

Anacardic acid, a histone acetyltransferase inhibitor, stimulates hemolysis and eryptosis through energy depletion and calcium accumulation

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ABSTRACT

Histone acetyltransferase (HAT) inhibitors are promising epigenetic anticancer therapeutics. However, challenges remain regarding tissue specificity and off-target toxicity. This study presents a preclinical safety assessment of the HAT inhibitor anacardic acid (AA) in erythrocytes, given that anaemia is a clinically significant complication of chemotherapy. Freshly isolated erythrocytes were cultured *in vitro* with and without 1–10 $\mu\text{mol L}^{-1}$ of AA, then markers and mechanisms of cell death were examined by fluorescence-activated cell sorting, spectrophotometry, and potentiometry. AA treatment induced marked hemolysis independently of oxidative stress, which was paralleled by Ca^{2+} accumulation and phosphatidylserine (PS) translocation. Morphological abnormalities were also observed upon AA treatment, including eryptotic loss of volume and elliptocyte phenotype. Co-treatment of AA with ATP, acetylsalicylic acid, NSC23766, or polyethylene glycol conferred significant cytoprotective effects. AA is cytotoxic to erythrocytes, causing both hemolysis and eryptosis, through non-genomic mechanisms involving Ca^{2+} signalling, metabolic exhaustion, K^{+} depletion, shrinkage and elliptocytic morphological aberrations. This toxic effect is sensitive to cyclooxygenase and Rac1 GTPase inhibition.

Keywords: anacardic acid, eryptosis, hemolysis, calcium, cyclooxygenase, Rac1 GTPase

INTRODUCTION

Anacardic acid (AA; also called ginkgolic acid C15:0) is a phenolic lipid derived from the oil of *Anacardium occidentale* (cashew) nutshell. AA has a broad range of biological effects, including antidiabetic, antibacterial, antiviral, antifibrotic, neuroprotective, and anticancer properties (1). Moreover, AA is a histone acetyltransferase (HAT) and protein SUMOylation inhibitor (2). The antiproliferative effects of AA have been reported in prostatic (3, 4), adenoma (5), hepatocellular, myeloma (6), lung (7), lymphoma (8), leukemia (9), pancreatic (10), breast, colorectal, cervical (11), and rhabdomyosarcoma (2) cancer cells. AA inhibits cancer cell proliferation through apoptosis and autophagy as it downregulates p300, Bcl-2, Bim, Bid, Akt/mTOR, survivin, XIAP, FOXO3a, Hsp70, and Strap, and stimu-

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lates Bax, Bak, AIF, PARP, caspase-9/-3/-8, p53, p21, LC3, beclin, ATF4, Atg7, eIF2 α , CHOP, BiP, and DAPK. In contrast, AA has also been reported to promote ovarian cancer cell proliferation (13).

Eryptosis is a physiological process that takes place in senescent red blood cells (RBCs). Eryptotic cells exhibit phosphatidylserine (PS) to facilitate efferocytosis. PS-exposing erythrocytes also bind to platelets and promote thrombosis and blood clotting (14). Cellular markers of eryptosis include intracytoplasmic Ca²⁺ accumulation, acetylcholinesterase (AChE) inhibition, reactive oxygen species (ROS) overload, membrane blebbing, perturbed ion trafficking, increased erythrocyte sedimentation rate (ESR), cellular dehydration, and ceramide accumulation (15).

Malignancy, infections, xenobiotics, and endogenous metabolites have been recognised as eryptosis inducers (16). Notably, eryptosis is linked to chemotherapy-related anemia, which may occur as a result of myelosuppression and inflammatory damage that results in impaired erythropoiesis (17). The cytotoxic effects of anticancer medications may also trigger eryptosis and hemolysis in cancer patients undergoing chemotherapy, which leads to anaemia (18). However, no reports so far exist regarding the off-target effects of AA on erythrocytes. Therefore, the objective of the current study is to examine the non-genomic mechanisms of AA toxicity in RBCs.

