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An *in vitro* evaluation of epigallocatechin gallate (eGCG) as a biocompatible inhibitor of ricin toxin

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ABSTRACT

The catechin, epigallocatechin gallate (eGCG), found in green tea, has inhibitory activity against a number of protein toxins and was investigated in relation to its impact upon ricin toxin (RT) *in vitro*. The IC_{50} for RT was 0.08 ± 0.004 ng/mL whereas the IC_{50} for RT + 100 μM eGCG was 3.02 ± 0.572 ng/mL, indicating that eGCG mediated a significant ($p < 0.0001$) reduction in ricin toxicity. This experiment was repeated in the human macrophage cell line THP-1 and IC_{50} values were obtained for RT (0.54 ± 0.024 ng/mL) and RT + 100 μM eGCG (0.68 ± 0.235 ng/mL) again using 100 μM eGCG and was significant ($p = 0.0013$). The documented reduction in ricin toxicity mediated by eGCG was found to be eGCG concentration dependent, with 80 and 100 μg/mL (*i.e.* 178 and 223 μM respectively) of eGCG mediating a significant ($p = 0.0472$ and 0.0232) reduction in ricin toxicity at 20 and 4 ng/ml of RT in Vero and THP-1 cells (respectively). When viability was measured in THP-1 cells by propidium iodide exclusion (as opposed to the MTT assays used previously) 10 ng/mL and 5 ng/mL of RT was used. The addition of 1000 μM and 100 μM eGCG mediated a significant ($p = 0.0015$ and <0.0001 respectively) reduction in ricin toxicity relative to an identical concentration of ricin with 1 μg eGCG. Further, eGCG (100 μM) was found to reduce the binding of RT B chain to lactose-conjugated Sepharose as well as significantly ($p = 0.0039$) reduce the uptake of RT B chain in Vero cells. This data suggests that eGCG may provide a starting point to refine biocompatible substances that can reduce the lethality of ricin.

1. Introduction

There is a clear and pressing need for improved first-line treatment and prophylaxis to combat the ever-increasing threat posed by agents of bioterrorism. Amongst these agents is ricin toxin (RT), a protein produced by the castor bean (*Ricinus communis*). RT is rated by the US Centres for Disease Control and Prevention as a level B biothreat [1] and has been used within the last 12 years to incite terror, with the intention of causing harm, morbidity and mortality within the human population [2]. RT is known to be of interest to organizations such as al-Qaeda, thought to be developing a ricin "bomb" *i.e.* wrapping ricin powder round explosive devices as a means of dissemination [3]. The availability of RT was underpinned by both the intoxication of Roger Bergendorff in 2008 [4] and the discovery of RT containing letters sent to Bill Frist in 2004, who was, at the time the US senate majority leader [5]. The tangibility (and consequent potency) of these threats is given greater urgency by the fact that RT is relatively easy to produce and weaponize in lethal quantities [2]. There is also no cure for intoxication with medical support being palliative [6]. The UK and US government exploited these observations during the production of "compound W" (RT) prior to the Biological and Toxin Weapons Convention of 1972 and the Chemical Weapons Convention of 1973 [7].

RT is composed of two protein chains joined *via* a single disulfide bond. The lectinic RT B chain (RTBC) is responsible for the binding of the toxin to the cell membrane. Binding is achieved *via* an interaction between RTBC and a terminal galactose or *N*-acetyl-galactosamine residues, which are then internalized [8]. RTBC has a bi-lobal structure, with each lobe having a lectinic activity [9] and mediates the translocation of RT A chain (RTAC) to the endoplasmic reticulum (ER) [10]. RTBC is

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; CD, circular dichroism; cRTAC, commercially acquired RTAC; cRTBC, commercially acquired RTBC; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; eGCG, epigallocatechin gallate; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMA, 1-ethyl-3-(3-dimethylamino-propyl) phorbol 12-myristate-13-acetate; rRTBC, recombinant RTBC; RT, ricin toxin; RTBC, RT B chain; RTAC, RT A chain; SEM, standard error of the mean.

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internalized *via* clathrin, caveolin and non-clathrin mediated endocytosis and the translocation of the RT molecule to the ER is achieved *via* the Golgi body. From the ER, the catalytic RTAC passes into the cytosol to mediate the depurination (and inactivation) of ribosomes [10,11]. Critical to RT activity is the ability of RTBC to interact with membrane components, as without RTBC, RTAC (and other type I ribosome-inactivating protein), is relatively non-toxic [12].

Resources have been directed towards developing treatments (aside from prophylactic immunization *i.e.* RiVax) [13], to prevent ricin intoxication post-exposure. To date, several strategies have emerged. The first employs a small molecule to inhibit ricin translocation out of the endosome *i.e.* Retro-2 [14]. The second seeks to block ricin uptake or intracellular trafficking *via* either antibody mediated steric hindrance [15,16] or by feeding milk-derived material (*i.e.* lactose) to exposed individuals [17]. RTBC has been documented to have a high lactose binding affinity and lactose is thought to compete for cell binding sites on RTBC [17,18]. In addition to the above-mentioned, the inhibition of a variety of protein toxins such as anthrax [19], tetanus [20], botulinum [21] and Shiga toxin [22] by polyphenols (found in tea), has also been reported. One such polyphenol is epigallocatechin gallate (eGCG) [23]. Here we present an evaluation of the inhibitory effect of eGCG upon RT *in vitro*. An evaluation of potential mechanisms of inhibitory activity was also undertaken and has been discussed.

2. Materials and methods

2.1. General chemicals and reagents

TRITON-X-100, propidium iodide, glycine, paraformaldehyde, leupeptin hydrochloride, bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylamino-propyl) phorbol 12-myristate-13-acetate (PMA), the bicinchoninic acid (BCA) assay kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and anhydrous cell culture grade dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (Dorset, UK). The (2R,3R)-2-(3,4,5-Trihydroxyphenyl)-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol-3-(3,4,5-trihydroxybenzoate) (eGCG) was purchased from Merck (Calbiochem, Nottingham, UK).

2.2. Texas Red-labeled cRTBC

Labeling was performed using the Texas Red®-X, succinimidyl ester, mixed isomers (Invitrogen, Paisley, UK) following the manufacturer's instructions and as previously described for labeling BSA [24]. Commercial cRTAC and cRTBC were from Vector labs (Peterborough, UK) and the refolded ricin was generated using a previously published protocol [25]. Re-folded ricin holotoxin was characterized by Western immunoblotting against a known quantity of cRTAC and (separately) cRTBC. Where refolded RT has been documented in the experimental section the amounts indicated refer to the amount of RTAC in the preparation and not to the total mass of protein. This is to control for small inter-batch variability in RTBC content required for RT refolding [25]. *Antibodies*: Monoclonal anti-TGN46 was from AbD Serotech (Kidlington, UK) and the polyclonal rabbit anti-RTAC and anti-RTBC were from AbCam, (Cambridge, UK). The anti-mouse and anti-rabbit secondary antibodies conjugated with Texas Red- or Alexafluor-488 were from Invitrogen (Paisley UK). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies for immunoblotting were from GE Healthcare (Little Chalfont, Bucks, UK).

