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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±0.004ng/mL where as the IC_{50} for RT + 100μM eGCG was 3.02±0.572ng/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±0.024ng/mL) and RT + 100μM eGCG (0.68±0.235ng/mL) again using 100μM 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 100μg/mL (i.e. 178 and 223μM respectively) of eGCG mediating a significant ($p=0.0472$ and 0.0232) reduction in ricin toxicity at 20 and 4ng/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) 10ng/mL and 5ng/mL of RT was used. The addition of 1000μM and 100μM eGCG mediated a significant ($p=0.0015$ and <0.0001 respectively) reduction in ricin toxicity relative to an identical concentration of ricin with 1μg eGCG. Further, eGCG (100μM) 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.

Keywords: ricin toxin, endocytosis, polyphenol, epigallocatechin gallate, eGCG, tea

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

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 Centre 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 disulphide 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 lectinic activity [9] and mediates the translocation of RT A chain (RTAC) to the endoplasmic reticulum (ER) [10]. RTBC is internalized via clathrin, caveolin and non-clathrin mediated endocytosis and 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, 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.

Materials and Methods: 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).

Texas Red-labeled cRTBC: labeling was performed using Texas Red®-X, succinimidyl ester, mixed isomers (Invitrogen, Paisley, UK) following the manufacturer's instructions and as previously described for labeling BSA [24]. Commercial (c) RTAC and cRTBC were from Vector labs (Peterborough, UK) and re-folded 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 re-folded RT has been documented in the experimental section the amounts indicated refer to the amount of RTAC in the preparation and not 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).

Cell culture and Microscopy: RPMI 1640 medium, Dulbecco's minimal essential medium, glutamax, penicillin/streptomycin, Dulbecco's Modified Eagles Medium without sodium pyruvate with 450mg/mL glucose, kanamycin and fetal calf serum (FCS) were from Invitrogen (Paisley, UK). The 6 well treated cluster plates and sterile 22×22x0.1mm 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).

Methods: Cloning and expression of recombinant (r)RTBC: A pUC19-derived plasmid encoding E.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 DNA Sequencing and Services at Dundee University, (Dundee, UK). Protein expression, enrichment from bacterial lysate and characterization by immunoblotting was performed as previously reported [27].

Cell culture: Immunofluorescent Microscopy: This methodology has been extensively described and discussed previously [24]. Briefly, after being exposed to either Texas Red-labeled RTBC (50µg/mL each treatment) or Texas Red-labeled RTBC with eGCG (100µM each treatment), and left for 4h 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 Alexa488-labelled secondary antibody. Following a subsequent wash step (3 x 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 x60 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).

In vitro toxicity assay: This methodology has been described extensively [28] and assays were conducted over 48 or 72h (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 10μM 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 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 10ng/mL propidium iodide. The cells were then subject to analysis at 488nm. 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.

RTBC uptake experiments: Here $5x10^5$ 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^oC in a humidified atmosphere containing 5%(v/v) CO_2). The next day, either RTBC (50μg/mL) or RTBC plus eGCG (1mM) were added along with a third treatment which had only RTBC added and was kept at 0^oC through out the experiment. The remaining two plates were placed back under standard culture conditions. After 4h 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 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 transfer had completed the blots were blocked with PBS containing 0.01% (v/v) TWEEN 20 and 5% (w/v) none fat dried milk. Blots were then cut in half to allow then 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 manufacturers instructions, using Pierce™ ECL reagent (Thermo Scientific; Waltham, MA USA).

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, 100μl 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 x 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).

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

Results: The characterization of RT, RTBC and RTAC was performed (figure 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 (figure 1; panel a). These data (figure 1; panel a) were important for the validation of the re-folded RT. When re-folded RT was characterized by immunoblotting, typically a 2:1 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]. Whilst this procedure was not optimal, it was necessary, given the current availability of commercial RT holotoxin in the UK.

