THERAPEUTIC AND TOXICOLOGICAL EVALUATION OF BROAD-SPECTRUM ANTIMICROBIAL TRICLOSAN AS A CHEMOTHERAPEUTIC AGENT

by

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April, 2019

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Abstract: Triclosan (TCS) is a phenolic antimicrobial incorporated in personal care products and medical devices. Interest in the antiproliferative properties of TCS has recently grown owing to its antilipogenic effects. Through the studies presented here, we provide an appraisal of TCS as a chemotherapeutic agent by investigating its influence on the growth and survival of lymphoma cells. We also examine the contribution of TCS to the development of anemia; a major side effect of chemotherapy with a prevalence as high as 90% in cancer patients. Finally, we identify nonionic detergents, often used as excipients in drug formulations, as potent inhibitors of TCS *in vivo*.

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A Dissertation

Presented to the Academic Faculty of the Department of Internal Medicine,

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in Biomedical Sciences

by

Mohammad Abdulmohsen Alfhili

April, 2019

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DEDICATION

I dedicate this work to my family, and the scientific and medical communities around the world.

ACKNOWLEDGEMENTS

I acknowledge the contribution of everyone who has made this work possible, most notably my advisor, Dr. Lee, and committee members.

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BL: Burkitt lymphoma

c-MYC: Cellular myelocytomatosis

EBV: Epstein-Barr virus

HIV: Human immunodeficiency virus

CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisolone.

TCS: Triclosan

FASN: Fatty acid synthase

RPMI: Roswell Park Memorial Institute

BTM: Glycine, *N*,*N'*-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2 [(acetyloxy)methoxy]-2-oxoethyl]]-, bis[(acetyloxy)methyl] ester

JNK: c-Jun N-terminal kinase

SB: SB2035080

zVAD: zVAD(OH)-fmk

DMSO: Dimethyl sulfoxide

AO/EB: Acridine orange/ethidium bromide

NAC: N-acetylcysteine

ERK: Extracellular-signal regulated kinase

RIP1: Receptor-interacting protein 1

Nec-1: Necrostatin-1

MLKL: Mixed lineage kinase domain-like pseudokinase

NSA: Necrosulfonamide

PS: Phosphatidylserine

ROS: Reactive oxygen species

$DCFH_2$-DA: 2-,7-dichlorodihydrofluorescin diacetate

MAPK: Mitogen-activated protein kinase

RIP1: Receptor-interacting protein 1

FAS: Fatty acid synthase

RBC: Red blood cell

AMPK: Adenosine monophosphate-activated protein kinase

JAK3: Janus kinase 3

COX: Cyclooxygenase

PGE_2: Prostaglandin E_2

PLA_2: Phospholipase A_2

PBS: Phosphate-buffered saline

RPM: Revolutions per minute

HEPES: *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid

EGTA: ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid

PKC: Protein kinase C

StSp: Staurosporine

CK1: Casein kinase 1

ELISA: Enzyme-linked immunosorbent assay

FSC: Forward scatter

FITC: Fluorescein isothiocyanate

FACS: Fluorescence-activated cell sorting

DCF: Dichlorodihydrofluorescin

SEM: Standard error of the mean

EDTA: Ethylenediaminetetraacetic acid

SCR: Scramblase

EDC: Endocrine-disrupting chemical

NIS: Nonionic surfactant

Tw: Tween

PMP3: Peroxisomal membrane protein

ABC: ATP-binding cassette

FabI: Enoyl-acyl carrier protein reductase

DIC: Differential interference contrast

NS: Not significant

TX100: Triton X-100

BPA: Bisphenol A

B4HB: Benzyl 4-hydroxybenzoic acid

SDS: Sodium dodecyl sulfate

CGC: Caenorhabditis Genetics Center

NGM: Nematode growth medium

LB: Lysogeny broth

OD: Optical density

PPM: Pumps per minute

SD: Standard deviation

SKN-1: Skinhead-1

NRF2: Nuclear factor (erythroid-derived 2)-like 2

EtOH: Ethanol

DNA: Deoxyribonucleic acid

GFP: Green fluorescent protein

HR96: Hormone receptor 96

CAR: Constitutive androstane receptor

PXR: Pregnane X receptor

VDR: Vitamin D receptor

CMC: Critical micelle concentration

ALS: ammonium lauryl sulfate

PCR: Polymerase chain reaction

Sal1: *Streptomyces albus* 1

BamH1: *Bacillus amyloliquefaciens* (H) 1

UTR: Untranslated region

DEET: *N*,*N*-Diethyl-3-methylbenzamide

WHO: World Health Organization

ORN: Olfactory receptor neuron

cGKI: Cyclic guanosine monophosphate-dependent protein kinase I

CPDA-1: Citrate-phosphate-dextrose-adenine

CXCL16: CXC chemokine ligand 16

CHAPTER I: LITERATURE REVIEW

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Adopted from “Triclosan: an update on biochemical and molecular mechanisms”

In press at *Oxid Med Cell Longev*.

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ABSTRACT:

Triclosan (TCS) is a synthetic, chlorinated phenolic antimicrobial agent commonly used in commercial and healthcare products. Items made with TCS include soaps, deodorants, shampoos, cosmetics, textiles, plastics, surgical sutures, and prosthetics. A wealth of information obtained from *in vitro* and *in vivo* studies have demonstrated the therapeutic effects of TCS, particularly against inflammatory skin conditions. Nevertheless, extensive investigations on the molecular aspects of TCS action have identified numerous adversaries associated with the disinfectant including oxidative injury and influence of physiological lifespan and longevity. This review presents a summary of the biochemical alterations pertaining to TCS exposure, with special emphasis on the diverse molecular pathways responsive to TCS that have been elucidated during the present decade.

KEYWORDS: Triclosan; Gene regulation; Membrane & Cytoskeletal Damage; Cellular Longevity; Oxidative stress; Genotoxicity; Immunity; Cellular signaling

Introduction

Triclosan (TCS), or 5-chloro-2-(2,4-dichlorophenoxy)phenol, is a synthetic broad-spectrum antimicrobial developed in the 1960s. As a polychlorinated bisphenolic compound, TCS has a perceptible aromatic odor, and is weakly soluble in water. It dissolves well in organic solvents including ethanol, dimethylsulfoxide (DMSO), and methanol (Montville and Schaffner, 2011), and the type of solvent and detergent availability seem to influence TCS activity (Kjaerheim *et al.*, 1994a; Kjaerheim *et al.*, 1996; Skaare *et al.*, 1997a). For example, TCS dissolved in oils (e.g., olive oil) and alkali (e.g., sodium carbonate) exhibits markedly reduced efficacy when compared to other solvents such as glycerol and polyethylene glycol (PEG) (Kjaerheim *et al.*, 1994a; Kjaerheim *et al.*, 1994b). In fact, using propylene glycol (PG) as a solvent renders TCS more effective than using PEG, which is probably due to micellar solubilization of TCS in the larger PEG molecules (Kjaerheim *et al.*, 1994a). Recently, we have shown that the presence of non-ionic detergents (e.g., Tween 20) inhibits TCS activity *in vivo*, most likely due to micelle formation (Alfhili *et al.*, 2018). In contrast, sodium dodecyl sulfate (SDS) has been reported to potentiate the antibacterial effect of TCS *in vitro* (Waaler *et al.*, 1993).

TCS has gained enormous popularity in commerce and in healthcare owing to its antibacterial, antiviral, and antifungal properties (Regos and Hitz, 1974; Regos *et al.*, 1979; Bellamy *et al.*, 1993). This efficacy has led to the widespread use of TCS as a preservative in a variety of consumer products, including cosmetics, soaps, mouthwashes, antiperspirants, kitchen utensils, clothing textiles, bedclothes, electronics, plastics, and toys (Triclosan White Paper prepared by The Alliance for the Prudent Use of Antibiotics (APUA)). In clinical practice, TCS is used as a disinfectant and an antiseptic in surgical

sutures, scrubs, implants, and medical devices (Rodricks *et al.*, 2010; Petersen, 2016). Annual global production of TCS was estimated at 1500 tons (Chen *et al.*, 2011), and a total of 132 million liters of TCS-containing products were consumed in a single year in the United States (Safety and Effectiveness of Consumer Antiseptics; Topical Antimicrobial Drug Products for Over-the-Counter Human Use; Proposed Amendment of the Tentative Final Monograph." 2013 https://www.fda.gov/downloads/AboutFDA/ReportsManualsForms/Reports/EconomicAnalyses/UCM379555.pdf).

The high demand for TCS has consequently led to substantial buildup in drinking and wastewater sources, and, more alarmingly, accumulation in body fluids (Schulze *et al.*, 1975; Adolfsson-Erici *et al.*, 2002; Hovander *et al.*, 2002; Geens *et al.*, 2012; Wu *et al.*, 2012; Olaniyan *et al.*, 2016; Weatherly and Gosse, 2017); establishing the antimicrobial as an environmental pollutant. Pharmacokinetic studies in man show that TCS reaches the systemic circulation by rapid absorption through the skin and mucous membranes of the oral cavity and gastrointestinal tract, and variations in the bioavailabilty of TCS unsurprisingly affect the rate of urinary excretion (Sandborgh-Englund *et al.*, 2006; Queckenberg *et al.*, 2010). TCS content in commercial products may reach as high as 17 mM, and comprise up to 1% of ingredients (Levy, 2001; Rodricks *et al.*, 2010; Weatherly and Gosse, 2017). Moreover, absorption of up to 25% of applied TCS has been recorded (Weatherly *et al.*, 2018), and metabolic studies in rats and mice revealed sulfation, glucuronidation, and hydroxylation products in tissues and excreta (Moss *et al.*, 2000; Fang *et al.*, 2016).

Since the advent of TCS, early studies on the antiseptic have shown evidence of symptomatic relief from acne (Franz and Weidner-Strahl, 1978; Lee *et al.*, 2003b) and contact dermatitis (Kalliomaki and Kuokkanen, 1979; Weitgasser *et al.*, 1983) with fewer, or at least comparable, side effects to other therapeutic alternatives (Aliaga *et al.*, 1983). Later, TCS was found to be effective against crural ulcer (Huber, 1991) and chemically induced dermatitis and desquamation (Barkvoll and Rolla, 1994; Skaare *et al.*, 1996), which could be attributed to its anti-inflammatory (Kjaerheim *et al.*, 1995a), hypoallergenic (Barkvoll and Rolla, 1995), and analgesic (Kjaerheim *et al.*, 1995b) properties. Moreover, a battery of studies collectively indicate that TCS is not a skin or oral mucosal irritant, has a very low sensitization potential (0.1-0.3% of 14,000 subjects), and is unlikely to be phototoxic to human skin (http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_166.pdf). This is in contrast to the reversible skin and eye irritation caused by up to 10% TCS reported in animals (http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_166.pdf). Also, in initial studies by Lyman and Furia, it was suggested that TCS is carcinogenic when orally administered to rats (Lyman and Furia, 1968; Lyman and Furia, 1969). Subsequent investigations in rats and mice disclosed that TCS perturbs microsomal detoxification (Arrhenius *et al.*, 1977), causes nephrotoxicity and hepatotoxicity (Chow *et al.*, 1977), reduces prenatal and postnatal survival (Russell and Montgomery, 1980), and leads to central nervous system suppression (Miller *et al.*, 1982) and hypothermia (Miller *et al.*, 1983). In humans, the earliest description of an adverse TCS reaction probably comes from a case report of two patients who developed contact dermatitis following application of deodorants containing 0.12% and 0.2% TCS (Roed-Petersen *et al.*, 1975). Since then,

several case reports of the same ailment have thus far been in congruence (Veronesi *et al.*, 1986; Steinkjer and Braathen, 1988; Wong and Beck, 2001; Storer *et al.*, 2004). It is important to mention that, as is the case with healthy subjects, in patients diagnosed with, or suspected to have, contact dermatitis, TCS was similarly found to have a very low sensitization potential (0.6-0.8% of 11,887 patients) (http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_166.pdf).

In light of the dichotomous debate surrounding TCS, the US Food and Drug Administration (FDA), following extensive examination of available data, has effectively banned antiseptic products containing TCS since September 2016 (Weatherly and Gosse, 2017). In Europe, TCS was approved for use in cosmetics by the European Community Cosmetic Directive in 1986 (http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_166.pdf). However, the European Commission disapproved the use of TCS for hygienic purposes in 2017, but maintained its legality as a preservative in select cosmetics and mouthwashes in concentrations up to 0.3% and 0.2%, respectively (http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_054.pdf; http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=OJ:L:2014:107:FULL&from=EN). Furthermore, the Scientific Committee on Consumer Safety (SCCS) expressed its concern over the continued use of TCS in cosmetics, but not in antiseptics, mainly due to the cumulative pattern of exposure (http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_054.pdf). Importantly, the European Chemicals Agency (ECHA) classifies TCS, under the

classification, labeling, and packaging (CLP) regulation, as an eye irritant 2 (causes serious eye irritation), skin irritant 2 (causes skin irritation), aquatic acute 1 (very toxic to aquatic life), and aquatic chronic 1 (very toxic to aquatic life with long-lasting effects) (https://echa.europa.eu/documents/10162/21680461/bpc_opinion_triclosan_pt1_en.pdf/efc985e4-8802-4ebb-8245-29708747a358). Because of the previously mentioned ecotoxic properties, TCS is currently a candidate for substitution under the Biocides European Union regulation (Reg 528/2012/EC) (https://echa.europa.eu/potential-candidates-for-substitution-previous-consultations/-/substance-rev/12/term?_viewsubstances_WAR_echarevsubstanceportlet_SEARCH_CRITERIA_EC_NUMBER=222-182-2&_viewsubstances_WAR_echarevsubstanceportlet_DISS=true).

Our aim in this review is to provide an update on current knowledge regarding TCS therapeutic and toxic potential. Emphasis is placed on the biochemical and molecular alterations, either brought about by, or in response to, TCS exposure. Data from both *in vitro* and *in vivo* studies, obtained from humans and other organisms, are incorporated into the analysis, with special attention being given to reports published during the present decade.

Membrane & Cytoskeletal Damage

Perhaps the earliest report describing the antimicrobial activity of TCS was by Vischer *et al.*, (Vischer and Regos, 1974) which was shown through topical application In a follow-up study, TCS was found to be more effective with the broadest spectrum against bacteria and fungi when compared to other antimicrobials such as gentamicin and clotrimazole (Regos *et al.*, 1979). Subsequent efforts, which continue to this day, have focused on

dissecting the diverse action mechanisms and cellular targets of TCS. Initially, it was thought that TCS interacts with the prokaryotic cell membrane nonspecifically (Regos and Hitz, 1974). This was corroborated by the resistance of Gram-negative bacteria to TCS, which was ascribed to their outer membrane (Heath *et al.*, 2000; Gilbert and McBain, 2002). Investigating the genetic response of *Mycobacterium tuberculosis* to TCS, Betts *et al.* (Betts *et al.*, 2003) identified perturbations in a wide assortment of genes involved in cell wall, transport, detoxification, and DNA replication and transcription. Also, *Klebsiella pneumoniae* with inactive efflux pump *KpnGH* exhibit pronounced susceptibility to multiple antibiotics including TCS (Srinivasan *et al.*, 2014). Several genes in the membrane stress response pathway were also studied in *Escherichia coli* and *Rhodospirillum rubrum* S1H (Pycke *et al.*, 2010; Gou *et al.*, 2014; Lu *et al.*, 2018). During the electro-Fenton transformation of TCS, significant changes in expression patterns of genes involved in cell wall and membrane structure, cell envelope, flagella, and multidrug efflux were observed (Table 1). These findings complement an earlier report describing enhanced resistance to TCS due to overexpressed *acrAB* multidrug efflux pump (McMurry *et al.*, 1998a). It was recently suggested that TCS binds to the transcriptional repressor AcrR, causing conformational changes, and prevent its binding to the efflux pump *AcrA* promoter in *Agrobacterium tumefaciens* (Nuonming *et al.*, 2018).

The interaction of TCS with the cell membrane was also studied in human red blood cells (RBCs; erythrocytes). TCS exposure led to K^+ leakage and overt hemolysis, indicating membrane damage, while antagonizing hypotonic lysis, which may be due to membrane expansion (Miller and Deinzer, 1980). TCS also inhibited membrane-bound Na^+,K^+,Mg^{2+}-ATPase enzymatic activity (Lorusso *et al.*, 1981). These observations

suggest that TCS causes membrane destabilization, perturbs monovalent ion transport, and modulates the overall osmoregulation of erythrocytes. Evidence for membrane damage is further confirmed in numerous studies by means of compromised stability and permeability (Villalain *et al.*, 2001). To directly observe how TCS interacts with the cell membrane, Guillén and coworkers utilized nuclear magnetic resonance (NMR) spectroscopy to demonstrate that TCS intercalates within hydrophobic pockets in the lipid bilayer, perpendicularly to phospholipid molecules (Guillen *et al.*, 2004). Furthermore, using neutral red to evaluate membrane integrity, diminished uptake of the dye in hemocytes of the clam *Ruditapes philippinarum* and mussel *Mytilus galloprovincialis*, was related to TCS-induced suppression of pinocytosis and disturbed phagocytosis (Canesi *et al.*, 2007; Matozzo *et al.*, 2012).

Along those lines, our recent findings indicate that TCS blunts the expression of *pmp3* membrane transporter in *Caenorhabitidis elegans* nematodes, and that *pmp3(ok1087)* mutants exhibit increased sensitivity to the disinfectant (Yoon *et al.*, 2017). Finally, a proteomic analysis of zebrafish (*Danio rerio*) larvae and gills of fresh water mussel *Dreissena polymorpha*, revealed alterations in cytoskeletal protein levels following TCS exposure (summarized in Table 1) (Riva *et al.*, 2012; Falisse *et al.*, 2017).

There is a consensus in the literature regarding the membranotropic nature of TCS in different membrane models across various species. The cell membrane is a primary target for TCS, and among the first cellular obstacles that must be overcome by the antiseptic to exert its effects. Although evidence implicating membrane-associated efflux pumps as part of the cellular response to TCS is strong, there is paucity in reports describing TCS modulation of structural or functional membrane components in human-based systems.

Similarly lacking is an understanding of the role of membrane receptors not only in pumping out TCS molecules, but also in transducing both inter- and intracellular signals as a consequence to TCS presence.

Table 1: Summary of membrane and cytoskeletal targets of TCS

Model	Target		Response
	Gene/Protein	**Molecular Identity**	
K. pneumoniae	*KpnGH*	Efflux pump	
E. coli	*AcrAB*	Efflux pumps	
	acrE		
	mdtE		
	acrF		
	mdtB		
	mdtC		
	yddA		
	emrA		
	emrE		
	sanA	Cell wall/membrane structure	

	dacB		
	amiC	Cell envelope	
	clsA		
	ompX	Membrane porin	
	motA	Flagellar	
	flgM		
R. rubrum S1H	*sugE*	Small multidrug resistance protein	
	mexF	RND efflux system, inner membrane transporter	
	mexB		
	mexE	RND efflux system, membrane fusion proteins	
	mexA		
	mexM		
	oprM	RND efflux system, outer membrane transporter	
	glmM	Cell envelope; phosphoglucosamine mutase	
	exoD	Cell envelope; exopolysaccharide synthesis protein D	
	wbpM	Cell envelope; polysaccharide biosynthesis protein M	

A. tumefaciens C58	*AcrA*	RND efflux system, periplasmic adaptor protein	
Human erythrocytes	Na^{+},K^{+},Mg^{2+}-ATPase	Membrane ion transporter	
C. elegans	*pmp-3*	Membrane ABC transporter	
D. rerio	Actin, cytoplasmic 2	Cytoskeleton	
	Actin α1, skeletal muscle		
	light polypeptide 3		
	Desmin	Cytoskeleton; muscular filament structure	
	Fast skeletal muscle myosin		
	Keratin, type I cytoskeletal 18		
	Tropomyosin α-1 chain		
	Type II cytokeratin		

	Lamin B1	Cytoskeleton; nuclear lamina	
D. polymorpha	Tubulin β-2/ α-4 chain	Cytoskeleton	
	Tubulin β-4 chain		
	Myosin light chain	Cytoskeleton; muscular filament structure	
Upregulated by TCS Downregulated by TCS Sensitive to TCS			
Abbreviation: RND, resistance-nodulation-division; ABC, ATP-binding cassette			

Cellular Longevity:

The interest in TCS and ultimate cell fate has originally stemmed from its use in oral hygiene products, which is reflected in two seminal studies on human gingival cells (Babich and Babich, 1997; Zuckerbraun *et al.*, 1998). TCS was shown to be cytotoxic to gingival fibroblasts and epithelial cells, identifying it as a novel stimulator of apoptosis in the latter.

Investigations have thus far followed a more comprehensive approach, relating cell death induced by TCS to other cellular adversaries, utilizing both human and non-human model systems. When TCS was treated to human choriocarcinoma placental cells (JEG-3), multiple dose- and time-dependent responses were observed (Honkisz *et al.*, 2012). While there was a proportional increase in estradiol and progesterone secretion, β-human

chorionic gonadotropin (β-hCG) release was nevertheless inhibited with increasing TCS concentrations (Honkisz *et al.*, 2012). In addition to blunted proliferation, significant cell death was recognized as apoptotic in nature evidenced by activated caspase-3 and Hoechst 33342-stained fragmented DNA (Honkisz *et al.*, 2012). Similarly, using anoikis-resistant H460 human lung cancer cells, Winitthana *et al.* (Winitthana *et al.*, 2014) demonstrated that 24-hour exposure to 10 uM TCS causes cell death and apoptosis. Nontoxic levels ($\leq$7.5 μM), however, enhanced cell growth (increased colony number and reduced size) without altering proliferation. TCS also promoted epithelial-to-mesenchymal transition (EMT), along with the migratory and invasive abilities of the cells (Winitthana *et al.*, 2014).

A research group performed a series of *in vivo* and *in vitro* studies on the effect of TCS on growth and proliferation of human BG-1 ovarian cancer cells. Results from these studies indicate that TCS increases cellular proliferation and both gene expression and protein levels of cyclin D1, and decreases p21 and Bax gene expression and protein levels (Kim *et al.*, 2014). These effects were significantly antagonized by the estrogen receptor (ER) antagonist ICI 182,780, implicating ER in TCS-induced cell cycle progression and in its anti-apoptotic role. Investigators from the same group also reported a similar response to TCS by MCF-7 breast cancer cells and LNCaP prostate cancer cells. In MCF-7 cells, 1 uM TCS enhanced growth and proliferation during a six-day period, which was associated with increased cyclin D1 and reduced p21 expression levels. When mice were treated with TCS for 8 weeks, brdU-positive breast tumor cells were significantly increased compared to the control group treated with corn oil (Lee *et al.*, 2014). Similar to BG-1 cells, TCS-promoted proliferation of MCF-7 cells was mediated through ERα signaling, demonstrated as antagonism by kaempferol and 3,3′-diindolylmethane (DIM); two phytoestrogens (Kim *et*

al., 2016). In addition to cyclin D1 and p21, TCS caused an increase in cyclin E and a decrease in Bax, and induced metastasis through elevated cathepsin D protein expression. These observations were paralleled *in vivo* using xenografted mouse models. Researchers from this report expanded their findings to VM7Luc4E2 cells, a variant of the MCF-7 model, to show that TCS (0.1-10 μM) is pro-proliferative and anti-apoptotic by inhibiting oxidative stress, with both effects being antagonized by kaempferol (Lee *et al.*, 2018). In LNCaP cells exposed to concentrations of TCS ranging from 0.01 to 10 uM for up to 5 days showed enhanced proliferation and migration, and reduced p21 protein expression (Kim *et al.*, 2015). In primary human syncytiotrophoblasts, TCS at 0.001 to 10 uM induced apoptosis as seen by condensed nuclei and fragmented DNA (Zhang *et al.*, 2015). TCS also reduced 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) via a caspase-dependent mechanism. Other targets included both Bax and Bcl-2 proteins.

Similar to human cells, both pro- and anti-apoptotic properties were observed in rodent cells treated with TCS. Beside its cytotoxicity, TCS caused caspase-dependent apoptosis in rat neural stem cells along with elevated Bax and reduced Bcl-2 (Park *et al.*, 2016). In a series of studies, Szychowski *et al.* (Szychowski *et al.*, 2015; Szychowski *et al.*, 2016; Szychowski *et al.*, 2018) used mouse neurons to show that TCS is apoptotic through the Fas receptor (FasR), aryl hydrocarbon receptor (AhR), and caspase activation involving N-Methyl-D-aspartate receptors (NMDARs). In agreement with the cytotoxicity data, TCS-treated mouse lung epithelial cells were deformed with reduced viability (Kwon *et al.*, 2013). Conversely, TCS stimulated the proliferation of mouse epidermis-derived JB6 Cl 41-5a cells, by increasing cyclins D1 and A and inhibiting p27(Kip1) protein levels (Wu *et al.*, 2015). Examining these effects *in vivo*, B6C3F1 mice exhibited epidermal

hyperplasia and focal necrosis following topical administration of TCS. Moreover, the pluripotency markers of mouse embryonic stem cells were analyzed following TCS exposure (Chen *et al.*, 2015). Alkaline phosphatase (*Alp*), *Sox2, Oct4, and Nanog* were all reduced, while miRNA-134 was elevated.

Unlike human and rodent cells, *in vivo* and *in vitro* studies on aquatic organisms uniformly agree that TCS is solely pro-apoptotic in these animals. Pyknotic apoptosis in the central nervous system of zebrafish *D. rerio* was observed following treatment with either TCS alone or TCS combined with derivatives 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4-dichlorophenol (2,4-DCP) (Kim *et al.*, 2018; Liu *et al.*, 2018). The TCS-derivative mixture caused pronounced deformities, behavioral abnormalities, and perturbed the expression of a panel of neurodevelopmental and apoptotic genes (Table 2). Also, TCS, following both *in vivo* and *in vitro* exposure, induced a dose- and time-dependent increase in apoptotic hemocytes of *D. polymorpha* (Binelli *et al.*, 2009b; Binelli *et al.*, 2009a). Likewise, when the saltwater clam *Ruditapes philippinarum* was treated with TCS, hemocytes exhibited significant cell death, blunted proliferation, reduced size and volume, and prominent apoptotic DNA fragmentation (Matozzo *et al.*, 2012). TCS-induced apoptosis, or apoptosis-like cell death, was also detected in unicellular organisms, such as the green alga *Chlamydomonas reinhardtii* and the pathogenic fungus *Cryptococcus neoformans* (Movahed *et al.*, 2016; Gonzalez-Pleiter *et al.*, 2017).

Collectively, studies on TCS influence on cell fate indicate estrogenic, proliferative, and apoptotic activities. Disturbances in genes and proteins governing the regulation of cell cycle and apoptosis are particularly sensitive to TCS modulation. The disparity in ultimate cell fate seem to point at an inter-species variation, and a dose-specific response, among

other experimental details such as cell type and duration of exposure. Elucidating the existence and the identity of a specific molecular 'switch' that may tip the scales in favor of either cell death or survival could be an important inquiry for future investigations.

Table 2: Summary of cell survival molecules modulated by TCS

Model	Target		Response
	Gene/Protein	Molecular Identity	
JEG-3 cells	Estradiol	Major female sex hormones	
	Progesterone		
	β-hCG	Maintenance of pregnancy	
	Caspase-3	Apoptosis regulator; pro-apoptotic	
BG-1 cells	Cyclin D1	Cell cycle regulators	
	p21		
	Bax	Apoptosis regulator; pro-apoptotic	
MCF-7 cells	Cyclin D1	Cell cycle regulators	
	Cyclin E		
	p21		
	Bax	Apoptosis regulator; pro-apoptotic	

	Cathepsin B	Metastasis markers	
	Cathepsin D		
	MMP-9		
	MMP-2		
	CXCR4		
	Snail	Mesenchymal markers	
	Slug		
LNCaP	p21	Cell cycle regulator	
Primary human syncytiotrophoblasts	11β-HSD2	Fetal development; anti-cortisol	
	Caspase-3	Apoptosis regulators; pro-apoptotic	
	Bax		
	Bcl-2	Apoptosis regulator; anti-apoptotic	
Rat neural stem cells	Caspase-3	Apoptosis regulators; pro-apoptotic	
	Bax		
	Bcl-2	Apoptosis regulator; anti-apoptotic	
Mouse neocortical neurons	*GluN1*	Ionotropic glutamate receptors; neurotransmission	
	GluN1		

	GluN2A		
	GluN2A		
	GluN2B		
	GluN2B		
	FasR	Apoptosis regulators; pro-apoptotic	
	Caspase-8		
	Caspase-9		
	Caspase-3		
	AhR	Ligand-activated receptor; detoxification	
JB6 Cl 41-5a cells	Cyclin D1	Cell cycle regulators	
	Cyclin A		
	p27		
B6C3F1 mice	*Alp*	Pluripotency markers; stem cell self-renewal and differentiation regulators	
	Oct4		
	Nanog		
	ALP		
	Oct 4		

	Nanog		
	Sox 2		
	miRNA-134	Transcriptional regulator of pluripotency markers	
D. rerio	*Oct4*	Pluripotency markers	
	Nanog		
	Sox2		
	p53	Cell cycle regulator; tumor suppressor	
	Casp3	Apoptosis regulators; pro-apoptotic	
	Casp8		
	Shha	Early neurogenesis	
	Ngn1		
	Nrd		
	Elavl3		
	α1-tubulin	Neural maturation	
	Gap43		
	Gfap		
	Mbp		

Upregulated by TCS Downregulated by TCS Sensitive to TCS

Abbreviation: *Shha,* Sonic hedgehog a; *Ngn1,* Neurogenin1; *Nrd,* NeuroD; *Elavl3,* ELAV like; neuron-specific RNA binding protein 3; *Gap43,* Growth associated protein 43; *Gfap,* Glial fibrillary acidic protein; *Mbp,* Myelin basic protein

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Oxidative Stress:

Overwhelming evidence has recently accumulated in support of the pro-oxidative action of TCS. It is prudent to provide an overview of human-based studies first before summarizing notable findings obtained from other model organisms.

In Porto Rican pregnant women, a correlation between exposure to TCS during pregnancy and oxidative damage, as measured by urinary 8-hydroxyguanosine (8-OHdG), and inflammation was suggested (Watkins *et al.*, 2015). Similar observations were also mirrored in Chinese and Brazilian children (Lv *et al.*, 2016; Rocha *et al.*, 2018). Conversely, in a global effort comprising nine countries from Asia, Europe, and North America, no relation between urinary TCS and 8-OHdG was established (Iyer *et al.*, 2018).

In vitro studies on human cells have also shed some light on the oxidative potential of TCS. In peripheral blood mononuclear cells (PBMC), 2,4-dichlorophenol (2,4-DCP) –a product of TCS transformation– promoted reactive oxygen species (ROS) generation, with subsequent lipid peroxidation and protein carbonylation (Bukowska *et al.*, 2016). Similarly, TCS caused elevated ROS in Nthy-ori 3-1 human follicular thyroid cells (Zhang *et al.*, 2018) and lipid peroxidation in retinoblastoma (Y79 RB) cells (Vandhana *et al.*, 2013). Our recent investigations on mesenchymal stem cells also showed TCS interference

with the activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), the 'master regulator' of detoxification, and its downstream targets, heme oxygenase 1 (HO-1) and NAD(P)H dehydrogenase [quinone 1] (NQO-1) (Yoon *et al.*, 2017). Consistently, TCS incorporated in mouthrinse did not exhibit antioxidant activity on fibroblasts (Battino *et al.*, 2002). In contrast, TCS reduced ROS levels in VM7Luc4E2 cells, which contributed to its anti-apoptotic activity in these malignant breast cells (Lee *et al.*, 2018).

Mitochondrial damage was also evident in multiple mammalian cells including human PBMC and keratinocytes, exposed to 3.5-350 uM TCS (Ajao *et al.*, 2015). At concentrations up to 100 uM, TCS caused depolarization of mitochondrial membrane, reduced oxidative phosphorylation, and suppressed ATP synthesis. Weatherly *et al*. (Weatherly *et al.*, 2016). utilized human HMC-1.2 mast cells and primary keratinocytes to show that TCS is a proton ionophore uncoupler, and interferes with ATP production

Animal studies conducted on mice and rats have revealed a profound response in the cellular antioxidant machinery upon TCS treatment. In rat thymocytes, superoxide anions were found to be elevated following TCS treatment (Tamura *et al.*, 2012) which, as Yueh *et al.* (Yueh *et al.*, 2014) showed, was met with increased expression of key antioxidant enzymes including HO-1, NQO-1, and glutathione S-transferase (GST) in mice liver. Evidence for testicular DNA damage, elevated malondialdehyde (MDA) and superoxide dismutase (SOD), in addition to diminished catalase (CAT), was related to TCS treatment in weanling rats (Riad *et al.*, 2018). Similarly, in lung homogenates of female albino rats, TCS was found to induce lipid peroxidation, and severely deplete the levels of other crucial antioxidants; SOD, CAT, and glutathione (GSH) (Mohammed *et al.*, 2017). Increased expression of glutathione peroxidase 1 (*Gpx1*) and aldehyde oxidase 1 (*Aox1*) was also

observed as a consequence to TCS exposure in C57BL/6 mice (Wang *et al.*, 2017). Most recently, Zhang *et al.* (Zhang *et al.*, 2018) showed downregulation of antioxidant enzymes, *Gpx3*, *Cat*, and *Sod2*, along with elevated MDA, in the hypothalamus of Sprague-Dawley rats. Moreover, it was found that TCS treatment leads to increased ROS and reduced GSH activity in rat neural stem cells (Park *et al.*, 2016). TCS also increased ROS levels in mouse neocortical neurons, along with perturbed regulation of cytochrome P450 family 1, subfamily a, member 1 (CYP1a1) and CYP1b1 (Szychowski *et al.*, 2016; Szychowski *et al.*, 2018). Effects of TCS on cytochromes and hepatic detoxification were also demonstrated in Sprague-Dawley rats, showing increased levels of UDP-glucuronosyltransferase 1-1 (Ugt1a), Ugt2b1, CYP1a1, CYP1a2, CYP2b1, CYP3a1, and sulfotransferase family 1E member 1 (Sult1e1) (Zhang *et al.*, 2018).

Several terrestrial organisms have been employed in the study of TCS toxicology. *Caenorhabtidis elegans* is among the best-studied animal models due to its ease of maintenance and high genetic homology to humans. We have recently shown that TCS leads to overproduction of ROS, inhibition of nuclear translocation of protein skinhead-1 (SKN-1) antioxidant transcription factor, and downregulation of gamma-glutamyl cysteine synthetase (*Gcs1*) (Yoon *et al.*, 2017). In a subsequent report, *Skn1* expression was found to be upregulated by TCS along with *Sod1*, *Sod4*, heat shock proteins (*Hsp*)*-3*, *-4*, *-16.2*, and *-70*, and cytochromes *Cyp29A2* and *Cyp34A9* (https://app.dimensions.ai/details/publication/pub.1103154992#readcube-epdf). TCS also enhanced nuclear translocation of stress-related factor DAF-16, suggesting the occurrence of oxidative stress (Lenz *et al.*, 2017). In the Earthworm *Eisenia fetida*, oxidative damage by TCS was manifested as a transient elevation in CAT and GST enzymes, increased

MDA, as well as DNA damage (Lin *et al.*, 2010). In a follow-up study by the same group, SOD was also increased and decreased by TCS depending on the concentration used (Lin *et al.*, 2012); a response mirrored by CAT in the snail *Achatina fulica* (Wang *et al.*, 2014). In that study, TCS caused diminished levels of SOD and peroxidase (POD), along with elevated MDA, among other morphological anomalies.

The ubiquity of TCS in aquatic environments has made animal models from that habitat the subject of extensive investigations on TCS toxicity. Perhaps the most relevant aquatic organism is the zebrafish *D. rerio*, owing to a strong structural and molecular resemblance to humans. Elucidating the interaction between TCS and the antioxidant system in ZFL liver cells, Zhou *et al.* (Zhou *et al.*, 2017) showed evidence of induced CYP1A activity along with a general trend of suppression in phase I and II detoxification enzymes. Elevated MDA, along with perturbed homeostasis of GSH, peroxiredoxin-2 (PRD-2) and HSPs were observed in zebrafish larvae grown in presence of TCS (Falisse *et al.*, 2017; Liu *et al.*, 2018).