2.3. Cell culture and microscopy

RPMI 1640 medium, Dulbecco's minimal essential medium, glutamax, penicillin/streptomycin, Dulbecco's modified Eagle's medium without sodium pyruvate with 450 mg/mL glucose, kanamycin and fetal calf serum (FCS) were from Invitrogen (Paisley, UK). The 6 -well treated cluster plates and sterile 22 × 22 × 0.1 mm coverslips were from Fisher Scientific (Loughborough, UK). Vero cells (ATCC number CCL-81), and THP-1 cells (ATCC number TIB-202), were from the American Type Culture Collection (ATCC) (Teddington, UK).

3. Methods

3.1. Cloning and expression of recombinant (r)RTBC

A pUC19-derived plasmid encoding *Escherichia coli* codon optimized RTBC was supplied by Biobasic Inc. (Markham, Ontario Ca) and was created using published sequences [26] RTBC was amplified from the aforementioned template using the following primers: (forward) ((5′-CAC CGC TGA TGT TTG TAT GGA TCC T and (reverse) 5′- TCA AAA TAA TGG TAA CCA TAT TTG). The resulting PCR product was ligated into pET151/D Topo (Invitrogen, Paisley, UK) following the manufacturer's instructions. PCR was performed using an Accuzyme PCR kit (Bioline Reagents Limited, London, UK). Sub-cloning was verified by DNA sequencing, performed by the DNA Sequencing and Services at Dundee University, (Dundee, UK). Protein expression, enrichment from bacterial lysate and characterization by immunoblotting were performed as previously reported [27].

3.2. Cell culture: immunofluorescent microscopy

This methodology has been extensively described and discussed previously [24]. Briefly, after being exposed to either Texas Red-labeled RTBC (50 μg/mL each treatment) or Texas Red-labeled RTBC with eGCG (100 μM each treatment), and left for 4 h under standard incubation conditions, the cells were fixed with 2% (*w*/*v*) formalin in PBS at room temperature for 20 min, prior to being quenched with 5% (*w*/*v*) glycine in PBS containing 0.05% (*w*/*v*) TRITON-X-100 which also served to permeabilized the cells. Following a blocking step using 1% (*v*/*v*) FBS in PBS, the cells were exposed to an anti-TGN46 primary antibody, (60 min at room temperature) and an Alexafluor® 488-labeled secondary antibody. Following a subsequent wash step (3× using PBS) the cells were mounted in 50% (*w*/*v*) glycerol in PBS containing 1% (*w*/*v*) n-propyl gallate. Microscopy was performed using an Eclipse 90i microscope (Nikon UK Ltd., Kingston Upon Thames, Surrey, UK) fitted with an Apo ×60 objective and a DS-Qi1Mc camera. Image acquisition was performed using Advanced Research Elements software version 3.2 (Nikon UK Ltd., Kingston Upon Thames, Surrey, UK).

3.3. In vitro toxicity assay

This methodology has been described extensively [28] and assays were conducted over 48 or 72 h (as stated) with the stated number of replicates [24,25] Statistical analysis was performed using the Prism 6.0b software package (GraphPad Inc. La Jolla, CA, USA) and t-tests were unpaired, two-tailed. Where concentrations of eGCG above 10 μM were used, care was taken to remove the eGCG from the culture and to wash the culture 3 times with PBS prior to adding MTT. This was necessary to minimize any false positive data resulting from an eGCG interaction with MTT (data not shown). Where eGCG was co-administered with RT the two substances were dissolved in complete media and left for 60 min at room temperature to come to equilibrium prior to being added to the cells. Flow cytometer data was acquired using an Accuri C6 flow cytometer (BD Bioscience, Oxford UK). THP-1 cells were incubated with RT and eGCG in PBS at the reported concentrations and left for the specified time prior to being re-suspended in sterile PBS containing 10 ng/mL propidium iodide. The cells were then subject to analysis at 488 nm. The IC_{50} values for refolded RT are also representative of an extended data set beyond the number of replicates stated as each batch of refolded ricin was characterized (in part) by examining its toxicity.

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3.4. RTBC uptake experiments

Here 5×10^5 Vero cells were used to seed individual wells in a 6 -well plate. The cells were then left overnight in complete media under standard incubation conditions (37 °C in a humidified atmosphere containing 5% (*v*/v) CO_2). The next day, either RTBC (50 μg/mL) or RTBC plus eGCG (1 mM) were added along with a third treatment which had only RTBC added and was kept at 0 °C throughout the experiment. The remaining two plates were placed back under standard culture conditions. After 4 h the cells were washed with ice-cold PBS 3 times and blotted dry, taking care not to disturb the monolayer. The monolayers were then dissolved in the Laemmli SDS page buffer containing 10% (*v*/v) 2-mercaptoethanol and run on a 12% (*w*/*v*) SDS PAGE gel prior to Western blotting using standard conditions. After the transfer had completed the blots were blocked with PBS containing 0.01% (*v*/v) TWEEN 20 and 5% (*w*/*v*) nonfat dried milk. Blots were then cut in half to allow them to be separately probed with antibodies specific for EEA1 (BD Bioscience; Oxford, UK) and RTBC (AbCam, Cambridge, UK) using HRP-conjugated secondary antibodies (GE Healthcare; Bucks, UK) following standard protocols and following the manufacturer's instructions, using the Pierce™ ECL reagent (Thermo Scientific; Waltham, MA USA).

3.5. In vitro RTBC binding assay

Lactose-conjugated Sepharose (Sigma Chemical Company, Dorset UK) was washed 3 times in PBS and either recombinant RTBC commercial RTBC (Vector Labs; Peterborough, UK) or commercial RTBC and eGCG was added. This was washed with 10 column volumes of PBS. To the beads, 100 μL of Laemmli SDS page buffer containing 10% BME) was added and this was compared to an equivalent amount of input protein. Following the sedimentation of the beads at 12,000 × G at room temperature for 2 min, the supernatant was analyzed by SDS PAGE and Western blotting. Detection was performed using an anti RTBC polyclonal antibody (AbCam; Cambridge, UK) and an anti-rabbit, HRP-conjugated secondary (Invitrogen, Paisley, UK) using Pierce™ ECL reagent (Thermo Scientific; Waltham, MA USA).

3.6. Assessment of protein conformation using circular dichroism (CD) Spectroscopy

Proteins (0.3 mg/ml in PBS) were analyzed using a Chirascan™ CD spectrometer, (Applied Photophysics, Surrey, UK), and data was acquired between 190 and 260 nm, (2 s per time point, 1 nm bandwidth) at 20 °C. Three repeats were taken and 6 M guanidine hydrochloride denatured samples were used as a control. A 0.1 mm path-length was used.