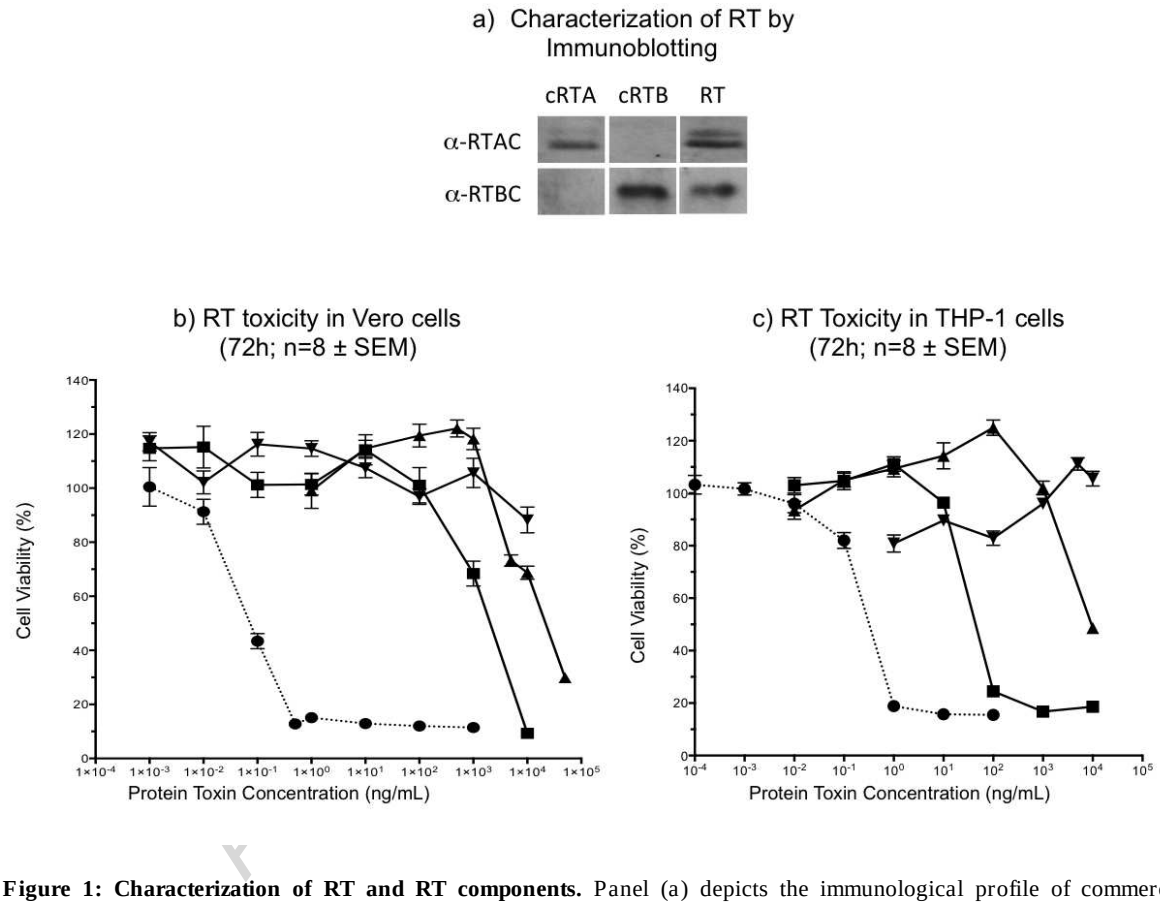


Figure 1: Characterization of RT and RT components. Panel (a) depicts the immunological profile of commercially obtained *("c")* RTAC, cRTBC, and refolded RT when probed (individually) with antibodies specific for RTAC or RTBC. Panel (b & c) document the in vitro toxicity profiles of refolded ricin holotoxin (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 72h 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.

To characterize refolded 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 (figure1; panel b & c) are summarized (in table 1 & 2) and in each instance the refolded RT holotoxin displayed the highest level of toxicity (IC_{50} Vero 0.08±0.004ng/ml; THP-1 0.54±0.024ng/mL), followed by RTAC (IC_{50} Vero 3.525±1.017µg/mL; THP-1 0.068±0.001µg/mL), cRTBC (IC_{50} Vero 28.625±2.917µg/mL; THP-1 9.750±0.744µg/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 (figure 2; panel 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 (figure 2). These data are shown for Vero cells (figure 2; panel c) and THP-1 cells (figure 2; panel d). Figure 2 (panel c) documents a statistically significant inhibition of RT by: eGCG (43.8µg/ml (i.e. 100µM)) ($p>0.0001$), when tested in Vero cells. The addition of lactose (3.42µg/ml (i.e. 10µM)) to RT prior to incubation with Vero cells resulted in an IC_{50} value that was greater than 10ng/ml (RTAC equivalent) under similar assay conditions to those reported in figure 2 (panel d). A similar concentration of lactose gave rise to an IC_{50} value of >10ng/mL (RTAC equivalent) when measured in THP-1 cells. Similarly figure 2 (panel d) documents a statistically significant inhibition of RT by eGCG ($p=0.0013$) when measured using THP-1 cells.

	THP-1 (72h) µg/ml	Vero (72h) 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: IC_{50} values of experimental materials evaluated herein

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

	THP-1 (72h) ng/ml	P value D RT IC_{50}	Vero (72h) 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: IC_{50} values describing the toxicity of ricin holotoxin and its inhibition by eGCG and lactose