EXPERIMENTAL

Chemicals

All chemicals were purchased from Solarbio Life Science (China). The stock preparation of 10 mmol L⁻¹ of AA (CAS: 16611-84-0) was made using DMSO as a solvent (equivalent to 3.50 mg mL⁻¹). This preparation was divided into aliquots and stored at -80 °C. Experiments were carried out on cells suspended in Ringer's buffer (19).

RBC culture

The study was approved by the Institutional Review Board of King Saud University (project #E-23-7685). The protocol for the current study is in line with the experimental guidelines published by the Eryptosis Study Consortium (19). A total of 21 whole blood samples were collected in EDTA vacutainer tubes. Individuals were between the ages of 26 and 34 with normal body mass index and complete blood count (BC-6200, Mindray, China). Donors provided written informed consent according to the Declaration of Helsinki. RBCs were purified as described earlier (20) and cultured *in vitro* in the presence and absence of 1–10 μ mol L⁻¹ of AA for 24 h at 37 °C. In all experiments, a positive control was set up by suspending cells in distilled water.

Hemolysis

Light absorbance at 405 nm was recorded as a function of leaked hemoglobin in the supernatants of cultured erythrocytes using a LMPR-A14 microplate reader (Labtron, UK). Percent hemolysis was derived as a fold change relative to control cells using the following equation (21):

$$\text{Hemolysis}(\%) = \frac{OD_{\text{test}} - OD_{\text{blank}}}{OD_{\text{pos}} - OD_{\text{blank}}} \times 100$$

Hemolysis was also assessed in hypotonic conditions to determine the effect of AA on erythrocyte osmotic resistance. Cells were incubated with 0.5 or 1.0 $\mu\text{mol L}^{-1}$ of AA in saline solutions containing 0.1, 0.3, 0.5, 0.7, or 0.9 % NaCl (17.1, 51.3, 85.6, 119.8, or 154 mmol L^{-1} , respectively) for 1 h at 37 °C (19).

Hemolytic markers

The enzymatic activities of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK) were assayed in supernatants of cultured erythrocytes using a Mindray BS-240Pro analyser.

Ionic release

Levels of K^+ , Na^+ , Ca^{2+} , and pH were detected in supernatants of cultured erythrocytes using an EXIAS e1 electrolyte analyser (EXIAS, Austria) (22). We measured Mg^{2+} levels in supernatants of cultured erythrocytes using a BS-240Pro analyser.

AChE activity

Hemolysates were assayed for AChE activity in Mindray's BS-240Pro clinical chemistry analyser. In the assay reaction, AChE hydrolyses butyrylthiocholine to butyrate and thiocholine, which reduces the yellow hexacyanoferrate(III) to the colourless hexacyanoferrate(II). The decrease in absorption at 405 nm as a function of hexacyanoferrate(III) reduction is directly proportional to AChE activity.

ESR

Westergren tubes were loaded with 1 mL of control and treated (5 $\mu\text{mol L}^{-1}$) cells up to the 0 mm mark. The sedimentation rate was recorded after 1 h at room temperature (23).

Cell membrane scrambling

All flow cytometric assays were performed using a Northern Lights flow cytometer (Cytek Biosciences, USA). All runs recorded and analysed 10,000 events. Translocated PS was stained with 1 % annexin-V-FITC for 10 min away from light (19).

Oxidative stress

Total ROS were analysed by incubating the cells with 10 $\mu\text{mol L}^{-1}$ of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 30 min at 37 °C, wrapped in foil, as described elsewhere (19). A total of 50 μL of cells was added to 150 μL of H_2DCFDA stock solution to achieve a final concentration of 10 $\mu\text{mol L}^{-1}$. Upon entering the cells, the non-fluorescent H_2DCFDA is cleaved by esterases into 2',7'-dichlorodihydrofluorescein (DCFH), which is oxidised by ROS into the green-fluorescent product 2',7'-dichlorofluorescein (DCF). After incubation, cells were washed in 300 μL of Ringer's buffer and analysed by flow cytometry.