4. Results

The characterization of RT, RTBC and RTAC was performed (Fig. 1; panel a) and with the exception of the re-folded RT preparation, there was no detectable RTBC in the RTAC preparations and no detectable RTAC in the RTBC preparations (Fig. 1; panel a). These data (Fig. 1; panel a) were important for the validation of the re-folded RT. When the re-folded RT was characterized by immunoblotting, typically a 2:1

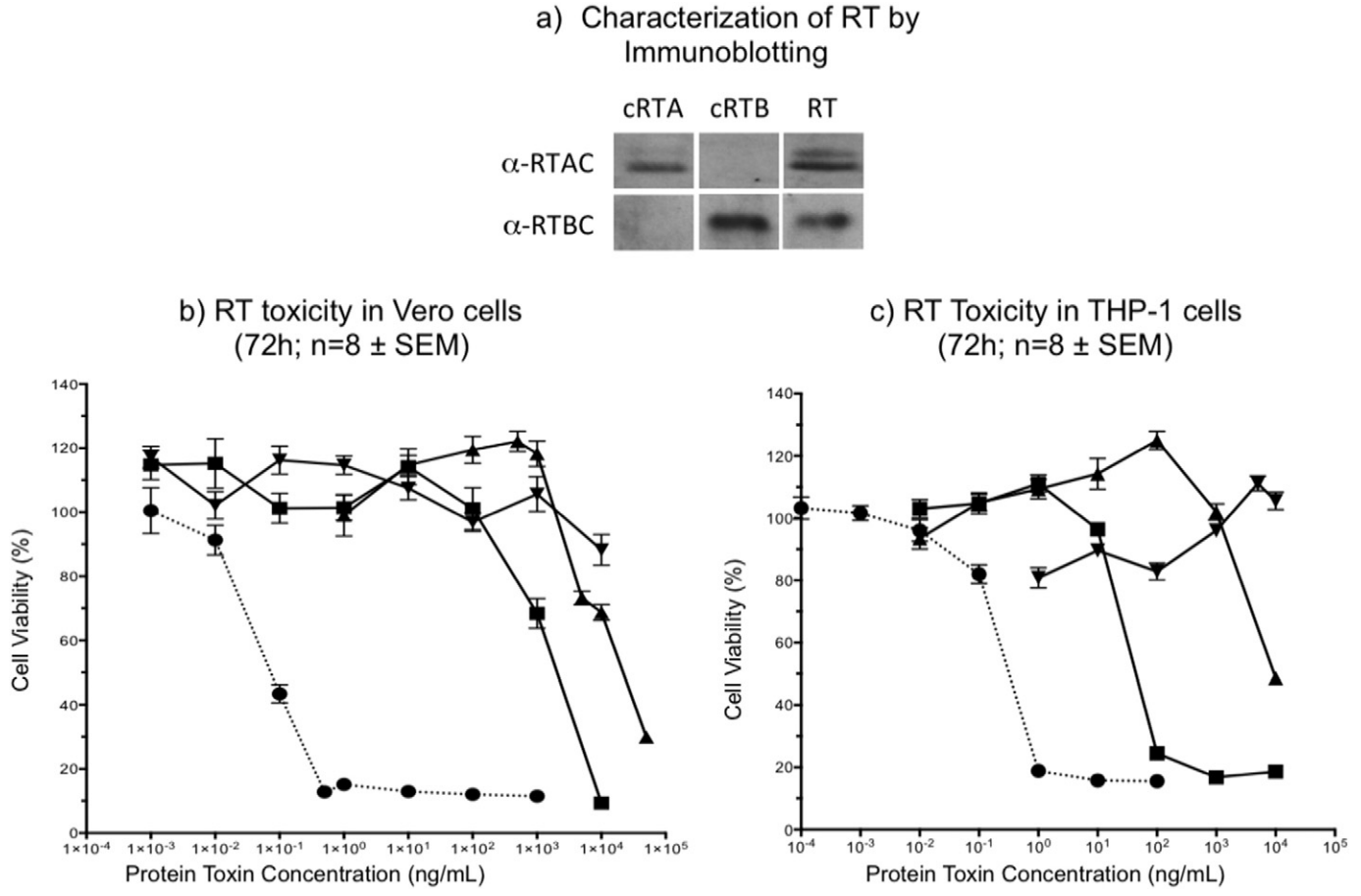

Fig. 1. Characterization of RT and RT components. Panel (a) depicts the immunological profile of commercially obtained ("c") RTAC, cRTBC, and refolded RT when probed (individually) with antibodies specific for RTAC or RTBC. Panels (b & c) document the *in vitro* toxicity profiles of refolded ricin holotoxin (black circles joined by a dotted line), cRTAC (black squares), cRTBC (black triangles, point up) and recombinant RTBC (black triangles, point down), in both Vero (panel b) and THP-1 (panel c) cells after 72 h when assessed using the MTT assay (in each instance n = 8; ±SEM). Data derived from these assays are summarized (Table 1) where calculated IC_{50} values are recorded.

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ratio of RTBC to RTAC was obtained (data not shown). This was an unavoidable consequence of the protocol that was necessary to ensure that the majority of the RT preparation was heterodimeric [25]. While this procedure was not optimal, it was necessary, given the current availability of commercial RT holotoxin in the UK.

To characterize re-folded RT (and its components) *in vitro* toxicity, both Vero (African green monkey kidney) and THP-1 (human macrophage) cell lines were used. These toxicity data (Fig. 1; panels b & c) are summarized (in Tables 1 & 2) and in each instance the refolded RT holotoxin displayed the highest level of toxicity (IC_{50} Vero 0.08 ± 0.004 ng/mL; THP-1 0.54 ± 0.024 ng/mL), followed by RTAC (IC_{50} Vero 3.525 ± 1.017 μg/mL; THP-1 0.068 ± 0.001 μg/mL), cRTBC (IC_{50} Vero 28.625 ± 2.917 μg/mL; THP-1 9.750 ± 0.744 μg/mL) with recombinant RTBC demonstrating the least toxicity (the IC_{50} was 100 + μg/mL in Vero and 100 + μg/mL in THP-1 cells). Having established base-line toxicities for re-folded ricin, the effect of a known inhibitor of ricin toxin (lactose) [17] as well as that of the polyphenol under investigation (eGCG) was investigated, with lactose serving as a positive control rather than a gold standard. However, before these interactions could be characterized, baseline toxicities for each of the potential inhibitors were also measured (Fig. 2; panels a & b) and the IC_{50} values calculated (Table 1). Initially RT inhibition was assessed using a static concentration of potential inhibitor in relation to a variable concentration of RT (Fig. 2). These data are shown for Vero cells (Fig. 2; panel c) and THP-1 cells (Fig. 2; panel d). Fig. 2 (panel c) documents a statistically significant inhibition of RT by: eGCG (43.8 μg/mL (*i.e.* 100 μM)) ($p > 0.0001$), when tested in Vero cells. The addition of lactose (3.42 μg/mL (*i.e.* 10 μM)) to RT prior to incubation with Vero cells resulted in an IC_{50} value that was greater than 10 ng/mL (RTAC equivalent) under similar assay conditions to those reported in Fig. 2 (panel d). A similar concentration of lactose gave rise to an IC_{50} value of >10 ng/mL (RTAC equivalent) when measured in THP-1 cells. Similarly Fig. 2 (panel d) documents a statistically significant inhibition of RT by eGCG ($p = 0.0013$) when measured using THP-1 cells.