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

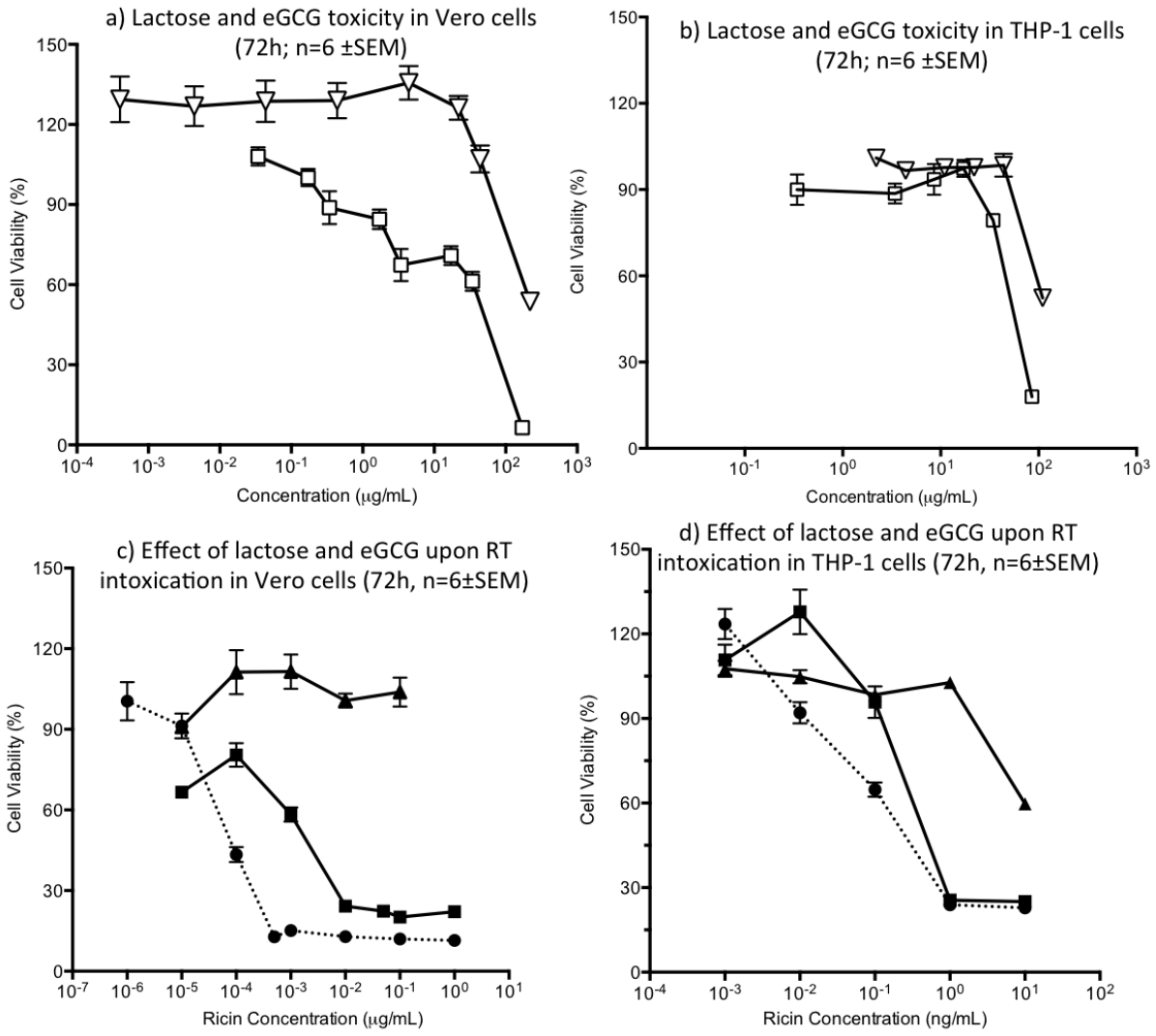


Figure 2: The effects of potential inhibitors upon RT intoxication. The in vitro toxicity profile of both lactose (white square) and eGGC (white triangle) upon Vero cell viability over 72h (panel a; n=6 ± SEM) was documented (panel a). A similar characterization was performed using activated THP-1 cells (panel b). The viability of cells exposed to a static concentration of either lactose (10μM i.e. 3.42μg/ml) (black triangles) or eGGC (100μM i.e. 43.8μg/ml) (black squares), and increasing concentrations of RT was documented in Vero cells using the MTT assay (72h; 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.

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 (figure 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; figure 2; panels c & d). A consequence of this was the choice of static concentrations of RT used to generate figure 3. Figure 3 (panel a) shows the effect of a lethal concentration of RT (20ng/ml) when incubated with increasing amounts of eGCG upon Vero viability after 48h. Figure 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 40μg/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. Figure 2 (panel b) shows the effect of eGCG (50-200μg/ml) upon THP-1 cell viability after incubation with RT (4ng/ml) at a lower concentration. Again a significant difference in viability is reported at a dose of eGCG of 100μg/ml (p=0.0232) and 200μg/ml (p=0.0076) relative to the RT only control (figure 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 (figure 3; panel c and d). This assay used more eGCG than had been previously assayed (figure 2; panels a-d) and shows an increase in cell viability (p=<0.0014) (1000μM eGCG relative to 1μM eGCG) when THP-1 cells were treated with 10ng/mL RT (figure 3; panel c). When a reduced concentration of RT was used (5ng/mL) (figure 3; panel c), a statistically significant increase in cell viability was recorded when a dose of 1000μM eGCG was compared to 1μM of eGCG (p>0.0001).

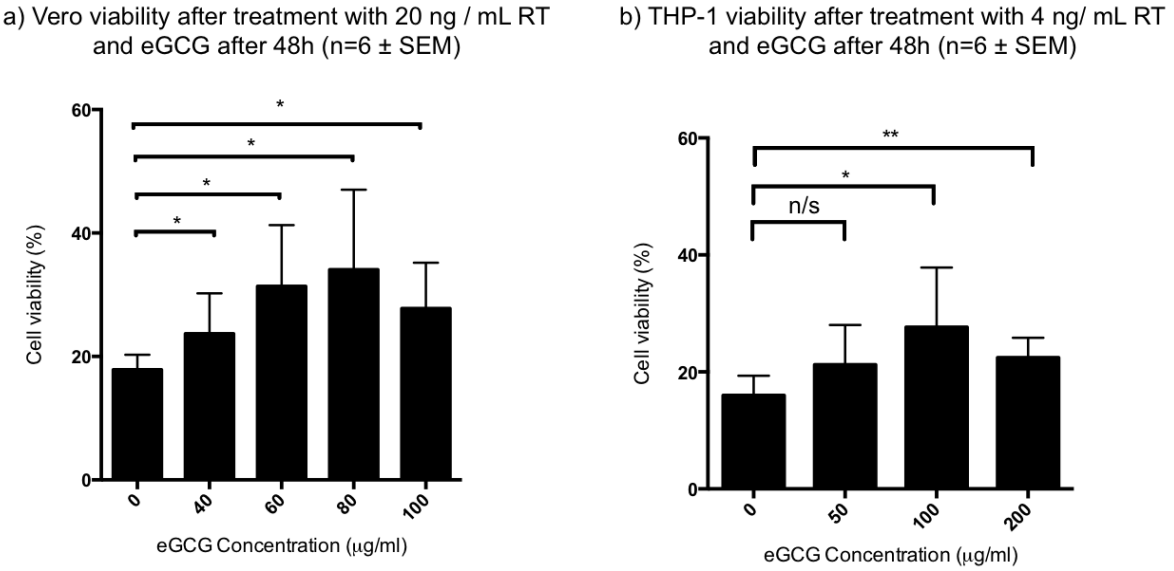
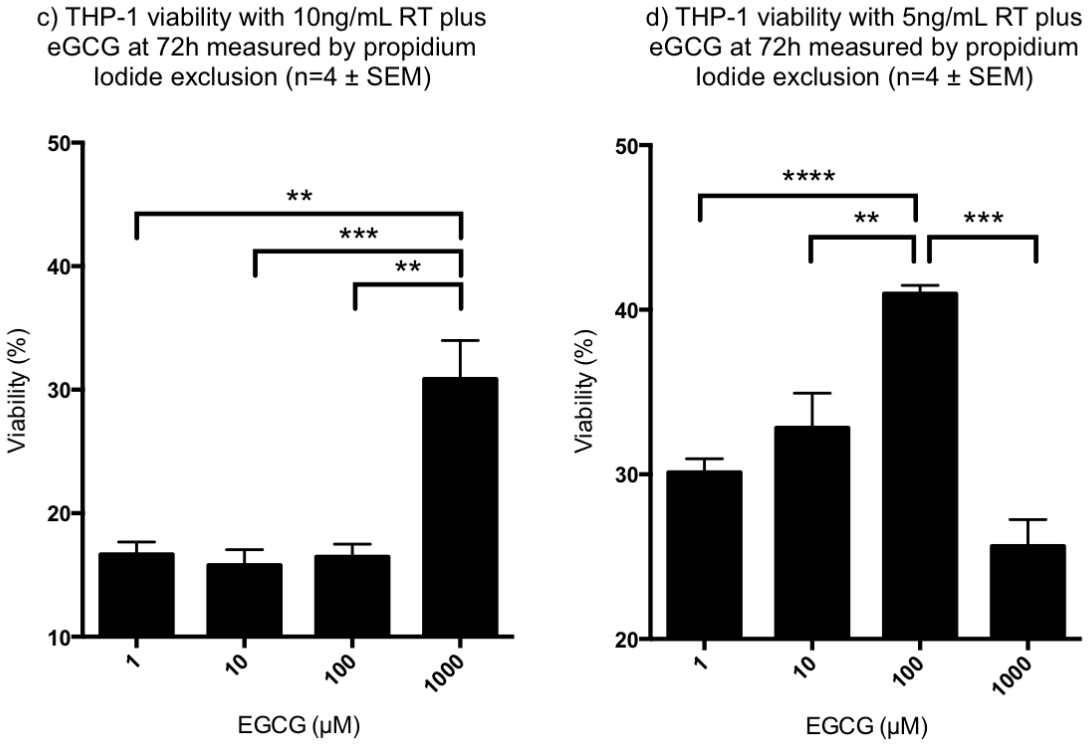