Intracellular Ca²⁺

Cytosolic Ca²⁺ was estimated by labelling the cells with 2 µmol L⁻¹ of Fluo4/AM for 30 min at 37 °C in foil as per standard protocols (24). A live-cell aliquot of 50 µL was added to 150 µL of Fluo4/AM stock solution to achieve a final concentration of 2 µmol L⁻¹. Upon entering the cells, the non-fluorescent Fluo4/AM is cleaved by esterases into Fluo4, whose fluorescence is proportional to Ca²⁺ activity. After incubation, cells were washed in 300 µL of Ringer's buffer and analysed by flow cytometry.

Cellular morphology

Cell size was measured by forward scatter (FSC). Cells were also processed for electron microscopy as previously detailed (25) using a JSM-7610F electron microscope (JEOL, Japan).

Functional inhibitor assays

Hemolysis was measured in cells exposed to either the vehicle control or 3 µmol L⁻¹ of AA. The following inhibitors were used in distinct experiments: ATP (500 µmol L⁻¹), Ca²⁺ chelator BAPTA-AM (10 µmol L⁻¹), melatonin (5 µmol L⁻¹), mixed-lineage kinase domain-like protein inhibitor necrosulfonamide (0.5 µmol L⁻¹), Rac1 inhibitor (NSC23766; 100 µmol L⁻¹), p38 MAPK inhibitor SB203580 (100 µmol L⁻¹), protein kinase C inhibitor staurosporine (1 µmol L⁻¹), casein kinase 1α inhibitor D4476 (20 µmol L⁻¹), nitric oxide synthase inhibitor L-NAME (20 µmol L⁻¹), vitamin C (1 mmol L⁻¹), and cyclooxygenase (COX) inhibitor acetylsalicylic acid (ASA; 25 µmol L⁻¹).

Statistical analysis

All experiments were performed three times in triplicate using cells from three different donors (*n* = 9). Figures display arithmetic means ± SEM. Analysis was carried out using Prism 9 (GraphPad, USA) with a *p*-value of less than 0.05 set as the threshold for significance. Student's *t*-test and one-way ANOVA followed by Dunnett's test were used as appropriate.

RESULTS AND DISCUSSION

Initial experiments were carried out to determine the hemolytic activity of AA, whose structure is shown in Fig. 1a. Compared to the control, our results revealed a significant (*p* < 0.0001) increase in hemolysis with increasing AA concentrations (Fig. 1b). As a consequence of membrane lysis, we measured the activity of CK (Fig. 1c), as a surrogate marker of metabolite leakage, most notably adenylate kinase, which was significantly increased at 5 µmol L⁻¹ (*p* < 0.01). Similarly, significant elevations in AST (*p* < 0.0001) and LDH (*p* < 0.0001) were noted after treatment with 5 µmol L⁻¹, as shown in Fig. 1d and 1e, respectively. No significant alteration was observed in K⁺ levels (Fig. 1f) or osmotic fragility (Fig. 1g). Na⁺, Ca²⁺, Mg²⁺, and pH also showed no significant changes compared to control cells following AA treatment at all concentrations tested (all *p* > 0.05; data not shown).

To identify the mechanism behind the observed cell death, we measured ROS. Unexpectedly, no significant increase in DCF fluorescence was seen in AA-treated eryth-