Given that eGCG could mediate a significant reduction of RT toxicity when assayed in both Vero and THP-1 cells, at a static concentration of eGCG, the next logical question was to ask if a variable concentration of eGCG could exert a dose dependent effect upon the toxicity of a static RT concentration (Fig. 3). As Vero and THP-1 cells displayed varying sensitivity to RT (Table 2), Vero being approximately 6 times more sensitive to RT than THP-1 cells, it was surprising to note that the effect of (eGCG mediated) RT inhibition was more profound within the populations of Vero cells (Table 2; Fig. 2; panels c & d). A consequence of this was the choice of static concentrations of RT used to generate Fig. 3. Fig. 3 (panel a) shows the effect of a lethal concentration of RT (20 ng/mL) when incubated with increasing amounts of eGCG upon Vero viability after 48 h. Fig. 3 (panel a) reports that in every instance, a statistical difference was observed relative to cells only treated with RT. Here 100, 80, 60 and 40 μg/mL of eGCG demonstrated significant (*i.e.*, $p = 0.0464$, $p = 0.072$, $p = 0.0233$, $p = 0.0337$ respectively) increases in cell viability relative to an untreated RT control. Fig. 2 (panel b) shows the effect of eGCG (50-200 μg/mL) upon THP-1 cell viability after incubation with RT (4 ng/mL) at a lower concentration. Again a significant difference in viability is reported at a dose of eGCG of 100 μg/mL ($p = 0.0232$) and 200 μg/mL ($p = 0.0076$) relative to the RT only control (Fig. 3; panel b). In an attempt to further rule out the possibility of false positive data resulting from an interaction between MTT and eGCG, a flow cytometer was used to measure propidium iodide exclusion (as an indication of cell viability) from non-activated (monocyte) THP-1 cells (Fig. 3; panels c and d). This assay used more eGCG than had been previously assayed (Fig. 2; panels a-d) and shows an increase in cell viability ($p = <0.0014$) (1000 μM eGCG relative to 1 μM eGCG) when THP-1 cells were treated with 10 ng/mL RT (Fig. 3; panel c). When a reduced concentration of RT was used (5 ng/mL) (Fig. 3; panel c), a statistically significant increase in cell viability was recorded when a dose of 1000 μM eGCG was compared to 1 μM of eGCG ($p > 0.0001$).

In an attempt to understand the mechanism(s) driving the observed reductions in RT toxicity, the effect of eGCG was examined upon RTBC. The effect of eGCG upon RTBC was documented (Fig. 4; panel a) where the ability of cRTBC to bind to lactose-conjugated Sepharose with and without eGCG (100 μM) relative to rRTBC (the negative control) was investigated. Reduced cRTBC lectinic activity in the presence of eGCG (100 μM) was recorded. This observation supported the hypothesis that the reduction in toxicity associated with RT in the presence of eGCG may be, at least in part, due to an interaction between eGCG and the RTBC, which resulted in relaxed RTBC conformation (impacting upon RTBC lectinic activity, cell uptake and RTAC intracellular trafficking).

This hypothesis was tested by monitoring the uptake of RTBC, with and without eGCG by Vero cells over 4 h (Fig. 4; panel b). On account of the relatively short timescale used, the dose of eGCG was increased and the results expressed relative to a housekeeper (early endosomal antigen (EEA) 1), to control for any variability in cell number. Fig. 4 (panel b) shows that there was a reduction in RTBC uptake by Vero cells ($p = 0.0039$) incubated with RTBC and 1 mM eGCG relative to cells only incubated with RTBC at 37 °C and at 0 °C. The 0 °C control was necessary as it controlled for non-specific interactions. It was possible that eGCG was inhibiting endocytosis at such a high concentration rather than reducing RTBC uptake *via* the eGCG mediated RTBC conformational relaxation, however, this point remains to be addressed. What was clear was that less RTBC was entering the cells at high concentrations of eGCG, which may account for the reduced toxicity observed earlier (Table 1). Were this hypothesis true, then it might be predicted that treatment with eGCG would alter levels of RTBC cellular uptake and Golgi translocation. The results of testing this hypothesis were documented (Fig. 4; panel c). Fig. 4 (panel c; micrographs i to iii) was captured in the absence of eGCG and shows the co-localization of Texas-Red labeled-cRTBC with a primary antibody specific for TGN46 (a Golgi marker), which was labeled with a secondary (anti-mouse) antibody conjugated to Alexafluor® 488. In contrast, Fig. 4 (panels c; micrographs iv–vi) documented no detectable co-localization between TGN46 and Texas Red-labeled cRTBC in the presence of eGCG (100 μM). These micrographs support the hypothesis that eGCG altered the activity of RTBC. Fig. 4 (panel d) shows the CD spectrum of eGCG at a concentration of 100 μM in PBS. It was of note that eGCG displayed a profound spectra particularly in the 200–230 nm band. This CD response of eGCG means that it may hide changes within the RTBC spectra, if any are present. This negative peak impacts upon the interpretation of the spectra derived from RTBC (panel e) and its summative effect suggests that there was a decrease in order, regarding the secondary (and tertiary) conformation of RTBC (panel e). This may represent a loss of (α-helical) secondary structure. This data was reinforced given the CD spectra of RTBC without eGCG, which remains very similar to those previously published [29].

Table 1
IC_{50} values of experimental materials evaluated herein.

	THP-1 (72 h) μg/mL	Vero (72 h) μg/mL
cRTAC	0.068 ± 0.001	3.525 ± 1.017
cRTBC	9.750 ± 0.744	28.625 ± 2.917
rRTBC	100+	100+
Lactose	58.625 ± 1.361	61.375 ± 11.361
eGCG	100+	200+

Table 1 documents the IC_{50} values characterizing the individual experimental components used herein with THP-1 and Vero cells after 72 h (n = 6 ± SEM). These data are derived from Fig. 1 (panels b & c) and Fig. 2 (panels a & b).

5. Discussion

RT toxicity has been assayed using a variety of methodologies, which have utilized many different cell lines. This variety and diversity of methodology, while underscoring the robust nature of these findings, makes direct comparisons between this work and those of others

Table 2
IC_{50} values describing the toxicity of ricin holotoxin and its inhibition by eGCG and lactose.