TCS has been shown to induce MDA and cause oscillations in CAT, Ethoxyresorufin-O-deethylase (EROD), erythromycin N-demethylase (ERND), and aminopyrine N-demethylase (APND) in *Daphnia magna* (Peng *et al.*, 2013). Moreover, elevated amino acids, including glutamine, glutamate, and proline, has been attributed to a general oxidative stress state in the daphnids (Kovacevic *et al.*, 2016). Also, stress-related proteins, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hsp-70, were modulated by TCS in *D. polymorpha*, in addition to lipid peroxidation (Riva *et al.*, 2012). TCS exposure demonstrated reduced oxyradicals and lipofuscin, and elevated oxidized glutathione (GSSG) in the digestive gland of swollen river mussels *Unio tumidus*

(Falfushynska *et al.*, 2014). In *Tigriopus japonicas* copepods treated with TCS, increased ROS, SOD, GST, GPx, and GSH content was noted (Park *et al.*, 2017). TCS also caused perturbations in expressional profiles of *Cyps*, *Sod*, *Gst*, and *Cat* proteins (Table 3) (Park *et al.*, 2017).

TCS treatment in the yellow catfish *Pelteobagrus fulvidraco* revealed induced CAT, EROD, ERND, and APND (Ku *et al.*, 2014). Expressional profiling of *Cyp1a*, *Cyp3a*, and *Gst* showed both up- and downregulation depending on TCS concentration and length of exposure; a pattern that was also seen with MDA formation. When another catfish, *Heteropneustes fossilis*, was treated with a cosmetic effluent rich in TCS, increased SOD and CAT activities, and reduced GSH, GST, and GPx were noted (Banerjee *et al.*, 2016).

Oxidative damage by TCS was also evident in the goldfish *Carassius auratus*, as MDA, CAT, and GSH were elevated in addition to a reduced total antioxidant capacity (Wang *et al.*, 2018b). Variable responses by antioxidant enzymes and in MDA levels were recorded in the goldfish's liver after TCS treatment under a pH range of 6 to 9 (Li *et al.*, 2018). The oxidative potential of TCS was also evident in the rotifer *Brachionus koreanus,* detected as ROS overproduction and enhanced GST activity, in addition to transcriptional modulation of cytochromes, antioxidant genes *Gst*, *Gpx*, *Sod*, and *Cat*, and chaperons (Table 3) (Han *et al.*, 2016). Moreover, TCS inhibited *Sod* and phospholipid hydroperoxide glutathione peroxidase (*Phgpx*) expression in the liver of *Bufo gargarizans* tadpoles (Chai *et al.*, 2017), and induced GST in *Pelophylax perezi* frog larvae (Martins *et al.*, 2017).

Sendra *et al.* (Sendra *et al.*, 2017) studied the combined effect of titanium dioxide (TiO_2) and a heterogenous mixture of organic compounds including TCS using the clam

Ruditapes philippinarum. Modulations in EROD, SOD, CAT, GPx, GST, and GR enzyme activities were noted in the clam's digestive gland, in parallel with increased lipid peroxidation. TCS exposure caused alterations in *Cat*, *Sod*, *Gpx1*, *Gpx2*, *Gsta*, *Hsp90bb*, *Hsp90ba*, and *Hsc70a* genes in rainbow trout *Oncorhynchus mykiss* (Capkin *et al.*, 2017). Although in one report TCS failed to elicit oxidative stress in the green algae *Chlamydomonas reinhardtii* (Almeida *et al.*, 2017), another report detected ROS formation following TCS exposure (Gonzalez-Pleiter *et al.*, 2017), which was also most recently confirmed by significantly increased MDA, downregulated *Gpx*, and upregulated *Sod* expression (Pan *et al.*, 2018).

The antimicrobial nature of TCS makes bacteria an appropriate target for mechanistic studies. Using *Rhodospirillum rubrum* S1H, Pycke *et al.* (Pycke *et al.*, 2010) detected upregulation in a host of TCS-induced oxidative response genes, most notably *Gpx*. In *E. coli* K12, MG1655, the electro-Fenton transformation of TCS caused activation of genes related to ROS sensing, along with reduced glutaredoxin (*Grx*), *Sod*, *Cat*, and alkyl hydroperoxide reductase (*Ahpr*) (Gou *et al.*, 2014). Very recently, ROS formation by TCS was associated with diminished expression of antioxidants in *E. coli* (Table 3); an event that preceded mutagenesis and enhanced drug resistance in that species (Lu *et al.*, 2018). TCS was also recently used to validate novel self-luminescent bioreporter strains of *Nostoc sp.* PCC 7120 using *Sod* promoters (Hurtado-Gallego *et al.*, 2018).

Collectively, monumental evidence demonstrates the pro-oxidant properties of TCS evident as both overproduction of ROS as well as interference with the cellular antioxidant defensome. TCS is toxic in part by inducing oxidative damage in a wide range of organisms and by targeting a defined cluster of proteins in a fashion that is conserved among diverse

species. Nonetheless, the vast majority of data are collected from non-human models, and, as is the case with other toxicological reports of TCS, studies conducted on man or human-derived tissues are severely lacking.

Table 3: Oxidative stress patterns elicited by TCS

Model	Target		Response
	Biomarker	**Molecular Identity**	
Humans (pregnant women; children)	Urinary 8-OHdG	Oxidized deoxyguanosine; DNA damage	
Nthy-ori 3-1 cells	ROS	Metabolic oxygen byproducts	
PBMC*	ROS		
	Lipid peroxidation	Oxidized lipids	
	Protein carbonylation	Oxidized proteins	
Y79 RB cells	Lipid peroxidation	Oxidized lipids	
Human bone marrow-derived mesenchymal stem cells	Nrf2	Antioxidant regulator	
	Ho-1	Antioxidant enzymes	
	Nqo-1		
VM7Luc4E2 cells	ROS	Metabolic oxygen byproducts	

Mouse liver	O_2^-	Antioxidant enzymes	
	HO-1		
	NQO-1		
	GST		
Weanling rats	MDA	Oxidized lipid marker	
	SOD	Antioxidant enzymes	
	CAT		
Female albino rat lung homogenates	Lipid peroxidation	Oxidized lipids	
	SOD	Antioxidants	
	CAT		
	GSH		
C57BL/6 mice liver	*Gpx1*	Antioxidant enzyme; glutathione homeostasis	
	Aox1	Superoxide and hydrogen peroxide formation	
Sprague-Dawley rat hypothalamus	MDA	Oxidized lipid marker	
	Gpx3	Antioxidant enzyme; glutathione homeostasis	
	Cat	Antioxidant enzymes	

	Sod2		
Rat neural stem cells	ROS	Metabolic oxygen byproducts	
	GSH	Antioxidant	
Mouse neocortical neurons	ROS	Metabolic oxygen byproducts	
	Cyp1a1	Cytochrome family enzymes; detoxification	
	CYP1a1		
	Cyp1b1		
	Cyp1b1		
Sprague-Dawley rat liver	*Cyp1a1*		
	Cyp1a2		
	Cyp2b1		
	CYP2b1		
	Cyp3a1		
	Ugt2b1	Glucuronidation enzymes; detoxification	
	Ugt2b1		
	Sult1e1	Sulfation enzyme; detoxification	
	Sult1e1		

C. elegans	ROS	Metabolic oxygen byproducts	
	Skn1	Stress response regulator	
	SKN-1		
	Gcs1	Antioxidant enzymes	
	Sod1		
	Sod4		
	Hsp-3	Stress response; protein stabilization	
	Hsp-4		
	Hsp-16.2		
	Hsp-70		
	Cyp29A2	Cytochrome family enzymes; detoxification	
	Cyp34A9		
	DAF-16	Stress response	
E. fetida	MDA	Oxidized lipid marker	
	CAT	Antioxidant enzymes	
	GST		
	SOD		

A. fulica	MDA	Oxidized lipid marker	
	CAT	Antioxidant enzymes	
	SOD		
	POD		
ZFL liver cells	CYP1A	Cytochrome family enzyme; detoxification	
D. rerio larvae	GPx	Antioxidant enzymes; glutathione homeostasis	
	GR		
	PRD-2	Antioxidant enzyme	
	hsp-5	Stress response; protein stabilization	
	hsp-90 β		
D. magna	MDA	Oxidized lipid marker	
	CAT	Antioxidant enzymes	
	EROD	Detoxification enzymes	
	ERND		
	APND		
	Glutamine	Amino acids; markers of protein oxidation/breakdown	
	Glutamate		

	Proline		
D. polymorpha gills	hsp-70	Stress response; protein stabilization	
U. tumidus digestive gland	GAPDH	Oxidoreductase; glucose metabolism	
	GSSG	Oxidized glutathione; antioxidant	
	Oxyradicals	Oxygen-containing radicals; pro-oxidants	
	Lipofuscin	Lysosomal pigment granules; toxicity marker	
T. japonicum	ROS	Metabolic oxygen byproducts	
	Sod	Antioxidant enzymes	
	SOD		
	Cat		
	Gst variants	Antioxidants; glutathione homeostasis	
	GST		
	GPx		
	GSH		
	Cyp3026a3		

	Cyp3037a1	Cytochrome family enzymes; detoxification	
P. fulvidraco	MDA	Oxidized lipid marker	
	CAT	Antioxidant enzyme	
	Gst	Antioxidant enzyme; glutathione homeostasis	
	EROD	Detoxification enzymes	
	ERND		
	APND		
	Cyp1a	Cytochrome family enzymes; detoxification	
	Cyp3a		
H. fossilis	CAT	Antioxidant enzymes	
	SOD		
	GSH	Antioxidants; glutathione homeostasis	
	GST		
	GPx		
C. auratus	MDA	Oxidized lipid marker	
	CAT	Antioxidant enzymes	
	SOD		

	GSH	Antioxidant; glutathione homeostasis	
Brachionus koreanus	ROS	Metabolic oxygen byproducts	
	Gst variants	Antioxidant enzyme; glutathione homeostasis	
	Gpx		
	GST		
	Sod	Antioxidant enzymes	
	Cat		
	Cyp3042a1	Cytochrome family enzymes; detoxification	
	Cyp43a1		
	Hsp10	Stress response; protein stabilization	
	Hsp21		
	Hsp27		
	Hsp30		
	Hsp40		
	Hsp40h		
	Hsp60		
	Hsp70		

	Hsc70		
	Hsp90α1		
	Hsp90α2		
	Hsp90β		
B. gargarizans liver	*Sod*	Antioxidant enzyme	
	Phgpx	Antioxidant enzyme; glutathione homeostasis	
P. perezi larvae	GST		
R. philippinarum digestive gland	MDA	Oxidized lipid marker	
	CAT	Antioxidant enzymes	
	SOD		
	GPx variants	Antioxidant enzymes; glutathione homeostasis	
	GST		
	GR		
	EROD	Detoxification enzyme	
O. mykiss liver and kidney	*Cat*	Antioxidant enzymes	
	Sod		
	Gpx vriants		

	Gsta	Antioxidant enzymes; glutathione homeostasis	
	Hsp90bb	Stress response; protein stabilization	
	Hsp90ba		
	Hsc70a		
C. reinhardtii	ROS	Metabolic oxygen byproducts	
	MDA	Oxidized lipid marker	
	Sod	Antioxidant enzyme	
	Gpx	Antioxidant enzyme; glutathione homeostasis	
R. rubrum S1H	*Gpx*		
	GrxC	Antioxidant enzymes; Glutathione homeostasis	
	TrxB		
	OsmC	Antioxidant enzyme	
	DnaJ	Heat shock protein; general stress marker	
	RpoN	RNA polymerase factor sigma-54; general stress marker	
	TerA	Tellurite resistance protein A; general stress marker	

	Psp variants	Phage shock proteins; general stress markers	
	ClpP	ATP-dependent protease, proteolytic subunit; general stress marker	
	HrcA	Heat-inducible transcription suppressor; general stress marker	
E. coli K12, MG1655	*OxyR*	ROS sensor proteins	
	Grx	Antioxidant enzymes; Glutathione homeostasis	
	Sod variants	Antioxidant enzymes	
	Cat variants		
	Ahp variants	Antioxidant enzymes	
E. coli	ROS	Metabolic oxygen byproducts	
	YgiW	Antioxidant proteins	
	SoxS		
	YhcN		

Upregulated by TCS Downregulated by TCS Sensitive to TCS

Abbreviation: *TrxB*, Thioredoxin; *OsmC*, Peroxiredoxin osmotically inducible protein C-like

*Effects of 2,4-DCP; a byproduct of TCS degradation.

Immunity and Inflammation:

TCS has, for a long time, been recognized as an effective therapy for infectious dermatitis (Kalliomaki and Kuokkanen, 1979; Aliaga *et al.*, 1983; Weitgasser *et al.*, 1983), and the observed curative capacity of the compound was solely attributed to its antimicrobial activity. It was not until the end of last century that associations between TCS exposure and remission of non-infectious inflammation were made (Barkvoll and Rolla, 1994; Barkvoll and Rolla, 1995; Kjaerheim *et al.*, 1995a), and the use of antibacterials as anti-inflammatory agents has gained deserved attention during the past two decades. For example, an appreciable number of antibiotics, including macrolides and quinolones, have been shown to possess ant-inflammatory activity (Korzeniowski, 1989; Iino *et al.*, 1992; Van Vlem *et al.*, 1996; Culic *et al.*, 2001; Uriarte *et al.*, 2004). Follow-up efforts have successfully provided solid evidence for the direct interaction of TCS with inflammatory pathways.

Gaffar *et al.* (Gaffar *et al.*, 1995) reported that TCS inhibits cyclooxygenase-1 (COX-1) and COX-2, 5-lipoxygenase and (LPO), 15-LPO, interleukin (IL)-1β-induced prostaglandin E2 (PGE2) in gingival cells. TCS was also shown to suppress a wider range of inflammatory mediators including IL-1β-induced prostaglandin I2 (PGI2) and arachidonic acid, tumor necrosis factor (TNF)α-induced PGE2, phospholipase A2 (PLA2), and COX (Modeer *et al.*, 1996). Moreover, in a double-blind crossover study, participants who used a mouthrinse with added 0.15% TCS developed significantly less oral erythematous lesions than those who used a TCS-free mouthrinse (Skaare *et al.*, 1997b). By then, the anti-inflammatory properties of TCS were established and were widely accepted within the scientific and medical communities.

TCS in prosthetic devices was found to have no influence on the acute phase response (Hernandez-Richter *et al.*, 2001), and only modest differences were seen between TCS and stannous fluoride dentrifice (Kumar, 2015). Nevertheless, TCS, when applied intracrevicularly, improved clinical parameters of ginigivits (Suresh *et al.*, 2001). In a recent double-blind, randomized, crossover study, it was concluded that TCS-containing toothpaste inhibits inflammation in peri-implant tissue (Ribeiro *et al.*, 2018).

To date, elaborations on the anti-inflammatory nature of TCS have been the focus of subsequent studies. Mustafa *et al.* (Mustafa *et al.*, 1998; Mustafa *et al.*, 2000; Mustafa *et al.*, 2005) identified IL-1β, interferon (IFN)γ, major histocompatibility complex (MHC) class II, and PGE synthase-1, as targets of TCS in human gingival fibroblasts. Of note, studies to discern the subcellular localization of TCS show preference for nuclear, as opposed to cytosolic, accumulation. Although initial uptake was considerably higher in the cytoplasm, a great proportion of cytosolic TCS was eliminated after repeated washing, while nuclear retention was observed (Mustafa *et al.*, 2003). This may explain the perturbed inflammatory signaling associated with TCS. Moreover, in primary human oral epithelial cells, TCS attenuated LPS-induced cytokine response including IL-8, IL-1α, and TNFα, and aggravated the anti-microbial response, which was mediated through micro RNA (miRNA) regulation of toll-like receptor (TLR) pathway (Wallet *et al.*, 2013). The findings were also reciprocated in cells derived from diabetic patients, with an exaggerated TLR response (Neiva *et al.*, 2014). It was revealed that TCS, nevertheless, abrogated LPS-induced TLR response, again, through regulating miRNAs (stimulating miR146a and inhibiting miR155s).

In skin and leukocytes of mice topically treated with TCS, alterations in inflammatory responses were mediated through TLR4 (Marshall *et al.*, 2017). Likewise, TCS downregulated parathyroid hormone (PTH)- or PGE2-stimulated matrix metalloproteinase-13 (MMP-13) expression in rat osteoblastic osteosarcoma cells (Barnes *et al.*, 2013). Since hyperactive MMP-13 is implicated in periodontal disease, it was suggested that TCS might have a protective role against oral inflammatory conditions through its action on that enzyme, among others (Pancer *et al.*, 2016).

Interestingly, favorable results have been observed for TCS against other inflammatory conditions including cardiovascular disease and hidradenitis suppurativa (Cullinan *et al.*, 2015; Hessam *et al.*, 2016). Moreover, the use of TCS-impregnated ureteral stents seems to be a promising approach to combat urinary tract infections (UTI) and associated inflammation (Cadieux *et al.*, 2006; Elwood *et al.*, 2007). Along those lines, an increased urinary TCS was related to increased serum IL-6 in pregnant women (Watkins *et al.*, 2015), pointing at a possible pro- or anti-inflammatory role.

In a unique effort by Barros *et al.* (Barros *et al.*, 2010), TCS modulation of the inflammatory response in an *ex vivo* whole blood stimulation assay was investigated. In that study, TCS inhibited multiple inflammatory mediators induced by LPS, including interleukins, most notably IL-1 & IL-6, IFNs, and colony-stimulating factor (CSF) 2. Activation of type 1 T helper lymphocytes was interrupted through the action of TCS on CD70. In a related report, TCS also reduced the capacity of natural killer (NK) lymphocytes to lyse chronic myelogenous leukemia K562 cells (Udoji *et al.*, 2010). Recently, chitosan-TCS particles reduced the expression of IL-1β-induced *Cox2* and *Il6*,

among other immune molecules in gingival fibroblasts (Table 4) (Pavez *et al.*, 2018), showcasing the vast amenability of this antimicrobial to nanoparticle manipulation.

Other *in vivo* studies on rodents and marine organisms clarified further the immunomodulatory properties of TCS. For instance, in mice subjected to an acute, systemic *E. coli* infection, Sharma *et al.* (Sharma *et al.*, 2003) demonstrated that co-treatment with TCS significantly reversed the damage caused by the bacteria. Specifically, TCS prolonged survival, lessened hepatic congestion, hemorrhage, and fatty changes, and reduced blood liver enzymes, serum TNFα, and the severity of bacteremia. In accordance with published data, TCS was similarly immunosuppressive in aquatic mussels (*M. galloprovincialis*) and clams (*R. philippinarum*) (Canesi *et al.*, 2007; Matozzo *et al.*, 2012).

Contrary to the overwhelming evidence of the anti-inflammatory function of TCS, a number of studies have nonetheless identified a pro-inflammatory role by the antiseptic. For example, upon intratracheal instillation of TCS in Sprague-Dawley rats, elevated total cell (TC) count, polymorphonuclear leukocytes (PMNs), total protein (TP), LDH, TNFα, and IL-6 were observed in bronchoalveolar lavage (BAL) fluid (Kwon *et al.*, 2013), which, except for TP, returned to baseline levels after 14 days of exposure. Consonantly, it has also been demonstrated that TCS exacerbates diethylnitrosamine-induced hepatocellular carcinoma in C57BL/6 mice (Yueh *et al.*, 2014). Likewise, TCS was very recently found to increase *Tlr4* expression to promote colitis and aggravate colitis-related cancer in C57BL/6 mice (Yang *et al.*, 2018).

It is evident from the wealth of information present that TCS is a modulator of immune and inflammatory reactions. The sum of data from *in vitro* and *in vivo* studies indicate that

TCS, on its own, is immunosuppressive. Nevertheless, increasing evidence seems to suggest that in the presence of an existing adverse condition, such as inflammation or tumor, TCS further potentiates and worsens the eventual outcome. Investigations into the molecular basis behind this unique behavior are particularly warranted.

Table 4: Inflammatory and immune mediators responsive to TCS

Model	Target		Response
	Biomarker	**Molecular Identity**	
Human gingival fibroblasts	COX-1/2	Inflammatory mediators	
	5/15-LPO		
	PGE2		
	PGI2		
	Arachidonic acid		
	PLA2		
	PGE synthase-1		
	IFNγ	Immune/inflammatory cytokines	
	IL-1β		
	MHC II	Cell surface proteins; adaptive immunity regulators	

	Cox2	Inflammatory mediator	
	Il6	Immune/inflammatory cytokines	
	Il1b		
	Tlr6	Innate immunity receptor	
Human primary oral epithelial cells	IL-8	Immune/inflammatory cytokines	
	IL-1α		
	TNFα		
	miR146a	Transcriptional regulators of TLR response	
	miR155s		
Mouse skin and leukocytes	S100A8/A9	Inflammatory modulator; Ca^{2+}-binding protein	
	Tlr4	Innate immunity receptors	
	TLR4		
	Tlr1		
	Tlr2		
	Tlr6		
Rat osteoblastic osteosarcoma cells	MMP-13	Endopeptidase; collagen degradation	
Human oral fluids	IL-1α		

	IL-1β	Immune/inflammatory cytokines	
	IL-8		
	MCP-1		
	TIMP-2	MMP regulator proteins	
	TIMP-1		
	MMP-8/9	Endopeptidases; extracellular matrix degradation	
Human urine	IL-6	Immune/inflammatory cytokines	
Sprague-Dawley rats	TNF-α		
	IL-6		
Human whole blood leukocytes	*Csf2*	Hematopoietic stem cell growth and maintenance	
	Ifna1	Immune/inflammatory cytokines	
	Ifna2		
	Ifna4		
	Ifna8		
	Il-1f10		
	Il-1f5		
	Il-1f7		

	Il-1f8		
	Il-1f9		
	Il-6		
	Il-11		
	Il-13		
	Il-25		
	Il-19		
	Il-21		
	Il-9		
	Cd70	Cell surface receptor/ligand; Activated lymphocytes	
	Bmp2	Growth factors; bone and cartilage development	
	Bmp6		
	Tnfrsf11b	TNFSF11 receptor	
	Gdf3	Growth/differentiation factors	
	Gdf2		
	Gdf5		
	Gdf9		

	Inhba	Hypothalamus-pituitary axis regulator	
	Lefty2	Left-right determination factor 2; Left-right asymmetry of organs	
Sprague-Dawley rats	TNF-α	Immune/inflammatory cytokine	
	IL-6		

Upregulated by TCS Downregulated by TCS Sensitive to TCS

Abbreviation: MCP, Monocyte chemoattractant protein; TIMP, Tissue inhibitor of metalloproteinase; *Bmp*, Bone morphogenetic protein; *Gdf*, Growth differentiation factor; *Inhba*, Inhibin beta A chain

Genotoxicity and Carcinogenicity:

Among the most important aspects of toxicological profiling of compounds is their interaction with the molecule of life – the DNA. Early efforts by Fahrig *et al.* (Russell and Montgomery, 1980; Kanetoshi *et al.*, 1992) point at a possible role for TCS in somatic mutations observed in mice. TCS also caused a significant reduction in global DNA methylation in human hepatocellular carcinoma HepG2 cells; a finding associated with liver tumor (Ma *et al.*, 2013). Similarly, TCS caused a dose-responsive increase in chromosomal aberrations in lung fibroblast V79 cells, but not in ovary CHO cells, of the Chinese hamster *Cricetulus griseus* (Rodricks *et al.*, 2010). In a comparative study on *Drosophila melanogaster* using three mouthwashes, namely Cepacol® (0.05% cetylpyridinium chloride), Periograd® (0.12% chlorhexidinedigluconate), and Plax®

(0.03% TCS), it was concluded that only the ethanol content in Cepacol®, but not other active ingredients, caused mitotic recombination between homologues chromosomes (Rodrigues *et al.*, 2007). On the other hand, TCS induced dose-responsive DNA damage in hemocytes of the zebra mussel *D. polymorpha* (Binelli *et al.*, 2009a), and strand breaks in the digestive gland of *U. tumidus* mussels (Falfushynska *et al.*, 2014). A similar dose-dependent DNA damage was also observed in the Earthworm *E. fetida* (Lin *et al.*, 2010; Lin *et al.*, 2012), but not in *E. andrei* (Chevillot *et al.*, 2018).

Comparing TCS to other toxicants in the larvae of freshwater insect *Chironomus riparius*, Martínez-Paz *et al.* (Martinez-Paz *et al.*, 2013) found TCS, along with nonylphenol, to be the most potent in causing DNA breakage. It was also noted that TCS, either alone or in combination with carbendazim, induced DNA damage in *D. magna* (Silva *et al.*, 2015). Using the brine shrimp *Artemia salina*, a time-dependent pattern of TCS-induced genotoxicity was identified (Xu *et al.*, 2015). Moreover, TCS was genotoxic in the catfish *Heteropneustes fossilis*, goldfish *C. auratus*, and rainbow trout *O. mykiss* (Banerjee *et al.*, 2016; Capkin *et al.*, 2017; Wang *et al.*, 2018b). Importantly, when TCS at an environmentally relevant concentration (3 nM) was treated to the freshwater protozoan *Tetrahymena thermophila*, notable DNA damage, without significant perturbation in growth or cell viability, was evident (Gao *et al.*, 2015). In a more detailed study on *E. coli*, Gou *et al.* (Gou *et al.*, 2014) revealed that the electro-Fenton transformation of TCS caused upregulation of a host of genes involved in the DNA repair machinery, indicative of DNA stress. These genes belong to base excision repair (*mutT* and *nfo*), nucleotide exision repair (*uvrA* and *uvrD*), mismatch repair (*uvrD* and *ssb*) and double-strand break repair (*ssb* and *recN*). Chromosomal stickiness, reduced mitotic activity, and ana-telophase bridges were

also noticeable in the bulb onion *Allium cepa* following TCS treatment (Herrero *et al.*, 2012).

In a recent proof-of-concept study, the promising potential of a toxicogenomic approach as a follow-up to positive *in vitro* genotoxicity data was evaluated. Using TCS as a testing compound, it was shown that the antimicrobial is non-DNA reactive, and that it is genotoxic solely *in vitro* as opposed to *in vivo* (Doktorova *et al.*, 2014).

Ambiguity surrounding the carcinogenicity of TCS still remains today. Investigators have generally been able to provide evidence for carcinogenic effects in animal models but not in humans. Of the earliest studies in this regard was a report by Lyman *et al.* (Lyman and Furia, 1969) identifying TCS as a carcinogen in mice. Other studies on mice have been in agreement with that conclusion. For example, it was noted that chronic TCS exposure increased the incidence of liver neoplasms (Rodricks *et al.*, 2010), and aggravated hepatocellular carcinoma (Yueh *et al.*, 2014). Furthermore, TCS caused colonic inflammation and worsened colitis or tumorigenesis induced by dextran sodium sulfate (Sanidad *et al.*, 2018). These findings, were, however, not paralleled in rats, hamsters, or baboons (Bhargava and Leonard, 1996; Rodricks *et al.*, 2010). More importantly, *in vivo* human studies of TCS are scarce, and aspects related to TCS-induced oncogenesis are lacking. Consequently, whether TCS poses a carcinogenic hazard to humans is unknown and requires further investigation. Nonetheless, the interaction of TCS with human-derived cancer cells *in vitro* has recently gained considerable attention (reviewed under Therapeutic Proposals).

In light of available data, TCS demonstrates carcinogenicity solely in mice, and within a narrow range of tissues (the liver and colon), which constitutes limited evidence of carcinogenicity according to ECHA (https://echa.europa.eu/documents/10162/23036412/clp_en.pdf/58b5dc6d-ac2a-4910-9702-e9e1f5051cc5). Hence, TCS is not classifiable as a carcinogen (http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_054.pdf). It must be noted that in case future assessment conclusively rules out TCS as a human carcinogen, caution with its use must still be exercised given the established carcinogenicity of its transformation products – dioxins, chloroform, and anilines (Halden *et al.*, 2017).

Table 5: TCS genotoxicity and carcinogenicity

Model	**Effect**	**Classification**
HepG2 cells	Global DNA hypomethylation	Limited evidence of carcinogenicity
V79 cells	Chromosomal aberrations	
Mouse	Somatic mutation (positive spot test) Increased incidence of liver tumors Aggravated hepatocellular carcinoma Exacerbated colon tumorigenesis	
D. polymorpha	DNA damage (positive Comet assay)	N/A

U. tumidus	DNA strand breaks (Hoescht 33342 fluorescence)	
E. fetida	DNA damage (positive Comet assay)	
D. magna	DNA damage (positive Comet assay)	
A. salina	DNA damage (positive Comet assay)	
H. fossilis	DNA damage (positive Comet assay)	
C. auratus	DNA damage (positive Comet assay)	
O. mykiss	DNA damage (positive Comet assay)	
T. thermophila	DNA damage (positive Comet assay)	
A. cepa	Chromosomal stickiness, reduced mitotic activity, and ana-telophase bridges (positive Feulgen reaction)	
N/A = Data from non-mammalian animals are not considered for ECHA mutagenicity/carcinogenicity classification		

Cellular Signaling:

Adaptations to the ever-changing intracellular and surrounding environments are achieved, in large part, by effective communication. Transmission of information that carry specific instructions is executed by messengers that function in tandem within a defined pathway. Tasks, however, are usually accomplished through the sequential transduction of multiple messages along a complex, intertwining network that involves a wide assortment

of mediators (Uings and Farrow, 2000). Hence, the participation of cell signaling cascades in the response to xenobiotics cannot be overlooked.

The use of human cell lines has provided a wealth of information particularly regarding the study of signaling molecules responsive to stressors and xenobiotics, including TCS. In H460 lung cancer cells, TCS promoted migration and invasion through focal adhesion kinase/ATP dependent tyrosine kinase (FAK/Akt) and Ras-related C3 botulinum toxin substrate 1 (Rac1) (Winitthana *et al.*, 2014). Evidence similarly exists for the classical mitogen-activated protein kinases (MAPK) as targets of TCS. For example, proliferation of JB6 Cl 41-5a cells as induced by TCS was accompanied by activation of extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 MAPKs, in addition to Akt (Wu *et al.*, 2015). Importantly, blocking either MEK1/2 or phosphoinositide 3-kinase (PI3K) significantly attenuated TCS-induced proliferation. In another study on rat neural stem cells, TCS-induced cytotoxicity and apoptosis were accompanied by activation of p38 and JNK, and suppression of ERK, Akt, and PI3K (Park *et al.*, 2016). This points at the involvement of these proteins in both cellular survival and death as brought about by TCS. Recently, TCS was shown to activate p38 and JNK *in vivo* as detected in the hypothalamus of Sprague-Dawley rats and *in vitro* utilizing human Nthy-ori 3-1 thyroid follicular cells (Zhang *et al.*, 2018). In that study, TCS stimulated thyrotropin-releasing hormone receptor through p38 MAPK, which, in turn, influenced thyroid peroxidase (TPO) level.

In suppressing TLR signaling in whole blood leukocytes, TCS downregulated the expression of several signaling mediators, most notably, NF-kB-inducing kinase (*Nik*) and *C-jun*, which accounted for the overall blunted inflammatory response to LPS in these cells

(Barros *et al.*, 2010). Furthermore, suppression of *Mmp-13* expression in mouse osteoblastic osteocarcinoma cells by TCS was possibly related to its inhibition of Fos/Jun and AP-1 sequence binding in both the *Mmp-13* and *C-fos* promoters (Barnes *et al.*, 2013).

The endocrine-disrupting activity of TCS, specifically its estrogenicity, has been of great interest to researchers. Kim *et al.* (Kim *et al.*, 2014) utilized BG-1 ovarian cancer cells to show that the proliferative effects of TCS were mediated through ERα. Confirming the ER role, the use of ICI 182,780 reversed the proliferative properties of TCS along with associated perturbations in cyclin D1, p21, and Bax expression and protein levels. Likewise, the ER is implicated in TCS-induced proliferation of MCF-7 cells and increased breast tumor mass in mice (Lee *et al.*, 2014; Kim *et al.*, 2016; Lee *et al.*, 2017b). This was similarly indicated by TCS inhibition with ICI 182,780 or kaempferol, and the stimulation of insulin-like growth factor (IGF) signaling, namely phosphorylated insulin receptor substrate (pIRS-1), pAkt, pMEK1/2 and pERK1/2 (Kim *et al.*, 2016). Notably, kaempferol also inhibited TCS-induced VM7Luc4E2 cell growth (Lee *et al.*, 2018). These observations are in congruence with an earlier report by Huang *et al.* (Huang *et al.*, 2014) describing the estrogenic activities of nanomolar concentrations of TCS in the same cells. Investigating ER-responsive genes on the transcriptional and translational levels, it was shown that TCS induced pS2 but blunted ERα mRNA and protein levels the latter of which was related to elevated miR-22, miR-206, and miR-193b miRNAs.

Recent studies have also argued for the dual effect of TCS on ER signaling. For example, Henry *et al.* (Henry and Fair, 2013) demonstrated that, when administered alone to MCF7 cells, TCS at 7 nM to 700 µM exhibits estrogenic activity but becomes anti-estrogenic in presence of E2. Along those lines, it was shown that TCS, on its own, lacked

any effect on rat uterine growth, but could still potentiate the effect of ethinylestradiol (EE) (Stoker *et al.*, 2010). In a follow-up investigation, it was reported that TCS promotes EE-induced inhibition of ERα and ERβ expression, and when given alone does not activate ER at concentrations from 30 nM to 100 µM (Louis *et al.*, 2013). Furthermore, TCS diminished E2 and estrogen sulfotransferase in sheep placenta (James *et al.*, 2010). This is in contrast to the increased activity of ERβ but not ERα caused by a TCS-derivative mixture, which led to neurological and behavioral abnormalities in zebrafish (Liu *et al.*, 2018). Also, Sprague-Dawley rats given TCS showed increased uterine weight and *Calbindin-d(9k)* (CaBP-9k) expression, which was also reciprocated in pituitary GH3 cells (Jung *et al.*, 2012). Reversal of both anomalies by ICI 182,780 and RU 486 points at a possible estrogenic role of the antimicrobial.

Very recently, Serra *et al.* (Serra *et al.*, 2018) challenged accumulating evidence of TCS estrogenicity by showing the lack of agonistic or antagonistic effect *in vivo* and *in vitro*. While up to 0.3 µM TCS did not modulate ER-dependent brain aromatase in zebrafish embryos, interference with the enzyme's activity, and with E2 activation of the enzyme observed at 1 µM, was not attributed to TCS-ER interaction. Moreover, up to 10 µM TCS lacked estrogenic effects in ER-expressing zebrafish liver cells as well as in MCF-7 cells (Serra *et al.*, 2018). Additionally, in a screening study of the estrogenicity of a group of endocrine-disrupting chemicals on fish species, TCS failed to significantly elicit a response in an *in vitro* ERα reporter gene assay (Miyagawa *et al.*, 2014).

In light of available evidence, the general consensus seems to indicate that the estrogenicity of TCS is contingent upon multiple factors, including concentration, species,

duration of exposure, and whether TCS is administered alone or in combination with other molecules.

With regard to the androgenic properties of TCS, it was revealed that TCS interferes with testosterone (TSN)-related transcription but promotes that dependent on androgen (Chen *et al.*, 2007; Christen *et al.*, 2010). In a recent *in vivo* study on weanling male rats, Riad *et al.* (Riad *et al.*, 2018) reported that TCS, either alone or combined with butylparaben, reduced TSN, leutinizing hormone (LH), and follicle-stimulating hormone (FSH), while increased E2 was observed upon single TCS administration Also, TCS-induced proliferation and migration of LNCaP cells were significantly reduced in presence of bicalutamide, an androgen receptor (AR) antagonist (Kim *et al.*, 2015). These findings support a previous report by Ahn *et al*. (Ahn *et al.*, 2008) in which 1 μM TCS reduced E2-induced ER activation by 50% and AR in human BG1Luc4E2 ovarian adenocarcinoma cells and T47D-ARE breast cancer cells, respectively. Evidence for TCS estrogenicity was detected in MCF7 cells when [(3)H]estradiol was successfully displaced from the ER by the antimicrobial (Gee *et al.*, 2008). Furthermore, 10 μM TCS attenuated E2-dependent ERE-CAT reporter gene induction, while 0.1 and 1 μM TCS inhibited TSN-stimulated LTR-CAT reporter gene in both T47D cells and S115 mouse mammary tumor cells (Gee *et al.*, 2008). TCS was also determined to have a weak effect on AhR in recombinant rat hepatoma (H4L1.1c4) cells. Finally, Forgacs *et al.* (Forgacs *et al.*, 2012) showed that TCS interferes with recombinant hCG stimulation of TSN in a novel BLTK1 murine Leydig cell model. Most recently, however, no significant influence on androgen synthesis or activity by TCS was observed in Wistar rats (Farmer *et al.*, 2018).

