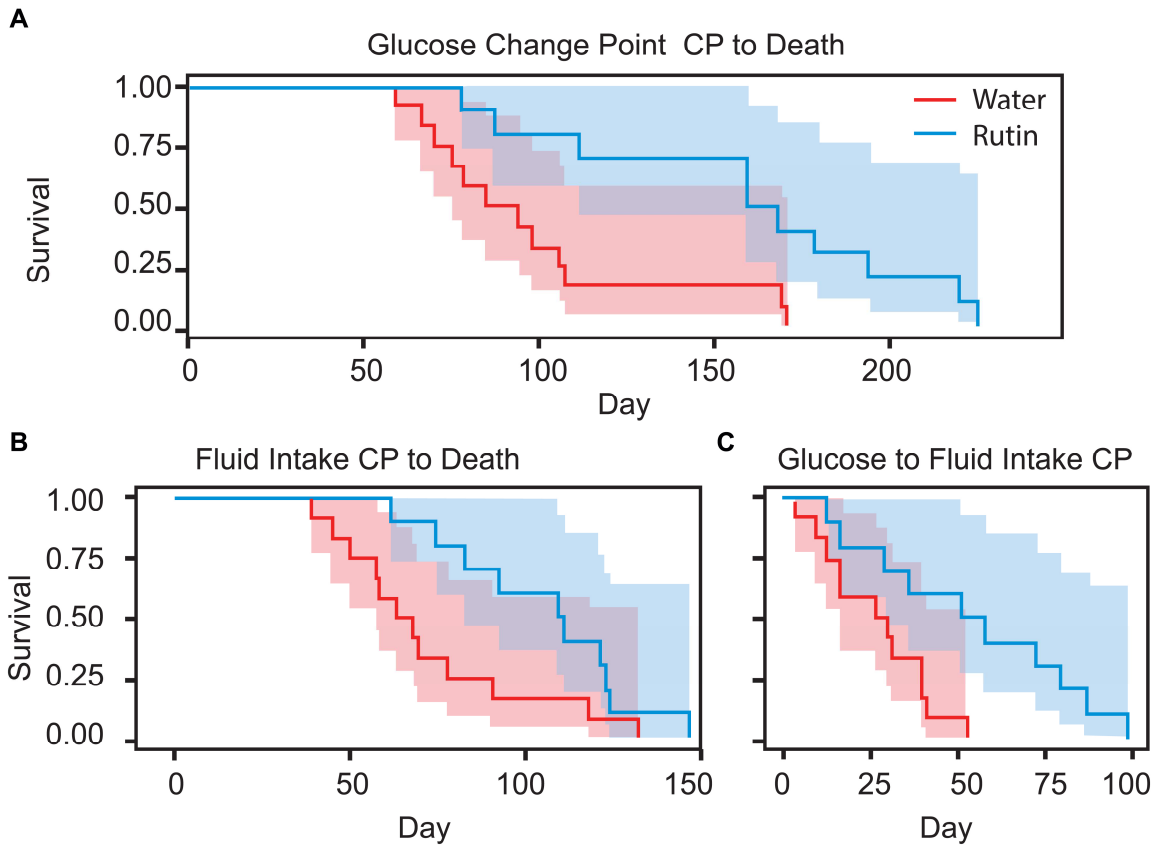

Highlights

- Defective islet beta-cell function is central to the causation of type 2 diabetes.
- Human amylin aggregation is closely linked to the mechanism of β-cell death.
- Rutin bound to amylin suppressed misfolding, and disaggregated amylin oligomers.
- Rutin markedly delays diabetes progression with doubling of lifespan in mice.