	THP-1 (75 h) ng/ml	p value D RT IC_{50}	Vero (75 h) ng/ml	p value D RT IC_{50}
Ricin toxin (RT)	0.54 ± 0.024	–	0.08 ± 0.004	–
Ricin + eGCG	0.68 ± 0.235	0.0013(**)	3.02 ± 0.572	<0.0001(****)
Ricin + Lactose	>10	–	>100	–

Table 2 documents IC_{50} values for RT *in vitro* with and without the proposed inhibitors after 72 h (n = 6 ± SEM). These data are derived from Fig. 1 (panels a & b) and Fig. 2 (panels c & d).

difficult. Previously, we evaluated commercial RT (isolated from *Ricinus communis*) toxicity using B16 cells *in vitro* [28] after 72 h using the same experimental parameters as reported here and the results are comparable. The IC_{50} values published for HUT102 cells [30] are also similar to the values presented herein, once experimental variables and the sensitivity associated with different cell lines are considered (Tables 1 & 2).

In order to gauge the efficiency of any inhibitory activity exerted by eGCG, a positive control was established. The literature suggested the use of lactose [17] as a suitable inhibitor. Above a lactose concentration of 3.4 μg/mL, some toxicity was documented (Fig. 2; panels a & b) presumably due to osmotic effects or the effects of lactose upon non-enzymatic glycosylation [31]. The IC_{50} resulting from treating THP-1 cells with escalating doses of RT and 10 μM lactose were beyond the maximum concentration of RT assayed (10 ng/mL), limited by the results of the re-folding procedure used to anneal the A and B chains. RT inhibition by lactose was also documented in Vero cells and was similar to those documented for THP-1 cells. Supplemental Fig. 1 also shows the inhibitory effect of lactose in a complex mixture (non-fat dried milk (NFDM)), which surprisingly hints at limited lactose bioavailability when NFDM is used in this context.

Treatment of both Vero and THP-1 cells with eGCG resulted in some toxicity above 10 μg/mL and this was to be expected as eGCG has previously been reported to induce apoptosis [32] (Fig. 2; panel a & b) and may be linked to eCGCs ability to switch from acting as a free radical

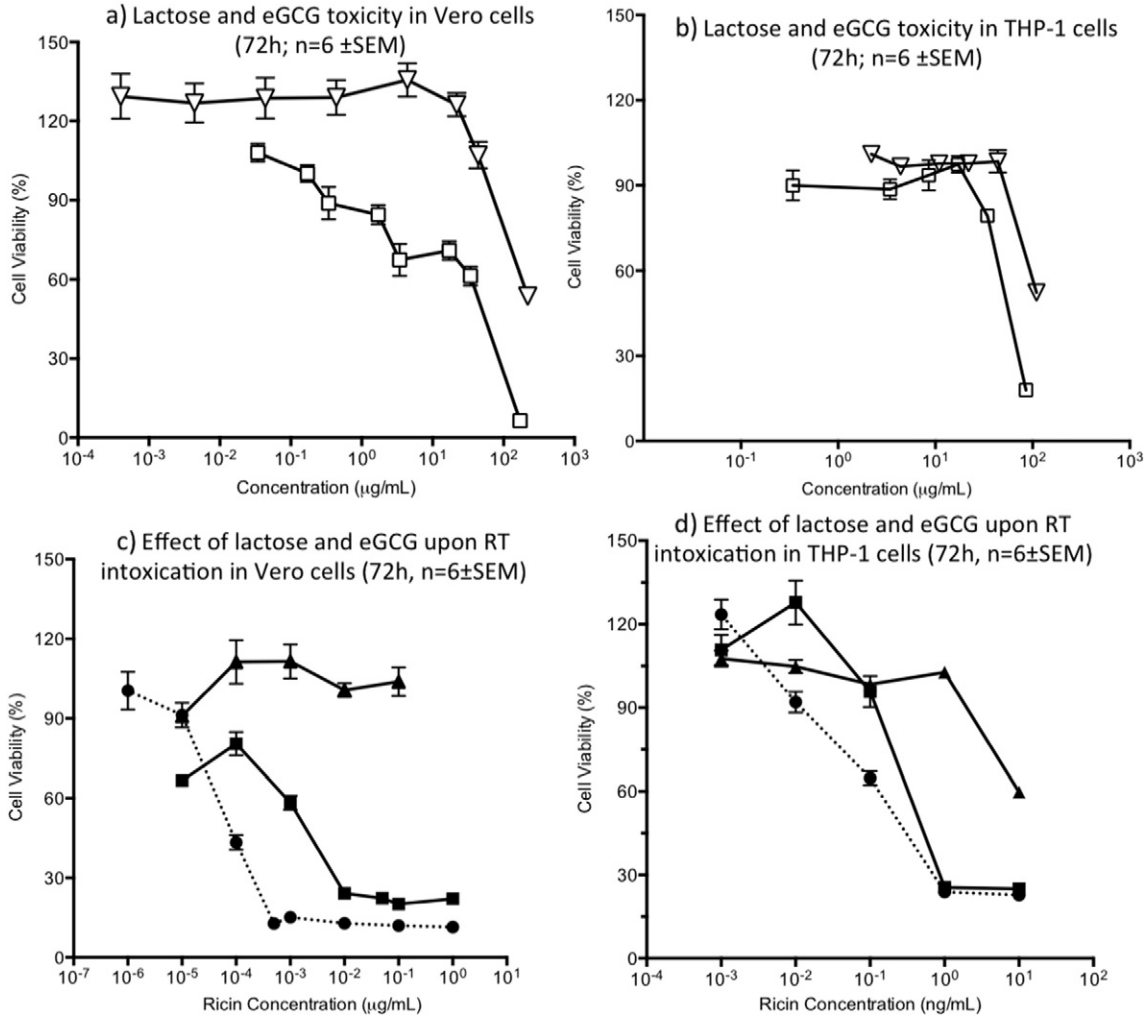

Fig. 2. The effects of potential inhibitors upon RT intoxication. The *in vitro* toxicity profile of both lactose (white square) and eGGC (white triangle) upon Vero cell viability over 72 h (panel a; n = 6 ± SEM) was documented (panel a). A similar characterization was performed using activated THP-1 cells (panel b). The viability of cells exposed to a static concentration of either lactose (10 μM *i.e.* 3.42 μg/mL) (black triangles) or eGGC (100 μM *i.e.* 43.8 μg/mL) (black squares), and increasing concentrations of RT was documented in Vero cells using the MTT assay (72 h; n = 6 ± SEM) (panel c). A similar experiment was performed using activated THP-1 cells (panel d). Data derived from these data sets are summarized (Table 2) where calculated IC_{50} values are recorded.