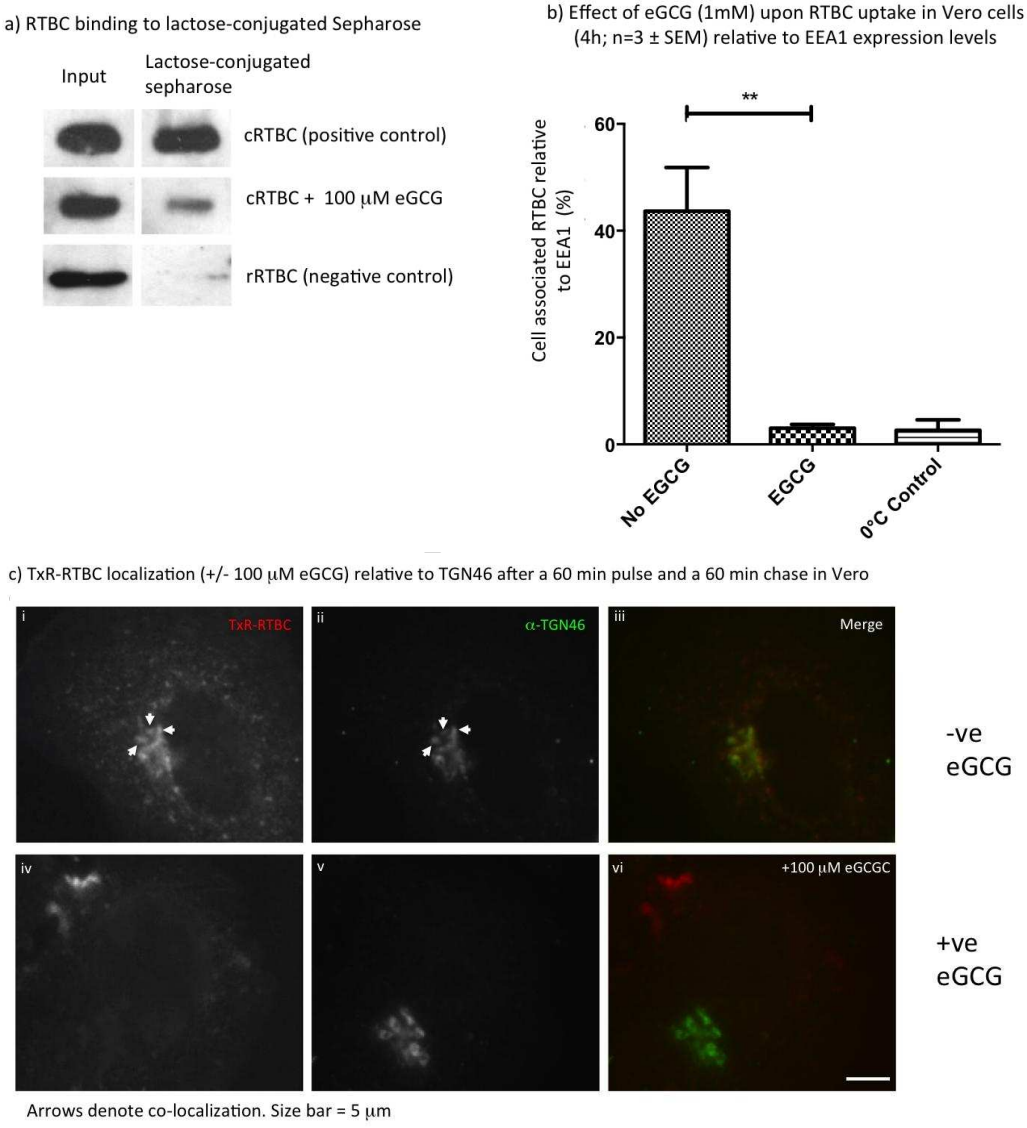
Figure 3: Effect of varying the concentration of eGCG upon RT lethality. Panel (a) documents the effect of varying concentrations of eGCG upon a static concentration (20ng/mL) of RT in Vero cells. Cell viability was documented and statistically significant differences in viability were recorded for; 40 (p=0.0464), 60 (p=0.0472), 80 (p=0.0233) and 100μg/ml (p=0.0337) eGCG relative to no eGCG and 20ng RT. Viability data in response to challenge with 4ng/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 (panel a & b) were acquired over 48h. 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) 10ng/ml and (panel d) 5ng/ml RT were used and data were gathered after a 72h exposure to both RT and eGCG. There was a statistically significant difference in cell viability between cells treated with 10ng/ml RT i.e. with: 1000μM eGCG (p=0.0014), 100μM eGCG (p=0.0009) and 10μM (p=0.0015) and cells treated with 1μM eGCG (panel c). A statistically significant amount of RT inhibition (5ng/ml) was mediated by 10 and 100μg/mL eGCG relative to 1μM eGCG (panel d). The p values were: 1000μM eGCG (p=0.0021), 100μM/ml (p=0.0021) and 10μM eGCG (p>0.0001) relative to 1μM. The flow cytometry assays were performed 4 times each and the data represents the mean ± SEM.