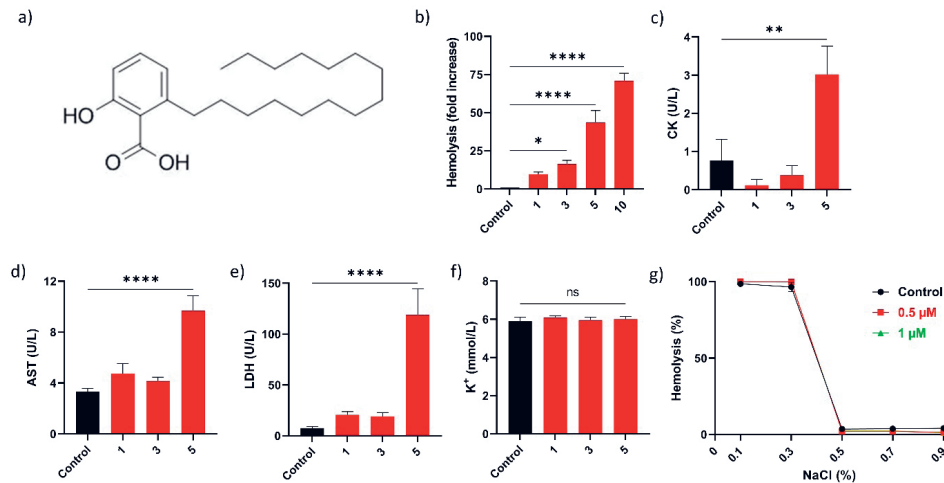


Fig. 1. Hemolytic activity of anacardic acid (AA): a) chemical structure of AA; b) hemolytic activity of AA; enzymatic activities of: c) CK; d) AST; e) LDH; f) potassium levels; g) effect of AA on hypotonic hemolysis. Data are shown as arithmetic means $\pm$ SEM ($n = 9$). ns – no statistical significance; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

rocytes (Fig. 2a). The potential effect of antioxidants against AA cytotoxicity was also investigated. The hemolytic activity of RBCs showed no significant change when cells

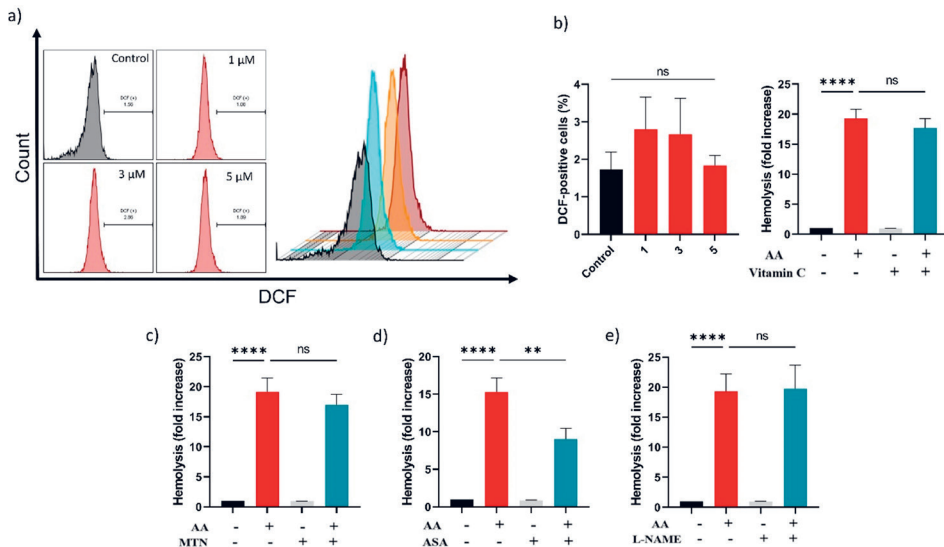


Fig. 2. Redox insensitivity of anacardic acid (AA): a) DCF fluorescence. Impact of: b) 1 mmol L⁻¹ of vitamin C; c) 25 μmol L⁻¹ of melatonin (MTN); d) 25 μmol L⁻¹ of acetylsalicylic acid (ASA); e) 20 μmol L⁻¹ of L-NAME on hemolysis. Data are shown as arithmetic means $\pm$ SEM ($n = 9$). ns – no statistical significance; ** $p < 0.01$; **** $p < 0.0001$.