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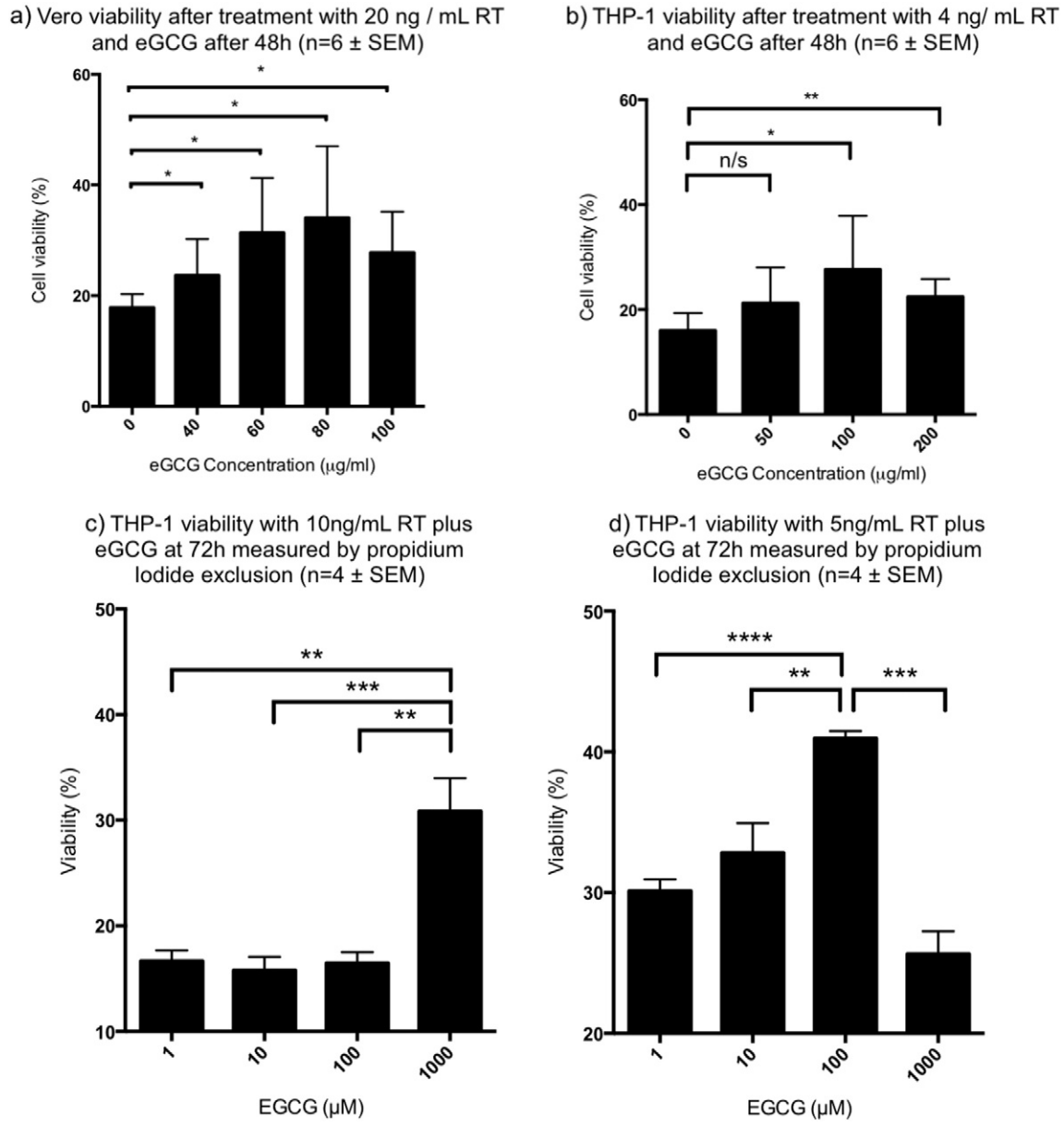

Fig. 3. Effect of varying the concentration of eGCG upon RT lethality. Panel (a) documents the effect of varying concentrations of eGCG upon a static concentration (20 ng/mL) of RT in Vero cells. Cell viability was documented and statistically significant differences in viability were recorded for; 40 ($p = 0.0464$), 60 ($p = 0.0472$), 80 ($p = 0.0233$) and 100 μg/mL ($p = 0.0337$) eGCG relative to no eGCG and 20 ng RT. Viability data in response to challenge with 4 ng/mL RT and varying concentrations of eGCG was also recorded using THP-1 cells (panel b). Statistically significant variation from THP-1 viability after treatment with RT and no eGCG was observed at 100 ($p = 0.0232$) and 200 ($p = 0.0076$) μg/mL of eGCG. These data sets (panels a & b) were acquired over 48 h. These data were acquired using the MTT assay. Flow cytometry and propidium iodide exclusion was also used to measure (non-activated) THP-1 viability to further control for any interaction between MTT and eGCG (panels c & d). Ricin concentrations of (panel c) 10 ng/mL and (panel d) 5 ng/mL RT were used and data were gathered after a 72 h exposure to both RT and eGCG. There was a statistically significant difference in cell viability between cells treated with 10 ng/mL RT *i.e.* with: 1000 μM eGCG ($p = 0.0014$), 100 μM eGCG ($p = 0.0009$) and 10 μM ($p = 0.0015$) and cells treated with 1 μM eGCG (panel c). A statistically significant amount of RT inhibition (5 ng/mL) was mediated by 10 and 100 μg/mL eGCG relative to 1 μM eGCG (panel d). The p values were: 1000 μM eGCG ($p = 0.0021$), 100 μM/mL ($p = 0.0021$) and 10 μM eGCG ($p > 0.0001$) relative to 1 μM. The flow cytometry assays were performed 4 times each and the data represents the mean ± SEM.

scavenger to a free radical generator as a function of pH [33]. Little inhibition of RT was recorded at an eGCG concentration of 10 μM in either cell line (data not shown). Consequently a concentration of 100 μM (43.8 μg/mL) was used. At 100 μM eGCG, Vero cells display significant ($p < 0.0001$) resistance to RT intoxication when IC_{50} values were compared (Table 2). Similarly eGCG also mediated significant inhibition to RT intoxication in THP-1 cells ($p = 0.0013$) upon a comparison of IC_{50} values relative to RT treated cells alone (Table 2). These data sets were

Fig. 4. The effect of eGCG upon RTBC activity. Panel (a) depicts the effect of eGCG (100 μM) upon RTBC's lectinic activity in relation to RTBC binding to lactose-conjugated Sepharose. Panel (b) documents the effect of eGCG upon RTBC uptake by Vero cells over 4 h relative to a 0 °C control. A statistically significant ($p = 0.0039$) inhibition of RTBC uptake was mediated by eGCG (1000 μM). Panel (c) documents the effect of 100 μM eGCG upon the Golgi localization of Texas Red-labeled RTBC after a 60 min (RTBC) pulse and a further 60 min chase. Micrographs (i–iii) show typical co-localization between Texas-Red labeled RTBC and the trans-Golgi specific antibody TGN46. Micrographs (iv–vi) demonstrate that in the presence of 100 μM eGCG, there is no detectable co-localization. Micrographs were representative of the majority of the population of cells observed. Arrows denote co-localization and the size bar represents approximately 5 μm. Panels (d & e) denote the effect of eGCG upon the CD spectra of RTBC. The spectra of eGCG at 100 μM in PBS (dotted line) and 10 μM is shown (panel d) as well as that of RTBC (solid line) as well as RTBC + 100 μM eGCG (dashed line), + 10 μM eGCG (dotted line) and + 5 μM eGCG (dotted and dashed line) in PBS (panel e).