Controversy surrounding the interaction between TCS and members of the peroxisome proliferator-activated receptors (PPARs) has gained considerable attention as of late. This has essentially stemmed from the apparent discrepancy between data obtained from humans and those from rodents. In comparing the differential modulation of TCS on PPARα in HepG2 cells and mouse hepatoma Hepa1c1c7 cells, distinct responses were observed by Wu *et al.* (Wu *et al.*, 2014). Protein levels of PPARα downstream target, acyl-coenzyme A oxidase, were decreased in HepG2 cells but were increased in Hepa1c1c7, which also showed higher DNA synthesis and blunted apoptosis through transforming growth factor (TGF-β). PPAR signaling was similarly identified as a target of TCS through genome-wide CRISPR-Cas9 screening in HepG2 cells (Xia *et al.*, 2016), zebrafish (Haggard *et al.*, 2016), and *Gallus gallus* chicken embryos (Guo *et al.*, 2018). In the latter model, PPAR signaling members *Cyp7a1*, fatty acid binding protein 1 (*Fabp1*), acyl-CoA synthetase long-chain family member 5 (*Acsl5*), acyl-CoA oxidase 2 (*Acox2*), perilipin 1 (*Plin1*) were upregulated, whereas angiopoietin like 4 (*Angptl*) was downregulated.

TCS administered to pregnant mice caused insulin resistance, hypothyroidism, diminished glucose transporter 4 (GLUT4) expression, and inhibition of Akt and mTOR phosphorylation (Cao *et al.*, 2017; Hua *et al.*, 2017). While thyroxine corrected these adversaries, PPARγ activator, rosiglitazone, solely reversed the decrease in Akt phosphorylation in adipose tissue and in muscle (Hua *et al.*, 2017). PPARγ is known to ameliorate mTOR suppression-induced glucose intolerance in rats (Festuccia *et al.*,2014), further underlining the far-reaching effects of TCS action.

Although TCS has been reported to promote hepatocyte proliferation in mice through PPAR (Rodricks *et al.*, 2010), Yueh *et al.* (Yueh *et al.*, 2014) found no appreciable

induction of PPARα following TCS treatment. Importantly, the authors also identified constitutive androstane receptor (CAR) as a possible aggravator of TCS-induced tumorigenesis, given the halved tumor number in *Car*$^{-/-}$ mice compared to their *Car*$^{+/-}$ counterparts. TCS, as is the case with PPARs, is reported to exhibit varying affinities for CAR and pregnane X receptor (PXR) in humans and rodents. A weak agonist for human CAR, TCS was found to be a reverse agonist for rodent CAR, an agonist for human PXR, and had no effect on rodent PXR (Paul *et al.*, 2013).

Calcium concentration within cells influences protein conformation and dynamics. Protein binding of Ca^{2+}, on the other hand, maintains the ion's content within a physiological range and sets forth diverse cellular activities related to gene expression, motility, secretion, and survival (Clapham, 2007). Beside proteins, intracellular Ca^{2+} levels are modulated by a variety of stimuli, including xenobiotic exposure. Through the Ca^{2+} channel ryanodine (Ry) receptor type 1 (RyR1), TCS increased cytosolic Ca^{2+} dose-dependently in primary skeletal myotubes irrespective of extracellular Ca^{2+} (Ahn *et al.*, 2008). Accordingly, muscle contractility was compromised upon TCS exposure *in vitro* and *in vivo* (Cherednichenko *et al.*, 2012). Results from this study indicate that TCS impaired excitation-contraction coupling (ECC) in cardiac and skeletal muscles, and enhanced electrically-induced Ca^{2+} transients in myotubes without depleting intracellular Ca^{2+} and notwithstanding RyR1 blockage. TCS also efficiently blocked excitation-coupled Ca^{2+} entry and interfered with the bidirectional signaling between RyR1 channels and Ca^{2+} ions. Likewise, TCS compromised ECC in larval fathead minnows *Pimephales promelas*, as evidenced by altered RyR and dihydropyridine receptor (DHPR) mRNA and protein

levels, and weakened ligand binding to both receptors in adult muscle homogenates (Fritsch *et al.*, 2013).

In rat thymocytes, TCS elevated intracellular Ca^{2+} levels, and opened Ca^{2+}-responsive K^+ channels, eventually leading to membrane hyperpolarization (Kawanai, 2011). Also, TCS prevented Ca^{2+}-induced mitochondrial swelling in rat liver (Teplova *et al.*, 2017). A more in-depth analysis of TCS modulation of Ca^{2+} homeostasis was conducted on rat basophilic leukemia (RBL) mast cells (Weatherly *et al.*, 2018). In this cell type, TCS caused mitochondrial fission, diminished membrane potential and translocation, with compromised ATP production and elevated ROS. These changes were associated with perturbed mitochondrial and endoplasmic reticulum Ca^{2+} and depleted cytosolic Ca^{2+} levels following antigen stimulation. Accordingly, TCS-induced degranulation of mast cell may at least in part be attributed to Ca^{2+} mobilization.

Calcium modulation by TCS has also been investigated in other organisms. In *C. reinhardtii* exposed to 14 µM TCS, increased Ca^{2+} levels with oxidative stress, cell and mitochondrial membrane depolarization, compromised photosynthesis, and caspase activation were noted (Gonzalez-Pleiter *et al.*, 2017). Importantly, chelation of intracellular Ca^{2+} ions by BAPTA-AM protected the algae from TCS-induced Ca^{2+} dysregulation. These observations strongly implicate Ca^{2+} as a mediator of a wide array of toxic anomalies attributed to TCS.

Literature concerning the xenobiotic response to TCS has revealed important signaling pathways activated or suppressed by TCS (Table 6). Distinct outcomes exist among species, and even within the same species based on experimental conditions and model

under investigation. Although important milestones in TCS signaling have been achieved so far, there remains a lot to be discovered, especially in human-based systems, about the modulatory effects of TCS on cellular physiology. In particular, the response of many human cell types and tissues to TCS treatment is unknown, and identification of signaling pathways and their roles in cellular growth, metabolism, and overall function, is therefore advised.

Table 6: TCS modulation of signaling pathways

Model	**Target**		**Response**
	Pathways	**TCS Role**	
H460 cells	FAK/Akt	Cellular migration and invasion	
	Rac1		
JB6 Cl 41-5a cells	ERK1/2	Cell proliferation	
	JNK		
	p38		
	Akt		
	PI3K		
Rat neural stem cells	JNK	Cytotoxicity and apoptosis	
	p38		

	ERK Akt PI3K		
Sprague-Dawley rats hypothalamus and Nthy-ori 3-1 cells	JNK	Reduced TPO; hypothyroidism	
	p38		
Whole blood leukocytes	*Nik*	Anti-inflammatory response	
	Cjun		
Mouse osteoblastic osteocarcinoma	*Fos*		
	Jun		
	Ap1		
BG-1	ERα	Cell proliferation	
MCF-7 cells	ERα*		
	pIRS-1		
	pAKT		
	pMEK1/2		
	pERK1/2		
VM7Luc4E2 cells	*Erα*		

	Ps2		
	ERα		
	pS2		
	miR-22		
	miR-206		
	miR-193b		
Sheep placenta	E2	Anti-estrogenicity	
	Estrogen sulfotransferase		
BG1Luc4E2 cells	ER*		
Sprague-Dawley rats and GH3 cells	*CaBP-9k*	Estrogenicity	
LNCaP	AR	Androgenicity; cell proliferation and migration	
T47D-ARE cells	AR	Anti-androgenicity	
H4L1.1c4 cells	AR	Pro(anti)-androgenicity	
HepG2 cells	Acyl-coenzyme A oxidase	Blunted lipid metabolism	
Hepa1c1c7 cells	Acyl-coenzyme A oxidase	Enhanced lipid metabolism and DNA synthesis	

	TGF-β	Anti-apoptosis	
D. rerio	PPARα	Enhanced lipid metabolism	
	PPARγ		
G. gallus embryo livers	PPARα		
ICR mice	Akt	Impaired glucose metabolism	
	mTOR		
C57BL/6 mice	CAR	Tumorigenesis	
HepG2 cells	CAR	Enhanced hepatic catabolism	
	PXR		
Rodent FAO hepatoma cells	CAR	Reduced hepatic catabolism	
Primary skeletal myotubes	Ca^{2+}	Diminished muscle contractility	
	RyR1		
P. promelas muscle homogenates	*Ryr2*		
	Ryr3		
	RyR		
Rat thymocytes	Ca^{2+}	Cell membrane hyperpolarization	

RBL cells	Ca^{2+}	Mast cell degranulation	
C. reinhardtii	Ca^{2+}	Dampened photosynthesis	
Upregulated by TCS Downregulated by TCS Sensitive to TCS			
*TCS is anti-estrogenic in presence of E2			

Therapeutic Proposals

The first specific action mechanism of TCS in prokaryotes was only demonstrated 20 years ago, when inhibition of fatty acid synthesis in *Escherichia coli* was noted following exposure to TCS (McMurry *et al.*, 1998b; McDonnell and Russell, 1999). TCS irreversibly inhibited the fatty acid biosynthesis enzyme, enoyl–acyl carrier protein reductase (ACP), by mimicking its natural substrate *in vivo*. Further, a mutated or an overexpressed ACP, encoded by *fabI*, was shown to confer TCS resistance in the bacterium. These findings established ACP as a specific, subcellular TCS target. Efforts have thus far revealed the susceptibility of a host of other pathogens to fatty acid synthesis by TCS. These include *Staphylococcus aureus*, *M. tuberculosis*, *Helicobacter pylori*, *Haemophilus influenzae*, *Plasmodium falciparum*, *Toxoplasma gondii*, *Leishmania spp.*, and *Trypanosoma spp.* ((Heath *et al.*, 2000; Parikh *et al.*, 2000; Beeson *et al.*, 2001; Marcinkeviciene *et al.*, 2001; McLeod *et al.*, 2001; Surolia and Surolia, 2001; Lee *et al.*, 2002; Roberts *et al.*, 2003). In humans, fatty acid synthase (FAS) is the only multi-enzyme complex that is responsible for the endogenous synthesis of saturated fatty acids from acetyl-CoA and malonyl-CoA (Lu and Archer, 2005; Lupu and Menendez, 2006). Although a BLAST analysis of *E. coli*

FabI protein and FAS showed no homology, appreciable sequence similarities were nevertheless found with polyketide synthase and type I FAS of *M. tuberculosis* (Liu *et al.*, 2002).

The success of cerulenin, a mycotoxin with fatty acid inhibitory action, in suppressing tumor progression *in vivo* has spawned several reports in support of fatty acid synthesis inhibition as an emerging target for chemotherapy (Pizer *et al.*, 1996). The earliest study in this regard investigated the cytotoxicity of TCS in MCF-7 and SKBr-3 breast cancer cells (Liu *et al.*, 2002). It was revealed that TCS at 10-50 uM is cytotoxic, anti-proliferative, induces morphological alterations, and inhibits FAS. These findings corroborate an earlier observation linking FAS inhibition with apoptotic death of breast cancer cells (Kuhajda *et al.*, 1994; Kuhajda *et al.*, 2000; Liu *et al.*, 2002). TCS was similarly found to inhibit the development of methylnitrosourea-induced breast cancer in Sprague-Dawley rats (Lu and Archer, 2005). In human A-375 melanoma cells, TCS inhibited growth at 40 uM (Ho *et al.*, 2007). TCS was similarly found to be dose-dependently pro-apoptotic in prostate cancer cells, with IC_{50} values as low as 4.5-7.8 uM (Sadowski *et al.*, 2014). Whereas no cytotoxicity was observed in NIH3T3 fibroblasts at concentrations up to 60 uM, values of IC_{50} ranging from 0.74-62 uM were nonetheless observed in non-malignant prostate cells. This suggests two things; first, that prostate cells are relatively more sensitive to TCS toxicity than fibroblasts and presumably other non-malignant cell types, and second, that malignant prostate cells exhibit higher chemosensitivity compared to their non-malignant counterparts. This differential susceptibility could be due to overexpressed FAS in malignant cells. However, in contrast to these reports, at concentrations up to 345 uM, TCS was found to be preferentially cytotoxic to Y79 RB cells over mouse 3T3 fibroblasts and

human MIO-M1 Müller glia cells as indicated by IC_{50} values, creating a large therapeutic index of 7.1 and 5.3 respectively (Deepa *et al.*, 2012). FAS suppression, depleted fatty acid content, lipid peroxidation, and apoptotic death were noted in Y79 RB cells at the same TCS concentration range (Vandhana *et al.*, 2013). Recently, TCS at 40 uM was also shown to be effective against MiaPaCa-2 and AsPC-1 pancreatic cancer cells suppressing proliferation, and eliciting apoptotic death (Nishi *et al.*, 2016). Of note, in a related study, TCS impeded mouse pre-adipocyte differentiation (Schmid *et al.*, 2005). Given the regulation of food intake by FAS, and the susceptibility of adipocyte development to TCS inhibition, it was suggested that TCS may possess anti-obesogenic properties.

The differential expression and activity of FAS in healthy and malignant tissues, where it is up-regulated in the latter (Pizer *et al.*, 1997; Wilentz *et al.*, 2000), indicates a possibly high therapeutic index. The long history of human use, and the ubiquity of TCS in consumer products, coupled with encouraging *in vivo* results, cements the antimicrobial as a promising candidate for chemotherapy. As noted earlier, it must be stressed that variations in the final outcome of TCS treatment largely depend on experimental setup. Moreover, limited data from animal studies suggest that in presence of a preexisting tumor, TCS administration seems to exacerbate the condition. This observation is concerning, and indeed warrants further investigation before TCS can be invested in for clinical trials.

Conclusion and specific aims:

TCS is a synthetic antimicrobial with a long history of human use. At concentrations well below those present in commercial products, data from *in vitro* and *in vivo* studies have provided evidence of adverse effects on diverse molecular pathways. Most alarmingly

is TCS enhancement of malignant cell proliferation *in vitro* and tumor growth *in vivo*. On the other hand, TCS has also been shown to be protective against malignant cell growth and proliferation, possibly opening the door for its use in chemotherapy. Clearly, dose and time dependence is an important factor in determining the eventual denouement of the chemical. In spite of the numerous publications dissecting the signaling pathways responsive to TCS, it is evident that a severe paucity surrounding human-based *in vivo* and *in vitro* studies still remains today. Future studies, thus, should focus on identifying signaling molecules differentially regulated by TCS, and characterize their roles in toxic or protective effects in different cell types. Insights gained from such revelations will be invaluable to possibly validate targets for drug development, or devise possible TCS adjuvants or inhibitors.

The objective of this work is to provide an appraisal of the utility of TCS in chemotherapy. Toward this aim, we will investigate the influence of TCS on the growth and proliferation of leukemia cells, and delineate the molecular mechanisms underlying such an effect. Also, given the high prevalence of chemotherapy-induced anemia in cancer patients, we will study the interaction of TCS with human erythrocytes to elucidate the potential hemolytic properties of the antimicrobial. Finally, we plan to model drug formulations by examining if and how TCS activity is modulated in presence of detergent excipients.

ACKNOWLEDGMENTS:

We thank the members of the Lee laboratory for helpful advice and discussion during this work. This work was supported in part by the Brody Brothers Grant (BBE216102),

NIH (1R15AG060373-01) to M-H.L. and the Saudi Government Graduate Scholarship (through King Saud University) to M.A.A.

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CHAPTER II: ANTILEUKEMIC EFFECT OF TRICLOSAN AND ASSOCIATED MOLECULAR MECHANISMS IN BURKITT LYMPHOMA: AN *IN VITRO* STUDY

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ABSTRACT:

Burkitt's lymphoma (BL) is the fastest growing human tumor. Current treatment consists of a multiagent regimen of cytotoxic drugs with serious side effects including tumor lysis, cardiotoxicity, hepatic impairment, neuropathy, myelosuppression, increased susceptibility to malignancy, and death. Furthermore, accessibility of therapeutic interventions in areas of BL prevalence are not as feasible as in high-income countries. Therefore, there exists an urgent need to identify new therapies with safer profile and accessibility. Triclosan (TCS), an antimicrobial used in personal care products and surgical scrubs, has gained considerable interest as an antitumor agent due to its interference with fatty acid synthesis. Here, we investigate the antitumor properties and associated molecular mechanisms of TCS in Burkitt lymphoma BJAB cells. A dose-dependent cell death was observed following treatment with 10-100 μM TCS for 24 h, which was associated with membrane phospholipid scrambling, compromised permeability, and cell shrinkage. TCS-induced cell death was accompanied by a elevated intracellular calcium, perturbed redox balance, chromatin condensation, and DNA fragmentation. TCS upregulated *Bad* expression and downregulated that of *Bcl2*. Moreover, caspase and JNK MAPK signaling were required for the full apoptotic activity of TCS. In conclusion, this report identifies TCS as an antileukemic agent and provides new insights into the molecular mechanisms governing TCS-induced apoptosis in BL cells.

KEYWORDS: Lymphoma; Chemotherapy; Triclosan; Apoptosis

INTRODUCTION:

Burkitt lymphoma (BL) is a malignant tumor of B lymphocytes, with metastatic dissemination to the nervous system, bone marrow, and abdominal tissue (Dozzo *et al.*, 2017). The most aggressive human tumor (Molyneux *et al.*, 2012), BL is often caused by a chromosomal translocation of immunoglobulin and *c-MYC* genes. Malignant transformation is strongly associated with Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), and malaria (Molyneux *et al.*, 2012), although BL still occurs in absence of infection. Affecting patients of all age groups, BL is the most common cancer in children where malaria is holoendemic (Orem *et al.*, 2007).

The currently approved treatment for BL consists of a multiagent regimen colloquially known as CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone). This standard approach remains insufficient, and short-intensive, periodical treatment plans are most often required, consisting of methotrexate, cytarabine, etoposide, ifosfamide, and carboplatin (Dozzo *et al.*, 2017). However, constrained by economic factors, therapeutic interventions in areas where BL is more prevalent, as in Africa, are not as feasible as in high-income countries (Molyneux *et al.*, 2012). Prognosis is similarly negatively influenced by such a reality (Hesseling *et al.*, 2005). Furthermore, serious side effects of existing BL treatment include tumor lysis, cardiotoxicity, hepatic impairment, neuropathy, hemorrhagic cystitis, myelosuppression, and increased susceptibility to malignancy (Molyneux *et al.*, 2012; Casulo and Friedberg, 2015; Ahlmann and Hempel, 2016; Kurauchi *et al.*, 2017; Luu *et al.*, 2018). More alarming has been the mortality attributed to therapy in about 5% of BL patients (Hesseling *et al.*, 2005; Hesseling *et al.*, 2009). Of note, antiretroviral therapy has thus far failed to reduce the incidence of

immunodeficiency-associated BL (Casulo and Friedberg, 2015). Therefore, there exists an urgent need to identify alternative therapies with preferentially lesser side effects and improved accessibility (Hesseling *et al.*, 2009; Schmitz *et al.*, 2012).

Triclosan (TCS; 2,4,4´-trichloro-2´-hydroxydiphenyl ether; CAS 3380-34-5) is a broad-spectrum antimicrobial used in a wide variety of commercial products including household items, cosmetics, soaps, toothpastes, lotions, deodorants, and surgical scrubs (Jones *et al.*, 2000). The mechanism of TCS action, aside from the disruption of cell membrane integrity (Guillen *et al.*, 2004), relies on its inhibition of fatty acid synthase (McMurry *et al.*, 1998b). This has ignited an interest among researchers to further explore the potential use of TCS as a chemotherapeutic agent by targeting fatty acid synthesis; a major source of energy in malignant cells (Schcolnik-Cabrera *et al.*, 2018).

To date, conflict in the literature regarding the effect of TCS on ultimate cell fate still exists. While several reports have described the antiproliferative activity of TCS in MCF-7 breast cancer cells (Liu *et al.*, 2002; Vandhana *et al.*, 2010), recent findings indicate that TCS promotes MCF-7 growth and invasion (Lee *et al.*, 2014; Lee *et al.*, 2017a; Lee *et al.*, 2017b). A similar response was also observed in LNCaP prostate cancer cells (Sadowski *et al.*, 2014; Kim *et al.*, 2015) and H460 lung cancer cells (Winitthana *et al.*, 2014). Thus, the contrasting reports on the denouement of TCS exposure and the duality of its role poses a viable and feasibly exploitable avenue to further explicate its antineoplastic potential. Herein, we investigate the interaction of TCS with BJAB Burkitt lymphoma cells, and delineate the molecular mechanisms governing the antitumor role of the antimicrobial.

MATERIALS AND METHODS:

Chemicals and reagents:

All chemicals are of analytical grade and were purchased from MilliporeSigma (Burlington, MA, USA) unless otherwise noted. A stock solution of TCS was prepared in ethanol at 10 mM, and further diluted to testing concentrations in RPMI-1640 medium. Intracellular Ca^{2+} chelator glycine, *N*,*N*′-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-, bis[(acetyloxy)methyl] ester (BTM) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), p38 MAPK inhibitor SB2035080 (SB) was from Selleckchem (Houston, TX, USA), while c-Jun N-terminal kinase (JNK) inhibitor XVI (JNK-IN-8) and pan-caspase inhibitor zVAD(OH)-fmk (zVAD) were from Cayman Chemical Company (Ann Arbor, MI, USA). All inhibitors were dissolved in dimethyl sulfoxide (DMSO) and then diluted to desired concentrations in RPMI-1640 prior to cell treatment.

Cell Culture:

B cell acute lymphoblastic leukemia BJAB cell line was grown at 37^0C with 5% CO_2 in RPMI-1640 medium supplemented with 0.3 g/L L-glutamnie, 10% heat-inactivated fetal bovine serum, and 1% antibiotic-antimycotic mixture (Thermo).

Cytotoxicity:

Dual acridine orange/ethidium bromide (AO/EB) staining was utilized to distinguish live and dead cells based on membrane integrity. Control and treated cells were mixed with an equal volume of AO/EB staining solution (AO = 1 µg/ml; EB = 20 µg/ml),

loaded on a hemocytometer, and live and dead cells were counted and imaged with EVOS® fl Digital Inverted Microscope (Thermo).

Phosphatidylserine (PS) externalization, membrane integrity, and cellular dimensions:

Identification of the mode of cell death was achieved using Annexin-V-FITC (AV) and propidium iodide (PI) supravital staining (Thermo). Following treatment, cells were washed twice in PBS, resuspended in Annexin binding buffer, and double-stained with 1.25% v/v Annexin-V-FITC and 20 µg/ml PI for 20 min at room temperature away from light. Two-dimensional analysis was subsequently carried out on a FACScan flow cytometer (Betcon Dickinson, Franklin Lakes, NJ, USA) at excitation and emission wavelengths of 488/530 (FITC) and 535/617 nm (PI), respectively. Forward scatter (FSC) and side scatter (SSC) properties were simultaneously determined.

Oxidative stress:

Generation of reactive oxygen species (ROS) was detected by the cell-permeant probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Thermo). This nonfluorescent probe is oxidized by intracellular ROS into DCF whose fluorescence is proportional to ROS levels. Cells were preloaded with 2 µM H_2DCFDA for 45 min at 37^0C. treated with TCS for 30 min, washed in PBS to remove excess, unbound dye, and fluorescence was analyzed on a FACScan at an excitation wavelength of 495 nm and an emission wavelength of 527 nm.

Intracellular calcium:

Cell-permeable Ca^{2+} indicator Fluo3/AM (Biotium, Fremont, CA, USA) was utilized to measure cytosolic Ca^{2+} content. Nonfluorescent Fluo3/AM is hydrolyzed by intracellular esterases into Fluo3 whose fluorescence increases upon Ca^{2+} binding. To measure cytoplasmic Ca^{2+} levels, control and treated cells were washed in PBS, suspended in 2.5 mM $CaCl_2$ solution containing 1 µM Fluo3/AM for 30 min at 37^0C in the dark, washed again in PBS, and finally subjected to FACS analysis. Fluo3 was excited by the 488 argon-ion laser and emitted fluorescence was detected at 530 nm. Control and experimental cells were imaged with EVOS® fl Digital Inverted Microscope following staining with 5 µM Fluo3/AM.

Nuclear chromatin condensation:

Control and treated cells were incubated with 100 ng/ml Hoechst 33342 for 30 min at room temperature in the dark, and then imaged with EVOS® fl Digital Inverted Microscope.

Oligonucleosomal DNA fragmentation:

Genomic DNA was isolated from control and treated cells using Qiagen's DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. A total of 200 ng of extracted DNA was fractioned by 0.7% agarose gel electrophoresis, visualized with EB, and documented with UVP MultiDoc-It™ UV trans-illuminator.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR):

Total RNA was extracted using Qiagen's RNeasy Mini Kit and assessed for quality and quantity on NanoDrop 1000 Spectrophotometer V3.7 (Thermo). A total of 1 µg of RNA was denatured and reverse transcribed to cDNA using SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Synthesized cDNA was amplified by qRT-PCR (Bio-Rad iQ^{TM}5 Multicolor RT-PCR Detection System) using PowerUp™ SYBR™ Green Master Mix (Applied Bioscience, Carlsbad, CA, USA) and Sigma's KiCqStart® SYBR® Green primers for *CAT, SOD, GPX, GSR, BAD, BCL2, H2AFX, PARP1,* and *ACTB.* Relative quantification of gene expression was achieved using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis:

Results are expressed as means $\pm$ S.E.M. Comparative analysis among the means was performed by Student's *t*-test or one-way ANOVA with Dunnett's *post hoc* test as analyzed by Prism 5.0 (GraphPad Software, San Diego, CA, USA). Significance was defined as a *P* value of <0.05.

RESULTS:

TCS induces cell death dose-dependently

The cell membrane is a primary target of TCS (Villalain *et al.*, 2001; Lygre *et al.*, 2003; Guillen *et al.*, 2004). To assess the cytotoxic potential of TCS, BJAB cells were treated with 10-100 µM TCS for 24 h at 37^0C, and live and dead cells were distinguished based on membrane integrity as determined by AO/EB double staining. While AO stains live and dead cells green, EB only penetrates through damaged membranes imparting a red fluorescence on dead cells. As shown in Figure 1B&C, an exponential increase in the

percentage of EB-stained cells is observed with increasing TCS concentrations, an effect attaining statistical significance at 30 µM. This indicates a dose-dependent cytotoxic effect by TCS against B-cell lymphoma cells.

TCS stimulates both AV and PI uptake

To identify the pattern of cell death inflicted by TCS, a FACS analysis with AV and PI labeling was employed. Whereas AV is taken up by PS-exposing cells, those with a ruptured membrane are permeable to PI. Figure 2 shows that while untreated cells exclude both dyes, significantly increased AV and PI uptake was observed for TCS-treated cells. At 50 µM, TCS caused a significant increase in the percentage of AV^-/PI^+ cells from 2% $\pm$ 0.499 to 49.36% $\pm$ 14.16. For cells treated with 75 µM TCS, AV^+/PI^+ cells represented a significant increase to 16.55% $\pm$ 4.98, compared to a control value of 5.37% $\pm$ 0.91, while those showing AV^-/PI^+ were 76.49% $\pm$ 2.58; a significant increase from 2% $\pm$ 0.499 in the case of control cells. Taken together, these data, coupled with the virtual absence of cells solely bound to AV, seem to implicate late apoptosis that has progressed to secondary necrosis as a mode of death.

TCS-induced cell death is accompanied by shrinkage and enhanced granularity

One of the morphological hallmarks of dying cells is shrinkage and increased granularity (Lizard *et al.*, 1995; Lizard, 2001), seen as a decrease in FSC due to cell condensation and an increase in SSC due to surface complexity. Light scatter properties were therefore analyzed in order to estimate cell size and granularity following TCS treatment.

As depicted in Figure 3A-D, TCS exposure caused a significant decrease in FSC geomean from an average of 525.1 ± 22.52 (control) to 422.9 ± 25.68 (50 µM) and 304.9 ± 27.35 (75 µM). The percentage of cells with a reduced FSC (<400) also increased from 3.92% ± 0.55 (control) to 15.65% ± 3.78 (50 µM) and 32.72% ± 3.07 (75 µM), while that of cells with FSC >800 significantly diminished following treatment with 75 µM TCS (11.43 ± 1.99 vs. 1.98 ± 0.79). As for SSC, significant elevations were noted following treatment with TCS (Figure 3E-G). At 75 µM TCS increased SSC geomean from 133 ± 9.30 to 175.72 ± 17.60. Also, the percentage of cells with enhanced SSC increased from 8.29 ± 0.28 to 19.73 ± 2.12 (50 µM) and 18.79 ± 1.79 (75 µM).

Collectively, the significant changes in cellular dimensions indicate that TCS treatment alters the light scatter behavior of cells, reflective of cellular shrinkage and increased surface complexity; two distinctive features of apoptotic cells.

TCS increases intracellular Ca^{2+} accumulation

Calcium is a major regulator of apoptosis. To assess the role of Ca^{2+} in TCS-induced cell death, cells were incubated with and without 25-75 µM TCS at 37^0C for 30 min and stained with Fluo3 as described earlier. Figure 4A-C show that Fluo3 fluorescence intensity rises to 16.63 ± 1.38 (50 µM) and 25.27 ± 4.02 (75 µM) relative to an average of 5.0 ± 0.65 in the case of control cells. A significant increase in the percentage of cells with enhanced Fluo3 fluorescence was also detected at 50 µM (34.31% ± 4.47) and 75 µM (42.85% ± 3.31) TCS compared to control cells (8.89% ± 1.25).

Because TCS lead to elevated Ca^{2+} levels, we were prompted to determine the contribution of Ca^{2+} mobilization to TCS-induced cell death. To this end, cells were incubated for 24 h at 37^0C in presence or absence of 50 µM TCS with and without 1 h pretreatment with 10 µM BTM, and cytotoxicity was then examined by AV/PI staining. As depicted in Figure 4 E&F, no significant difference in cell death was observed in presence of BTM compared to its absence (59.45 $\pm$ 3.56 vs. 56.67 $\pm$ 5.76), which indicates that TCS-induced Ca^{2+} increase was not necessary for the full apoptotic activity of TCS.

TCS causes oxidative stress

Perturbations in cellular redox state and excessive ROS production are associated with apoptosis. In order to examine the effect of TCS on ROS levels, cells were preloaded with 2 µM H_2DCFDA for 45 min at 37^0C and then either left untreated or exposed to 25-75 µM TCS for an additional 30 min, before DCF fluorescence was subsequently detected as described earlier. Data in Figure 5A-C indicate that, compared to a control value of 2.89 $\pm$ 0.17, TCS at 50 and 75 µM significantly increased the average geomean DCF fluorescence to 5.90 $\pm$ 0.49 and 8.0 $\pm$ 0.62, respectively. The percentage of cells with enhanced fluorescence also significantly increased from an average of 2.95% $\pm$ 0.24 (control) to 14.01% $\pm$ 2.24 (50 µM TCS) and 22.23% $\pm$ 1.80 (75 µM TCS).

Given the observed oxidative stress, it was of interest to identify the effect of TCS on redox enzymes regulating ROS levels. For this purpose, gene expression of key antioxidant enzymes was examined by qRT-PCR. As shown in Figure 5D-G, TCS significantly downregulated *CAT*, *SOD*, *GPX*, and *GSR* expression at all concentrations tested. Furthermore, we evaluated the contribution of oxidative stress in TCS-induced cell

death by incubating the cells for 24 h with and without 50 µM TCS in presence or absence of 1 mM ROS scavenger N-acetylcysteine (NAC). As seen in Figure 5 H&I, the percentage of cell death in presence of NAC is not significantly different from its absence (54.41% $\pm$ 3.47 vs. 55.45% $\pm$ 2.97). This indicates that TCS treatment induces oxidative stress which is nevertheless apparently not required for its full cytotoxic effect.

TCS perturbs *BAD* and *BCL2* gene expression

The expression pattern of Bcl-2 genes regulates apoptosis and participates in tumor progression. To interrogate the influence of TCS on *BAD* and *BCL2* gene expression, control and treated cells were subjected to qRT-PCR analysis as described earlier. Figure 6A shows that TCS significantly upregulates the proapoptotic *BAD* gene expression and significantly downregulates that of the antiapoptotic *BCL2*. This pattern is consistent with apoptosis and complements other findings in this study.

TCS triggers DNA damage

A prominent feature of apoptotic cells is systematically fragmented DNA. To elucidate the effect of TCS on DNA integrity, cells were treated with 25-75 µM for 24 h, and multiple methods were subsequently employed. Initially, nuclear condensation was evaluated by Hoechst 33342; a widely used DNA-binding dye whose fluorescence intensity is proportional to chromatin condensation. Figure 7A shows enhanced fluorescence in presence of TCS compared to the control cells, reflective of pyknotic nuclei.

Next, we examined the degree of DNA fragmentation as caused by TCS exposure. The electrophoretic pattern in Figure 7B depicts oligomers of DNA extracted from control

and TCS-treated cells. While no significant DNA degradation is observed in untreated cells, those exposed to TCS exhibit pronounced fragmentation. Finally, in an attempt to investigate impaired or responsive DNA repair pathways, we analyzed mRNA expression profiles of major repair genes including *γH2AFX* and *PARP1*. TCS upregulated *H2AX* expression (Figure 7C) and downregulated that of *PARP1* (Figure 7D). Taken together, these data provide evidence of compromised DNA integrity and repair mechanisms characteristic of cellular death.

TCS-induced cell death is mediated through caspase and JNK signaling

Multiple signaling pathways are implicated in the regulation of cell survival and response to stress stimuli. To identify signaling mediators required for the full cytotoxic activity of TCS, cells were preincubated with 100 µM zVAD(OH)-fmk, 100 µM Nec-1, 50 nM NSA, 5 µM SB203580, or 5 µM U0126 for 1 h, or with 5 µM JNK-IN-8 for 3 h, before they were treated with 50 µM TCS for 24 h.

As shown in Figure 8A, TCS-induced cell death was significantly, but not thoroughly, ameliorated in presence of zVAD (58.08 $\pm$ 2.49 vs. 41.64 $\pm$ 3.02), indicating that TCS is cytotoxic in caspase-dependent and independent mechanisms. Similarly, Figure 8D shows that JNK inhibition significantly protects the cells against TCS toxicity (44.29 $\pm$ 4.12 vs. 35.25 $\pm$ 2.21) identifying JNK as a requirement for the full cytotoxic effect of TCS. Notably, blockade of key elements of necroptosis signaling failed to significantly reverse TCS-induced cell death. These observations, along with other findings in this study, are strongly indicative of apoptosis as the type of cell death stimulated by TCS in BJAB Burkitt lymphoma cells.

DISCUSSION:

TCS is a widely used antimicrobial with a long history of use in commercial products and clinical practice. Although previous reports have discerned both pro- and antitumor activities, the effect of TCS on blood malignancies has largely been overlooked. To the best of our knowledge, this report is the first to identify TCS as an anti-leukemic/anti-lymphoma agent. It was revealed that TCS induced apoptosis in Burkitt lymphoma cells at least in part through dysregulated calcium homeostasis, oxidative stress, and nuclear fragmentation. Moreover, the antitumor effect of TCS was mediated through caspase and JNK signaling. TCS concentrations used in this study are several orders of magnitude lower than those present in consumer products (Rodricks *et al.*, 2010) and are within the range shown to be cytotoxic to a variety of cells (Liu *et al.*, 2002; Ho *et al.*, 2007; Sadowski *et al.*, 2014).

During apoptosis, cells display a reduced volume (Figure 3A-D) as the cytosolic accumulation of Ca^{2+} (Figure 4A-D) activates Ca^{2+}-responsive K^+ channels leading to loss of KCl and osmotically obliged water (Lang *et al.*, 2006b). A reduced FSC may also indicate dying cells as they fragment into smaller apoptotic bodies (Bortner and Cidlowski, 2007). Although it is unknown what advantage this morphological hallmark gives to dying cells, it is thought to facilitate their engulfment by larger phagocytes. A coarser and more granular cell surface (Figure 3E-G) may result from wrinkled membrane or internal complexity reflecting changes in number and shape of intracellular organelles (Ramirez *et al.*, 2013).

One important distinction that must be borne in mind is one between necrotic cell rupture and the disruption of cell membrane by TCS. As a lipophilic compound, TCS readily diffuses into the membrane where it is positioned perpendicularly to the phospholipid bilayer (Guillen *et al.*, 2004). Consequently, alterations in membrane permeability ensue. Along those lines, our results show that TCS-treated cells become permeable to EB and PI (Figures 1 & 2), indicating compromised membrane integrity. Therefore, caution must be exercised in interpreting EB/PI-positive cells as necrotic especially in light of solid evidence of apoptotic transformation. In fact, apoptotic cells are recognized by phagocytes via the display of the "eat me" signal (i.e. PS externalization). However, given the absence of phagocytes *in vitro*, the membrane of apoptotic cells eventually loses its permeability becoming permissive to vital dyes (secondary necrosis) (Silva, 2010).