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 (figure 4; panel a) where the ability of cRTBC to bind to lactose-conjugated Sepharose with and without eGCG (100µM) relative to rRTBC (the negative control) was investigated. Reduced cRTBC lectinic activity in the presence of eGCG (100µM) 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 4h (figure 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. Figure 4 (panel b) shows that there was a reduction in RTBC uptake by Vero cells ($p=0.0039$) incubated with RTBC and 1mM eGCG relative to cells only incubated with RTBC at 37^{o}C and at 0^{o}C. The 0^{o}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 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 (figure 4; panel c). Figure 4 (panel c; micrographs i to iii) were captured in the absence of eGCG and show 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, figure 4 (panels c; micrographs iv-vi) documented no detectable co-localization between TGN46 and Texas Red-labeled cRTBC in the

presence of eGCG (100μM). These micrographs support the hypothesis that eGCG altered the activity of RTBC. Figure 4 (panel d) shows the CD spectrum of eGCG at a concentration of 100μM 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].



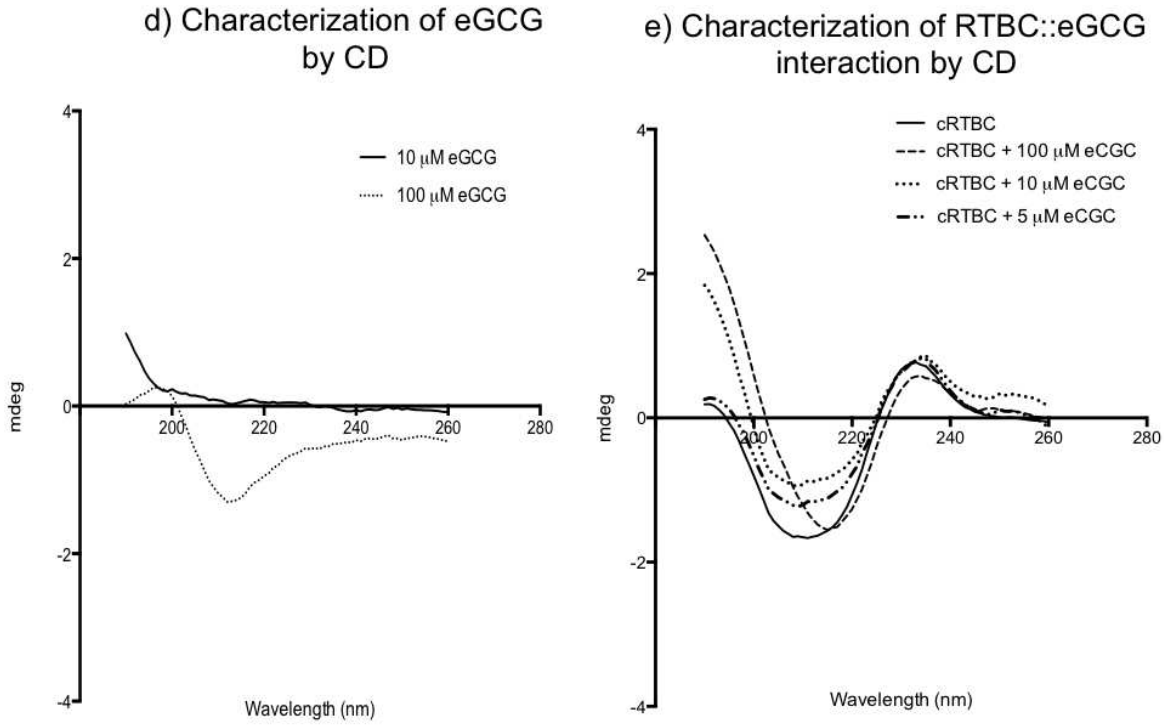


Figure 4: The effect of eGCG upon RTBC activity. Panel (a) depicts the effect of eGCG (100μM) upon *RTBC's* lectinic activity in relation to RTBC binding to lactose-conjugated Sepharose. Panel (b) documents the effect of eGCG upon RTBC uptake by Vero cells over 4h relative to a 0^{o}C control. A statistically significant ($p=0.0039$) inhibition of RTBC uptake was mediated by eGCG (1000μM). Panel (c) documents the effect of 100μM 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 100μM 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 microns. Panels (d & e) denote the effect of eGCG upon the CD spectra of RTBC. The spectra of eGCG at 100μM in PBS (dotted line) and 10μM is shown (panel d) as well as that of RTBC (solid line) as well as RTBC +100μM eGCG (dashed line), +10 μM eGCG (dotted line) and +5μM eGCG (dotted and dashed line) in PBS (panel e).