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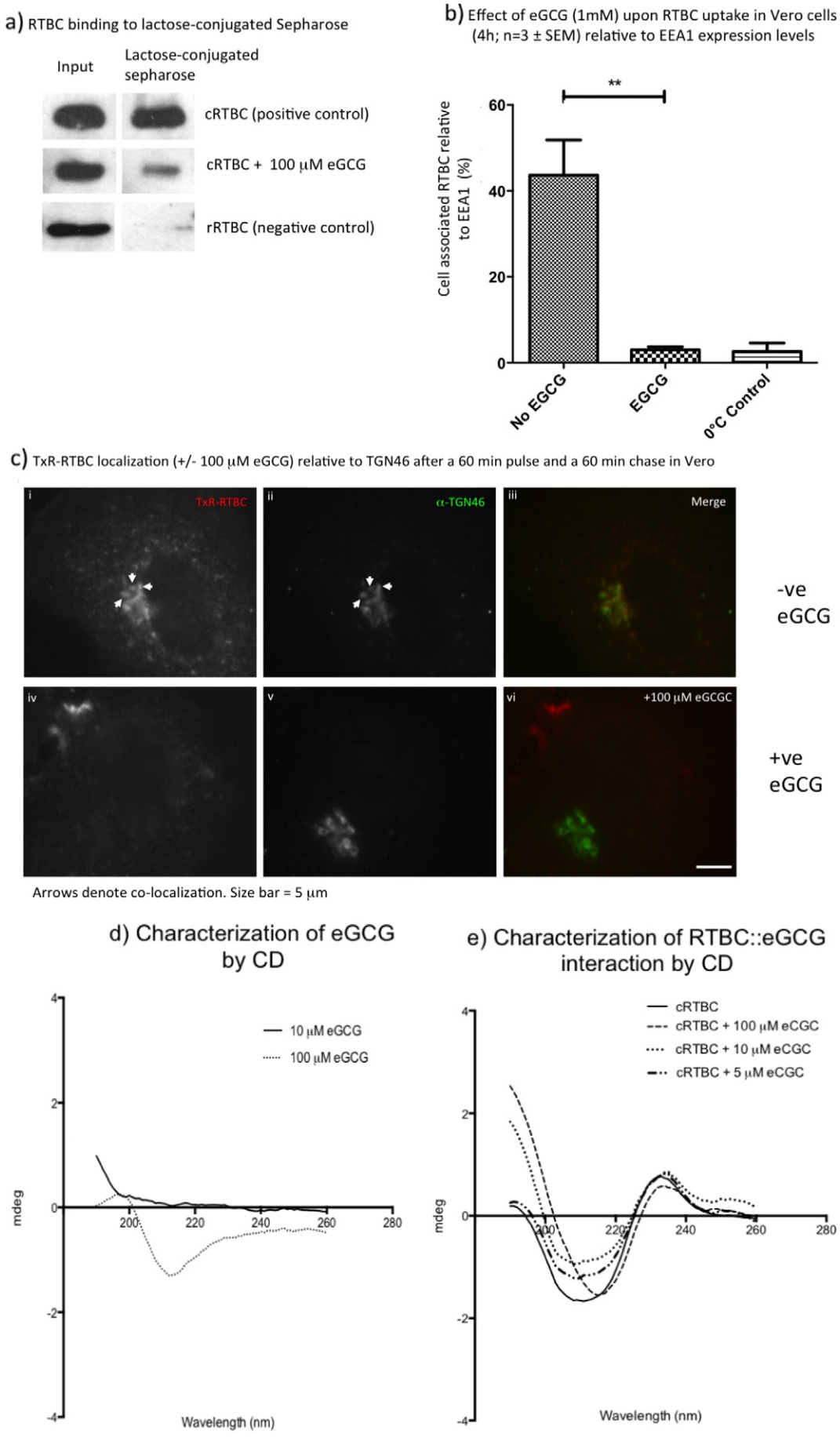

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reinforced when the effect of eGCG upon RT intoxication was measured using a static concentration of RT and variable concentrations of eGCG (Fig. 3; panels a–d). It is of note that protection from RT intoxication by eGCG was observed in an assay that did not require MTT (Fig. 2; panels c & d) negating the possibility that the previous data sets could be subject to false positives as a result of an interaction between eGCG and MTT. It is also important to note that during the assays reported herein, the cells were being stressed by both eGCG and RT and it is for this reason we conjecture that 100% protection from RT intoxication was not documented.

The reported reduction in RT toxicity in the presence of eGCG required some mechanistic explanation. It was hypothesized that the reduction in RT toxicity was due to an alteration in RTBC conformation as a result of either a direct or indirect interaction with eGCG. This interaction has not been defined herein. However, others have reported the ability of thearubigin fractions to bind to both tetanus toxin [20] and botulinum toxin [34]. Given that in the presence of 100 μM eGCG, (i) cRTBC loses its ability to efficiently bind to lactose conjugated-Sepharose (Fig. 4; panel a), (ii) that eGCG reduces RTBC cellular uptake (Fig. 4; panel b) either *via* an interaction with RTBC, an interaction with the cell, reducing endocytic internalization or both, and that (iii) after exposure of Vero cells to Texas Red-conjugated cRTBC, little Golgi localization was documented, it was possible that an interaction between eGCG and cRTBC leads to a change in RTBC conformation which renders it less able to bind to sugars (receptors) on the cell membrane. A consequence of impaired RTBC cell binding would predict that cellular uptake was dramatically reduced as it is likely that RTBC would undergo fluid-phase capture as opposed to a more efficient receptor mediated means of cellular entry facilitating Golgi localization. This hypothesis is shown diagrammatically (Fig. 5). It is of note that there was some interaction between eGCG upon RTAC, which may also hint at the possibility of eGCG activity post-RTAC cytosolic translocation *i.e.* once RTAC has translocated to the cytosol (Fig. 5). However if a synergy between lactose and eGCG is considered, it is unlikely that any effect of eGCG upon RTAC would be measurable *in vitro*. This conjecture is based upon the efficient way lactose prevents RTBC cell uptake and Golgi translocation *i.e.* the RTAC would be prevented from reaching the cytosol by virtue of the interaction of both lactose and eGCG with the B chain. This observation is further underscored by the published data documenting only 5% of internalized ricin in the Golgi apparatus [35]. While both eGCG and lactose inhibited RTBC may well enter the cell (inefficiently) by fluid phase endocytic capture, it is unlikely that this cargo could escape endolysosomal translocation and destruction. These data/observations do lead to questions about the specificity of eGCG with regards to RT A and B chains, however it is worth mentioning at this juncture that eGCG is not ubiquitously active against all protein toxins as the cytotoxicity of *Clostridium difficile* toxin A is unaffected by 100 μg/mL of eGCG (data not shown) in Vero cells after 72 h.