Calcium plays a crucial role in innumerable cellular processes, including signal transduction and regulated cell death. The asymmetrical architecture of phospholipids in the cell membrane is controlled by Ca^{2+}-dependent scramblases, and Ca^{2+} accumulation (Figure 4) stimulates PS exposure (Figure 2) possibly through dysregulated activity of enzymes and transmembrane proteins (Suzuki *et al.*, 2010). It has also been observed that inordinate liberation of sequestered Ca^{2+} ions from the endoplasmic reticulum into the mitochondria contributes to apoptosis by dephosphorylating Bad and opening of mitochondrial permeability transition pore (Zhivotovsky and Orrenius, 2011). Sustained pore opening causes a spill out of mitochondrial content, most notably Ca^{2+} ions and apoptosome components, and water influx, eventually leading to mitochondrial swelling and rupture. Furthermore, cytosolic Ca^{2+} accumulation leads to systematic DNA

fragmentation (Figure 7B) by activating Ca^{2+}- and Mg^{2+}-dependent endonuclease (Wyllie, 1980). Similarly, the intranuclear presence of Ca^{2+} has been shown to influence chromatin organization (Figure 6A), modulate gene expression, and activate caspases (Zhivotovsky and Orrenius, 2011).

We have also shown that TCS-induced apoptosis of B lymphoid cells is partially mediated through oxidative stress (Figure 5). Excessive generation of ROS contributes to nucleic acid (Figure 7), protein, lipid, and organelle damage, which in turn initiates the cellular death machinery (Redza-Dutordoir and Averill-Bates, 2016). This is in congruence with our previous report of TCS interference with the antioxidant master regulator, Nrf2, in primary human mesenchymal stem cells (Yoon *et al.*, 2017). Recent studies have also described the prooxidative effect of TCS in thyroid follicular epithelial Nthy-ori 3-1 cells (Zhang *et al.*, 2018) and retinoblastoma Y79 cells (Vandhana *et al.*, 2013). Interestingly, TCS rather diminished ROS levels in MCF-7 variant breast carcinoma VM7Luc4E2 cells (Lee *et al.*, 2018) as part of its antiapoptotic activity.

Moreover, ROS activate p53 or JNK which unbalances mitochondrial Bcl2 proteins in favor of apoptosis. ROS similarly depolarize the mitochondrial membrane which facilitates the liberation of cytochrome *c* from to the cytoplasm where it forms the apoptosome in association with Apaf-1 and procaspase-9. Consequently, effector caspases (e.g., caspase 3) execute apoptosis by cleaving cellular proteins (Redza-Dutordoir and Averill-Bates, 2016). Indeed, we found that TCS upregulates *BAD* expression and downregulates that of *BCL2* (Figure 6). *BAD* contributes to tumorigenesis (Marchion *et al.*, 2011) and overexpression sensitizes tumor cells to apoptotic stimuli (Mok *et al.*, 1999; Taghiyev *et al.*, 2003) and reduces tumorigenicity (Jiang *et al.*, 2013). *BCL2* overexpression has been

observed in follicular lymphomas (Fernandez *et al.*, 2019) and is known to favor prostate cancer progression and metastasis (Furuya *et al.*, 1996; Zellweger *et al.*, 2005), and resistance to apoptosis in pancreatic tumor (Bold *et al.*, 2001).

A late feature of apoptotic cell death is systematic DNA cleavage by endonucleases downstream of caspase-3 (Collins *et al.*, 1997). Apoptosis through the extrinsic or intrinsic pathways converge at activated caspase-3 which initiates DNA fragmentation. Nevertheless, apoptosis-inducing factor (AIF) and endonuclease G are both liberated from the mitochondria and may degrade the DNA independent of caspase activation (Elmore, 2007). ROS may also directly oxidize the DNA (Redza-Dutordoir and Averill-Bates, 2016). Since caspase (Figure 8A), but not ROS (Figure 5H&I), inhibition significantly rescued the cells from TCS-induced apoptosis, it follows then that DNA fragmentation (Figure 7B) in B-cell lymphoblasts, as caused by TCS, is most likely mediated through caspase stimulation.

Our results also show that TCS upregulates *H2AX* expression (Figure 6C). *H2AX* encodes for H2A Histone Family Member X whose phosphorylation (γ-H2AX) is induced by double-strand breaks and a stalled replication fork (Gagou *et al.*, 2010). In fact, phosphorylation at Ser-139 is induced by DNA fragmentation during apoptosis (Rogakou *et al.*, 2000). Also, knockdown of both copies of *H2AX* in mice caused lymphoma and solid tumors (Bassing *et al.*, 2003), suggesting that *H2AX* could be possess a tumor suppressing role (Kuo and Yang, 2008) which may therefore be targeted for therapy (Shay and Roninson, 2004). Some treatment modalities activate DNA repair to induce senescence in cancer cells (Shay and Roninson, 2004). It is important to note that radiation therapy is

known to induce DNA breaks, and γ-H2AX formation serves as a biomarker for radiosensitivity of cancer cells (Taneja *et al.*, 2004).

PARP1 encodes for poly(ADP-ribose) polymerase 1, an enzyme involved in chromatin remodeling, DNA repair, differentiation, proliferation, and tumorigenesis (Ahel *et al.*, 2008). As shown in Figure 6D, TCS downregulated *PARP1* expression which indicates impeded proliferation and impaired DNA repair. Interference with DNA damage response has recently gained considerable interest for the development of novel chemotherapeutics, some of which are currently used in the clinic for various cancers (Ray Chaudhuri and Nussenzweig, 2017). Likewise, targeting *PARP1* shows promise for cardiovascular and nervous disease (Pillai *et al.*, 2006; Reinemund *et al.*, 2009)).

Our small-molecule inhibitor studies demonstrate that TCS-induced cell death is significantly, but not thoroughly, ameliorated under conditions of caspase inhibition (Figure 8A). Thus, TCS induces apoptosis in B cell lymphoma cells through caspase-dependent and independent mechanisms. Similarly, caspase activation by TCS has been detected in human primary placental syncytiotrophoblasts (Zhang *et al.*, 2015) and choriocarcinoma JEG-3 cells (Honkisz *et al.*, 2012).

MAPKs are involved in the regulation of many cellular functions including proliferation, differentiation, apoptosis, and response to various stimuli. Activation of p38 induces apoptosis (Wada and Penninger, 2004), and cells not rescued by ERK inhibition (Figure 7C) is congruent with its described role as a survival factor (Harada *et al.*, 2004). On the other hand, Figure 8D shows that JNK inhibition attenuates TCS-induced cell death, identifying JNK as a target for TCS and as a requirement for its full apoptotic activity in

lymphoblastic leukemia B cells. JNK promotes proliferation and paradoxically many types of programmed cell death, including apoptosis (Wada and Penninger, 2004), depending on cell type and stimulus (Dhanasekaran and Reddy, 2008; Dhanasekaran and Reddy, 2017). In fact, maintenance of JNK in a dephosphorylated state has been shown to prevent apoptosis and hence contribute to therapy resistance (Candas *et al.*, 2014). Accordingly, JNK activation accompanied TCS-induced apoptosis in rat neural stem cells (Park *et al.*, 2016) and hypothyroidism in Sprague-Dawley rats (Zhang *et al.*, 2018). Conversely, TCS-induced proliferation of mouse epidermal JB6 Cl 41-5a cells was associated with, but did not require, JNK activity (Wu *et al.*, 2015).

Because impaired apoptosis is a hallmark of carcinogenesis, targeting apoptotic defects is an effective intervention that forms the basis behind the action mechanism of numerous chemotherapeutic agents (Wong, 2011). Drugs that modulate the Bcl-2 family of proteins and those that activate caspases are currently being investigated in clinical trials for different tumors including leukemias and lymphomas (Wong, 2011; Jiang *et al.*, 2013). Other apoptotic hallmarks, such as Ca^{2+} overload and oxidative stress, have similarly been investigated as therapeutic targets (Giorgi *et al.*, 2010; Ndombera *et al.*, 2016). Furthermore, monoclonal antibody (mAb)-based therapies, administered either in isolation or conjugated to chemotherapeutic drugs, have also demonstrated efficacy against various diseases with more than 30 antibodies currently approved for treatment (Liu, 2014). Of particular relevance to BL is rituximab, an anti-CD20 mAb, which significantly improved BL prognosis in young and adult populations alike (Dozzo *et al.*, 2017). Finally, microRNAs have recently been identified as regulators of cell death, survival, and

chemosensitivity in B-cell tumors (Leivonen *et al.*, 2017), suggesting they could be pursued as pharmaceutical targets for prospective therapies.

ACKNOWLEDGMENTS:

COMPETING INTERESTS:

The authors declare they have no competing interests relevant to this manuscript.

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FIGURE LEGENDS:

Figure 1. TCS induces cell death dose-dependently. (A) Molecular structure of TCS. (B) BJAB cells were exposed to 10-100 µM for 24 h at 37°C, double-stained with AO/EB to distinguish live and dead cells. Cytotoxicity is expressed as the percentage of dead cells compared to untreated control cells. (C) Micrographs showing AO and EB uptake by live and dead cells, respectively.

Figure 2. TCS stimulates AV and PI uptake. (A) Representative two-dimensional dot plots depicting the distribution of control cells treated with the vehicle and those treated with 25-75 µM TCS for 24 h at 37°C. (B) Arithmetic means $\pm$ SEM of AV/PI uptake in control and TCS-treated cells.

Figure 3. Effect of TCS on cellular morphology. (A) Representative histogram overlay showing FSC of control cells (black line) and those exposed to 75 µM TCS (blue line) for 24 h at 37°C. (B) Arithmetic means $\pm$ SEM of FSC in control cells and cells treated with 25-75 µM TCS. (C) Arithmetic means $\pm$ SEM of the percentage of cells with reduced FSC in control and treated groups. (D) Arithmetic means $\pm$ SEM of the percentage of cells with increased FSC in control and treated groups. (E) Representative histogram overlay showing SSC of control cells (black line) and those exposed to 75 µM TCS (pink line) for 24 h at 37°C. (F) Arithmetic means $\pm$ SEM of SSC of control cells and cells treated with 25-75 µM TCS for 24 h at 37°C. (G) Arithmetic means $\pm$ SEM of the percentage of cells with enhanced SSC in control and treated groups. (H) Representative two-dimensional dot plots depicting FSC and SSC patterns in control and treated cells.

Figure 4. TCS increases intracellular Ca^{2+} levels. (A) Representative histogram of Fluo3 fluorescence in control cells (black line) and those treated with 75 µM TCS (orange line) for 30 min at 37°C. (B) Arithmetic means ± SEM of Fluo3 fluorescence in control cells and cells treated with 25-75 µM TCS. (C) Arithmetic means ± SEM of the percentage of cells with increased Fluo3 fluorescence in control and treated groups. (D) Micrographs showing Fluo3 fluorescence intensity in control and experimental conditions. (E) Representative two-dimensional dot plots of AV/PI uptake in cells treated with 50 µM TCS with (orange plot) or without (red plot) 1 h pretreatment with 10 µM BTM. (F) Arithmetic means ± SEM of the percentage of dead cells in presence and absence of BTM.

Figure 5. TCS causes oxidative stress. (A) Representative histogram of DCF fluorescence in control cells (green line) and those treated with 75 µM TCS (brown line) for 30 min at 37°C. (B) Arithmetic means ±SEM of DCF fluorescence in control cells and cells treated with 25-75 µM TCS. (C) Arithmetic means ± SEM of the percentage of cells with increased DCF fluorescence in control and treated groups. (D-G) Relative mRNA expression levels of antioxidant enzyme genes *CAT, SOD, GPX,* and *GSR* in control and TCS-treated cells. (H) Representative two-dimensional dot plots of AV/PI uptake in cells treated with 50 µM TCS with (turquoise plot) or without (red plot) 1 h pretreatment with 1 mM NAC. (F) Arithmetic means ± SEM of the percentage of dead cells in presence and absence of NAC.

Figure 6. TCS unbalances *BAD* and *BCL2* gene expression. Relative mRNA expression levels of *BAD* (A) and *BCL2* (B) genes in control cells and cells treated with 50 µM TCS for 24 h at 37°C.

Figure 7. TCS induces genotoxicity. (A) Micrographs of Hoechst fluorescence intensity in control and experimental conditions. (B) DNA laddering pattern in control and treated cells following electrophoretic migration through 0.75% agarose gel. (C) Relative mRNA expression levels of *H2AX* gene in control cells and those exposed to 50 µM TCS for 24 h at 37°C. (D) Relative mRNA expression levels of *PARP1* gene in control cells and those exposed to 50 µM TCS for 24 h at 37°C.

Figure 8. Effect of small-molecule inhibitors on TCS-induced cell death. Representative two-dimensional dot plots of AV/PI uptake and arithmetic means $\pm$ SEM of the percentage of dead cells following treatment with 50 µM TCS in absence (red plot) and presence of 100 µM zVAD (purple plot; A), 5 µM SB (blue plot; B), 5 µM U0126 (tan plot), 5 µM JNK-IN-8 (green plot; D), 100 µM Nec-1 (brown plot; E), or 50 nM NSA (pink plot).

Figure 9. A working model for TCS-induced apoptosis in BJAB cells. TCS disrupts membrane asymmetry and permeability, elevates cytosolic Ca^{2+} levels, perturbs redox balance, activates caspase and JNK signaling, causing imbalance of Bcl-2 family of proteins, and nuclear fragmentation.

FIGURES:

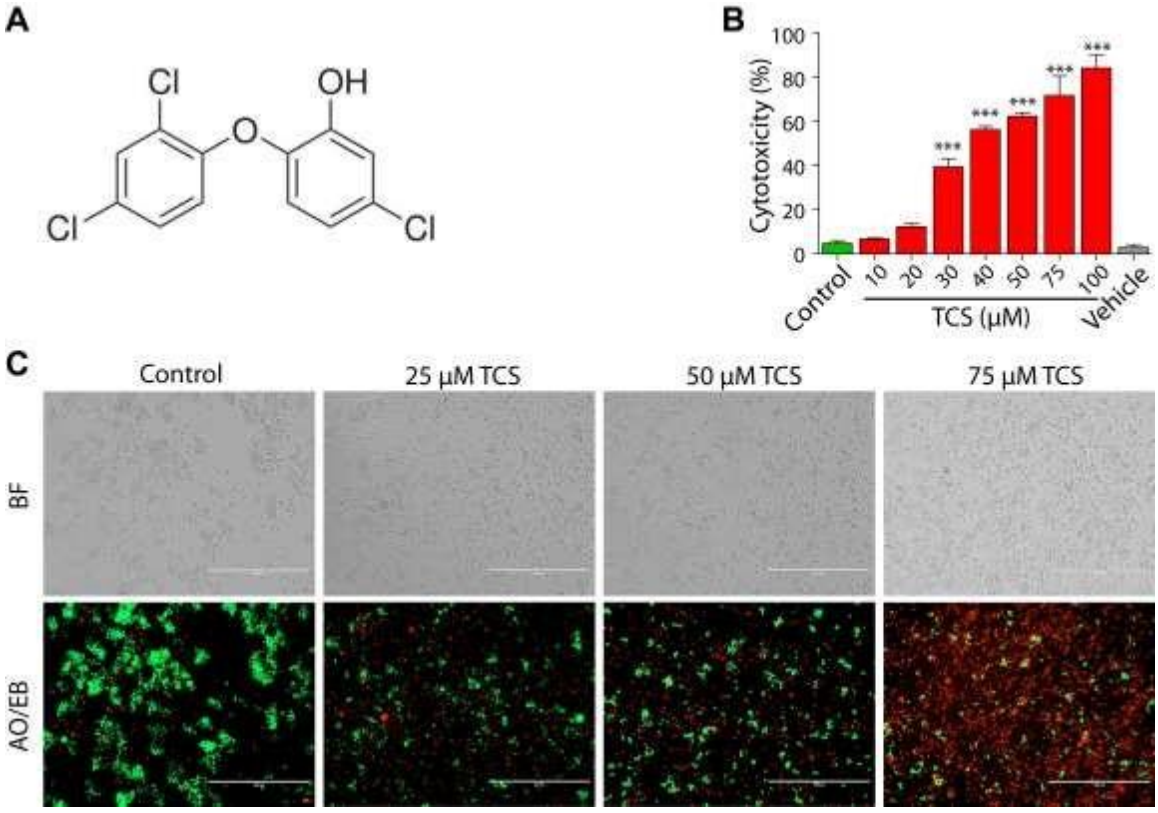

Figure 1

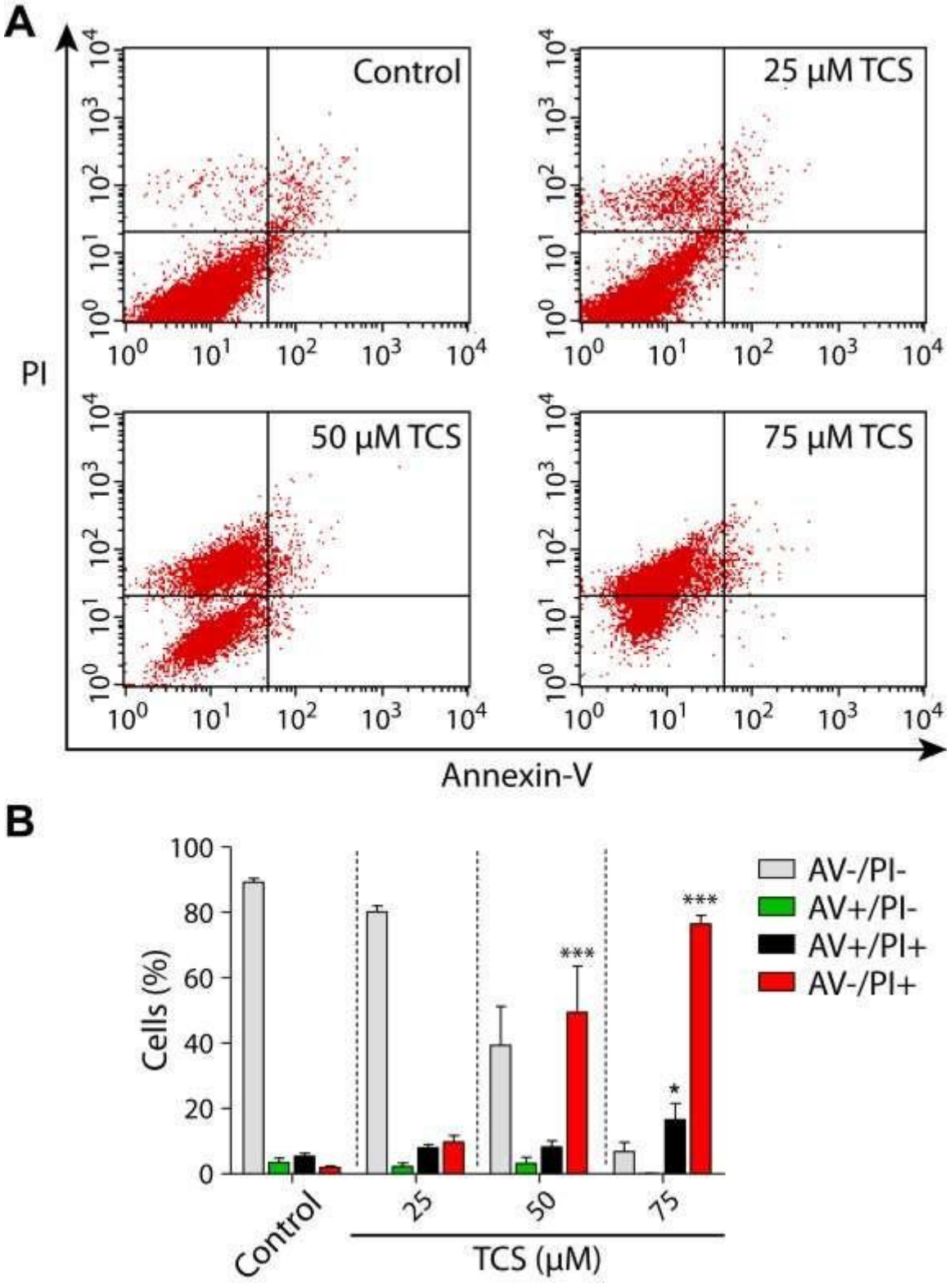
A
Control
25 µM TCS
50 µM TCS
75 µM TCS
PI
Annexin-V
B
Cells (%)
AV-/PI-
AV+/PI-
AV+/PI+
AV-/PI+
***
***
*
Control
25
50
75
TCS (µM)

Figure 2

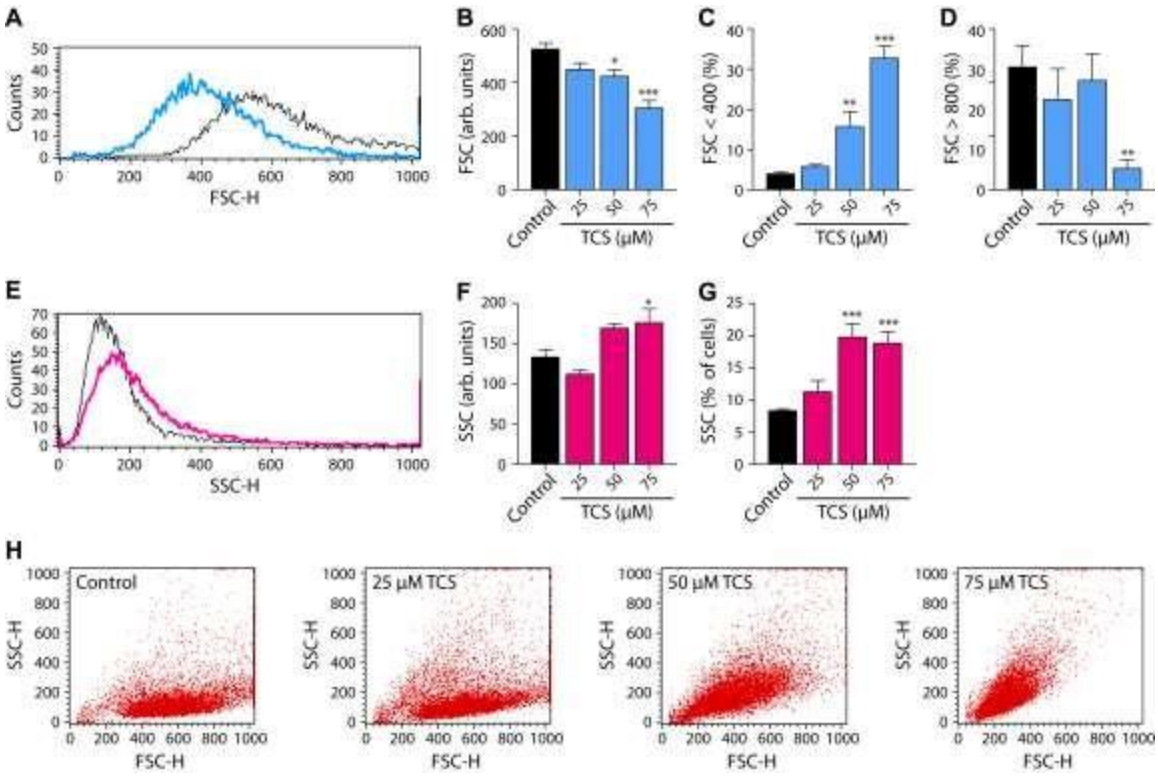
A
Counts
FSC-H
B
FSC (arb. units)
Control
TCS (μM)
C
FSC < 400 (%)
D
FSC > 800 (%)
E
SSC-H
F
SSC (arb. units)
G
SSC (% of cells)
H
Control
25 μM TCS
50 μM TCS
75 μM TCS

Figure 3

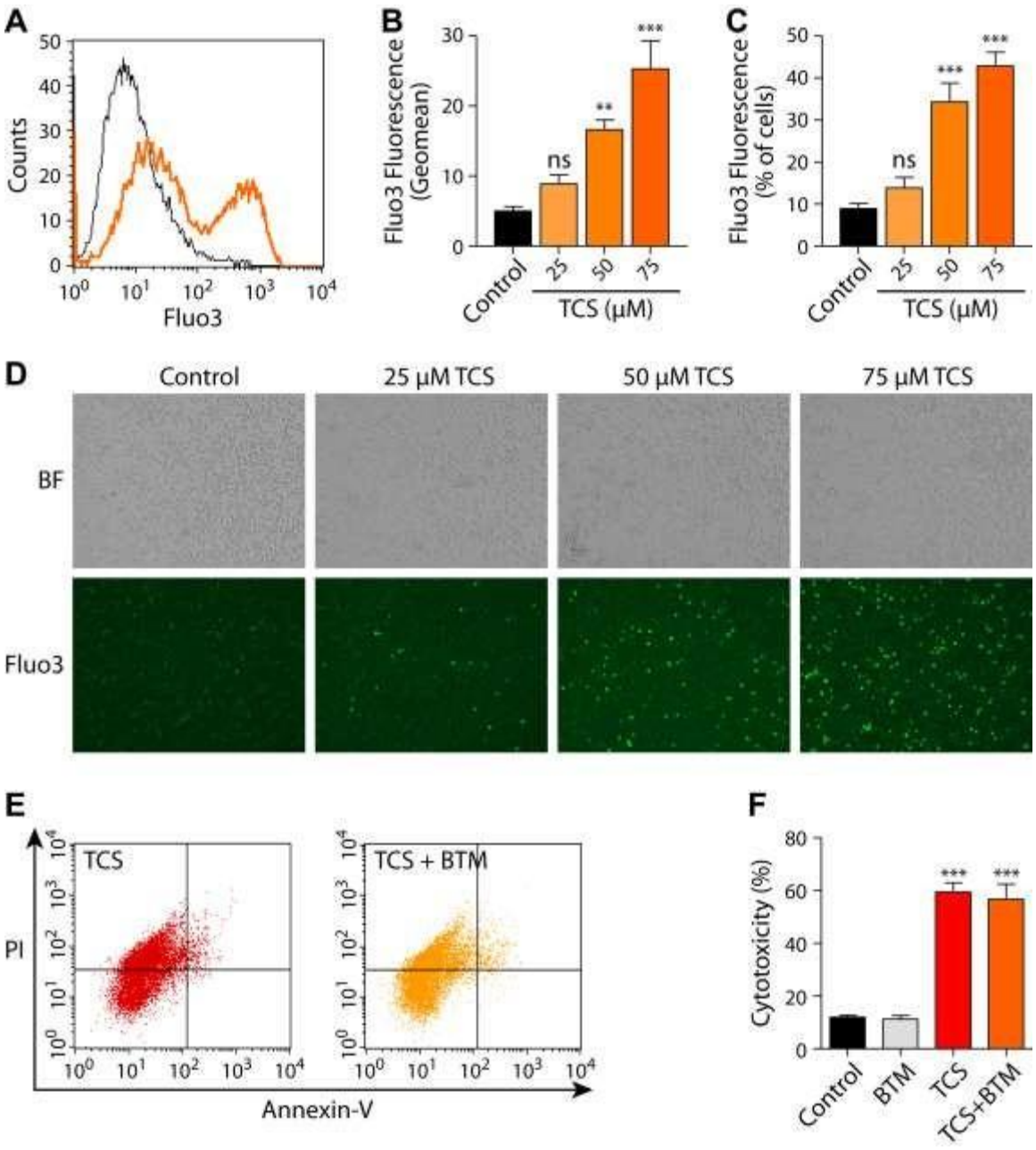
A
Counts
Fluo3
B
Fluo3 Fluorescence (Geomean)
ns
**
***
Control
25
50
75
TCS (μM)
C
Fluo3 Fluorescence (% of cells)
ns
***
***
Control
25
50
75
TCS (μM)
D
Control
25 μM TCS
50 μM TCS
75 μM TCS
BF
Fluo3
E
TCS
TCS + BTM
PI
Annexin-V
F
Cytotoxicity (%)
***
***
Control
BTM
TCS
TCS+BTM

Figure 4

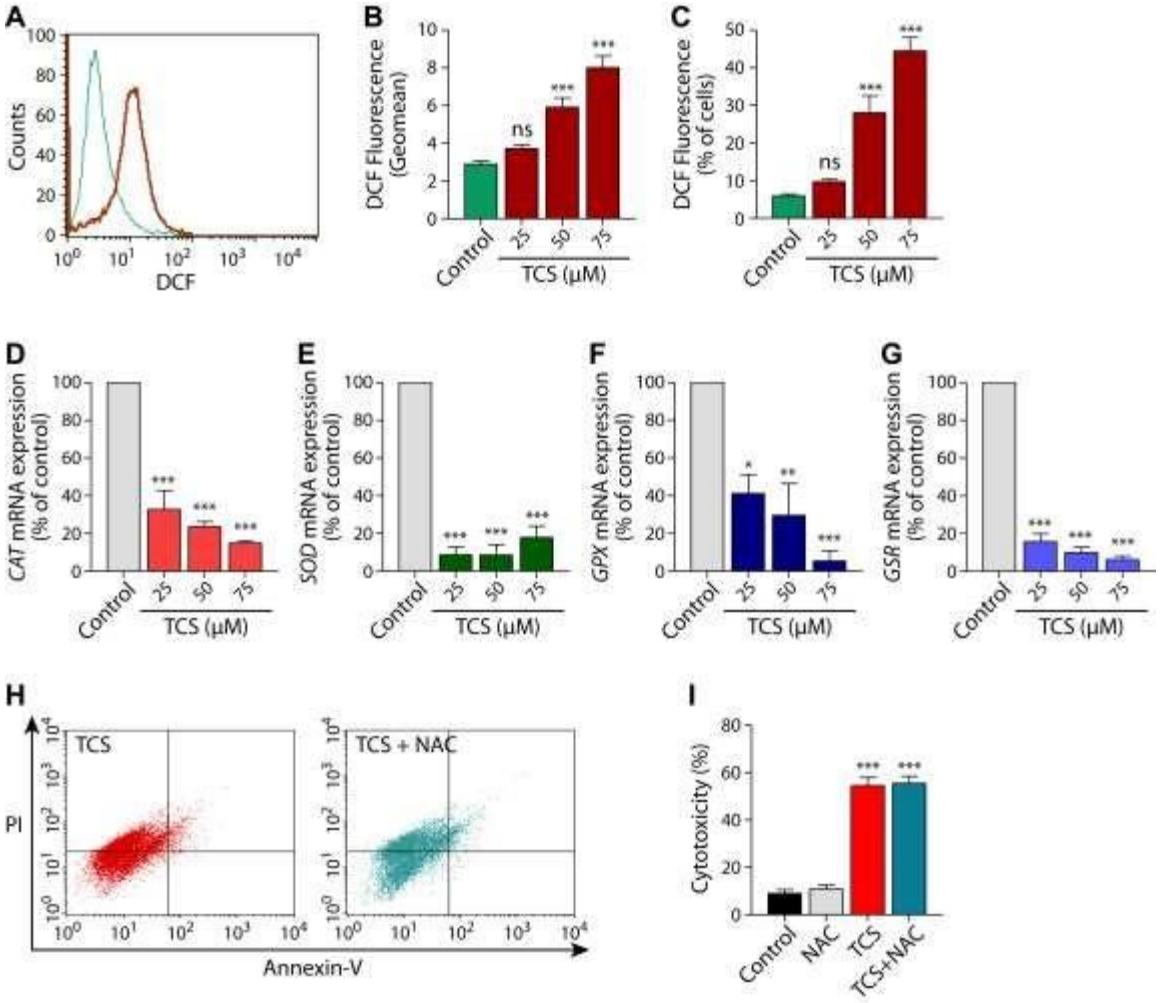
A
Counts
DCF
B
DCF Fluorescence (Geomean)
ns
***
Control
TCS (μM)
C
DCF Fluorescence (% of cells)
D
CAT mRNA expression (% of control)
E
SOD mRNA expression (% of control)
F
GPX mRNA expression (% of control)
G
GSR mRNA expression (% of control)
H
TCS
TCS + NAC
PI
Annexin-V
I
Cytotoxicity (%)
NAC
TCS
TCS+NAC

Figure 5

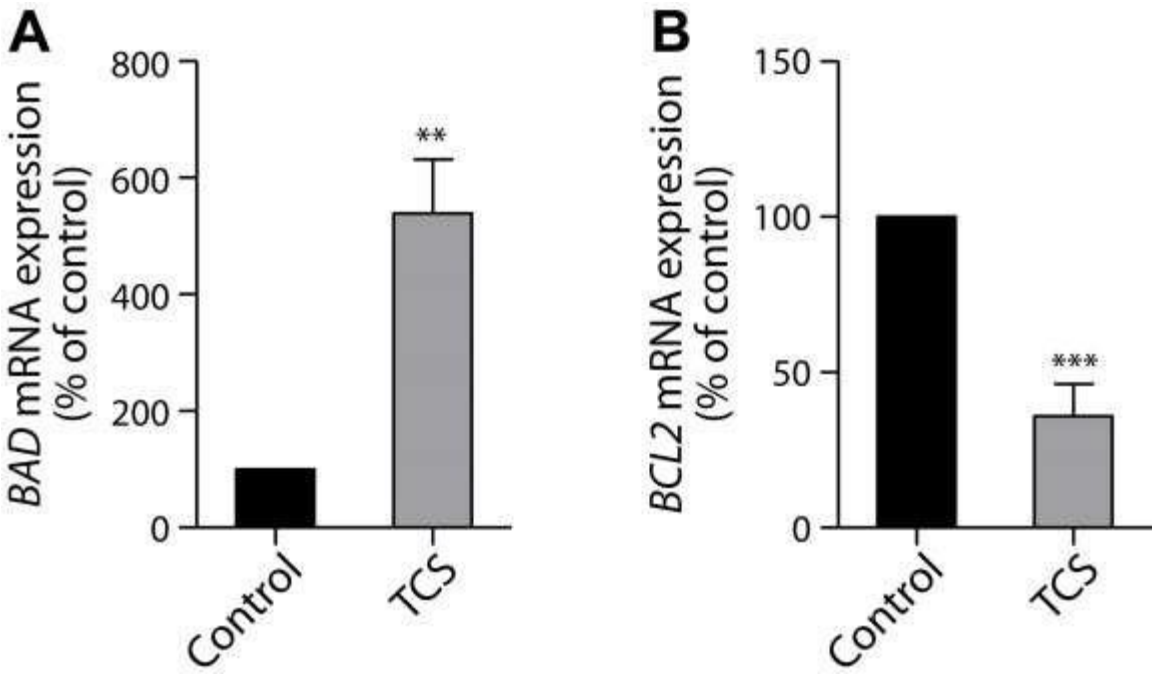
A
BAD mRNA expression
(% of control)
800
600
400
200
0
**
Control
TCS
B
BCL2 mRNA expression
(% of control)
150
100
50
0
***
Control
TCS

Figure 6

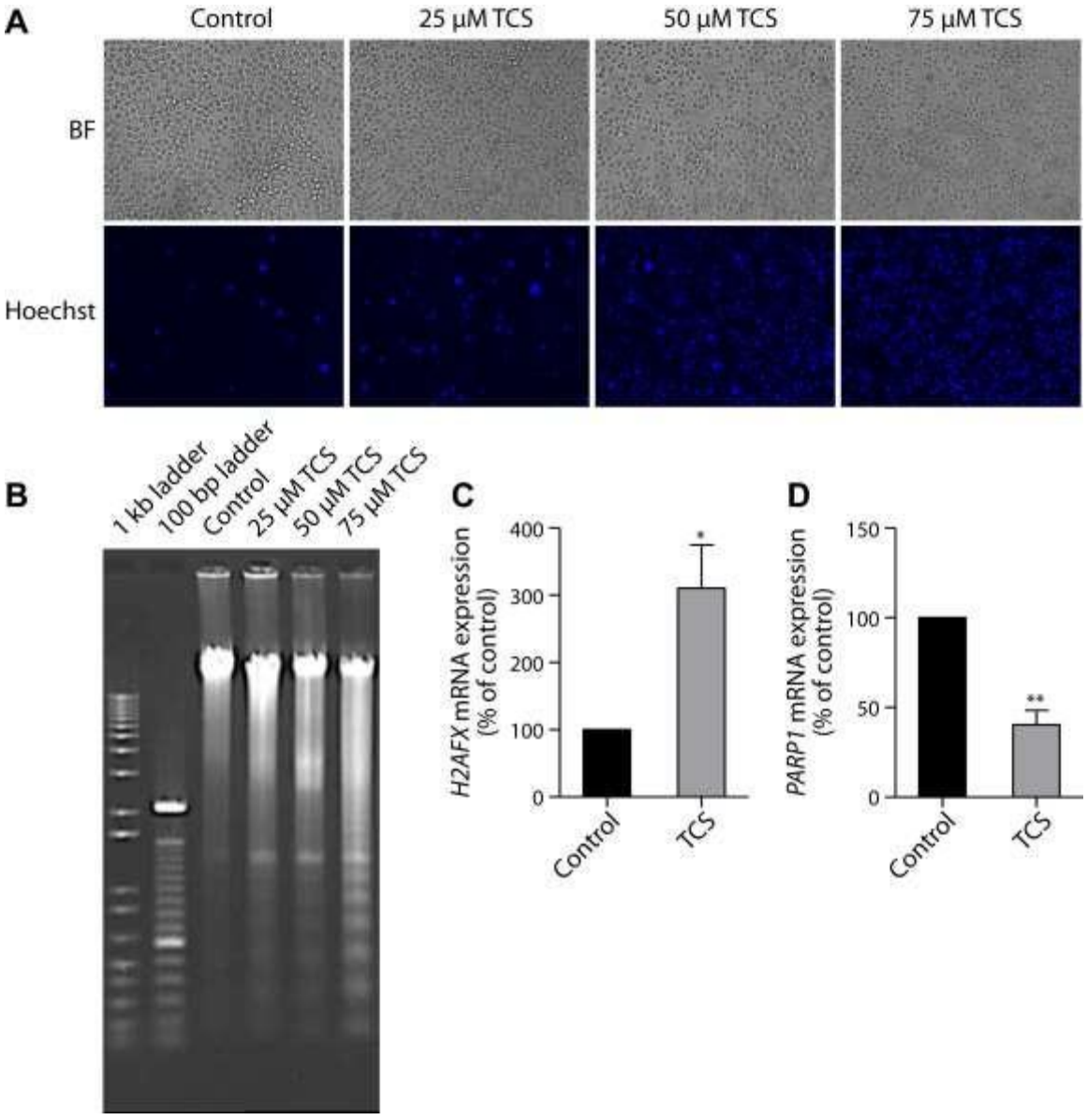
A
Control
25 μM TCS
50 μM TCS
75 μM TCS
BF
Hoechst
B
1 kb ladder
100 bp ladder
Control
25 μM TCS
50 μM TCS
75 μM TCS
C
H2AFX mRNA expression (% of control)
400
300
200
100
0
*
Control
TCS
D
PARP1 mRNA expression (% of control)
150
100
50
0
**
Control
TCS