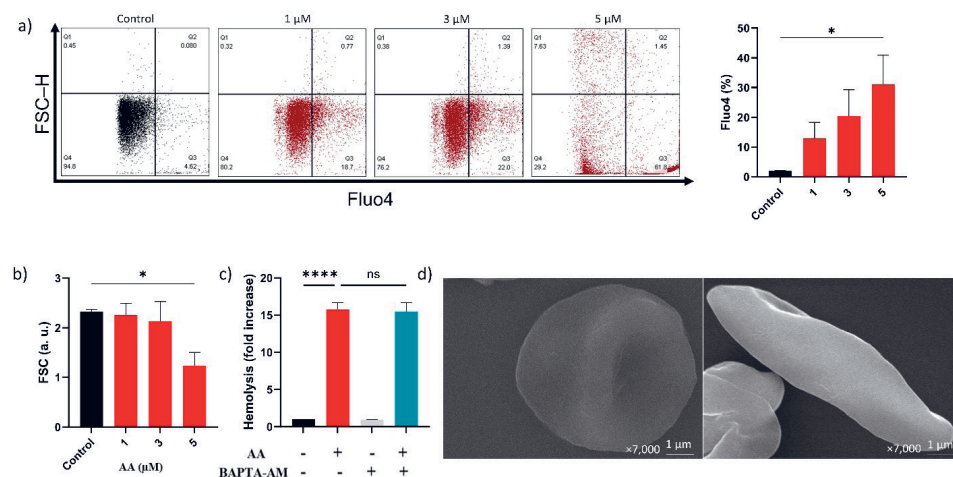


Fig. 3. Anacardic acid (AA) elevates intracellular Ca^{2+} to trigger morphological abnormalities: a) FSC-H and Fluo4 fluorescence; b) FSC in arbitrary units (a.u.); c) effect of $10 \mu\text{mol L}^{-1}$ of BAPTA-AM on hemolysis; d) scanning electron micrographs. Data are shown as arithmetic means $\pm$ SEM ($n = 9$). ns – no statistical significance; * $p < 0.05$; **** $p < 0.0001$.

were incubated with vitamin C (Fig. 2b) or melatonin (Fig. 2c). Co-treatment with ASA (Fig. 2d) significantly reduced the lytic activity of AA ($p < 0.01$), whereas L-NAME (Fig. 2e) did not offer any protection.

The maintenance of intracellular Ca^{2+} homeostasis is crucial to preserve membrane integrity and asymmetry. Elevated intracellular Ca^{2+} levels were noted upon AA exposure ($p < 0.05$) as depicted in Fig. 3a. Ca^{2+} influx leads to eryptotic loss of volume, and, congruently, Fig. 3b shows that FSC was significantly reduced ($p < 0.05$). This indicates a decrease in cell size consistent with cell shrinkage following Ca^{2+} increase. However, in Fig. 3c, chelation of cytosolic Ca^{2+} using BAPTA-AM was ineffective in reversing AA-mediated hemolysis. In order to evaluate the morphological changes associated with AA exposure, we examined the cells using electron microscopy. We found that AA led to the formation of elliptocytes revealing significant cytoskeletal damage.

Increased Ca^{2+} and cell shrinkage are features of eryptosis. Thus, PS translocation was measured (Fig. 4a) and found to be significantly increased ($p < 0.05$). Although increased adherence is characteristic of eryptotic cells, the ESR of cells exposed to $5 \mu\text{mol L}^{-1}$ of AA did not show any significant change (Fig. 4b). In contrast, AChE activity was significantly elevated ($p < 0.01$), as seen in Fig. 4c. Moreover, because metabolic exhaustion leads to cell death, we restored metabolic energy by supplementing the cells with ATP (Fig. 4d), which significantly blunted the hemolytic activity of AA ($p < 0.0001$).

Further, the elimination of extracellular Ca^{2+} (Fig. 5a), prevention of K^{+} loss (Fig. 5b), or addition of sucrose (Fig. 5c) offered no significant cytoprotective effects. However, hemolysis was significantly minimised by PEG 8000 ($p < 0.0001$, Fig. 5d). Combinations of modified Ringer buffers to provide distinct physiological states were unsuccessful in discernibly protecting the cells against hemolysis (Fig. 5e-i).