The CD spectrum of RTBC has been previously published [29] and agrees with the spectra of commercial RTBC documented herein (Fig. 4; panel e). The CD spectra of eGCG (100 μM), was recorded and

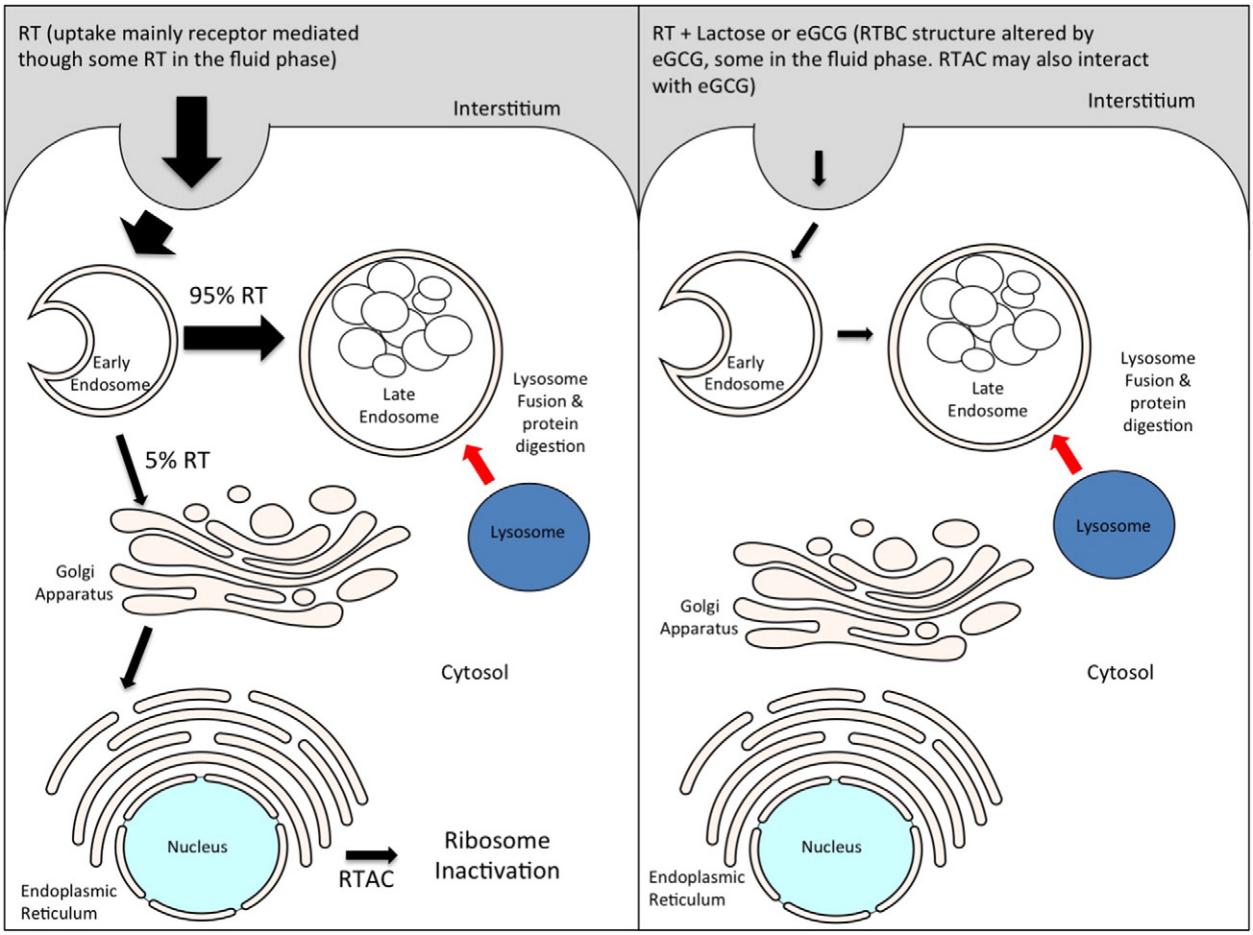

Fig. 5. Cartoon depicting the intracellular trafficking of RT with and without an inhibitor (eGCG or lactose). This cartoon proposes a mechanism to explain the inhibition of RT toxicity mediated by eGCG.
These data are adapted from: [16,35].

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surprisingly a negative peak was evident between 200 and 220 nm. This observation helps when interpreting the CD spectra of RTBC upon the addition of eGCG (100 μM), which may have an additive effect. As the 190–210 nm region of the CD spectra is sensitive to alterations in the amount of helix present, it is likely that upon the addition of eGCG there was a marked change in the amount of disorder associated with RTBC *i.e.* the addition of eGCG results in a decrease in the amount of α-helix within the RTBC. In addition, there was little significant change (given the presence of a contribution to the spectra from eGCG), in the RTBC CD spectra at 232 nm, previously reported to be due to disulfide bond transition [29]. This may indicate that eGCG was not reducing disulfide bonding or the environments of the disulfide bonds between RTBC and RTAC during toxicity experiments, which would result in RTAC behaving like a type I RIP. As no large structural observations were documented in the spectra of RTAC upon the addition of eGCG (data not shown), it is difficult to draw any conclusions about the effects of eGCG upon RTAC structure using this methodology. Further, an investigation into the ability of eGCG to inhibit RTAC using an *in vitro* translation assay was also inconclusive. Although all of the components of this assay behaved as the literature would suggest, the assay itself was inhibited by eGCG at 100 μM (though not at an eGCG concentration of 10 μM) (data not shown). This makes attributing specificity between an eGCG and RTBC interaction difficult and doesn't rule out an inhibition of RTAC by eGCG, contributing to the reduced levels of RT toxicity observed in the presence of eGCG (Table 2). The reduction in biological activity observed was not simply a consequence of protein precipitation and this was reflected not only in the CD data, but also visually during the execution of experiments. No clouding of solutions or increase in turbidity was observed at any time in response to the addition of eGCG. Given that the CD experiments required a protein concentration of 0.3 mg/mL, if protein precipitation was occurring it would have been easy to detect (as it is when 6-His tagged recombinant proteins all too frequently precipitate during dialysis against PBS).

Tetley have estimated that the level of flavonoids in their classic blend to be approximately 156 mg/cup and that of these flavonoids eGCG was predominant [36]. This is in contrast to a recent study evaluating the total flavonoid content of the green tea (16.3 mg/g ± 0.9 mg/g (±SE; n = 38) while the average for the black teas was lower at 12.9 mg/g ± 0.8 mg/g (±SE; n = 34) [37] Given the eGCG activities recorded here, it is unlikely that a cup of tea could provide a feasible antidote to RT intoxication. Given that lactose would only be effective prior to the cellular uptake of RT and the cytosolic translocation of RTAC, and that there are a significant number of people who are lactose intolerant, these intriguing results suggest that there may be value in further investigating eGCG's ability to reduce RTs toxicity with a view to isolating active groups or moieties within eGCG in order to improve its RT inhibitory potential.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2016.03.024.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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