Figure 7

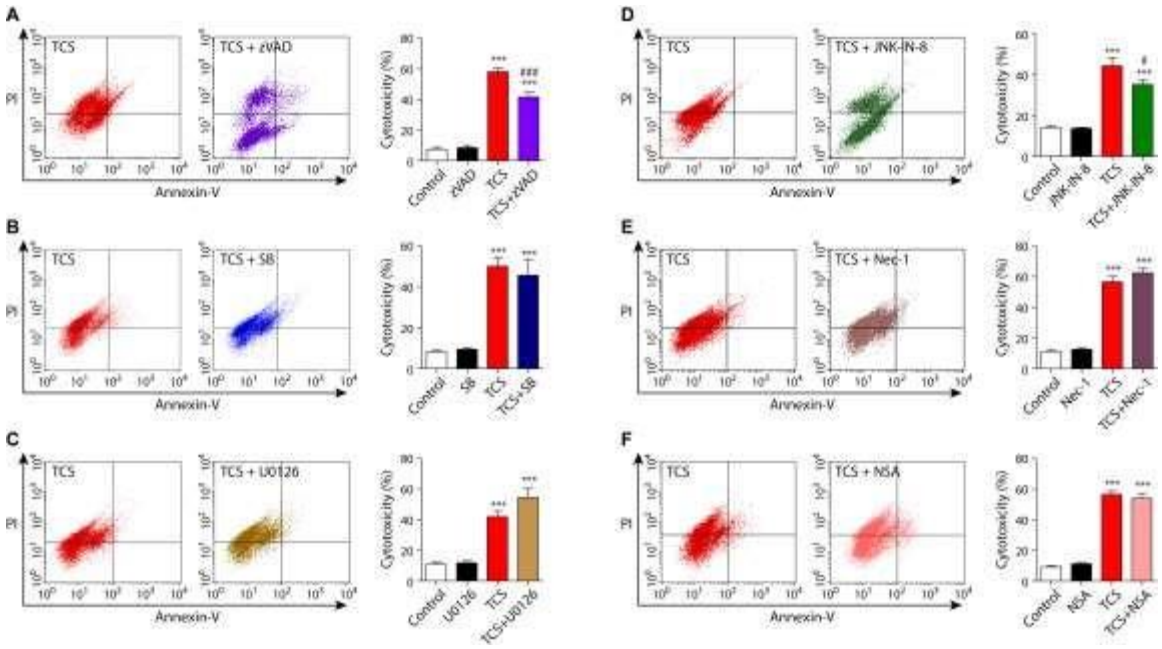
A
TCS
TCS + zVAD
PI
Annexin-V
Cytotoxicity (%)
Control
zVAD
TCS
TCS+zVAD
B
TCS
TCS + SB
SB
TCS+SB
C
TCS
TCS + U0126
U0126
TCS+U0126
D
TCS
TCS + JNK-IN-8
JNK-IN-8
TCS+JNK-IN-8
E
TCS
TCS + Nec-1
Nec-1
TCS+Nec-1
F
TCS
TCS + NSA
NSA
TCS+NSA

Figure 8

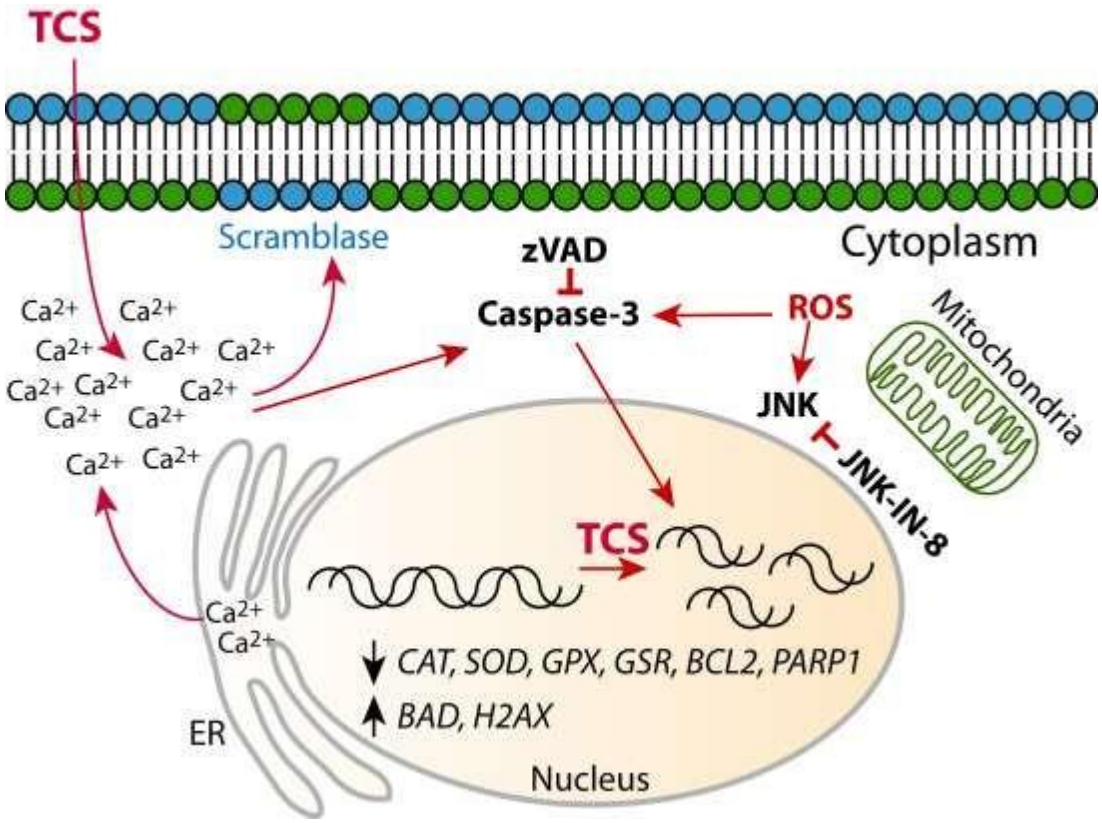
TCS
Scramblase
Cytoplasm
zVAD
Caspase-3
ROS
Mitochondria
JNK
JNK-IN-8
Ca2+
TCS
CAT, SOD, GPX, GSR, BCL2, PARP1
BAD, H2AX
ER
Nucleus

Figure 9

CHAPTER III: DISRUPTION OF ERYTHROCYTE MEMBRANE ASYMMETRY BY TRICLOSAN IS PRECEDED BY CALCIUM DYSREGULATION AND P38 MAPK AND RIP1 STIMULATION

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In press in *Chemosphere*

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ABSTRACT

Triclosan (TCS) is a broad-spectrum antimicrobial used in personal care products, household items, and medical devices. Owing to its apoptotic potential against tumor cells, TCS has been proposed for the treatment of malignancy. A major complication of chemotherapy is anemia, which may result from direct erythrocyte hemolysis or premature cell death known as eryptosis. Similar to nucleated cells, eryptotic cells lose membrane asymmetry and Ca^{2+} regulation, and undergo oxidative stress, shrinkage, and activation of a host of kinases. In this report, we sought to examine the hemolytic and eryptotic potential of TCS and dissect the underlying mechanistic scenarios involved therein. Hemolysis was spectrophotometrically evaluated by the degree of hemoglobin release into the medium. Flow cytometry was utilized to detect phosphatidylserine (PS) exposure by annexin-V binding, intracellular Ca^{2+} by Fluo-3/AM fluorescence, and oxidative stress by 2-,7-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA). Incubation of cells with 10-100 μM TCS for 1-4 h induced time- and dose-dependent hemolysis. Moreover, TCS significantly increased the percentage of eryptotic cells as evident by PS exposure (significantly enhanced annexin-V binding). Interestingly, TCS-induced eryptosis was preceded by elevated intracellular Ca^{2+} levels but was not associated with oxidative stress. Cotreatment of erythrocytes with 50 μM TCS and 50 μM SB203580 (p38 MAPK inhibitor), or 300 μM necrostatin-1 (receptor-interacting protein 1 (RIP1) inhibitor) significantly ameliorated TCS-induced PS externalization. We conclude that TCS is cytotoxic to erythrocytes by inducing hemolysis and stimulating premature death at least in part through Ca^{2+} mobilization, and p38 MAPK and RIP1 activation.

Keywords: Triclosan; phosphatidylserine; eryptosis; hemolysis; p38 MAPK; RIP1

INTRODUCTION

Triclosan (TCS), or 5-chloro-2-(2,4-dichlorophenoxy) phenol, is a broad-spectrum antimicrobial extensively used in personal care and hygiene products, clothing and textiles, kitchenware, and medical devices (Yueh and Tukey, 2016) (Figure 1A). A recognized endocrine disruptor, exposure to TCS may be implicated in a myriad of serious disease conditions including immune and thyroid disorders (Clayton *et al.*, 2011). At the cellular level, TCS induces cytotoxicity, membrane damage, oxidative stress, and apoptosis (Yueh and Tukey, 2016). In particular, the antibacterial and cytotoxic effects of TCS are attributed in part to its inhibitory action on *de novo* fatty acid synthesis by inactivating fatty acid synthase (FAS). Because FAS is differentially upregulated in a variety of tumors (Wang *et al.*, 2001), TCS along with other FAS inhibitors has been proposed as a promising antineoplastic agent against breast, epithelial, and prostatic cancer cells (Sadowski *et al.*, 2014).

Owing to widespread exposure in humans, TCS has been shown to accumulate in various tissues and body fluids including the brain and blood (Geens *et al.*, 2012). Red blood cells (RBCs), also known as erythrocytes, are highly specialized, terminally differentiated cells responsible for oxygen delivery and carriage of immune complexes. Whereas RBCs have an average lifespan of 100-120 days, various stimuli, including xenobiotics, may trigger eryptosis; the suicidal death of erythrocytes. Distinctive features of eryptotic cells include cell shrinkage, membrane blebbing, and lipid bilayer scrambling leading to phosphatidylserine (PS) externalization to the outer membrane leaflet. Adverse conditions that often precede eryptosis include energy depletion, osmotic shock, hyperthermia, and oxidative stress (Lang *et al.*, 2012).

Accelerated eryptosis constitutes an integral part of the multifaceted pathophysiology of a plethora of diseases. These include metabolic syndrome, diabetes mellitus, anemia, renal disease, and cancer (Lang and Lang, 2015b; Lang *et al.*, 2017). Moreover, the presence of eryptotic cells in the circulation is detrimental because they aggravate anemia and adhere to platelets and endothelial cells, giving rise to intravascular coagulation and thrombosis (Borst *et al.*, 2012; Walker *et al.*, 2014). Therefore, mature erythrocytes have developed an intricate and elaborate machinery to regulate survival and senescence. This regulatory network has calcium homeostasis at its core, in addition to intracellular mediators such as p38 mitogen-activated protein kinase (MAPK), caspases, AMP-activated protein kinase (AMPK), Janus kinase 3 (JAK3), and receptor-interacting protein 1 (RIP1), among others (LaRocca *et al.*, 2014; Lang and Lang, 2015a). Cytosolic accumulation of calcium eventually leads to membrane scrambling, calpain-dependent blebbing, and cell shrinkage following KCl and water loss (Al Mamun Bhuyan *et al.*, 2017b). Similarly, eryptosis may be initiated through signaling cascades involving either the cyclooxygenase-prostaglandin E2 (COX-PGE2) pathway or the stimulation of phospholipase 2 (PLA2) and ceramide formation (Lang *et al.*, 2012).

Despite the revitalized interest in TCS as a cause for concern (Dann and Hontela, 2011; Yueh and Tukey, 2016), and the prevalence of chemotherapy-induced anemia in cancer (Rodgers *et al.*, 2012; Lang *et al.*, 2017), very little emphasis has been placed on the interaction of TCS with erythrocytes. Thus, the objective of this study was to characterize the hemolytic and eryptotic potential of TCS in this cell type. It was revealed that TCS triggers premature cell death through membrane damage, evident as overt hemolysis, and loss of membrane asymmetry. Mechanistically, TCS-induced PS exposure

was characterized by cytosolic Ca^{2+} accumulation along with p38 MAPK and RIP1 stimulation.

MATERIALS AND METHODS

Erythrocytes, chemicals, and solutions

Fresh, lithium heparin RBC samples from consented, healthy adults were obtained from ZenBio (Research Triangle Park, NC, USA). Samples were washed in phosphate-buffered saline (PBS; 0.9% NaCl, 1 mM KH_2PO_4, 5.6mM Na_2HPO_4; pH 7.4) at 3,000 rpm for 10 min at 21°C, and TCS exposure was conducted *in vitro* at 5% hematocrit in Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4. To test for the dependence of TCS-mediated PS exposure on extracellular Ca^{2+} influx, or intracellular Ca^{2+} availability, cells were incubated in Ca^{2+}-free Ringer solution in which $CaCl_2$ was substituted with 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) (Chem-Impex Intl., Wood Dale, IL, USA), or were cotreated with 50 μM TCS and 50 μM Ca^{2+} chelator BAPTA-AM. Signaling kinases were evaluated by treating the cells to a combination of 50 μM TCS and 50 μM p38 MAPK inhibitor SB203580 (Selleckchem, Houston, TX, USA), 100 μM pan-caspase inhibitor z-VAD-fmk (Selleckchem), 1 μM protein kinase C (PKC) inhibitor Staurosporine (StSp; Cayman Chemical Company, Ann Arbor, MI, USA), 100 μM casein kinase 1 (CK1) inhibitor (D4476; Cayman), 300 μM RIP1 inhibitor Necrostatin-1 (Nec-1), or 1 μM mixed lineage kinase domain-like (MLKL) peudokinase inhibitor Necrosulfonamide (NSA). All chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA)

unless otherwise noted. An ethanolic stock solution of TCS was prepared at 10 mM and diluted to desired concentrations in Ringer solution.

Hemolysis

RBCs at 5% hematocrit were exposed to 10-100 μM of TCS in Ringer solution for 1, 2, and 4 h at 37°C. Following treatment, samples were centrifuged at 13,300 RPM for 1 min, and the degree of hemoglobin release into the medium was measured by absorbance (A) at 405 nm using VersaMax™ ELISA microplate reader (Molecular Devices, San Jose, CA, USA). Cells suspended in distilled water constituted 100% hemolysis, and the relative percent hemolysis was calculated according to the formula:

% Hemolysis = (As/Aw) x 100

where As = absorbance of test sample, and Aw = absorbance of positive (distilled water) control.

Detection of PS externalization and forward scatter (FSC)

Following TCS treatment, 50 μl of cells were washed in Ringer solution containing 5 mM $CaCl_2$ and resuspended in a total volume of 200 μl. The resulting RBC suspension was stained with a 1% v/v solution of Annexin V-FITC (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature away from light. PS exposure and forward scatter FSC were subsequently determined by flow cytometry using a FACScan (Betcon Dickinson, Franklin Lakes, NJ, USA) at excitation and emission wavelengths of 488 nm and 530 nm, respectively.

Confocal microscopy

Control and TCS-treated cells were stained with Annexin-V-FITC as detailed above and a homogeneous 20 μl cell suspension was spread on a glass slide before it was immediately examined with a Zeiss LSM 700 laser scanning microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, USA) under a water immersion Plan-Neofluar 40/1.3 NA DIC objective.

Determination of intracellular calcium

Cytosolic Ca^{2+} activity was determined by Fluo3/AM fluorescence (Biotium, Fremont, CA, USA). The membrane-permeant Fluo3/AM ester is hydrolyzed intracellularly by esterases into Fluo3 whose fluorescence increases upon binding to Ca^{2+} ions, thus serving as an indicator of Ca^{2+} content. Following TCS treatment, 50 μl of cell suspension was washed in 5 mM $CaCl_2$ Ringer buffer and incubated with 5 μM of Fluo3/AM for 30 min at 37°C under protection from light. Cells were washed twice to remove excess stain, resuspended in 200 μl of 5 mM $CaCl_2$ Ringer solution, and finally analyzed by a FACScan at 488 nm excitation and 530 nm emission wavelengths. The geomean of Fluo3-dependent fluorescence was subsequently determined.

Measurement of ROS generation

Oxidative stress was assayed by measuring the generation of reactive oxygen species (ROS) using the probe 2-,7-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA) (Thermo Fisher Scientific, Waltham, MA, USA). $DCFH_2$-DA is a cell-permeant indicator that remains non-fluorescent until it is cleaved by intracellular esterases and in turn oxidized by ROS into the fluorescent DCF. Following treatment with 50 μM TCS, 50 μl of cell suspension was washed in Ringer buffer, resuspended in a final volume of 200 μl,

and incubated with 10 μM $DCFH_2$-DA for 30 min at 37°C in total darkness. DCF fluorescence was measured on a FACScan at excitation and emission wavelengths of 488 nm and 530 nm, respectively.

Statistical analysis

Data are shown as arithmetic means ± S.E.M. of at least three independent experiments conducted on RBC samples obtained from three different donors. The Student *t* test was employed to analyze differences among the means, and a value of $P < 0.05$ was defined as the cutoff for statistical significance. *n* denotes the number of technical replicates tested. To control for individual variation and differential susceptibility to external stimuli, only control and experimental cells from the same RBC specimen were compared.

RESULTS

TCS induces hemolysis time- and dose-dependently

Various xenobiotics have been shown to exhibit a hemolytic activity against human RBCs (Lang and Lang, 2015b), and the cytotoxicity of TCS was demonstrated in various cell types (Honkisz *et al.*, 2012; Zhang *et al.*, 2015; Park *et al.*, 2016) . To assess the hemolytic potential of TCS, erythrocytes were incubated with 10-100 μM TCS for 1, 2, and 4 h at 37°C, and hemoglobin release into the medium was measured as a function of cell lysis relative to cells lysed in distilled water. As depicted in Figure 1B, incubation of RBCs at the tested concentrations resulted in a dose- and time-dependent increase in hemolysis, an effect reaching statistical significance at 25 μM following 1 h exposure. This

indicates that TCS exerts a hemolytic effect on RBCs that is proportionally related to length of exposure time and concentration used.

TCS causes membrane phospholipid scrambling

Previous studies have demonstrated that TCS induces apoptosis in a variety of human cell types including prostatic and placental cells (Sadowski *et al.*, 2014; Zhang *et al.*, 2015). In RBCs, among the distinctive features of eryptosis are PS exposure and cell shrinkage. To investigate the ability of TCS to stimulate eryptosis, RBCs were incubated in Ringer solution containing 10-50 μM TCS for 4 h at 37°C. Cells were then stained with Annexin-V-FITC and analyzed using flow cytometry. Cell size was simultaneously estimated from FSC. Our results show that TCS increased PS externalization with a statistical significance starting at 25 μM (Figure 2A-C). Membrane scrambling was, however, not accompanied by alterations in cell volume as indicated by the unchanged FSC under control and experimental conditions (Figure 2D & E). Taken together, these data suggest that TCS leads to enhanced PS translocation characteristic of eryptotic cells without concurrent reduction in cell volume.

TCS disturbs calcium homeostasis

Membrane scrambling is initiated by increased activity of cytosolic Ca^{2+} (Lang *et al.*, 2012). To test whether Ca^{2+} accumulation is precedent to PS exposure, cells were treated with 10-50 μM TCS in Ringer solution for 4 h at 37°C, stained with Fluo3/AM, and the fluorescence intensity was analyzed by flow cytometry. It was noted that TCS treatment induced intracellular Ca^{2+} levels, an effect attaining statistical significance at 50 μM (Figure 3).

Elevated intracellular Ca^{2+} may be due to influx into the cell through Ca^{2+}-permeable cation channels. To examine whether the increase in cytosolic Ca^{2+} was due to extracellular Ca^{2+} entry, cells were incubated with or without 50 μM TCS for 4 h at 37°C in Ringer solution or in Ca^{2+}-free Ringer solution, and the ion's activity was estimated as described earlier. As seen in Figure 4, Fluo3 fluorescence was not significantly altered in presence or absence of extracellular Ca^{2+}, suggesting that TCS-induced elevated cytosolic Ca^{2+} was not consequent to extracellular influx of the ion.

Next, we sought to assess the role of Ca^{2+} in TCS-mediated PS translocation. To this end, Annexin-V binding was detected in control and TCS-treated cells in presence and absence of extracellular Ca^{2+}, or with and without 50 μM BAPTA-AM (a selective Ca^{2+} chelator) for 4 h at 37°C. Compared to unaltered Ca^{2+} conditions, the percentage of cells exposing PS was not significantly reduced neither when extracellular Ca^{2+} was removed (Figure 5A-C) nor when intracellular Ca^{2+} was chelated with BAPTA-AM (Figure 5D-F). Thus, preventing extracellular Ca^{2+} influx or depleting intracellular Ca^{2+} are apparently not required for the full PS-exposing activity of TCS.

TCS does not induce oxidative stress

The generation of excessive amounts of ROS is a recognized aggravator of eryptosis (Lang *et al.*, 2014). We have previously shown that damage associated with TCS exposure is in part due to oxidative stress (Yoon *et al.*, 2017). Thus, to test whether TCS-mediated PS translocation is preceded by ROS generation, cells were incubated with or without 50 μM TCS in Ringer solution for 4 h at 37°C, and ROS levels were assessed by DCF fluorescence using flow cytometry. Figure 6 demonstrates that ROS levels are not

significantly changed by TCS treatment, suggesting that TCS-induced eryptosis is not mediated through oxidative stress.

Involvement of kinases

Multiple signaling pathways have been implicated in eryptosis, including p38 MAPK, caspases, PKC, and CK1 (Lang and Lang, 2015a). RIP1 and MLKL have also been recently described, revealing necroptosis as a distinct death pathway in RBCs (LaRocca *et al.*, 2014). To identify kinases stimulated in response to TCS exposure, cells were incubated in Ringer solution with or without 50 μM TCS in presence and absence of 50 μM SB203580 (p38 MAPK inhibitor), 100 μM zVAD-fmk (pan-caspase inhibitor), 1 μM StSp (PKC inhibitor), 100 μM D4476 (CK1 inhibitor), 300 μM Nec-1 (RIP1 inhibitor), or 1 μM NSA (MLKL inhibitor) for 4 h at 37°C. PS translocation was subsequently evaluated as previously described. As seen in Figure 7, both SB203580 and Nec-1 significantly but not thoroughly blunted PS translocation, while no statistically significant inhibition was observed under caspase, PKC, CK1, or MLKL blockage. This identifies p38 MAPK and RIP1 not only as molecular targets of TCS, but also as essential requirements for its full eryptotic activity.

DISCUSSION

TCS is a high-volume, antimicrobial phenolic compound commonly used as a preservative in personal care products, textiles, medical devices, and food contact materials (Yueh and Tukey, 2016). Once absorbed, TCS is distributed and deposited in a variety of tissues and body fluids, including the liver, brain, and blood. Toxicological profiling of TCS has discerned its apoptotic potency in a variety of tumor cells, which could be exploited for therapeutic purposes. Thus, complementary to previous studies, this work not

only expands on current understanding of TCS toxicity, but also offers assessment of its therapeutic potential as an anticancer agent. It is worth mentioning that pathogens whose tropism involves the RBC are highly likely to transform infected cells into eryptotic corpses, as is the case with *Plasmodium spp.* (Foller *et al.*, 2009). Thus, elucidating the effects of TCS on RBCs becomes more relevant considering that, in addition to its antimalarial properties, TCS is known to be effective against important hemoparasites, including *Babesia*, *Trypanosoma*, *Leishmania*, and *Toxoplasma* (Bork *et al.*, 2003; Roberts *et al.*, 2003; Otero *et al.*, 2014).

To the best of our knowledge, this is the first study to report that TCS stimulates eryptosis; the suicidal erythrocyte death. TCS elicited hemolysis and eryptosis in micromolar concentrations that are lower than the millimolar levels present in consumer products, and which are within the range shown to possess antitumor activity (Rodricks *et al.*, 2010; Sadowski *et al.*, 2014). It is important to keep in mind that TCS adversaries described herein are based on RBCs obtained from healthy individuals. Such findings may parallel effects of lower doses in RBCs from cancer patients considering the augmented susceptibility of those cells to eryptosis (Lang *et al.*, 2017). Pending *in vivo* confirmation, data from this exploratory study provides evidence of a novel eryptotic sequela of TCS that may warrant careful consideration of its use for chemotherapy.

Similar to findings reported by Miller *et al.* (Miller and Deinzer, 1980), TCS was shown to be highly toxic to erythrocytes causing conspicuous hemolysis indicative of direct membrane damage. The lack of ROS generation seems to rule out oxidative stress as a contributing factor in TCS-induced hemolysis, as was observed for *para*-hydroxyanisole, and in contrast to bisphenol A (BPA), two related phenolic compounds (Nohl and Stolze,

1998; Macczak *et al.*, 2016). Circulating, naked hemoglobin is highly reactive and can participate in oxidative damage manifested as perturbations in endothelial cell function, hypertension, thrombosis, and atherosclerotic lesions (Miller and Shaklai, 1999; Reiter *et al.*, 2002; Studt *et al.*, 2005; Silva *et al.*, 2009). Similarly, extravascular presence of free hemoglobin is associated with dysregulated iron homeostasis, renal tubular injury, and neuronal damage (Tracz *et al.*, 2007; Lara *et al.*, 2009; Pantopoulos *et al.*, 2012).

Our data also show that TCS causes a significant increase in eryptotic cells as detected by PS externalization, which is in congruence with its apoptotic activity observed in nucleated cells (Deepa *et al.*, 2012; Honkisz *et al.*, 2012; Szychowski *et al.*, 2016). Several other compounds structurally related to TCS, such as BPA and other chlorophenols, have recently been shown to also trigger eryptosis (Macczak *et al.*, 2016; Michalowicz *et al.*, 2018). Under physiological conditions, eryptosis may be perceived as a counterpoise to erythropoiesis, preventing both anemia and polycythemia. This is because exposure of PS serves as a conserved flag on RBCs undergoing eryptosis for recognition and removal by the monocyte-macrophage system (Lang *et al.*, 2003), thus acting as a safeguard against hemolysis.

Inordinate eryptosis constitutes a common theme in a variety of life-threatening conditions including diabetes, hepatic failure, and malignancy (Lang and Lang, 2015a). In these cases and many others, eryptotic cells may adhere to endothelial cells and platelets, obstruct microcirculatory flow, and lead to thrombosis (Borst *et al.*, 2012; Walker *et al.*, 2014). This is a consequence of the negative impact the eryptotic RBC membrane exerts on the cell's deformability and aggregability. When RBCs form larger aggregates with rigid membranes, blood viscosity increases, causing enhanced flow resistance, and

eventually diminished tissue perfusion (du Plooy *et al.*, 2018; Pretorius, 2018). The limited elasticity characteristic of eryptotic membranes may also hinder the cell's ability to reassume its original biconcave shape following passage through the microvasculature (Pretorius, 2018). Therefore, identifying the impact of xenobiotics on RBC rheology is among the most important aspects of pharmaceutical assessment of potential therapies.

The importance of Ca^{2+} activity in mediating eryptosis cannot be overstated, and the process has been referred to in the literature as "Ca^{2+}-dependent" programmed cell death (LaRocca *et al.*, 2014). Activated K^+ channels in response to increased cytosolic Ca^{2+} lead to K^+ efflux, membrane hyperpolarization causing Cl^- outflow, dehydration due to water loss, and eventual cell shrinkage (Lang *et al.*, 2012). Although TCS has been shown to cause K^+ efflux prior to hemolysis (Miller and Deinzer, 1980), we observed no significant change in cell volume among healthy and eryptotic cells despite a significant increase in intracellular Ca^{2+}. Likewise, the ionophoric effect of clofazimine was not accompanied by cell shrinkage (Officioso *et al.*, 2015).

Because both scramblase and flippase are Ca^{2+}-sensitive, perturbations in the ion's activity are associated with loss of membrane asymmetry (Lang *et al.*, 2006a). In our study, we noted that TCS was able to cause membrane phospholipid scrambling with a significant increase in cytosolic Ca^{2+} at 50 μM. This is in consonance with recent findings demonstrating Ca^{2+} dysregulation caused by TCS both *in vitro* and *in vivo* (Ahn *et al.*, 2008; Cherednichenko *et al.*, 2012; Popova *et al.*, 2018). Our data also indicate that neither PS exposure nor cytosolic Ca^{2+} was significantly blunted by Ca^{2+} depletion, underlining the dispensability of the ion in TCS-mediated eryptosis, and pointing at possible additional mechanisms. Similar findings were recently reported by Gao *et al*. for betulinic acid (Gao

et al., 2014). Presumably, due to their hydrophobicity, both TCS and betulinic acid may readily permeabilize through the membrane to exert their effects (Guillen *et al.*, 2004). On the other hand, some xenobiotics such as regorafenib rather depleted intracellular Ca^{2+} while still inducing eryptosis (Zierle *et al.*, 2016). It is important to note that the use of EDTA to chelate Ca^{2+} does not provide total elimination of the ion, which must be taken into consideration when evaluating the contribution of extracellular Ca^{2+} (Al Mamun Bhuyan *et al.*, 2017b).

In RBCs, oxidative stress participates in Ca^{2+} entry by opening cation channels in the cell membrane. We have previously shown that TCS perturbs the antioxidant response in human mesenchymal stem cells (Yoon *et al.*, 2017), and oxidative damage by TCS has been detected in a variety of cell types (Ma *et al.*, 2013; Szychowski *et al.*, 2016). Nevertheless, we found that TCS-induced eryptosis was not accompanied by changes in ROS levels. Interestingly, other compounds such as carnosic acid, perifosine, and micafungin rather diminished ROS production as part of their eryptotic manifestations (Stockinger *et al.*, 2015; Peter *et al.*, 2016c; Egler and Lang, 2017). It is comprehensible to surmise that such an event is reflective of a suppressed metabolic rate and cellular adaptivity, which is compatible with the anti-inflammatory role of TCS (Barros *et al.*, 2010).

The use of small-molecule inhibitors has allowed us to reveal the identity of molecular mediators targeted by TCS in erythrocytes. We observed that TCS-induced PS exposure was significantly inhibited by blockade of either p38 MAPK or RIP1. Inhibition of caspases, PKC, and CK1 provided some degree of protection against PS externalization, the extent of which, however, failed to attain statistical significance. Most recently, Zhang

et al. demonstrated that TCS promotes p38 phosphorylation *in vitro* and *in vivo* (Zhang *et al.*, 2018), which is in accord to reports of TCS-induced p38 activation detected in Raw264.7 macrophages (Wang *et al.*, 2018a), and rat neural stem cells (Park *et al.*, 2016).

Whereas p38 is a major orchestrator of eryptosis, RIP1 is known to be critical for necroptosis (LaRocca *et al.*, 2014). Nonetheless, RIP1 may also signal for apoptosis, and, in fact, acts upstream of p38 MAPK (Lee *et al.*, 2003a). Furthermore, necroptosis is under regulation by caspase-8 (LaRocca *et al.*, 2014), adding to the complexity and further highlighting the interplay between apoptosis and necroptosis. Therefore, based on the identified role of RIP1, we were prompted to probe the involvement of the necroptosis executioner, MLKL, in TCS-induced RBC death. The results (Fig. 7G & H) demonstrated a lack of significant reduction in PS externalization under MLKL inhibition, thus possibly exonerating necroptosis as a mode of cell death. Notably, both pathways were recently found to be activated in HepG2 cells in response to Tanshinone IIA, a component of the red sage plant *Salvia miltiorrhiza* (Lin *et al.*, 2016), which also possesses eryptotic activity (Zelenak *et al.*, 2012).

In conclusion, this report shows that TCS adversely affects the physiology and survival of RBCs by triggering premature cell death. It was revealed that the integrity of the RBC membrane is perturbed by TCS leading to phospholipid scrambling at least in part through loss of Ca^{2+} homeostasis and p38 MAPK- and RIP1-mediated mechanisms (Figure 8).

ACKNOWLEDGMENTS

We thank the members of the Lee laboratory for helpful advice and discussion during this work. This work was supported in part by the Brody Brothers Grant (21602-

664261) to M-H.L. and the Saudi Government Graduate Scholarship (from King Saud University) to M.A.A.

COMPETING INTERESTS

The authors declare they have no competing interests relevant to this manuscript.

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FIGURE LEGENDS

Fig. 1. TCS induces hemolysis dose and time responsively. A. Chemical structure of TCS. **B.** Arithmetic means ± SEM (*n* = 9) of RBC hemolysis following incubation for 1-4 h in Ringer solution without (black bars) or with 10–100 μM TCS (grey bars). ns indicates not significant; ***($p<0.001$) indicates significant difference from control (Student's *t*-test).

Fig. 2. Effect of TCS on phosphatidylserine exposure and forward scatter. **A.** Representative histogram showing annexin-V-binding of RBCs incubated for 4 h in Ringer solution without (black line) or with (red line) 50 μM TCS. **B.** Arithmetic means ± SEM (*n* = 9) of the percentage of annexin-V-binding RBCs following incubation for 4 h in Ringer solution without (black bar) or with 10–50 μM TCS (grey bars). **C.** Confocal microscopy images demonstrating control and eryptotic cells with increased FITC fluorescence reflective of enhanced PS exposure. **D.** Representative histogram showing erythrocyte FSC after 4 h incubation in Ringer solution without (grey peak) or with (black line) 50 μM TCS. **E.** Arithmetic means ± SEM (*n* = 9) of erythrocyte FSC after 4 h incubation without (black bar) or with 10–50 μM TCS (grey bars). ns indicates not significant; ***($p<0.001$) indicates significant difference from control (Student's *t*-test).

Fig. 3. TCS causes intracellular Ca^{2+} mobilization. **A.** Representative histogram showing Fluo3 fluorescence as a function of cytosolic free Ca^{2+} in RBCs incubated for 4h in Ringer solution without (black line) or with (brown line) 50 μM TCS. **B.** Arithmetic means ± SEM (*n* = 9) of Fluo3 fluorescence in RBCs following incubation for 4 h in Ringer solution without (black bar) or with 10–50 μM TCS (grey bars). ns indicates not significant; ***($p<0.001$) indicate significant difference from control (Student's *t*-test).

Fig. 4. Effect of extracellular Ca^{2+} chelation on TCS-induced Ca^{2+} mobilization. **A,B**. Representative histograms showing Fluo3 fluorescence as a function of cytosolic free Ca^{2+} in RBCs incubated for 4 h without (black line) or with 50 μM TCS in presence (brown line, A) and absence (yellow line, B) of extracellular Ca^{2+}. **C.** Arithmetic means ± SEM (n = 9) of Fluo3 fluorescence in RBCs following incubation for 4 h without (black bars) or with 50 μM TCS (grey bars) in presence or nominal absence of extracellular Ca^{2+}. ***($p<0.001$) indicates significant difference from control (Student's *t*-test).

Fig. 5. Effect of extracellular Ca^{2+} chelation on TCS-induced PS exposure. **A,B**. Representative histograms showing annexin-V-binding RBCs incubated for 4 h without (black line) or with 50 μM TCS in presence (red line, A) and absence (blush line, B) of extracellular Ca^{2+}. **C.** Arithmetic means ± SEM (n = 9) of the percentage of annexin-V-binding cells incubated for 4 h without (black bars) or with 50 μM TCS (grey bars) in presence and absence of extracellular Ca^{2+}. **D,E**. Representative histograms showing annexin-V-binding RBCs incubated for 4 h without (black line) or with 50 μM TCS in absence (blue line, D) and presence (sky blue line, E) of 50 μM BAPTA-AM. **F.** Arithmetic means ± SEM (n = 9) of the percentage of annexin-V-binding cells incubated for 4 h without (black bars) or with 50 μM TCS (grey bars) in absence and presence of 50 μM BAPTA-AM. ***($p<0.001$) indicates significant difference from control (Student's *t*-test).