Discussion: RT toxicity has been assayed using a variety of methodologies, which have utilized many different cell lines. This variety and diversity of methodology, whilst underscoring the robust nature of these findings, makes direct comparisons between this work and those of others difficult. Previously, we evaluated commercial RT (isolated from Ricinus communis) toxicity using B16 cells in vitro [28] after 72h 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 (table 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 3.4µg/mL, some toxicity was documented (figure 2; panels a & b) presumably due to osmotic effects or the effects of lactose upon none-enzymatic glycosylation [31]. The IC_{50} resulting from treating THP-1 cells with escalating doses of RT and 10µM lactose were beyond the maximum concentration of RT assayed (10ng/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 were similar to those documented for THP-1 cells. Supplemental figure 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 10µg/ml and this was to be expected as eGCG has previously been reported to induce apoptosis [32] (figure 2; panel a & b) and may be linked to eCGCs ability to switch from acting as a free radical scavenger to a free radical generator as a function of pH [33]. Little inhibition of RT was recorded at an eGCG concentration of 10µM in either cell line (data not shown). Consequently a concentration of 100µM (43.8µg/ml) was used. At 100µM 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 reinforced when the effect of eGCG upon RT intoxication was measured using a static concentration of RT and variable concentrations of eGCG (figure 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 (figure 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 100µM eGCG, (i) cRTBC loses its ability to efficiently bind to lactose conjugated-Sepharose (figure 4; panel a), (ii) that eGCG reduces RTBC cellular uptake (figure 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 (figure 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 (figure 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]. Whilst 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 100µg/mL of eGCG (data not shown) in Vero cells after 72h.

The CD spectrum of RTBC has been previously published [29] and agrees with the spectra of commercial RTBC documented herein (figure 4; panel e). The CD spectra of eGCG (100µM), was recorded and surprisingly a negative peak was evident between 200-220nm. This observation helps when interpreting the CD spectra of RTBC upon the addition of eGCG (100µM), which may have an additive effect. As the 190-210nm 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 232nm, previously reported to be due to disulphide bond transition [29]. This may indicate that eGCG was not reducing disulphide bonding or the environments of the disulphide 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 10µM) (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 0.3mg/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) whilst 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.

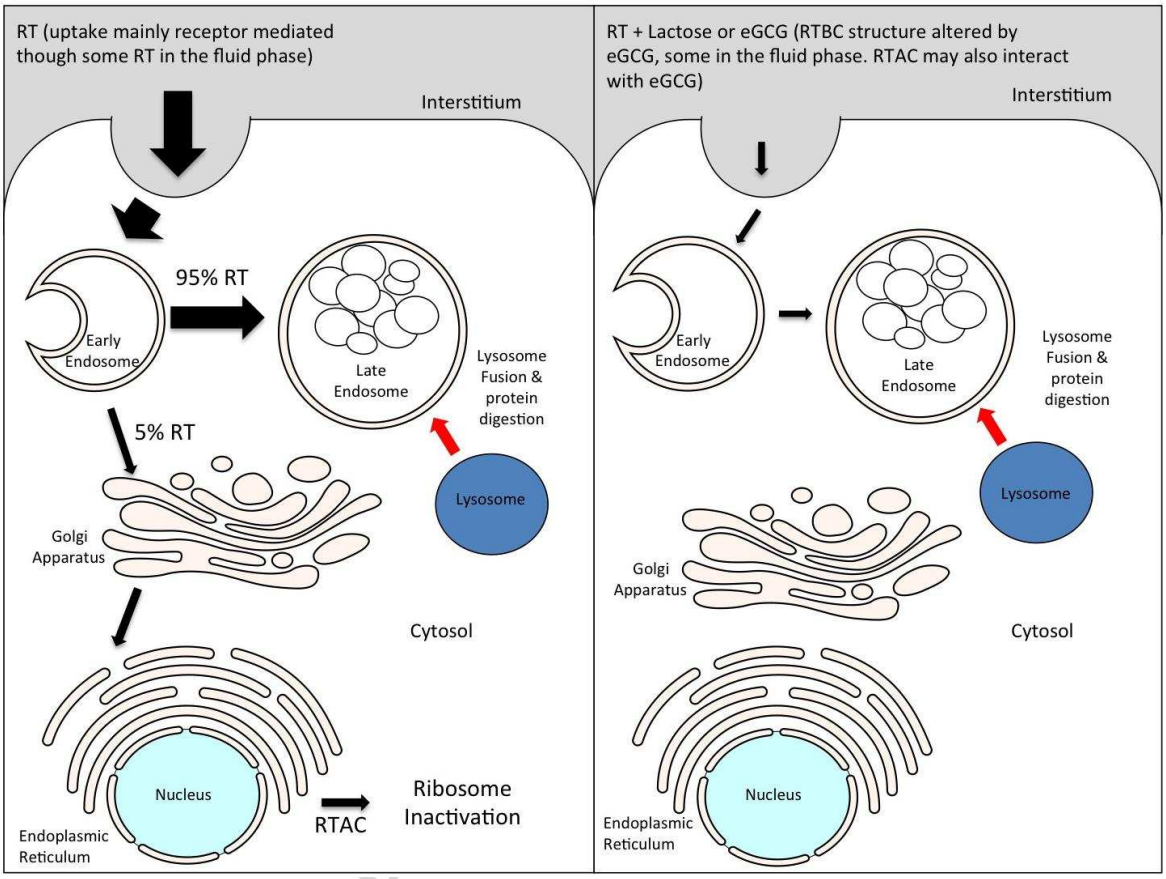


Figure 5: Cartoon depicting the intracellular trafficking of RT with and without an inhibitor (eGCG or lactose). This cartoon proposes a mechanism to explain the inhibition of RT toxicity mediated by eGCG. These data are adapted from: [16, 35].