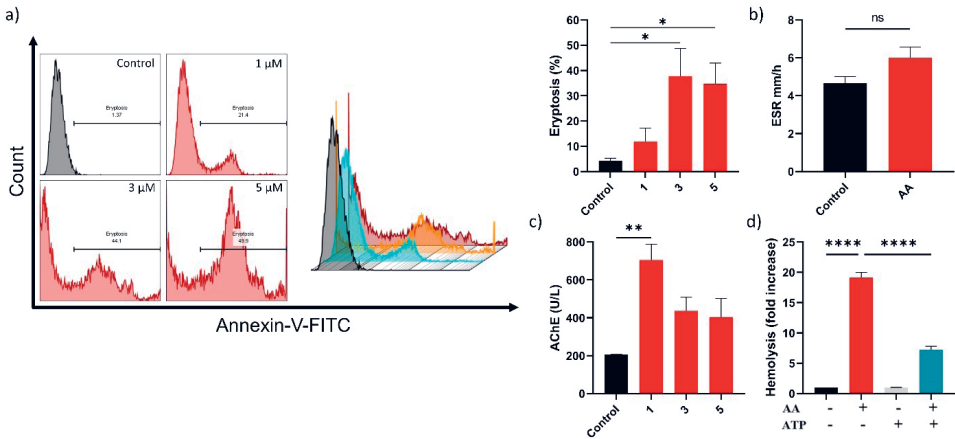


Fig. 4. Eryptotic potential of anacardic acid (AA): a) annexin-V-FITC fluorescence; b) ESR; c) AChE activity; d) Impact of 500 μmol L⁻¹ of ATP on AA toxicity. ns – no statistical significance; **p* < 0.05; ***p* < 0.01; *****p* < 0.0001.

Finally, we identified the underlying pathways implicated in AA toxicity by utilising specific inhibitors. In particular, only NSC23766 (*p* < 0.05, Fig. 6a) showed a significant

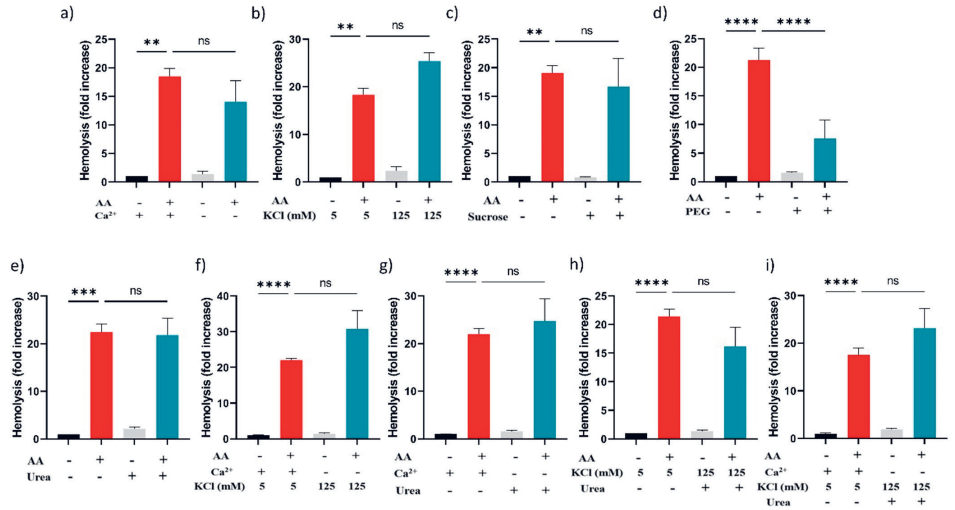


Fig. 5. Impact of extracellular environment on anacardic acid (AA) cytotoxicity. Modification of hemolysis by: a) extracellular Ca²⁺ deprivation; b) membrane depolarisation; c) 250 mmol L⁻¹ of sucrose; d) 10 % PEG; e) 300 mmol L⁻¹ of urea; f) extracellular Ca²⁺ deprivation and membrane depolarisation; g) extracellular Ca²⁺ deprivation and urea; h) membrane depolarisation and urea; i) extracellular Ca²⁺ deprivation, membrane depolarisation, and urea. Data are shown as arithmetic means ± SEM (*n* = 9). ns – no statistical significance; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

antihemolytic activity, whereas SB203580 (Fig. 6b), D4476 (Fig. 6c), staurosporine (Fig. 6d), and necrosulfonamide (Fig. 6e) resulted in no statistically significant inhibition.