Fig. 6. Effect of TCS on ROS levels. A. Representative histogram showing DCF fluorescence after 4 h incubation in Ringer solution without (black line) or with (purple line) 50 μM TCS. **B.** Arithmetic means ± SEM (n = 9) of DCF geomean fluorescence after 4 h incubation without (black bar) or with 50 μM TCS (grey bar). ns indicates not significant.

Fig. 7. TCS-induced phosphatidylserine exposure is suppressed by SB203580 and necrostatin-1. A-G Representative histograms showing annexin-V-binding RBCs incubated for 4 h without (black line) or with 50 μM TCS in absence (red line, A) and presence of 50 μM SB203580 (blue line, B), 100 μM zVAD-fmk (purple line, C), 1 μM StSp (turquoise line, D), 100 μM D4476 (brown line, E), 300 μM necrostatin-1 (lime line, F), or 1 μM NSA (green line, G). **H.** Arithmetic means ± SEM (*n* = 3) of the percentage of annexin-V-binding cells incubated for 4 h in Ringer solution without (black bar) or with 50 μM TCS (grey bars) in absence and presence of 50 μM SB203580, 100 μM zVAD-fmk, 1 μM StSp, 100 μM D4476, 300 μM Nec-1, or 1 μM NSA. ns indicates not significant; **($p<0.01$) and ***($p<0.001$) indicate significant difference from TCS-only cells (Student's *t*-test).

Fig. 8. A working model for TCS-induced premature erythrocyte death: TCS causes calcium ion dysregulation resulting in elevated intracellular Ca^{2+} activity. Stimulation of p38 MAPK and RIP1 signaling in response to TCS culminates in phosphatidylserine translocation to the outer membrane leaflet; an event significantly abrogated by pharmacological interference with either enzyme.

FIGURES

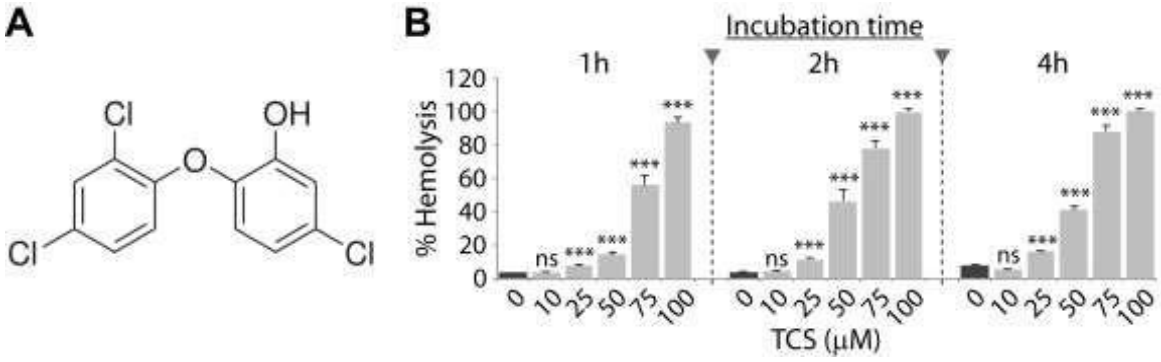

Figure 1

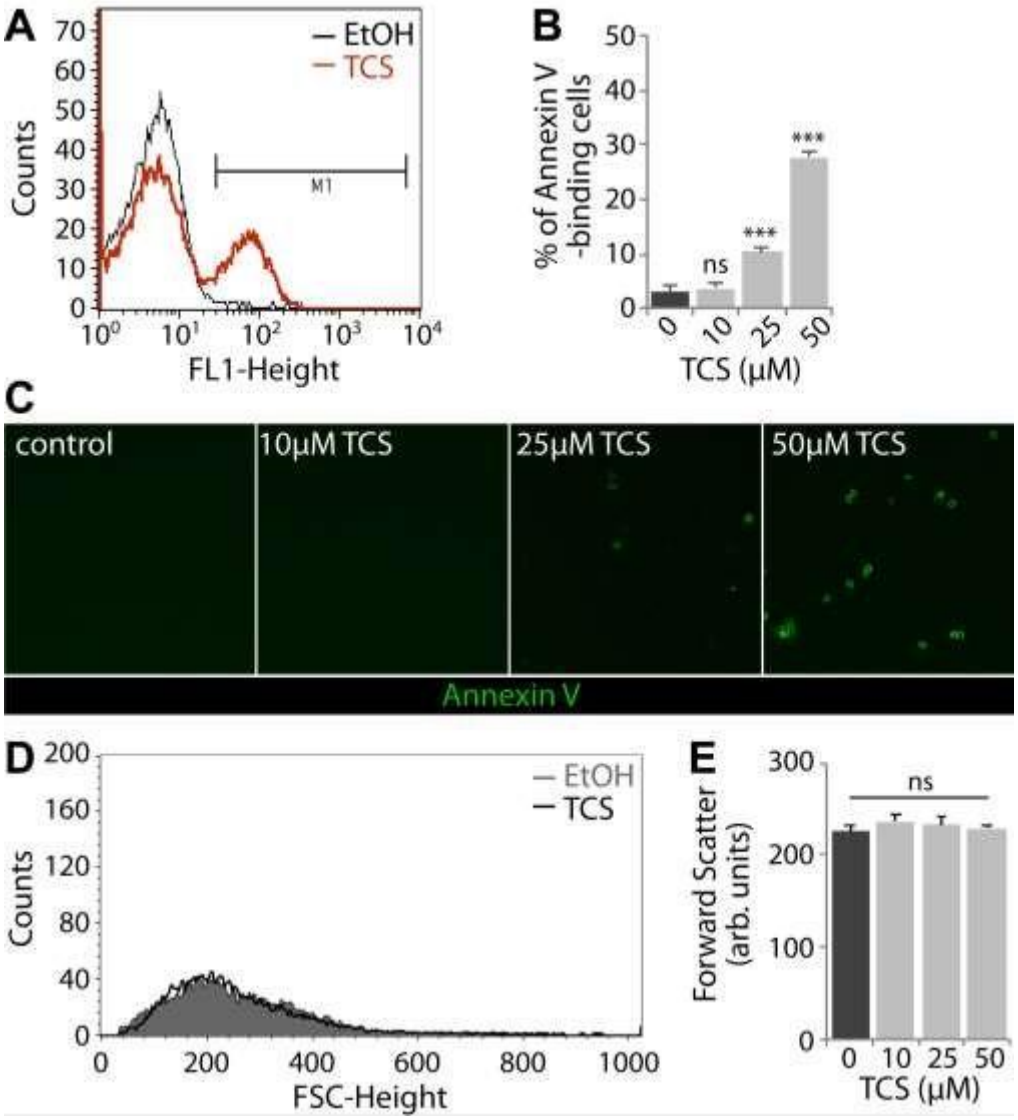
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Figure 2

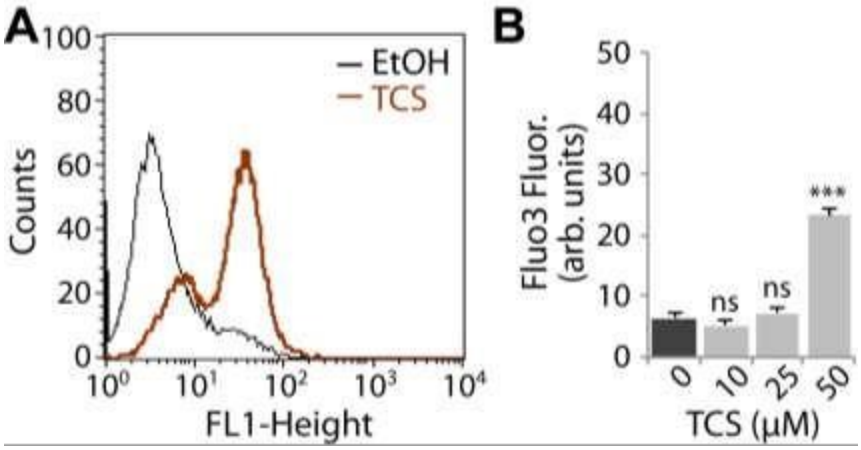
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Figure 3

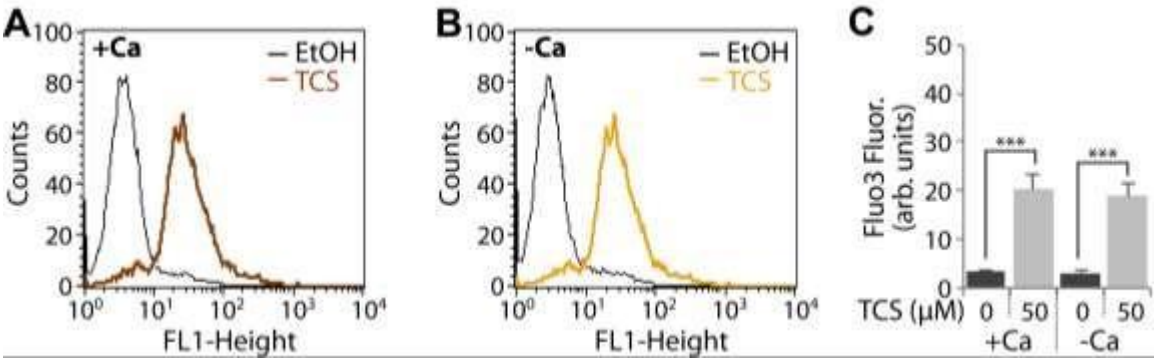
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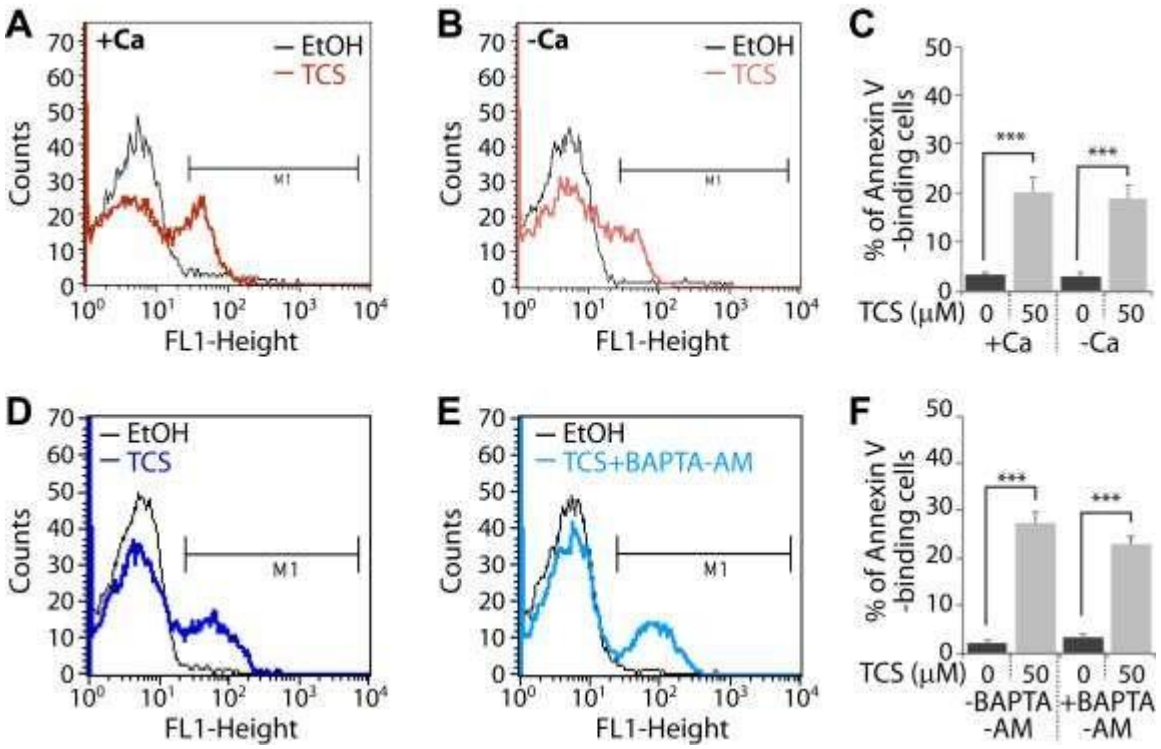
Figure 4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Counts
FL1-Height
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% of Annexin V -binding cells
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-BAPTA -AM
+BAPTA -AM

Figure 5

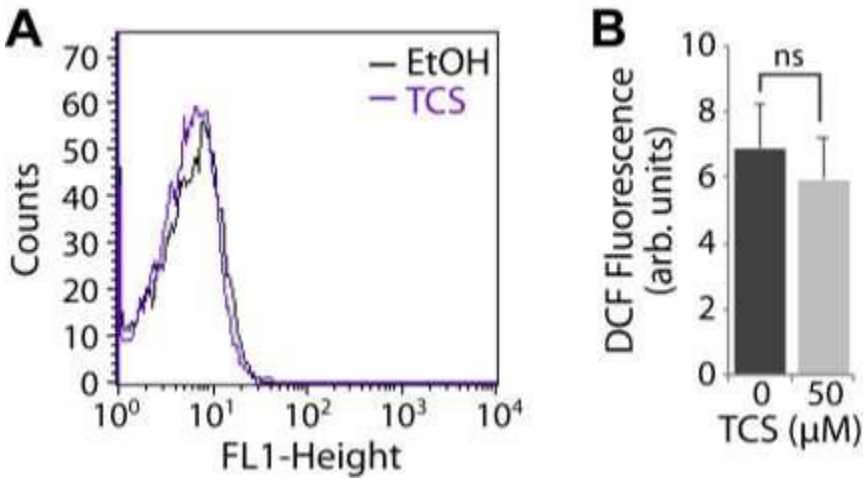
A
Counts
70
60
50
40
30
20
10
0
EtOH
TCS
10^0
10^1
10^2
10^3
10^4
FL1-Height
B
DCF Fluorescence (arb. units)
10
8
6
4
2
0
ns
0
50
TCS (μM)

Figure 6

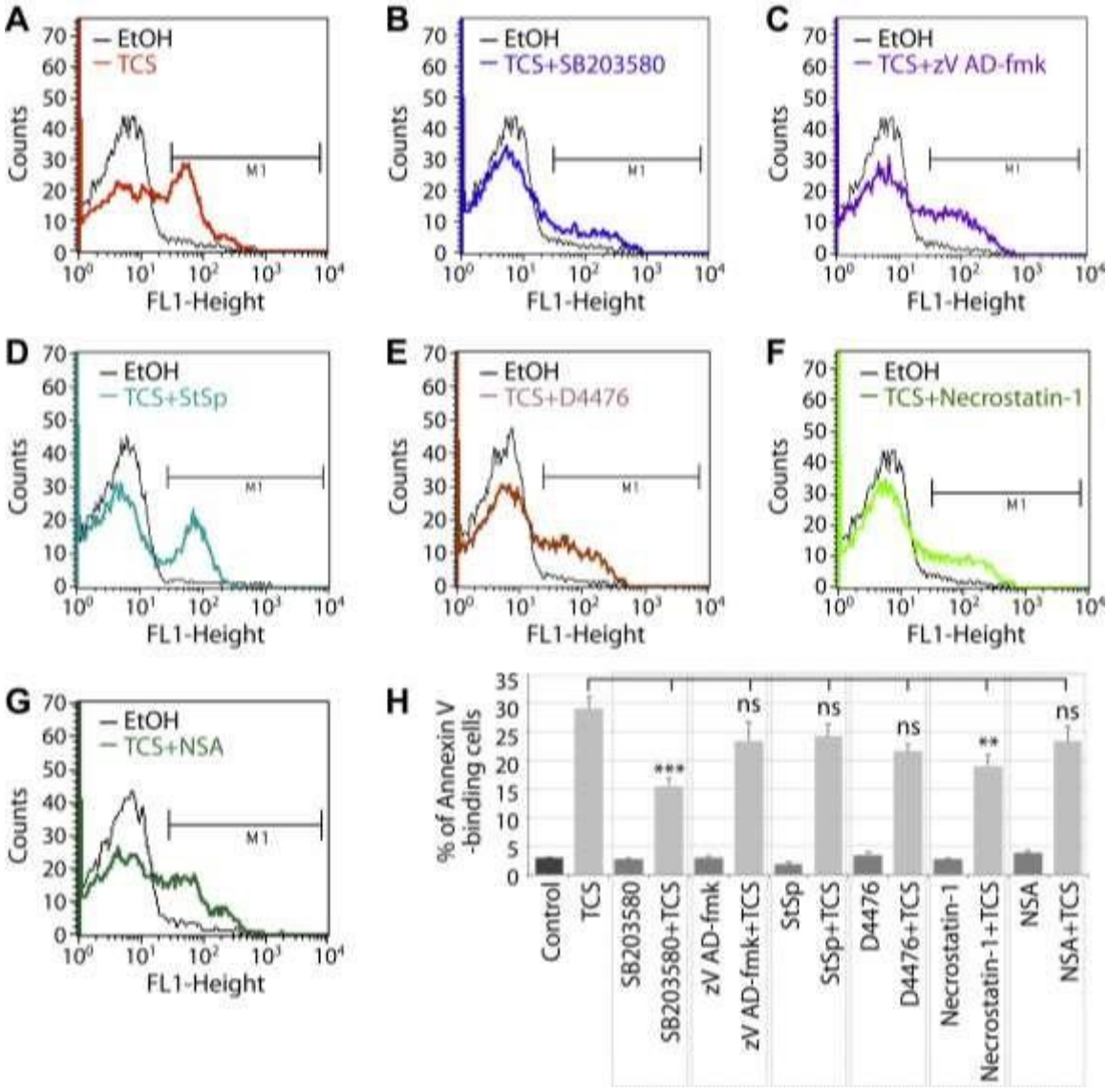
A
EtOH
TCS
B
EtOH
TCS+SB203580
C
EtOH
TCS+zV AD-fmk
D
EtOH
TCS+StSp
E
EtOH
TCS+D4476
F
EtOH
TCS+Necrostatin-1
G
EtOH
TCS+NSA
Counts
FL1-Height
M1
H
% of Annexin V -binding cells
Control
TCS
SB203580
SB203580+TCS
zV AD-fmk
zV AD-fmk+TCS
StSp
StSp+TCS
D4476
D4476+TCS
Necrostatin-1
Necrostatin-1+TCS
NSA
NSA+TCS
***
**
ns

Figure 7

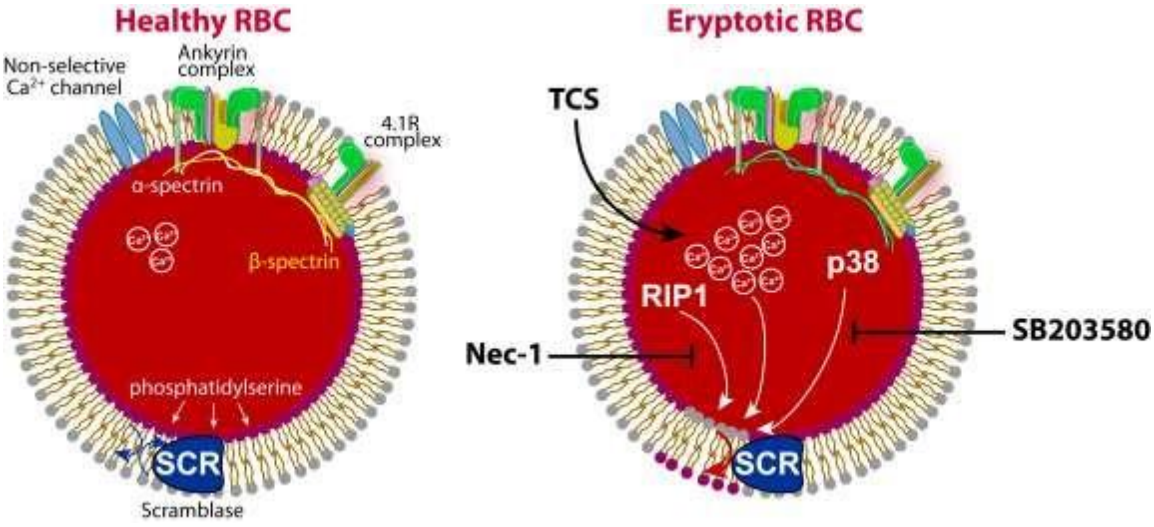
Healthy RBC
Non-selective
Ca2+ channel
Ankyrin
complex
4.1R
complex
α-spectrin
β-spectrin
phosphatidylserine
SCR
Scramblase
Eryptotic RBC
TCS
RIP1
p38
Nec-1
SB203580
SCR

Figure 8

CHAPTER IV: NON-IONIC SURFACTANTS ANTAGONIZE TOXICITY OF POTENTIAL PHENOLIC ENDOCRINE-DISRUPTING CHEMICALS, INCLUDING TRICLOSAN, IN *CAENORHABTIDIS ELEGANS*

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As published in *Mol Cells*, 2018 Dec 31; 41(12): 1052–1060.

doi: 10.14348/molcells.2018.0378

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ABSTRACT

Triclosan (TCS) is a phenolic antimicrobial chemical used in consumer products and medical devices. Evidence from in vitro and in vivo animal studies has linked TCS to numerous health problems, including allergic, cardiovascular, and neurodegenerative disease. Using Caenorhabditis elegans as a model system, we here show that short-term TCS treatment (LC50: ~0.2 mM) significantly induced mortality in a dose-dependent manner. Notably, TCS-induced mortality was dramatically suppressed by co-treatment with non-ionic surfactants (NISs: e.g., Tween 20, Tween 80, NP-40, and Triton X-100), but not with anionic surfactants (e.g., sodium dodecyl sulfate). To identify the range of compounds susceptible to NIS inhibition, other structurally related chemical compounds were also examined. Of the compounds tested, only the toxicity of phenolic compounds (bisphenol A and benzyl 4-hydroxybenzoic acid) was significantly abrogated by NISs. Mechanistic analyses using TCS revealed that NISs appear to interfere with TCS-mediated mortality by micellar solubilization. Once internalized, the TCS-micelle complex is inefficiently exported in worms lacking PMP-3 (encoding an ATP-binding cassette (ABC) transporter) transmembrane protein, resulting in overt toxicity. Since many EDCs and surfactants are extensively used in commercial products, findings from this study provide valuable insights to devise safer pharmaceutical and nutritional preparations.

KEYWORDS: *Caenorhabditis elegans*; PMP-3/ABC transporter; endocrine-disrupting chemicals; micelle; non-ionic surfactants; phenolic compound; triclosan

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are exogenous compounds that perturb the physiology of the endocrine glandular tissue (Swedenborg *et al.*, 2009). These compounds can disturb hormone production, release, transport, and metabolism (Kabir *et al.*, 2015). Routes of human exposure are varied owing to the wide array of applications and sources rich in EDCs. Transdermal absorption from cosmetics and personal hygiene products, ingestion in drinking water and food packaging material, and inhalation in dust represent the major and most common forms of exposure that carry the greatest risk potential (Diamanti-Kandarakis *et al.*, 2009). Furthermore, the developing neuroendocrine tissue of neonates is constantly being exposed to high concentrations of EDCs in breast milk and infant formula (Fang *et al.*, 2010; Azzouz *et al.*, 2016), incriminating these xenobiotics in developmental, neurological, and reproductive anomalies (Schug *et al.*, 2011).

Classification of EDCs is complicated as the number of newly identified, and erroneously recognized compounds, continues to steadily grow. Although many remain insufficiently characterized, phenolic EDCs are among the most common and well-studied classes. A prominent example is triclosan (TCS); an antimicrobial extensively used in the manufacture of plastics, toys, cosmetics, and kitchenware (Figure 1A). TCS has also been used for decades in hospital settings as an antiseptic and a disinfectant (Rodricks *et al.*, 2010; Dann and Hontela, 2011). The antimicrobial activity of TCS is attributed to the compound's interference with the enzyme enoyl-acyl carrier protein reductase (FabI), which is required for fatty acid and biotin biosynthesis (Rodricks *et al.*, 2010). Beside its antimicrobial properties, TCS toxicity has been studied in various living systems including humans, and the chemical has been shown to build up in body fluids including blood, urine,

and breast milk (Fang *et al.*, 2010; Rodricks *et al.*, 2010). Due to its widespread use and high chlorine content, TCS and its derivatives are ubiquitous in soil and aquatic environments, and have been detected in wastewater treatment systems as well as drinking water sources (McAvoy *et al.*, 2002; Escalada *et al.*, 2005; Benotti *et al.*, 2009; Li *et al.*, 2010).

The nematode *Caenorhabditis elegans* (*C. elegans*) has emerged as an attractive model animal for the functional analysis of various bioactive compounds (Tejeda-Benitez and Olivero-Verbel, 2016; Honnen, 2017; Hunt, 2017). Recent reports have shown that TCS exposure reduced the viability and fertility of wild-type *C. elegans* worms in a dose-dependent manner (Lenz *et al.*, 2017; Yoon *et al.*, 2017; Garcia-Espineira *et al.*, 2018; Vingskes and Spann, 2018). To date, although significant progress has been made in our understanding of TCS toxicity, studies devoted to the identification of clinically or industrially relevant TCS inhibitors are extremely scarce. In this study, we demonstrate that non-ionic surfactants (NISs), such as Tween 20 (Tw20), Tween 80 (Tw80), NP-40, and Triton X-100 (TX100), act as potent antagonists of phenolic EDCs including TCS, bisphenol A (BPA), and benzyl 4-hydroxybenzoic acid (B4HB). Mechanistic analyses revealed that NISs inhibit TCS-induced mortality by micellar solubilization, and that internalized TCS-micelle complex appears to be exported by PMP-3 (encoding an ATP-binding cassette (ABC) transporter) protein. Given the concerns surrounding TCS exposure, our findings may provide an innovative approach to reduce the burden of TCS and other phenolic EDCs on ecosystems and human health alike.

METHODS:

Chemicals and reagents

All chemicals used in this study were purchased from Sigma Aldrich (MO, USA) and were of analytical grade. TCS and benzyl 4-hydroxybenzoic acid (B4HB) were prepared in ethanol as 0.1 M stock solutions. Bisphenol A (BPA) was dissolved in methanol to obtain a 0.1 M stock solution, while 0.1 M stock solutions of sodium dodecyl sulfate (SDS) and sodium azide (NaN_3) were made in distilled water.

Strains and maintenance

C. elegans wild-type Bristol isolate (N2) and *pmp-3(ok1087)* mutant worms were obtained from the Caenorhabditis Genetics Center (CGC). All strains were cultured at 20°C in nematode growth medium (NGM) as previously described (Brenner, 1974).

Toxicity assays

Embryos were obtained by sodium hypochlorite (0.5 M NaOH and 1.2% NaClO) treatment of gravid hermaphrodites and incubated in M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO, 86 mM NaCl, and 1 mM $MgSO_4$) at 20°C overnight, as described elsewhere (Yoon *et al.,* 2016). Hatched L1 animals were either exposed to toxicants or were allowed to grow to adults on NGM plates for 3 days at 20°C before exposure. All chemicals were diluted in either M9 or M9/0.1% NISs to the final testing concentrations. Treatment groups were compared to the vehicle control, which did not exceed 0.2% in each case. The mortality rate was calculated visually by counting live and dead worms using a bright field

microscope (Figure 1B). Live worms exhibited normal locomotive behavior (Figure 1C), whereas dead worms were nonmotile and appeared rod-like in shape (Figure 1D).

Antimicrobial susceptibility testing

E. coli OP50 bacteria were grown at 37°C for 5 hours in Lysogeny Broth (LB) medium. Exposure was conducted in the same medium supplemented with TCS ranging from 0.001 mM to 0.05 mM with or without 0.1% Tween 20 (Tw20) for 24 hours at 25°C. The optical density (OD_{600}) was measured spectrophotometrically every two hours as an indicator of bacterial growth.

Pharyngeal pumping rate

Wild-type adult worms were incubated for 1 hour at 25°C in M9 buffer with or without 0.1% Tw20, before they were plated on NGM and examined for pumping using a dissecting microscope. Grinder movements were monitored for one minute, and the number of pumps per minute (ppm) was recorded.

Disruption of intracellular micelles

NIS Micelles were heat-disrupted at 35°C. Following TCS treatment with or without 0.1% Tw20, two approaches were followed for micelle disruption (Figure 4A). In method I, worms were immediately incubated at 35°C for an additional hour, whereas in method II, removal of extracellular TCS-Tw20 complexes by sequential washing in M9 buffer preceded incubation at 35°C.

Statistical analysis

Results are expressed as arithmetic means ± SD of at least three independent replicates (n>300). Comparative assessments between control and treatment groups were conducted using the paired *t*-student test. Statistical significance was determined by a *p* value of less than 0.05.

RESULTS

TCS increases mortality dose-dependently

Amongst phenolic EDCs, we initially investigated TCS due to its widespread occurrence and well-documented toxicity (Rodricks *et al.*, 2010). In eukaryotes, TCS disrupts mitochondrial oxidative phosphorylation and leads to profound increase reactive oxygen species (ROS) (Weatherly *et al.*, 2016). We recently reported that TCS induces toxicity, at least in part, by disrupting SKN-1(SKiNhead-1)/NRF2 (erythroid-2-related factor 2)-mediated oxidative stress response in both *C. elegans* and human stem cells (Yoon *et al.*, 2017).

To initially determine the effect of TCS on *C. elegans* viability, synchronized L1 larvae were treated with varying concentrations of TCS (0, 0.125, 0.25, 0.5, 1, 2, and 4 mM) for 1 hour at 25°C. As shown in Figure 1E, the mortality rate of L1 worms increased in a dose-dependent manner with a TCS concentration of half maximal response (EC_{50}) of ~ 0.2 mM. A similar dose-dependent pattern of increased mortality was also observed in adult worms (Figure 1F). These results suggest that TCS significantly increased the mortality of wild-type (N2) worms in a dose-dependent manner.

NISs abrogate TCS-induced mortality

Hydrophobic substances can be emulsified in micelles formed by NISs such as Tw20 (Lu and Park, 2013), and the benzene ring in TCS imparts a hydrophobic nature to the antiseptic (Petersen, 2016). To test the hypothesis that TCS-induced mortality could be neutralized by NISs, both L1 larvae and adult worms were treated with 0.125-8 mM TCS in presence or absence of 0.1% Tw20 for 1 hour at 25°C, and the mortality rate of both groups was calculated. Interestingly, co-treatment of TCS and Tw20 led to a profound decrease in the mortality of both stages when compared to TCS alone (Figure 2A and S1A). The highest TCS concentration susceptible to 0.1% Tw20 was around 8 mM and 4 mM for L1 larvae and adult worms, respectively (Figure 2A and S1A).

To determine the minimum effective concentration of Tw20 required to confer protection against lethal concentrations of TCS, L1 larvae were incubated for 1 hour with 1, 2, and 4 mM TCS in the absence or presence of 0.1, 0.02, 0.004, and 0.0008% Tw20. As for L1 larvae, at least 0.02% Tw20 was sufficient to protect against 4 mM TCS, while 0.004% Tw20 was sufficient against 1 mM TCS (Figure 2C). Parallel to L1 larvae, the mortality rate of adult worms at 1 and 2 mM TCS was significantly abrogated with at least 0.004% and 0.02% Tw20, respectively (Figure S1B).

Next, we were prompted to test the effects of other NISs, including Tw80, NP-40, and TX100 on TCS-induced mortality. To this end, L1 staged wild-type worms were incubated with or without 0.5 mM TCS (minimum concentration with >80% mortality) in the absence or presence of 0.1-0.0008% Tw80, NP-40, or TX100 for 1 hour at 25°C, and

the mortality rate was calculated as described earlier. As seen in Figure 2E, all NISs significantly protected the worms against 0.5 mM TCS dose-dependently.

In industrial settings, anionic surfactants such as sodium dodecyl sulfate (SDS) are also added to commercial products to solubilize TCS (Babich and Babich, 1997). In order to determine if SDS is also capable of antagonizing TCS toxicity, we incubated L1 larvae with 0.5 mM TCS in the presence of 1, 10, and 20 mM SDS for 1 hour at 25°C and scored the mortality rate as outlined before. Figure 2E shows the wormicidal activity of SDS at 10 and 20 mM, and, more importantly, the failure of SDS at 1 mM to protect against TCS-induced mortality. Collectively, these observations suggest that TCS-induced mortality is abrogated by co-treatment of NISs (i.e. Tw20, Tw80, NP-40, and TX100), but not by SDS.

We then sought to inquire whether Tw20 could also neutralize the antimicrobial activity of TCS. To this end, *E. coli* bacteria were cultured with or without 0.001-0.2 mM TCS in the absence or presence of 0.1% Tw20 for 24 hours at 25°C. We specifically cultured *E. coli* bacteria at 25°C, instead of 37°C, to preserve the micellar state of Tw20. Optical density at 2-hour intervals was measured as a function of bacterial growth. Although TCS unsurprisingly inhibited bacterial growth at all concentrations tested, significant resistance was observed under conditions of a combination of 0.001 mM TCS and 0.1% Tw20 (Figure 2D, compare (3) and (7)). These findings suggest that the inhibitory action of NISs against TCS is also observed in bacteria.

Ingestion in *C. elegans* is accomplished through the pharynx and requires two sequential events; pumping and peristalsis. Pharyngeal movement is related to food intake, and is influenced by environmental conditions in the immediate vicinity of the worm (Song

and Avery, 2013). Thus, we examined if the protective role of Tw20 is related to restricted pharyngeal pumping, which would hinder TCS uptake. To this end, adults were exposed to 0.1% Tw20 or left untreated for 1 hour at 25°C. Worms were then plated and grinder movements (number of pumps per minute) were recorded under a differential interference contrast (DIC) microscope. Our results showed no significant difference in the rate of pharyngeal pumping in presence or absence of Tw20 (Figure 2B), which indicates that Tw20 does not neutralize TCS toxicity by reducing its physical intake.

NISs mitigate the mortality induced by other phenolic EDCs: BPA and B4HB

To assess the range of compounds sensitive to NIS interference, we tested the effect of NISs on other toxicants that are not recognized as EDCs (Figure 3A, *left*). Sodium azide (NaN_3) is a polar, ionic salt commonly used as a solution preservative owing to its biocidal properties (Ishikawa *et al.*, 2006). It interferes with mitochondrial oxidative phosphorylation by chelating iron ions required for cytochrome oxidase activity (Ishikawa *et al.*, 2006). We evaluated the ability of NISs to subvert sodium azide toxicity by incubating L1 larvae with or without 0.2-1.6 mM sodium azide in the absence or presence of 0.1% NISs for 24 hours at 25°C. Figure 3B shows that the dose-responsive increase in mortality was not nullified by co-treatment with NISs. Ethanol (EtOH) is another polar compound with disruptive behavioral effects on *C. elegans* (Davies *et al.*, 2004) (Figure 3A, *left*). To test whether NISs could protect against EtOH mortality, L1 larvae were incubated with or without 10%-20% EtOH in the absence or presence of 0.1% NISs for 1 hour at 25°C, and the mortality rate was subsequently scored. Our data show that 20% EtOH resulted in 100% mortality in wild-type worms which was not reversed by co-treatment with 0.1% NISs (Figure 3C).

Although we cannot exclude all other possibilities, these results indicate that molecular similarity among compounds maybe a determining factor in their susceptibility to NISs. Hence, we examined two other chemicals that are closely related to TCS in terms of both their chemical nature (a common phenol ring) and activity (endocrine disruption) – BPA and B4HB (Figure 3A, *right*). The xenoestrogenic activity of BPA is associated with increased proliferation of ovarian and breast cancer cells (Dong *et al.*, 2011), genotoxicity (Pupo *et al.*, 2012), and elevated prolactin, estradiol, and progesterone levels in females (Miao *et al.*, 2015). B4HB is a paraben widely used as a preservative in cosmetics and food processing (Ye *et al.*, 2006). Exposure to parabens has been strongly linked to human health concerns mainly due to their estrogenicity and proliferative stimulation of breast cancer cells (Byford *et al.*, 2002). Moreover, butylparaben has been shown to cause DNA damage in human sperm (Meeker *et al.*, 2011).

To test if NISs could protect against BPA-induced mortality, L1 larvae were incubated with or without 0.5-2.0 mM BPA in presence and absence of 0.1% NISs for 24 hours at 25°C. In agreement with previous reports (Watanabe *et al.*, 2005), BPA caused a dose-dependent increase in mortality, and, interestingly, co-treatment with 0.1% NISs significantly ablated BPA-induced mortality (Figure 3D). We also determined the wormicidal potential of B4HB and its sensitivity to NIS inhibition. To this end, L1 larvae were incubated with or without 0.25-1.0 mM B4HB in the absence or presence of 0.1% NISs for 1 hour at 25°C. As shown in Figure 3E, B4HB resulted in a significant, dose-dependent increase in mortality at all concentrations tested. Importantly, a similar pattern of inhibition to TCS and BPA was also observed in worms co-treated with B4HB and NISs

(Figure 3E). Taken together, these results suggest that NISs may protect against phenolic EDCs that share structural similarity to TCS.