References:

CDC (2000) Biological and Chemical terrorism; strategic plan for preparedness and response recommendations of the CDC Strategic Planning Workgroup MMWR 49(RR-4); 1-14. http://www.cdc.gov/mmwr/preview/mmwrhtml/rr4904a1.htm.

2. Maman M, and Yehezkelli Y, (2009) Ricin a possible non-infectious biological weapon. In: Bioterrorism and Infectious Agents. Eds Fong I. W. and Alibek K. Springer Dordrecht, Heidelberg, London, New York. ISBN 978-1-4419-1265-7 pp 205-216. http://link.springer.com/chapter/10.1007%2F0-387-23685-6_8.

3. Goddard J, (2011) Yemen: Barack Obama warned that al-Qaeda planning ricin attack. The Telegraph. Aug 13th.http://www.telegraph.co.uk/news/worldnews/middleeast/yemen/8699400/Yemen-Barack-Obama-warned-that-al-Qaeda-planning-ricin-attack.html#

4. Roxas-Duncan VI, and Smith LA, (2012) Of beans and bread; Ricin and Abrin in bioterrorisum and biocrime. J Bioterr Biodef S7; 002. http://omicsonline.org/of-beans-and-beads-ricin-and-abrin-in-bioterrorism-and-biocrime-2157-2526.S2-002.php?aid=4686?aid=4686. doi: 10.4172/2157-2526.S2-002

5. Hulse C. (2004) Tests indicate poison in senate mail room of majority leader. New York Times, late ed., p12. February 3rd. http://www.nytimes.com/2004/02/03/us/tests-indicate-poison-in-senate-mail-room-of-majority-leader.html.

6. Reisler RB. and Smith LA. (2012) The need for continued development of ricin countermeasures. Adv. Prev. Med. 2012:149737. http://www.hindawi.com/journals/apm/2012/149737/. http://dx.doi.org/10.115 5/2012/149737.

7. Franz DR, and Jaax NK, (1997) Ricin Toxin In:Textbook of military medicine: Medical Aspects of Chemical and Biological Warfare. Eds Sidell, F. R. Takafuji E. T. Franz D. R. TMM Publications, Washington DC. http://www.bvsde.ops-oms.org/tutorial1/fulltex/armas/textos/chebio/chebio.pdf.

8. Rutenber E, Ready M, Robertus JD, (1987) Structure and evolution of Ricin B chain. Nature 326; 624-626. http://www.ncbi.nlm.nih.gov/pubmed/3561502.

9. Swimmer C, Lehar SM, McCafferty J, Chiswell DJ, Blatter WA, et al., (1992) Phage display of ricin B chain and its single binding domain: System for screening galactose binding mutants. PNAS USA 89; 3756-3760. http://www.ncbi.nlm.nih.gov/pubmed/1373889.

10. Sandvig, K. and van Deurs, B. (2005) Delivery into cells: lessons learned from plant and bacterial toxins. Gene Ther. **12**(11); 865-72. http://www.ncbi.nlm.nih.gov/pubmed/15815697.

11. Spooner RA, and Lord JM, (2012) How Ricin and Shiga toxin reach the cytosol of target cells: retrotranslocation from the ER. Curr Top. Microbiol. Immunol. **357**; 19-40. http://www.ncbi.nlm.nih.gov/pubmed/21761287. doi: 10.1007/82_2011_154.

12. Soler-Rodriguez AM, Uhr JW, Richardson J, Vitetta ES, (1992) The toxicity of chemically deglycosylated ricin A chain in mice. International Journal of Immunopharmacology **14**(2); 281-291. http://www.ncbi.nlm.nih.gov/pubmed/1624227.

13. Smallshaw JE, Richardson JA, and Vitetta ES. (2007) RiVax, a recombinant ricin subunit vaccine, protects mice against ricin delivered by gavage or aerosol. Vaccine, **25**(42); 7459–7469. http://www.ncbi.nlm.nih.gov/pubmed/17875350.

14. Stechmann B, Bai SK, Gobbo E, Lopez R, Merer G, et al., (2012) Inhibition of retrograde transport protects mice from lethal ricin challenge. Cell, **141**(2); 231-242. http://www.ncbi.nlm.nih.gov/pubmed/20403321. doi: 10.1016/j.cell.2010.01.043.

15. McGuinness CR, and Mantis NJ, (2006), Characterization of a Novel High-Affinity Monoclonal Immunoglobulin G Antibody against the Ricin B Subunit. Infect and Immunity. **74**(6); 3463–3470. http://www.ncbi.nlm.nih.gov/pubmed/16714577.

16. Yermakova A, Klokk TI, Cole R, Sandvig K, Mantis NJ, (2014) Antibody-Mediated Inhibition of Ricin Toxin Retrograde Transport. MBio. **5**(2); e00995-13. http://mbio.asm.org/content/5/2/e00995-13.short. doi:10.1128/mBio.00995-13.

17. Rasooly R, He X, and Friedman M, (2011) Milk Inhibits the Biological Activity of Ricin. J. Biol Chem. **287**(33); 27924-9. http://www.ncbi.nlm.nih.gov/pubmed/22733821. doi: 10.1074/jbc.M112.362988

18. Nicolson GL, and Blaustein J, (1972) The Interaction of Ricin Communis agglutinin with normal and tumor cell surfaces. Biochemica et Biophysica acta, **226**; 543-547. http://www.ncbi.nlm.nih.gov/pubmed/4338881.