A growing number of epigenetic compounds that target HAT action are increasingly being recognised as effective therapeutic options for numerous clinical conditions such as cancer and neurodegenerative disease. Challenges still remain, however, particularly with regard to the non-genomic, off-target toxicity of HAT inhibitors (26). Herein, we present experimental evidence of the cytotoxic effects of AA in erythrocytes, which warrants further safety evaluation. In fact, AA induced erythrocyte death at concentrations lower than those reported to inhibit cancer cell proliferation (4). Notably, it is expected that reduced concentrations of cytostatic drugs cause adverse events in cancer patients due to elevated sensitivity of RBCs in this cohort (27).

The current study demonstrated that AA has a high capacity to induce hemolysis with significant leakage of LDH and AST (Fig. 1). This indicates that AA physically disrupts the RBC membrane, releasing haemoglobin, which is highly toxic outside the protective environment of the red cell. Besides undergoing auto-oxidation, naked hemoglobin releases ferrous iron and scavenges nitric oxide (28, 29). Additionally, heme serves as a damage-associated molecular pattern that leads to widespread inflammation and tissue injury (30). The loss of membrane integrity implies cytoskeletal damage which was further confirmed by the appearance of elliptocytes in treated cells (Fig. 3). Abnormal cells lose their elastic recoil, resulting in compromised deformability, which has functional ramifications relevant to blood rheology, ischemia, anaemia, and inflammation (31, 32). Examination of

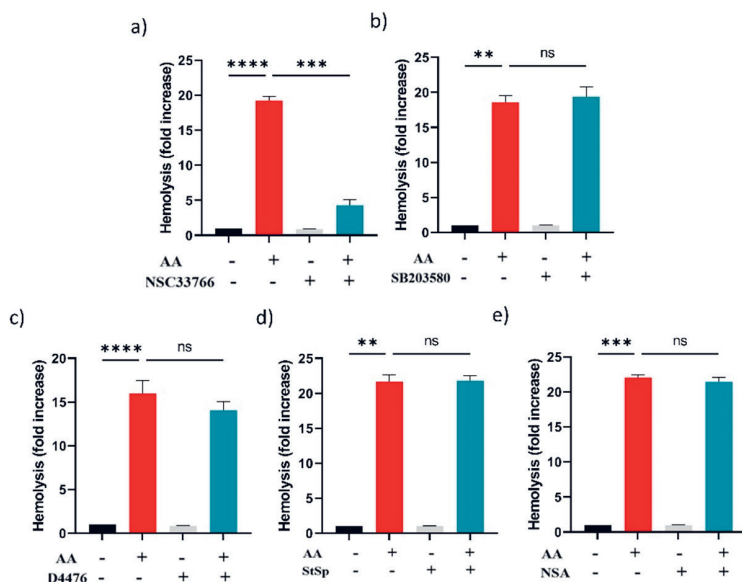


Fig. 6. Anacardic acid (AA) targets Rac1 GTPase. Impact of: a) NSC23766 (100 $\mu\text{mol L}^{-1}$); b) SB203580 (SB; 100 $\mu\text{mol L}^{-1}$); c) D4476 (20 $\mu\text{mol L}^{-1}$); d) staurosporine (StSp; 1 $\mu\text{mol L}^{-1}$); e) necrosulfonamide (NSA; 0.5 $\mu\text{mol L}^{-1}$) on the hemolytic activity of AA. Results are shown as arithmetic means $\pm$ SEM ($n = 9$). ns – no statistical significance; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

spectrin, protein 4.2, band-3, and ankyrin in treated cells may reveal pharmacological targets that enhance the clinical prospects of AA.

AA is reported to have antioxidant activity (33). Additionally, it was discovered that enhanced ROS levels lead to ion channel dysfunction and increased cytosolic Ca^{2+} concentration, thus modulating proliferation, gene expression, and channel-mediated transport (34). Nonetheless, our data show that AA-induced RBC death is not mediated through oxidative stress (Fig. 2), unlike doxorubicin (35) but similar to lead (36) and micafungin (37). Still, co-treatment with ASA provided partial but significant protection (Fig. 2), which indicates the involvement of COX signalling in AA toxicity. In RBCs, COX serves a central role in Ca^{2+} elevation (Fig. 3) by opening nonspecific cation channels through the synthesis of prostaglandin E_2 from arachidonic acid. This observation offers an alternative pathway, independently of ROS, through which AA disrupts membrane integrity.