Micellar solubilization is required for NIS-mediated protection

We next tested if NISs could inhibit the toxicity of TCS via micelle formation. Tw20 was chosen as a representative NIS as it showed potent inhibitory action against TCS concentrations with 100% mortality (Figure 2A). To this end, L1 larvae were incubated with or without 1 mM TCS in the absence or presence of 0.1% Tw20 for 1 hour at 25°C (Figure 4A). As observed earlier, 1 mM TCS resulted in 100% mortality, which was reversed by co-treatment with 0.1% Tw20 (Figure 4B, *Pre-incubation*).

To evaluate the importance of micellar solubilization for the anti-toxic activity of Tw20, micelles were heat-disrupted by upshifting exposure temperature to 35°C (Markovic-Housley and Garavito, 1986) for an additional hour (Figure 4A**,** *Method (I)*). Notably, the mortality of worms co-treated with TCS and Tw20 significantly increased following the temperature upshift (Figure 4C, *Method (I)*). This seems to indicate that sequestered TCS molecules within Tw20 micelles were released upon temperature-mediated micelle disruption and regained their wormicidal activity. Furthermore, to rule out the contribution of extracellular TCS in the observed mortality following the upshift, extracellular TCS-Tw20 complexes were removed by repeated washing and L1 larvae were then upshifted to 35°C for an additional hour (Figure 4A, *Method (II)*). Under these conditions, up to ~ 30% increase in mortality in comparison to Tw20-treated worms (Figure 4C, *Method (II)*) was observed, which is attributed mostly to the internalized TCS-Tw20 complexes.

The PMP-3/ABC transporter modulates the absorption, metabolism, and cytotoxicity of pharmacological agents (Das *et al.*, 2006). Of recent, we have reported that lack of PMP-3 increases susceptibility to TCS (Yoon *et al.*, 2017). A reporter gene analysis showed that *pmp-3 (promoter)::GFP* is expressed in the pharynx, muscles, intestine, and stem cell niche (Figure S2). To ask if internalized TCS-Tw20 micelle complexes are exported out of the worms' bodies through PMP-3, L1 stage *pmp-3(ok1087)* loss-of-function mutant worms (*pmp-3(-)*) were treated as described in Figure 4A. As is the case with wild-type worms (Figure 4B), 1 mM TCS caused 100% mortality in *pmp-3(-)* mutants, which was significantly ameliorated in the presence of 0.1% Tw20 (Figure 4D, *Pre-incubation*). Importantly, the mortality of *pmp-3(-)* mutants was significantly enhanced following heat-mediated micelle disruption, an effect that was significantly higher than that of their wild-type counterparts ($p<0.01$, compare Figure 4C and 4E, *Method (I)*).

Next, to test if accumulated intracellular TCS could increase the mortality in *pmp-3(-)* mutant worms, we co-treated L1 staged *pmp-3(-)* mutant worms with 1 mM TCS and 0.1% Tw20 for 1 hour at 25°C, before the temperature was upshifted to 35°C following washing for three times (Figure 4A, *Method (II)*). Interestingly, TCS molecules released from Tw20 micelles at 35°C significantly increased the mortality (76 ± 3.7%) of *pmp-3(-)* mutant worms more than that (34 ± 3.6%) seen in wild-type worms ($p<0.01$, compare *Method(II)* in Figure 4C and 4E).

Taken together, these results point at two possible conclusions: First, Tw20 may inhibit the wormicidal properties of TCS by micellar solubilization. Second, export of internalized TCS-Tw20 micellar complex may be facilitated, at least in part, through PMP-

3-mediated detoxification mechanism. Although only TCS was evaluated under these conditions, it is reasonable to suggest that a similar pattern is likely mirrored by other phenolic EDCs and NISs.

DISCUSSION:

EDCs are ubiquitous in the environment and pose a global threat to human and wildlife health. To date, studies elucidating the toxicity of EDCs have received greater attention from researchers, while investigations devoted to the identification of EDC inhibitors are only recently emerging. For instance, just a few years ago, Sengupta *et al.* reported that atrazine inhibits TCS toxicity by activating the nuclear receptor HR96 (an ortholog of CAR/PXP/VDR) in *Daphnia magna* (Sengupta *et al.*, 2015). In addition, the interaction of surfactants with other antibacterials such as amoxicillin and moxifloxacin has previously been examined (Schwameis *et al.*, 2013). However, no studies have investigated the protective role of NISs against EDC toxicity in eukaryotic model systems.

This work establishes the nematode *C. elegans* as a model for studying the toxicity of phenolic EDCs, and also demonstrates the potent ameliorative potential of NISs against the wormicidal properties of TCS and other phenolic EDCs, facilitated through micellar solubilization. The TCS-micelle complex appears to be exported out of the worms' bodies at least in part through a PMP-3/ABC transporter (Figure 5A and 5B). However, following micelle disruption, released TCS seems to regain its activity and in turn perturbs the survival of worms (Figure 5C). In pharmaceutical and nutritional preparations, NISs have been used as solubilizers and stabilizers, but their potential effects on the detoxification of

EDCs have largely been overlooked. Therefore, our findings present broad insights into EDC intoxication, detoxification, and product formulation strategies.

The activity of phenolic compounds is influenced by their percent saturation in solution (Ogata and Shibata, 2000). Micelle aggregates are formed when surfactants are dissolved in solutions at or above their critical micelle concentration (CMC). Surfactants can solubilize phenolics in the micellar phase and thus reduce their thermodynamic activity (Allawala and Riegelman, 1953). To put things into perspective, a saturated water solution of chloroxylenol, a phenolic disinfectant, was shown to exhibit comparable biocidal efficacy to a saturated surfactant solution with concentrations of many orders of magnitude higher (Mitchell, 1964). Moreover, Taylor *et al.* compared the efficacy of TCS against *E. coli* at 100% saturation in ammonium lauryl sulfate (ALS) solutions of varying concentrations (Taylor *et al.*, 2004). Interestingly, the degree of bacterial growth reduction when ALS was increased was similar to that observed when less ALS and twice as much TCS were used (Taylor *et al.*, 2004). This indicates that surfactant to EDC ratio, but not EDC concentration, determines the overall fate of EDC activity. Similarly, other phenolic antimicrobial agents, most notably rifampicin and isoeugenol, were found to be highly susceptible to inactivation by Tw80 (Nielsen *et al.*, 2016). In *E. coli,* our data show that TCS retains its antibacterial activity at a minimum concentration of 0.001 mM when co-administered with 0.1% Tw20, suggesting that the bioavailable portion of TCS was sufficient to exhibit its bactericidal effect under these saturation conditions (see Figure 2D). This is corroborated by the contrasting synergistic effect of Tw80 on water-soluble antimicrobials such as polymyxin B and benzalkonium chloride (Toutain-Kidd *et al.*, 2009). It is important to note that, because TCS inhibits the synthesis of fatty acids, which

are abundant in surfactants, it has been surmised that TCS-resistant *Staphylococcus aureus* compensate for the anti-lipogenic effect of TCS by utilizing exogenous fatty acids presumably provided by the Tween surfactants (Morvan *et al.*, 2016). In *C. elegans*, manipulating the NIS-TCS ratio showed that NISs are potent inhibitors of phenolic EDCs at very low concentrations. Our results revealed that NIS concentrations as low as 0.0008% significantly reduced the mortality caused by a lethal TCS dose of 0.5 mM (see Figure 2C). This remarkable inhibitory efficiency, compared to that seen in *E. coli*, could be ascribed to the outer cuticle that encapsulates the worms and imparts environmental and anti-toxic protection.

In conclusion, the current study identifies NISs as potent inhibitors of phenolic EDCs in an eukaryotic model organism. The findings presented herein may pave the way for devising and developing potentially effective preventive and therapeutic strategies to control the widespread dissemination of phenolic EDCs, while still maintaining their beneficial antimicrobial properties. The observations presented here, along with those from previous studies, mandate further investigations based on a multidisciplinary approach, combining physicochemical and biological aspects, to fully characterize the direct interaction between NISs and EDCs. Future efforts should be directed toward investigating the complex interplay between NIS solubilization and its net effect on drug digestion, absorption, and overall activity in highly relevant vertebrate model systems.

ACKNOWLEDGEMENTS

We thank the members of the Lee laboratory, Jiwoo Lee, and Jiah Lee for helpful advice and discussion during this work. This work was supported in part by the Brody

Brothers Grant (21602-664261) to M-H.L. and the Saudi Government Graduate Scholarship (from King Saud University) to M.A.A. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

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FIGURE LEGENDS

Figure 1. TCS induces mortality of wild-type worms. (A) Chemical structure of TCS. (B) Strategy for chemical treatment. (C and D) DIC pictures of wild-type worms in the absence or presence of TCS. (E) Percent mortality in TCS-treated wild-type L1 larvae. Standard deviation bars were calculated from at least three independent experiments (n>300). $p<0.05$(*); $p<0.01$(**); $p<0.001$(***); Not statistically significant (n.s.).

Figure 2. Protective role of NISs against TCS. (A) Effect of Tw20 on TCS-induced mortality in L1 larvae. (B) Effect of Tw20 on pharyngeal pumping. (C) Dose-dependence effect of TCS and Tw20 on mortality. (D) Effect of Tw20 on the antimicrobial activity of TCS. (E) Effect of NISs on TCS-induced mortality. Standard deviation bars were calculated from at least three independent experiments (n>300). $p<0.05$(*); $p<0.01$(**); $p<0.001$(***); Not statistically significant (n.s.).

Figure 3. NISs suppress the mortality induced by other phenolic EDCs. (A) Chemical structures of non-EDCs and phenolic EDCs. (B-E) Effect of NISs on toxicant-induced mortality. For NaN_3 and BPA, total exposure period was 24 hours at 25°C. For EtOH and B4HB, worms were treated for 1 hour at 25°C. Standard deviation bars were calculated from at least three independent experiments (n>300). $p<0.05$(*); $p<0.01$(**); $p<0.001$(***); Not statistically significant (n.s.).

Figure 4. Tw20 inhibits TCS-induced mortality via micelle formation. (A) Exposure strategy. (B & C) Effect of Tw20 micelle formation on TCS-induced mortality in wild-type worms. (D & E) Role of PMP-3 in the export of TCS-Tw20 micellar complex. Standard

deviation bars were calculated from at least three independent experiments (n>300). $p<0.05$(*); $p<0.01$(**); $p<0.001$(***); Not statistically significant (n.s.).

Figure 5. A working model for NIS amelioration of EDC-induced mortality. (A) EDCs can act via receptor-based mechanism, but at high doses, EDCs may employ receptor-independent mechanisms. EDCs (e.g., TCS) may also inhibit PMP-3-mediated detoxification mechanisms (Yoon *et al.*, 2017). (B) EDCs could be inactivated *in vivo* by NIS-mediated micellar solubilization and the EDC-NIS complex may be exported at least in part by PMP-3/ABC transporters. (C) Upon micelle disruption, liberated EDC molecules regain their toxicity and may inhibit PMP-3-mediated detoxification.

FIGURES

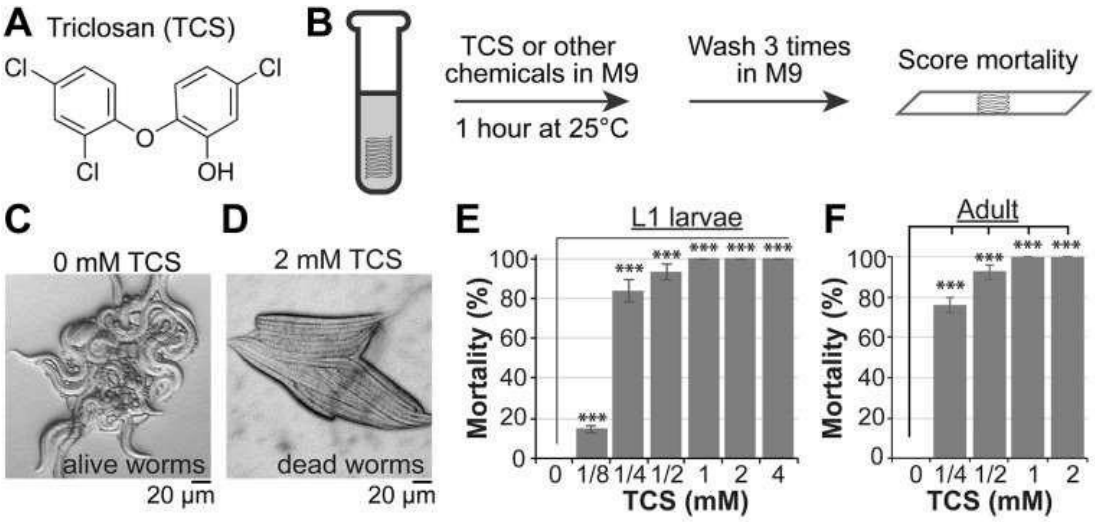

Figure 1

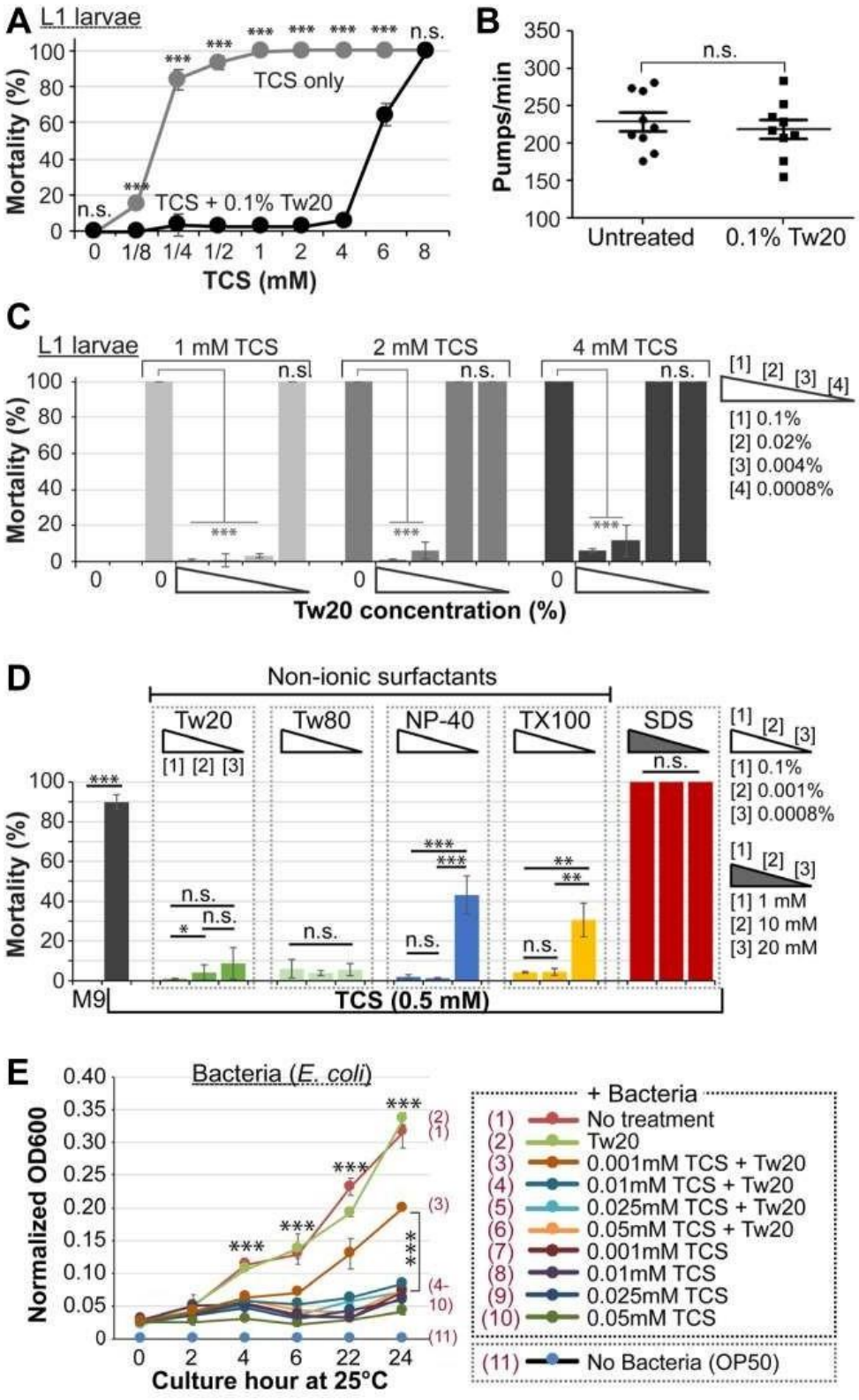
A
L1 larvae
Mortality (%)
TCS only
TCS + 0.1% Tw20
TCS (mM)
B
Pumps/min
n.s.
Untreated
0.1% Tw20
C
L1 larvae
1 mM TCS
2 mM TCS
4 mM TCS
[1] 0.1%
[2] 0.02%
[3] 0.004%
[4] 0.0008%
Tw20 concentration (%)
D
Non-ionic surfactants
Tw20
Tw80
NP-40
TX100
SDS
[1] 0.1%
[2] 0.001%
[3] 0.0008%
[1] 1 mM
[2] 10 mM
[3] 20 mM
M9
TCS (0.5 mM)
E
Bacteria (E. coli)
Normalized OD600
Culture hour at 25°C
+ Bacteria
(1) No treatment
(2) Tw20
(3) 0.001mM TCS + Tw20
(4) 0.01mM TCS + Tw20
(5) 0.025mM TCS + Tw20
(6) 0.05mM TCS + Tw20
(7) 0.001mM TCS
(8) 0.01mM TCS
(9) 0.025mM TCS
(10) 0.05mM TCS
(11) No Bacteria (OP50)

Figure 2

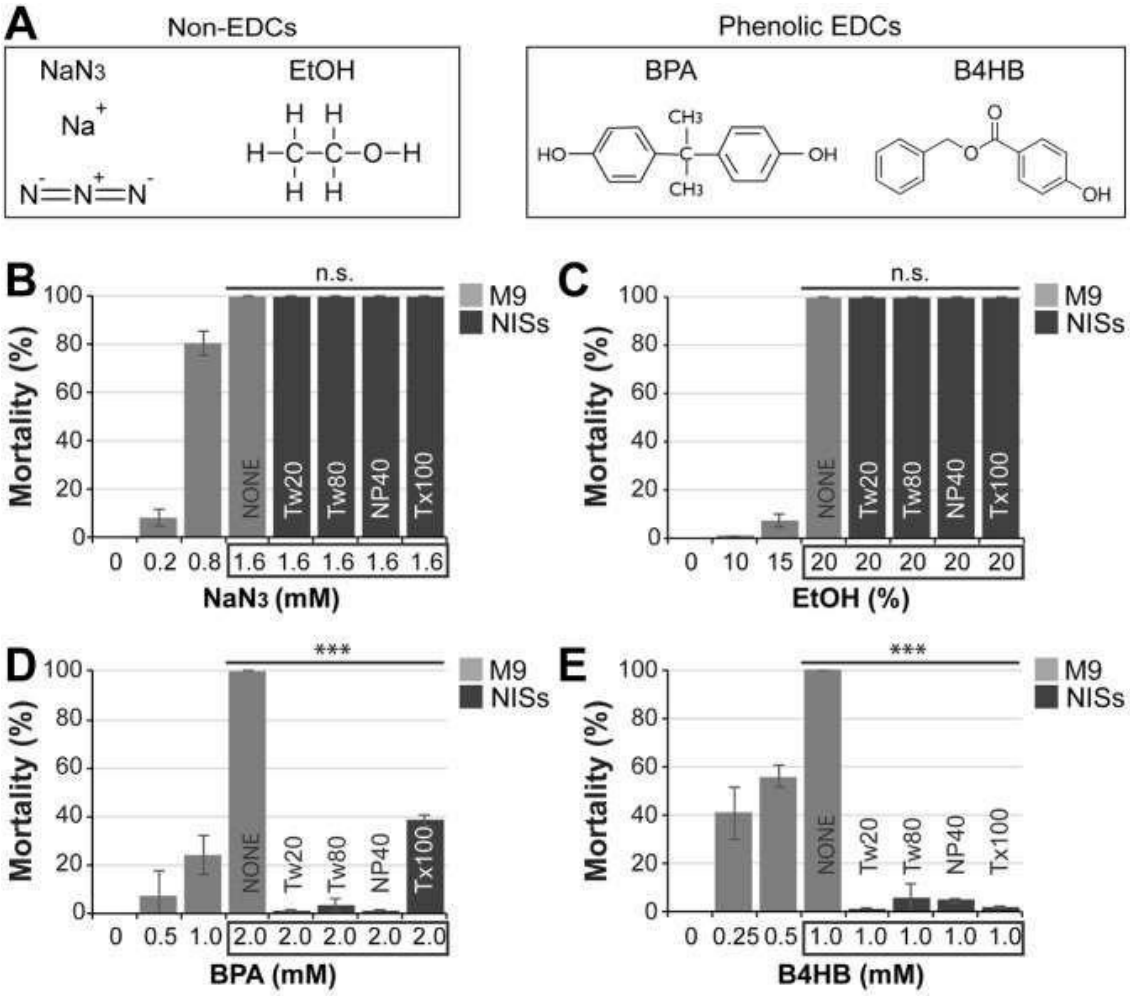
A
Non-EDCs
NaN3
Na+
EtOH
Phenolic EDCs
BPA
B4HB
B
n.s.
M9
NISs
Mortality (%)
NONE
Tw20
Tw80
NP40
Tx100
0 0.2 0.8 1.6 1.6 1.6 1.6 1.6
NaN3 (mM)
C
n.s.
0 10 15 20 20 20 20 20
EtOH (%)
D
***
0 0.5 1.0 2.0 2.0 2.0 2.0 2.0
BPA (mM)
E
***
0 0.25 0.5 1.0 1.0 1.0 1.0 1.0
B4HB (mM)

Figure 3

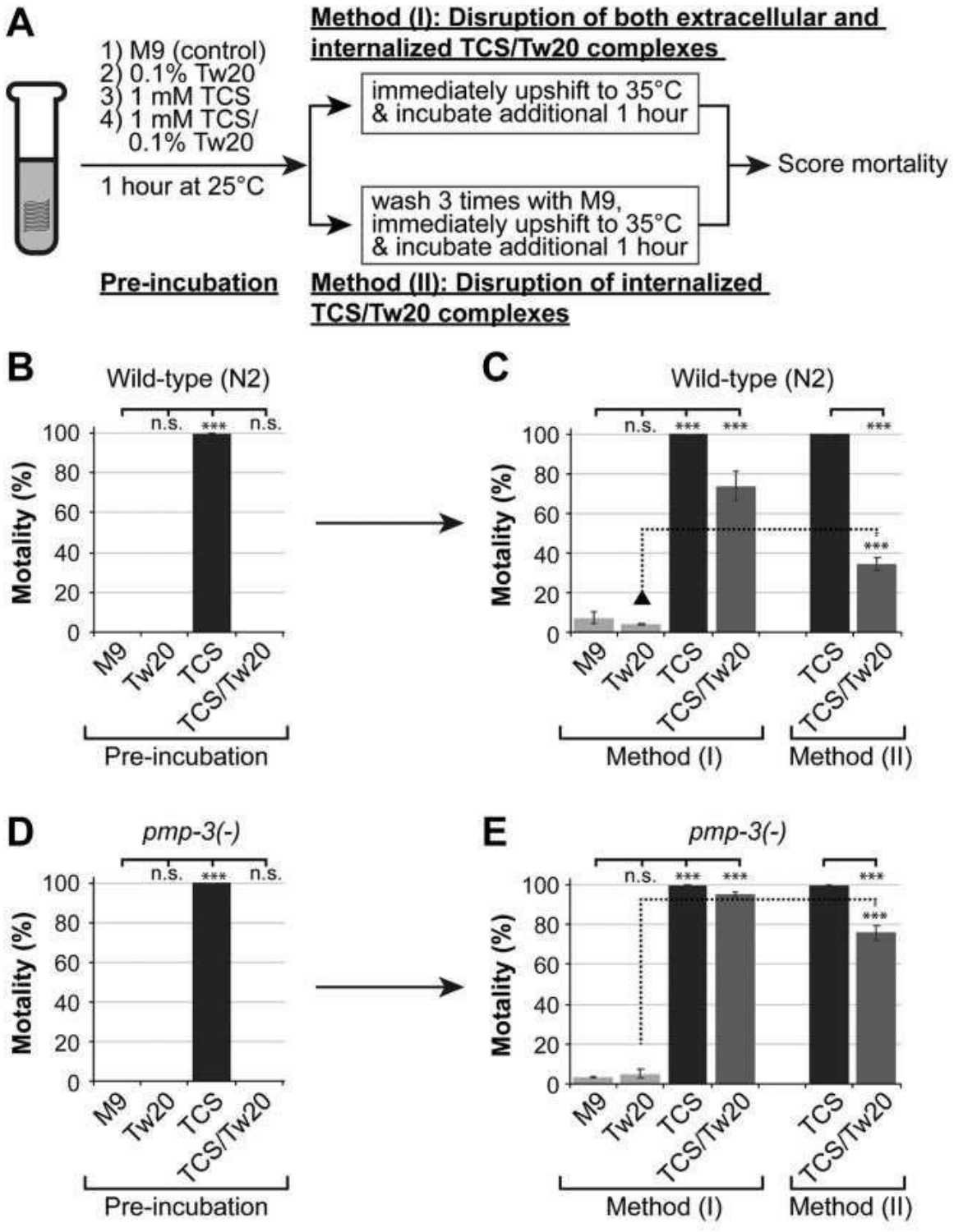
A
1) M9 (control)
2) 0.1% Tw20
3) 1 mM TCS
4) 1 mM TCS/
0.1% Tw20
1 hour at 25°C
Pre-incubation
Method (I): Disruption of both extracellular and internalized TCS/Tw20 complexes
immediately upshift to 35°C & incubate additional 1 hour
wash 3 times with M9, immediately upshift to 35°C & incubate additional 1 hour
Score mortality
Method (II): Disruption of internalized TCS/Tw20 complexes
B
Wild-type (N2)
Motality (%)
n.s.
***
M9
Tw20
TCS
TCS/Tw20
Pre-incubation
C
Wild-type (N2)
Method (I)
Method (II)
D
pmp-3(-)
E
pmp-3(-)

Figure 4

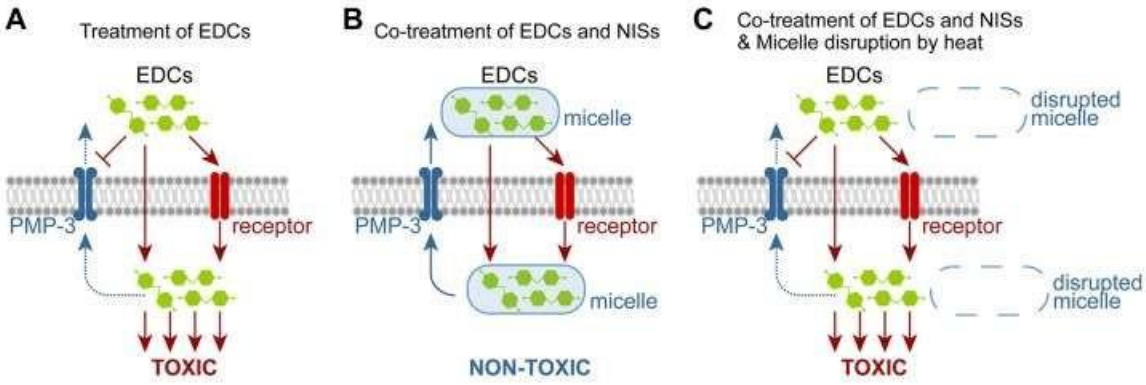
A
Treatment of EDCs
EDCs
PMP-3
receptor
TOXIC
B
Co-treatment of EDCs and NISs
EDCs
micelle
PMP-3
receptor
micelle
NON-TOXIC
C
Co-treatment of EDCs and NISs
& Micelle disruption by heat
EDCs
disrupted micelle
PMP-3
receptor
disrupted micelle
TOXIC

Figure 5

CHAPTER V – ADDITIONAL STUDIES: STIMULATION OF ERYPTOSIS BY BROAD-SPECTRUM INSECT REPELLENT *N*,*N*-DIETHYL-3-METHYLBENZAMIDE (DEET)

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As published in *Toxicol & Appl Pharmacol*, 2019 Mar 14;370:36-43.

doi: 10.1016/j.taap.2019.03.011.

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ABSTRACT

N,*N*-Diethyl-3-methylbenzamide (DEET) is the most widely used insect repellent in the world. Adverse effects following DEET exposure are well documented. Moreover, DEET has been shown to possess cytotoxic and apoptotic properties in nucleated cells. Although red blood cells (RBCs) lack intracellular organelles, they nevertheless undergo programmed cell death termed eryptosis. Compromised RBC health contributes to the development of anemia; a condition affecting 25% of the global population. This study investigated the interaction between DEET and human RBCs, and explored accompanying biochemical and molecular alterations. RBCs at 5% hematocrit were incubated in presence and absence of 1-5 mM (0.02%-0.1%) of DEET for 6 h at 37°C. Hemolysis was spectrophotometrically determined by hemoglobin release, while major eryptotic events were analyzed by flow cytometer. Phosphatidylserine (PS) exposure was detected with Annexin-V-FITC, cell volume by forward scatter (FSC) of light, intracellular calcium with Fluo-3/AM, and reactive oxygen species with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). DEET caused slight hemolysis at 4 and 5 mM, and significantly increased Annexin-V-FITC and Fluo3 fluorescence, with reduced FSC at 5 mM. Removal of extracellular Ca^{2+} abolished DEET-induced Fluo3 fluorescence but had no effect on Annexin-V binding. Importantly, blockade of eryptotic signaling mediators p38 MAPK, caspases, protein kinase C, casein kinase 1, or necroptotic kinases receptor-interacting protein 1 and mixed lineage kinase domain-like protein, with small molecule inhibitors, did not ameliorate DEET-mediated PS externalization. In conclusion, DEET elicits suicidal erythrocyte death; an event characterized by loss of membrane asymmetry, cell shrinkage, and elevations in intracellular Ca^{2+} mainly through dysregulated Ca^{2+} influx.

KEYWORDS: N,N-Diethyl-3-methylbenzamide (DEET); Red blood cells (Erythrocytes); Phosphatidylserine; Eryptosis

INTRODUCTION

The World Health Organization (WHO) reports that a little less than one-fifth of the global burden of infections is caused by vector-borne disease, which accounts for around 700,000 deaths annually (WHO. Global vector control response 2017–2030. Geneva: World Health Organization; 2017. http://apps.who.int/iris/bitstream/handle/10665/259205/9789241512978-eng.pdf?sequence=1). Among the most infamous vectors are mosquitoes in the *Anopheles*, *Aedes*, and *Culex* genera. These blood-sucking insects are responsible for the transmission of serious diseases and pathogens including malaria, filariasis, yellow fever, dengue fever, chikungunya, Zika virus, West Nile virus, Japanese encephalitis, and St. Louis encephalitis (WHO. Vector-borne diseases: Report of an informal expert consultation. New Delhi: World Health Organization; 2014. http://apps.who.int/iris/bitstream/handle/10665/206531/B5139.pdf?sequence=1; WHO: Yellow fever fact sheet. http://www.who.int/en/news-room/fact-sheets/detail/yellow-fever) (Mackenzie *et al.*, 2004; Mulatier *et al.*, 2018). Given the lack of vaccination, prophylaxis by insect repellents remains one of the most efficient and widely used methods to prevent the spread of vector-borne disease worldwide.

N,*N*-Diethyl-3-methylbenzamide (DEET) is the most commonly used insect repellent in the world (Fig. 1A) (Chen-Hussey *et al.*, 2014). Since its introduction over half a century ago (Nentwig, 2003), DEET has demonstrated efficacy in reducing the transmission of

vector-borne disease. The repellence properties of DEET may be attributed to its activation of olfactory receptor neurons (ORNs) in mosquito antennae, among other possible mechanisms (Leal, 2014). Products containing up to 100% DEET are available to consumers in a variety of formulations including sprays, aerosols, gels, lotions, sticks, and wipes (Diaz, 2016).

Pharmacokinetic studies in animals indicate significant variability in oral and dermal absorption of DEET. In humans, although absorption rates may reach as high as 20%, extensive variations observed in animals may still be mirrored in man (Feldmann and Maibach, 1970; Blomquist and Thorsell, 1977; Reifenrath *et al.*, 1980; Reifenrath *et al.*, 1981; Snodgrass *et al.*, 1982; Moody *et al.*, 1989; Moody and Nadeau, 1993; Taylor *et al.*, 1994; Selim *et al.*, 1995). DEET reaches the systemic circulation, crosses the placenta (McGready *et al.*, 2001; Barr *et al.*, 2010; Diaz, 2016), and is chiefly eliminated in the urine. Major DEET metabolites excreted are *N*,*N*-diethyl-mhydroxymethylbenzamide and *N*-ethyl-m-hydroxymethylbenzamide, representing up to 42% of applied dose (Selim *et al.*, 1995). The unchanged parent compound has also been detected in urine (Smallwood *et al.*, 1992). Nevertheless, based on the inherent diversity of human use and consumption, variations in DEET bioavailability and metabolism are expected to be not uncommon.

Intoxication related to DEET is well documented in animals and humans, with dermal, neurologic, ocular, psychotic, respiratory, gastrointestinal, and cardiovascular manifestations (Heick *et al.*, 1980; Snyder *et al.*, 1986; Heick *et al.*, 1988; McKinlay *et al.*, 1998; Briassoulis *et al.*, 2001; Bell *et al.*, 2002; Jortner, 2006). Cases of accidental and suicidal death have also been reported following oral and dermal exposure to DEET (Veltri *et al.*, 1994; Bell *et al.*, 2002; Wiles *et al.*, 2014). Populations at higher risk of adverse

DEET effects include children, workers in parks and manufacturing plants, and individuals with preexisting skin conditions (https://www.atsdr.cdc.gov/toxprofiles/tp185.pdf; https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+hsdb:@term+@DOCNO+1582). To date, there exists a paucity in the literature concerning the cellular and molecular effects of DEET, and the potential toxic effects of the repellent on human red blood cell (RBC; erythrocyte) lifespan have not yet been reported. Responsible for gas exchange and transfer of immune complexes, RBCs have a lifespan of 120 days, after which they are removed from the circulation by the monocyte-macrophage system (Lang and Lang, 2015b). Although they lack intracellular organelles, most notably the nucleus and mitochondria, RBCs nonetheless undergo a specific form of programmed cell death known as eryptosis. Characteristics of eryptotic cells include membrane blebbing and phospholipid scrambling, cell shrinkage, intracellular accumulation of calcium and reactive oxygen species (ROS), ceramide formation, and energy exhaustion (Lang *et al.*, 2012). As in apoptosis of nucleated cells, multiple signaling mediators similarly regulate eryptosis. Although still in their infancy, discovery efforts have discerned the existence of caspases, p38 mitogen-activated protein kinase (MAPK), casein kinase 1 (CK1), protein kinase C (PKC), receptor-interacting protein 1 (RIP1), mixed lineage kinase domain-like protein (MLKL), Janus kinase 3 (JAK3), 5' AMP-activated protein kinase (AMPK), and cGMP-dependent protein kinase (cGKI), (Lang *et al.*, 2012; LaRocca *et al.*, 2014; Lang and Lang, 2015b).

Various xenobiotics and pathological conditions have been identified as modulators of eryptosis (Pretorius *et al.*, 2016) and the effect of DEET on RBC lifespan remains an uncharted territory. In this report, we sought to investigate the interaction between DEET and human RBCs, which represent an excellent model to assess the toxicity of xenobiotics

(Hinderling, 1997; Schrijvers, 2003; Farag and Alagawany, 2018). Here, we show that DEET causes premature RBC death characterized by loss of membrane asymmetry and phosphatidylserine (PS) externalization, profound intracellular calcium accumulation secondary to dysregulated influx, and cell shrinkage. Interestingly, the eryptotic effects of DEET were not mediated through oxidative stress, did not depend on calcium availability, and are not significantly ameliorated by blocking of major cell death signaling pathways.