19. Dell'Aica I, Dona M, Tonello F, Piris A, Mock M, et al., (2004) Potent inhibitors of anthrax lethal factor from green tea. EMBO Reports **5**(4); 418-22. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1299029/. doi: 10.1038/sj.embor.7400118

20. Satoh E, Ishii T, Shimizu Y, Sawamura S and Nishimura M. (2002) A mechanism of the thearubigin fraction of black tea (Camellia sinensis) extract protecting against the effect of tetanus toxin. J. Toxicol. Sci. **27**(5); 441-7. http://www.ncbi.nlm.nih.gov/pubmed/12533914.

21. Satoh E, Ishii T, Shimizu Y, and Nishimura M, (2001) Black tea extract, thearubigin fraction, counteracts the effects of botulinum neurotoxins in mice. Br J. Pharmacol. **132**; 797-798. http://www.ncbi.nlm.nih.gov/pubmed/11181419.

22. Isogai E, Isogai H, Hirose K, Hayashi S, Oguma K, (2001) In vivo synergy between green tea extract and levofloxacin against enterohemorrhagic Escherichia coli O157 infection. Curr Microbiol **42**; 248-25. http://www.ncbi.nlm.nih.gov/pubmed/11178724.

23. Friedman M, (2007) Overview of antibacterial, antitoxin, antiviral and antifungal activities of tea flavonoids and teas. Mol. Nutr. Food Res **51**; 116-134. http://www.ncbi.nlm.nih.gov/pubmed/17195249.

24. Richardson SCW, Wallom K-L, Ferguson EL, Deacon SPE, Davies MW, et al., (2008) The use of fluorescence microscopy to define polymer localisation to the late endocytic compartments in cells that are targets for drug delivery. Journal of Controlled Release **127**; 1–11. http://www.ncbi.nlm.nih.gov/pubmed/18281120. doi: 10.1016/j.jconrel.2007.12.015

25. Smith DC, Marsden CJ, Lord MJ, Roberts LM. (2003) Expression, Purification and Characterization of Ricin vectors used for exogenous antigen delivery into the MHC Class I presentation pathway. Biol. Proced. Online. **5**(1); 13-19. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC150387/. doi: 10.1251/bpo42

26. Challing K, Challing A, Murray E, Fladin B, Houston LL, et al., (1985) Genomic cloning and characterization of a ricin gene from Ricinus communis. Nucleic Acids Research, **13**(22); 8019-8033. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC322107/.

27. Richardson SCW, Winistorfer SC, Poupon V, Luzio JP, and Piper RC, (2004) Mammalian Late Vacuole Protein Sorting Orthologues Participate in Early Endosomal Fusion and Interact with the Cytoskeleton. *Molecular Biology of the Cell,* **15**; 1197–1210. http://www.ncbi.nlm.nih.gov/pubmed/14668490.

28. Pattrick NG, Richardson SC, Casolaro M, Ferruti P and Duncan R. (2001) Poly(amidoamine)-mediated intracytoplasmic delivery of ricin A-chain and gelonin. *Journal of Controlled Release* **77**; 225–232. http://www.ncbi.nlm.nih.gov/pubmed/11733090.

29. Wawrzynczak EJ, Drake AF, and Thorp PE, (1988) Circular dichroism of isolated ricin A- and B-chains. Biophys Chem, **31** (3); 301-305. http://www.ncbi.nlm.nih.gov/pubmed/3233304.

30. Tagge EP, Chandler J, Harris B, Czako H, Marton L, et al., (1996) Preproricin expressed in Nicotiana tabacum cells in vitro is fully processed and biologically active. Protein Expr Purif. **8**(1); 109-118. http://www.ncbi.nlm.nih.gov/pubmed/8812841.

31. Ledesma-Osuna AI, Ramos-Clamont G, Vázquez-Moreno L. (2008) Characterization of bovine serum albumin glycated with glucose, galactose and lactose. Acta Biochim Pol. **55**(3); 491-7. http://www.ncbi.nlm.nih.gov/pubmed/18797521.

32. Siddiqui IA, Bharali DJ, Nihal M, Adhami VM, Khan N, et al., (2014) Excellent anti-proliferative and pro-apoptotic effects of (-)-epigallocatechin-3-gallate encapsulated in chitosan nanoparticles on human melanoma cell growth both in vitro and in vivo. Nanomedicine. S1549-9634(14)00220-2. http://www.ncbi.nlm.nih.gov/pubmed/24965756. doi: 10.1016/j.nano.2014.05.007.

33. Hagerman AE, Dean RT, Davies MJ. (2003) Radical chemistry of epigallocatechin gallate and its relevance to protein damage, Arch Biochem Biophys. **414**(1); 115-20. http://www.ncbi.nlm.nih.gov/pubmed/12745262.

34. Satoh E, Ishii T, Shimizu Y, Sawamura S. and Nishimura M. (2002b) The mechanism underlying the protective effect of the thearubigin fraction of black tea (Camellia sinensis) extract against the neuromuscular blocking action of botulinum neurotoxins. Pharmacol. Toxicol. **90**(4); 199-202. http://www.ncbi.nlm.nih.gov/pubmed/12076314.

35. van Deurs B., Sandvig K., Petersen O.W., Olsnes S., Simons K., Griffiths G., (1988) Estimation of the amount of internalized ricin that reaches the trans-Golgi network, J Cell Biol. **106**(2): 253-67.

36. www.tetleyusa.com/Over-a-cuppa/tetley-tea-faq#FAQ1.

37. McCully W, (2013) The Antibacterial Activity of Tea Infusions and Their Effect Against The Hospital Pathogen Clostridium difficile. PhD Thesis, Cardiff University. http://orca.cf.ac.uk/52337/.

Highlights

Epigallocatechin gallate (eGCG) has inhibitory activity against ricin toxin (RT).

The reduction in ricin toxicity mediated by eGCG was concentration dependent.

EGCG was found to reduce the binding of RT B chain to lactose-conjugated Sepharose.

EGCG significantly reduce the uptake of RTB chain in Vero cells.

EGCG may provide a starting point to refine substances that reduce ricin toxicity.