Translocation of PS (Fig. 4) implies the breakdown of membrane asymmetry through the inhibition of flippase activity. Membrane stress is also indicated by the increased activity of the membrane-bound AChE enzyme in an attempt to restore cellular homeostasis (Fig. 4). In the case of AA, Ca^{2+} accumulation (Fig. 3) acts upstream of flippase inhibition, most likely triggered by COX activity (Fig. 2). Furthermore, Gardos channels respond to Ca^{2+} influx by depleting the cytosolic K^+ pool; a signal that is permissive of eryptosis. This cascade culminates in the loss of corpuscular volume due to cellular dehydration (Fig. 3). PS binds stabilin-2 and tim-4 on macrophages (38) to foster eryptotic cell clearance in order to prevent or limit hypercoagulability, intravascular hemolysis, or anemia (39).

ATP generation within RBCs is greatly significant due to its integral role in the regulation of cellular structure and ionic balance. Our findings indicate that ATP replenishment significantly alleviated AA-induced hemolysis (Fig. 4), revealing energy as a main target of toxicity in erythrocytes. This is consistent with findings reported by Radde *et al.* (40) which indicate that AA decreases ATP-dependent oxygen consumption in breast cancer mitochondria. Interestingly, nitric oxide is anti-eryptotic upon glucose depletion possibly through nitrosylation and thioredoxin activation (41) while adenosine prevents eryptosis following glucose depletion and protein kinase C activation (42). In our study, neither nitric oxide synthase (Fig. 2) nor protein kinase C (Fig. 6) seemed to be required for AA toxicity, but this does not rule out their participation in depleting ATP stores (Fig. 2). Instead, our results identify Rac1 GTPase (Fig. 6) as a main target of AA, which is in line with its demonstrated function as a membrane stabilizer through actin organization. Thus, Rac1 activity triggered by AA may very well be related to the morphological alterations shown in treated cells (Fig. 3), given its potential role in the formation of sickle cells (43).

CONCLUSIONS

In conclusion, AA is a potent inducer of erythrocyte injury through non-genomic mechanisms involving Ca^{2+} modulation, cytoskeletal damage, energy depletion, and COX/Rac1 GTPase signalling. Further delineation of other off-target effects using *in vivo* models in addition to toxicological profiling of other HAT inhibitors, such as C646 (44), is invaluable to better contextualise current evidence regarding the safety profile of this class of epigenetic therapeutics.

Acronyms, abbreviations, symbols. – AA – anacardic acid, AChE – acetylcholinesterase, AIF – apoptosis-inducing factor, ANOVA – analysis of variance, ASA – acetylsalicylic acid, AST – aspartate

aminotransferase, ATP – adenosine triphosphate, BAPTA-AM – 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester, CBC – complete blood count, CK – creatine kinase, COX – cyclooxygenase, DCF – 2',7'-dichlorofluorescein, DCFH – 2',7'-dichlorodihydrofluorescein, DMSO – dimethyl sulfoxide, EDTA – ethylenediaminetetraacetic acid, ESR – erythrocyte sedimentation rate, FITC – fluorescein isothiocyanate, FSC – forward scatter, HAT – histone acetyltransferase, H₂DCFDA – 2',7'-dichlorodihydrofluorescein diacetate, IRB – institutional review board, LDH – lactate dehydrogenase, L-NAME – *N* ω -nitro-L-arginine methyl ester, MAPK – mitogen-activated protein kinase, MLKL – mixed-lineage kinase domain-like protein, NSA – necrosulfonamide, PEG – polyethylene glycol, PS – phosphatidylserine, RBC – red blood cell, ROS – reactive oxygen species, SEM – standard error of the mean, StSp – staurosporine, SUMO – small ubiquitin-like modifier.

Data availability. – The data that support the findings of this study are available from the corresponding author, J.A., upon reasonable request.

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Conflict of interest. – The authors declare no conflict of interest.

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