MATERIALS AND METHODS

Erythrocytes, chemicals, and solutions

Fresh, citrate-phosphate-dextrose-adenine (CPDA-1)-anticoagulated RBC samples from consented, healthy adults were obtained from ZenBio (Research Triangle Park, NC, USA). Samples were stored at 4^0C according to standard blood banking procedures (Fernandes da Cunha *et al.,* 2005) and used within 20 days of collection. Cell viability and validity for experimentation was verified by low ($\leq$5%) Annexin-V-FITC binding (Lupescu *et al.*, 2014). Prior to experiments, RBCs were washed in phosphate-buffered saline (PBS; 0.9% NaCl, 1 mM KH_2PO_4, 5.6mM Na_2HPO_4; pH 7.4) at 2500 rpm for 15 min at 21°C, and were then incubated at 5% hematocrit in Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4. For Ca^{2+}-free Ringer solution, $CaCl_2$ was replaced with 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) (Chem-Impex Intl., Wood Dale, IL, USA).

Preceded DEET treatment by 30 min was the use of 50 µM BAPTA-AM (cell-permeable Ca^{2+} chelator), 2 µM of PKC inhibitor, Staurosporin (StSp) (Cayman Chemical

Company, Ann Arbor, MI, USA), 100 μM of CK1α inhibitor, D4476 (Cayman), 50 μM of p38 MAPK inhibitor, SB203580 (Selleckchem, Houston, TX, USA), 100 μM of pan-caspase inhibitor, Z-VAD(OMe)-FMK (Cayman), 100 μM RIP1 inhibitor, necrostatin-1 (Nec-1), or 1 μM of mixed lineage kinase domain-like pseudokinase (MLKL) inhibitor, necrosulfonamide (NSA). All inhibitors were dissolved in dimethyl sulfoxide (DMSO). Where indicated, cells were co-treated with DEET and 1 mM of N-acetylcysteine (NAC).

All chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. An ethanolic stock solution of DEET was prepared at 1 M (20% DEET) and diluted to desired concentrations (1-5 mM; 0.02-0.1%) in Ringer solution. This concentration range reflects DEET blood levels encountered in cases of accidental and intentional intoxication in humans (Tenenbein, 1987; Wiles *et al.*, 2014); Handbook of Pesticide Toxicology; https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+hsdb:@term+@DOCNO+1582). Moreover, DEET at this range has previously been shown to inhibit erythrocyte cholinesterase activity *in vitro* (Wille *et al.*, 2011).

Hemolysis

RBCs (5% hematocrit) were treated with 1-5 mM of DEET in Ringer solution at 37°C for 6 h, and the degree of hemoglobin leakage was determined spectrophotometrically using VersaMax™ ELISA microplate reader (Molecular Devices, San Jose, CA, USA). Following DEET treatment, cells were pelleted by centrifugation at 13,300 × *g* for 1 min. The resulting supernatant was assayed for hemoglobin content by measuring light absorbance at 405 nm. Results are presented as percent hemolysis compared to cells suspended in distilled water (100% hemolysis).

PS externalization and forward scatter (FSC)

Annexin-V-FITC binding was utilized as a measure of exposed PS. Briefly, following DEET treatment, the cells were washed in 5 mM $CaCl_2$ Ringer solution, and a homogeneous 50-µl cell suspension was stained with 150 µl of 1% solution of Annexin-V-FITC (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min away from light. FITC fluorescence and FSC were subsequently obtained on a FACScan (Betcon Dickinson, Franklin Lakes, NJ, USA) at excitation/emission wavelengths of 488/530.

Determination of intracellular calcium

Calcium was determined by Fluo3/AM cleavage (Biotium, Fremont, CA, USA). Upon intracellular hydrolysis of Fluo3/AM by esterases, Fluo3 fluoresces relative to Ca^{2+} binding. To determine cytosolic Ca^{2+} content, 50 µl of control and DEET-treated cells were washed and resuspended in 5 mM $CaCl_2$ Ringer buffer, and then incubated in total darkness with 5 µM Fluo3/AM at 37°C for 30 min in a final volume of 200 µl. Fluorescence was then determined on a FACScan at excitation and emission wavelengths of 488 and 530 nm, respectively.

Measurement of ROS generation

ROS accumulation was measured by staining the cells with 2-,7-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA) (Thermo Fisher Scientific, Waltham, MA, USA). $DCFH_2$-DA is non-fluorescent until cleaved by esterases and subsequently oxidized by ROS. A 50-µl homogeneous cell suspension was washed in Ringer buffer, loaded with 10 µM $DCFH_2$-DA in a final volume of 200 µl, and incubated for 30 min at 37°C in the dark. DCF fluorescence was then quantified by FACScan as a measure of ROS formation (Ex = 488, Em = 530).

Statistical analysis

Data are represented as arithmetic means ± S.E.M. of triplicate measurements of three independent experiments conducted on RBC samples from different donors. Prism 5 GraphPad was used for statistical analysis using Student's *t*-test to compare two groups, and one-way ANOVA followed by Dunnett's post-hoc test to compare treatment groups with the vehicle control. To account for individual variation, comparisons between untreated and treated groups were only carried out on samples from the same donor.

RESULTS

DEET induces weak hemolysis in human RBCs

Cell-free hemoglobin is a marker of hemolysis. To determine the hemolytic potential of DEET, RBCs were treated with 1-5 mM of DEET for 6 h at 37°C, and hemoglobin leakage into the medium was subsequently assayed. Fig. 1B shows that DEET causes a modest, yet statistically significant increase in hemolysis at both 4 and 5 mM. This indicates that the cell membrane could be a major action site targeted by the repellent.

DEET causes PS externalization

Membrane phospholipid scrambling, the principal feature of eryptotic cells, was identified by binding of externalized phosphatidylserine to Annexin-V. To identify PS-exposing cells, RBCs were incubated in presence and absence of 1-5 mM DEET for 6 h at 37°C, and the percentage of Annexin-V-binding cells was determined by flow cytometry. As shown in Fig. 2, DEET causes a statistically significant increase in Annexin binding,

reflective of PS exposure. Thus, DEET stimulated eryptotic transformation of RBCs.

DEET reduces erythrocyte cell volume

Cell size was determined by FSC. Fig. 3 shows light scatter properties of vehicle-only and DEET-treated cells. A significant reduction in FSC was observed in cells exposed to 5 mM DEET compared to the vehicle control (Fig. 3A,B). DEET also caused an increasing trend in the percentage of cells with reduced FSC (Fig. 3C), and a significant decrease in the percentage of cells with an enlarged volume (Fig. 3D). Taken together, our data show that DEET-induced PS exposure is accompanied by cell shrinkage – a prominent feature of eryptotic RBCs.

DEET leads to elevations in intracellular Ca^{2+}

Dysregulated Ca^{2+} homeostasis is a key event during eryptosis. To investigate perturbations in Ca^{2+} homeostasis caused by DEET, erythrocytes were incubated with or without 1-5 mM DEET for 6 h at 37°C, and mean Fluo3 fluorescence intensity was measured as an indicator of intracellular Ca^{2+}. Fig. 4 reveals that a statistically significant elevation in intracellular Ca^{2+} was only observed at 5 mM DEET. This is consistent with the PS-exposing (Fig. 2B,C) and volume-reducing (Fig. 3A,B) effects observed at the same concentration. Hence, DEET induces PS externalization with accompanying cell shrinkage and intracellular Ca^{2+} accumulation.

Next, to probe whether the observed increase in Ca^{2+} is secondary to extracellular Ca^{2+} influx, cells were incubated for 6 h with either the vehicle or 5 mM DEET in presence or nominal absence of Ca^{2+}, and Fluo3 fluorescence was then measured. While DEET increased Ca^{2+} levels when extracellular Ca^{2+} was available (Fig. 5A), the increase was completely abolished when Ca^{2+} was removed from the medium (Fig. 5B). This suggests

that Ca^{2+} influx accounted for DEET-induced cytosolic Ca^{2+} accumulation.

Finally, to determine the importance of Ca^{2+} availability for the PS-exposing effect of DEET, cells were incubated for 6 h with the vehicle or 5 mM DEET in presence and absence of extracellular Ca^{2+}, or were pretreated for 30 min with 50 μM of intracellular Ca^{2+} chelator BAPTA-AM before DEET exposure. The results shown in Fig. 6 indicate that DEET-induced PS exposure is not significantly rescinded neither by removal of extracellular Ca^{2+} (Fig. 6A-C) nor by chelation of cytosolic Ca^{2+} (Fig. 6D-F). Therefore, PS exposure by DEET apparently does not require Ca^{2+}, an observation possibly pointing at other additional mechanisms.

DEET does not increase ROS generation

The buildup of ROS aggravates eryptosis. As a measure of ROS levels, DCF fluorescence was determined after 6 h treatment of RBCs to 1-5 mM DEET. Fig. 7A,B shows that no significant increase in DCF fluorescence was observed between vehicle and 5 mM-treated cells. As supportive evidence, the eryptotic activity of DEET was also examined in presence and absence of 1 mM of free radical scavenger NAC. As can be seen in Fig. 7C-E, DEET-induced PS exposure was not reversed by cotreatment with NAC. These findings suggest that DEET-induced eryptosis is not mediated through oxidative stress.

DEET-induced eryptosis is not dependent on a specific signaling pathway

The role of signaling mediators is fundamental to various cellular functions and responses, most importantly cell survival. To dissect the involvement of signaling enzymes in DEET-induced PS exposure, cells were pretreated for 30 min with specific inhibitors and then exposed to 5 mM DEET for 6 h at 37°C. As seen in Fig. 8, DEET-induced PS

externalization is not significantly reduced in presence of respective inhibitors of caspases (100 µM Z-VAD(OMe)-FMK), p38 MAPK (50 µM SB203580), PKC (2 µM StSp), or CK1 (100 µM D4476). Furthermore, blockade of necroptosis mediators, RIP1 (100 µM Nec-1) and MLKL (1 µM NSA), have similarly failed to protect RBCs from DEET-induced cell death (Fig. 9). Collectively, our data indicate that DEET-induced suicidal RBC death does not require the activity of a specific cell death pathway.

DISCUSSION

DEET is considered the gold standard of insect repellents, and formulations containing DEET are more effective than those without it (Tintinalli and Stapczynski, 2011). Environmental and public health agencies encourage DEET use, and more consumption is indeed on the horizon (Chen-Hussey *et al.*, 2014). Although no adverse effects on RBCs following DEET exposure have been observed in humans, reduced hemoglobin and hematocrit found in small-scale animal studies were either unreliable or not associated with DEET treatment (https://www.atsdr.cdc.gov/toxprofiles/tp185.pdf; https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+hsdb:@term+@DOCNO+1582). In particular, DEET exposure was reported to increase RBC count in *Cyprinus carpio* fish (Slaninova *et al.*, 2014). Nevertheless, our data show that DEET reduces human RBC lifespan by stimulating premature eryptosis, characterized by PS externalization, Ca^{2+} influx, and cell shrinkage. It must be stressed, however, that these observations were only evident at DEET concentrations that parallel those detected in the blood of individuals following accidental and suicidal exposure. Use of DEET at the recommended doses is, therefore, unlikely to be detrimental to erythrocyte health.

Although minimal, the statistically significant increase in hemolysis brought about by DEET suggests that the repellent is capable of inflicting direct membrane damage and eventual cell destruction. This hemolytic effect could be augmented in cases of enhanced RBC vulnerability such as diabetes, cancer, and hemoglobinopathies (Rytting *et al.*,1996; James and Meyers, 1998; Kato *et al.*, 2017). Hemolysis leads to a spill out of cellular contents, most notably hemoglobin, which participates in both acute and chronic injuries to vital organs. Nitric oxide depletion and oxidative damage, hypertension, and vascular occlusion are consequences of circulating hemoglobin (Doherty *et al.*, 1998). Prolonged presence of hemoglobin in the circulation eventually leads to endothelial dysfunction and atherosclerosis (Minneci *et al.*, 2005; Nagy *et al.*, 2010). When hemoglobin escapes the circulation, impaired hepatic iron metabolism, tubular injury and renal failure, and immune dysfunction may follow (Schaer *et al.*, 2013).

The central finding in this report is the ability of DEET to cause loss of the RBC membrane asymmetry culminating in PS externalization. The presence of PS on the outer membrane leaflet represents a binding site for receptors on phagocytes for elimination of defective RBCs (Niemoeller *et al.*, 2006). Therefore, physiologic eryptosis may be perceived as an insurance policy safeguarding against hemolysis. However, excessive and premature RBC death, as observed in the case of DEET, may outweigh their rate of clearance, thereby leading to hemolysis and compromised tissue oxygenation. Similarly, inordinate RBC death may outpace erythropoiesis in the bone marrow eventually causing anemia (Lang *et al.*, 2008).

Recent efforts also recognize the effects of eryptosis on hemorheology. For RBCs to efficiently carry out oxygen transport, they must withstand mechanical forces in the

circulation, which requires extreme structural flexibility. This is mediated by remarkable deformability and aggregability of healthy erythrocytes which is significantly compromised in eryptotic corpses (Pretorius, 2018). The alterations associated with eryptosis limit the capacity of the RBC membrane to deform, leading to increased blood flow resistance, hemodynamic stasis, and ultimately reduced tissue perfusion (du Plooy *et al.*, 2018; Pretorius, 2018). Moreover, eryptotic RBCs adhere to transmembrane CXC chemokine ligand 16 (CXCL16) present on vascular endothelial cells (Borst *et al.*, 2012), which have been shown to be susceptible to the proangiogenic activity of DEET (Legeay *et al.*, 2016). Thus, eryptosis may have ramifications beyond the development of anemia, which include vaso-occlusive thromboembolic lesions and ischemic injury. At the molecular level, DEET has been found to cause DNA damage in primary human nasal mucosal cells (Tisch *et al.*, 2002), and to modulate transcriptional activities of metabolic, oxidoreductive, and signal transduction genes in primary human hepatocytes (Das *et al.*, 2008; Mitchell *et al.*, 2016).

Parallel to PS exposure, we have also detected significant cell shrinkage – a distinctive feature of eryptotic cells. Diminished cell size is thought to be a consequence of Ca^{2+} channel opening and the ion's intracellular accumulation, an event also detected in our study (Fig. 4 & 5). The buildup of Ca^{2+} leads to opening of Ca^{2+}-sensitive K^+ channels, membrane hyperpolarization due to KCl efflux, water loss by osmotic forces, and cell shrinkage (Lang and Lang, 2015a). Presumably, smaller, eryptotic cells may facilitate engulfment and degradation by larger phagocytes thus expediting their clearance from the circulation.

Calcium signaling is a major orchestrator of programmed cell death. Scramblases are

Ca^{2+}-sensitive enzymes responsible for the bidirectional movement of phospholipids within the lipid bilayer. Accumulation of Ca^{2+} therefore impinges on scramblase regulation and leads to loss of membrane asymmetry (Weiss *et al.*, 2012). Our experiments revealed an increase in Ca^{2+} as part of DEET-mediated eryptosis (Fig. 4), that was chiefly through influx from the medium (Fig. 5). Based on the dispensability of the ion to DEET-induced PS externalization (Fig. 6), other possible mechanisms leading up to membrane scrambling cannot be excluded. In this regard, DEET resembles the eryptotic behavior of caspofungin (Peter *et al.*, 2016a), micafungin (Peter *et al.*, 2016c), and exemestane (Al Mamun Bhuyan *et al.*, 2017a).

Oxidative stress is among the major manifestations of eryptotic cells, and a number of life-threatening conditions lie at the intersection of oxidative stress and enhanced eryptosis, most notably diabetes, hepatic failure, and malignancy (Bissinger *et al.*, 2018). It has previously been demonstrated that ROS accumulation stimulates the activity of Ca^{2+}-permeable cation channels and thus promotes intracellular Ca^{2+} overload with subsequent PS externalization (Lang *et al.*, 2012). Also secondary to oxidative stress is caspase activation (Pretorius *et al.*, 2016). Our data in Fig. 7 indicate that DEET-induced PS exposure occurs independently of oxidative stress as was observed for caspofungin (Peter *et al.*, 2016a), micafungin (Peter *et al.*, 2016c), anidulafungin (Peter *et al.*, 2016b), and sclareol (Signoretto *et al.*, 2016). It is worth mentioning that various endo- and xenobiotics have been shown to possess a protective role against eryptosis in part by counteracting oxidative stress (Lang and Lang, 2015b).

In order to identify the molecular pathways through which DEET reduces the RBC lifespan, we have employed a series of small-molecule inhibitors to scan for signaling

mediators involved in two cell death pathways; eryptosis and necroptosis. Our attempts in this regard were not fruitful as DEET still exposed PS under inhibition of all major cell death pathways (Fig. 8 & 9). It may be, however, that DEET does not require the specific action of a signaling mediator, or that a prominent role of such a player only becomes apparent at a later point during cell death. Another possibility is that DEET may activate multiple pathways such that inhibition of one is still outweighed by others.

In conclusion, this investigation identified DEET as a stimulator of erythrocyte membrane scrambling with concurrent cell shrinkage and elevated intracellular Ca^{2+} mainly through Ca^{2+} entry from the extracellular space. Recent analysis concludes that DEET is both more toxic and less efficacious compared to other repellents including IR 3535, Picaridin, and oil of lemon eucalyptus (p-menthane-3, 8-diol; PMD) (Diaz, 2016; Tavares *et al.*, 2018). In fact, the existence of a DEET-sensitive receptor makes it possible to devise potentially safer alternatives (Leal, 2014). Thus, combined with entomological and epidemiological studies, examination of the biochemical and molecular interactions of current DEET competitors, Picaridin, IR 3535, and essential oils, and new repellents (e.g., ethyl anthranilate (Islam *et al.*, 2017), is particularly warranted.

ACKNOWLEDGMENTS

We thank the members of the Lee laboratory for helpful advice and discussion during this work. This work was supported in part by the Brody Brothers Grant (BBE216102) to M-H.L. and the Saudi Government Graduate Scholarship (through King Saud University) to M.A.A.

COMPETING INTERESTS

The authors declare they have no competing interests relevant to this manuscript.

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FIGURE LEGENDS

Figure 1. DEET induces minor hemolysis. (A) Molecular structure of DEET. **(B)** RBCs were exposed to 1-5 mM DEET for 6 h at 37°C after which the supernatant was spectrophotometrically tested for hemoglobin content at a wavelength of 405 nm. Data are means ± S.E.M. (n = 9). *(P < 0.05) indicates significant difference from vehicle-treated cells (ANOVA).

Figure 2. DEET stimulates PS exposure. (A) Representative histograms showing Annexin-V-FITC fluorescence of RBCs treated with either the vehicle or 1-5 mM DEET for 6 h at 37°C. **(B)** Overlay histogram showing Annexin-V-FITC fluorescence by vehicle and 5 mM DEET-treated RBCs. **(C)** Arithmetic means ± S.E.M. (n = 9) of the percentage of cells bound to Annexin-V following exposure either to the vehicle or to 1-5 mM DEET for 6 h at 37°C. ***(P<0.001) indicates significant difference from the absence of DEET (ANOVA).

Figure 3. DEET reduces forward scatter. (A) Overlay histogram showing FSC of RBCs treated with the vehicle (grey peak) and those with 5 mM DEET (orange peak) for 6 h at 37°C. **(B)** Arithmetic means ± S.E.M. (n = 9) of mean FSC values for cells exposed to the vehicle or to 1-5 mM DEET for 6 h at 37°C. *(P < 0.05) indicates significant difference from vehicle-treated cells (ANOVA). **(C)** Arithmetic means ± S.E.M. (n = 9) of the percentage of cells, exposed to the vehicle or to 1-5 mM DEET for 6 h at 37°C, with mean FSC of <200. **(D)** Arithmetic means ± S.E.M. (n = 9) of the percentage of cells, exposed to the vehicle or to 1-5 mM DEET for 6 h at 37°C, with mean FSC of >400. *(P < 0.05) indicates significant difference from vehicle-treated cells (ANOVA).

Figure 4. DEET increases intracellular Ca^{2+} levels. (A) Representative histograms showing Fluo3 fluorescence of RBCs treated with either the vehicle or 1-5 mM DEET for 6 h at 37°C. **(B)** Overlay histogram showing Flou3 fluorescence by vehicle and 5 mM DEET-treated RBCs. **(C)** Arithmetic means ± S.E.M. (n = 9) of mean Fluo3 fluorescence by RBCs exposed to the vehicle or to 1-5 mM DEET for 6 h at 37°C. ***(P<0.001) indicates significant difference from the absence of DEET (ANOVA).

Figure 5. Effect of extracellular Ca^{2+} removal on cytosolic Ca^{2+} levels. (A) Overlay histogram showing Fluo3 fluorescence of RBCs exposed to the vehicle only (black line) or to 5 mM DEET (turquoise line) in Ringer solution for 6 h at 37°C. **(B)** Overlay histogram showing Fluo3 fluorescence of RBCs exposed to the vehicle only (black line) or to 5 mM DEET (green line) in Ca^{2+}-free Ringer solution for 6 h at 37°C. **(C)** Arithmetic means ± S.E.M. (n = 9) of mean Fluo3 fluorescence by RBCs exposed to the vehicle or to 1-5 mM DEET for 6 h at 37°C in presence and absence of extracellular Ca^{2+}. ***(P <0.001) indicates significant difference from the absence of DEET. ##(P <0.01) indicates significant difference from the corresponding treatment condition in presence of Ca^{2+} (ANOVA).

Figure 6. Effect of Ca^{2+} removal on PS externalization. (A) Overlay histogram showing Annexin-V-FITC fluorescence of RBCs exposed to the vehicle (black line) only or to 5 mM DEET (purple line) in Ringer solution for 6 h at 37°C. **(B)** Overlay histogram showing Annexin-V-FITC fluorescence of RBCs exposed to the vehicle only (black line) or to 5 mM DEET (pink line) in Ca^{2+}-free Ringer solution for 6 h at 37°C. **(C)** Arithmetic means ± S.E.M. (n = 9) of the percentage of cells bound to Annexin-V following treatment with the vehicle or with 1-5 mM DEET for 6 h at 37°C in presence and absence of extracellular

Ca^{2+}. ***(P <0.001) indicates significant difference from the absence of DEET (ANOVA). **(D)** Overlay histogram showing Annexin-V-FITC fluorescence of RBCs exposed to the vehicle only (black line) or to 5 mM DEET (blue line) for 6 h at 37°C. **(E)** Overlay histogram showing Annexin-V-FITC fluorescence of RBCs pretreated with 50 µM BAPTA-AM for 30 min at 37^0C and then exposed to the vehicle only (black line) or to 5 mM DEET (rose line) for 6 h at 37°C. **(F)** Arithmetic means ± S.E.M. (n = 9) of the percentage of cells bound to Annexin-V following treatment with the vehicle or with 1-5 mM DEET for 6 h at 37°C without and with BAPTA-AM pretreatment. ***(P <0.001) indicates significant difference from the absence of DEET (ANOVA).

Figure 7. Lack of oxidative stress in DEET-induced PS externalization. (A) Overlay histogram showing DCF fluorescence of RBCs exposed to the vehicle only (black line) or to 5 mM DEET (green line) in Ringer solution for 6 h at 37°C. **(B)** Arithmetic means ± S.E.M. (n = 9) of DCF fluorescence following treatment with the vehicle or with 5 mM DEET for 6 h at 37°C. ns (P >0.05) indicates insignificant difference from the absence of DEET (t-test). **(C)** Overlay histogram showing Annexin-V-FITC fluorescence of RBCs exposed to the vehicle only (black line) or to 5 mM DEET (red line) for 6 h at 37°C. **(D)** Overlay histogram showing Annexin-V-FITC fluorescence of RBCs exposed to the vehicle only (black line) or to a combination of 1 mM NAC and 5 mM DEET (turquoise line) for 6 h at 37°C. **(E)** Arithmetic means ± S.E.M. (n = 9) of the percentage of cells bound to Annexin-V following treatment with the vehicle, NAC, 5 mM DEET, or following cotreatment with NAC and 5 mM DEET for 6 h at 37°C. ***(P <0.001) indicates significant difference from the absence of DEET (ANOVA).

Figure 8. Effect of eryptosis inhibitors on DEET-induced PS exposure. (A-E) Overlay histograms showing Annexin-V fluorescence of RBCs exposed for 6 h at 37°C to the vehicle only (black line) or to 5 mM DEET without **(**red line, **A)** or with pretreatment for 30 min with 100 µM Z-VAD(OMe)-FMK **(**purple line, **B)**, 2 µM StSp **(**orange line, **C)**, 100 µM D4476 **(**blue line, **D)**, or 50 µM SB **(**green line, **E)**. **(F)** Arithmetic means ± S.E.M. (*n* = 9) of the percentage of cells bound to Annexin-V following treatment for 6 h at 37°C with the vehicle or 5 mM DEET with and without pretreatment with Z-VAD(OMe)-FMK, StSp, D4476, or SB. ***(*P* <0.001) indicates significant difference from the absence of DEET (ANOVA).

Figure 9. Effect of necroptosis inhibitors on DEET-induced PS exposure. (A-C) Overlay histograms showing Annexin-V fluorescence of RBCs exposed for 6 h at 37°C to the vehicle only (black line) or to 5 mM DEET without **(**blue line, **A)** or with pretreatment for 30 min with 100 µM Nec-1 **(**rose line, **B)** or 1 µM NSA **(**pink line, **C). (D)** Arithmetic means ± S.E.M. (*n* = 9) of the percentage of cells bound to Annexin-V following treatment for 6 h at 37°C with the vehicle or 5 mM DEET with and without pretreatment with either Nec-1 or NSA. ns (*P* >0.05) indicates insignificant difference. ***(*P* <0.001) indicates significant difference from the absence of DEET (ANOVA).

FIGURES

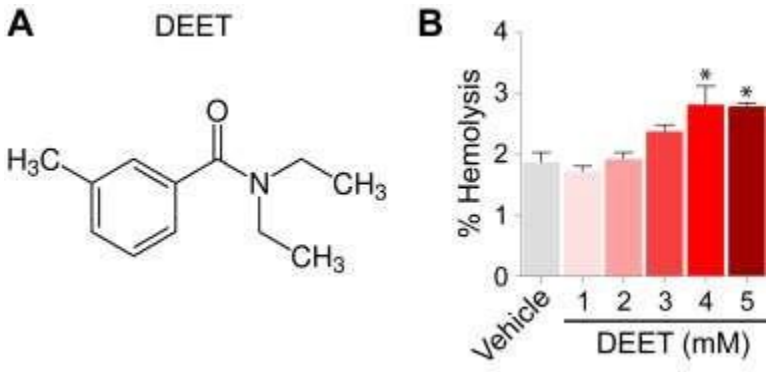

Figure 1

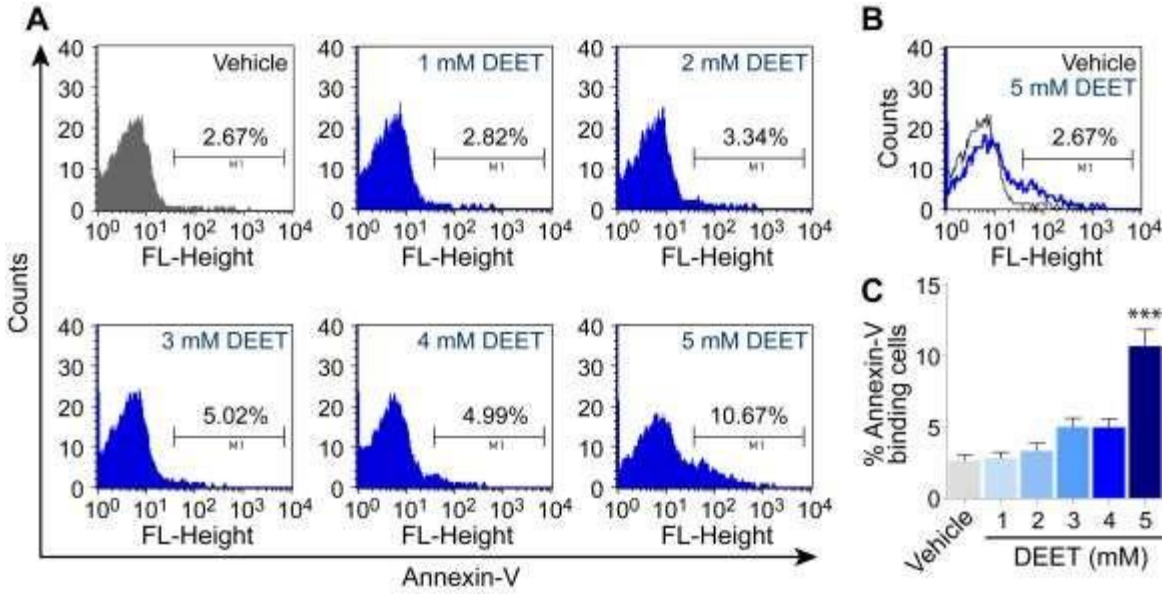
A
Vehicle
2.67%
1 mM DEET
2.82%
2 mM DEET
3.34%
3 mM DEET
5.02%
4 mM DEET
4.99%
5 mM DEET
10.67%
FL-Height
Counts
Annexin-V
B
Vehicle
5 mM DEET
2.67%
Counts
FL-Height
C
% Annexin-V binding cells
***
Vehicle
DEET (mM)

Figure 2

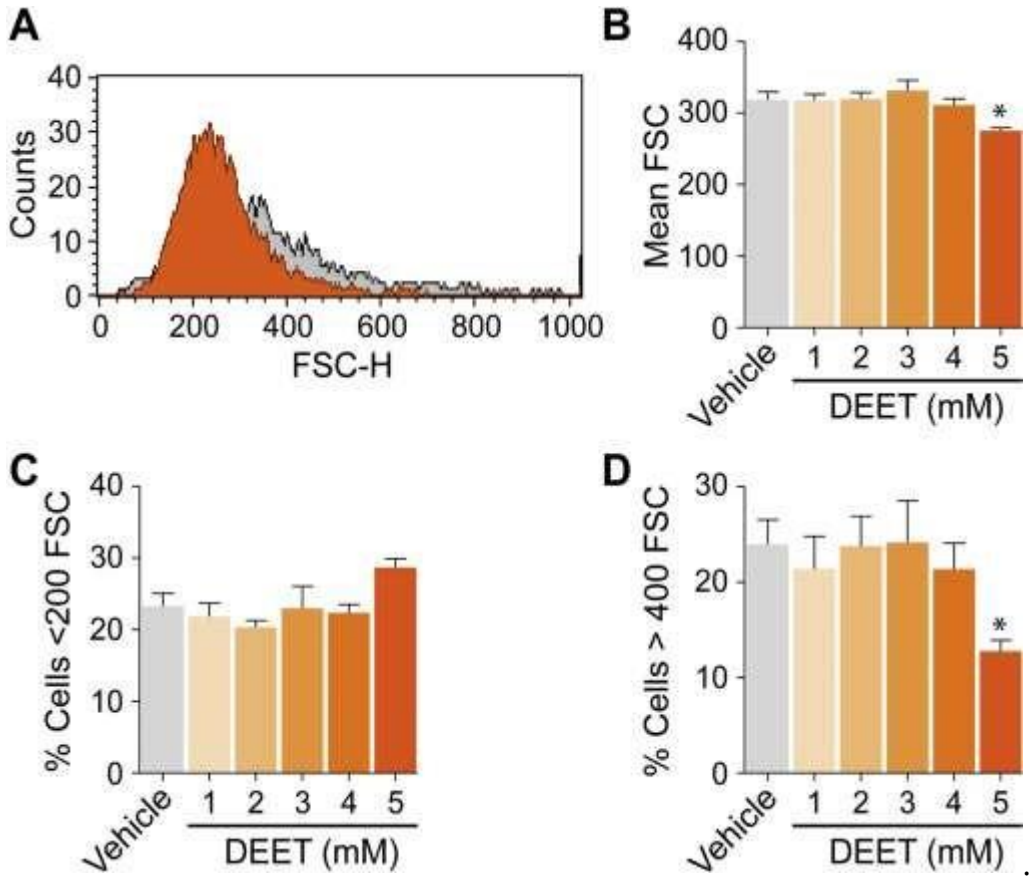
A
Counts
FSC-H
B
Mean FSC
Vehicle
DEET (mM)
C
% Cells <200 FSC
Vehicle
DEET (mM)
D
% Cells > 400 FSC
Vehicle
DEET (mM)

Figure 3

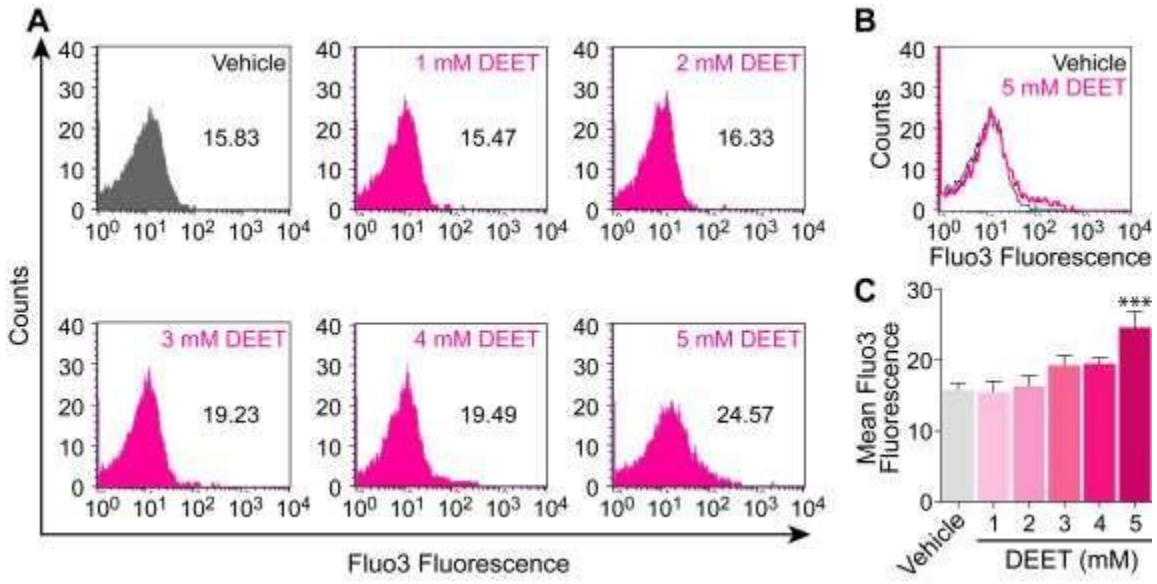
A
Vehicle
15.83
1 mM DEET
15.47
2 mM DEET
16.33
3 mM DEET
19.23
4 mM DEET
19.49
5 mM DEET
24.57
Counts
Fluo3 Fluorescence
B
Vehicle
5 mM DEET
Counts
Fluo3 Fluorescence
C
Mean Fluo3 Fluorescence
***
Vehicle
1 2 3 4 5
DEET (mM)

Figure 4

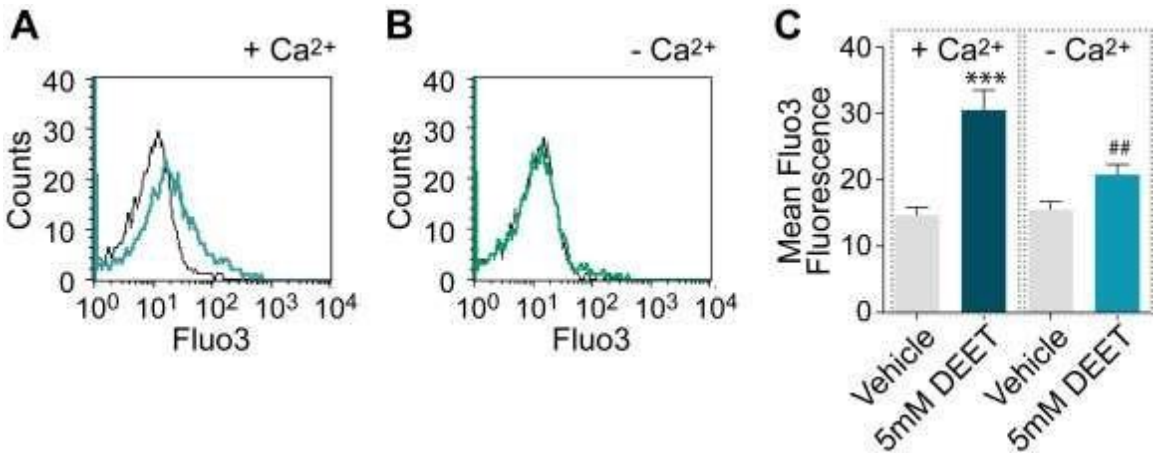
A
+ Ca2+
Counts
40
30
20
10
0
10^0 10^1 10^2 10^3 10^4
Fluo3
B
- Ca2+
Counts
40
30
20
10
0
10^0 10^1 10^2 10^3 10^4
Fluo3
C
+ Ca2+
- Ca2+
***
##
Mean Fluo3 Fluorescence
40
30
20
10
0
Vehicle
5mM DEET
Vehicle
5mM DEET

Figure 5

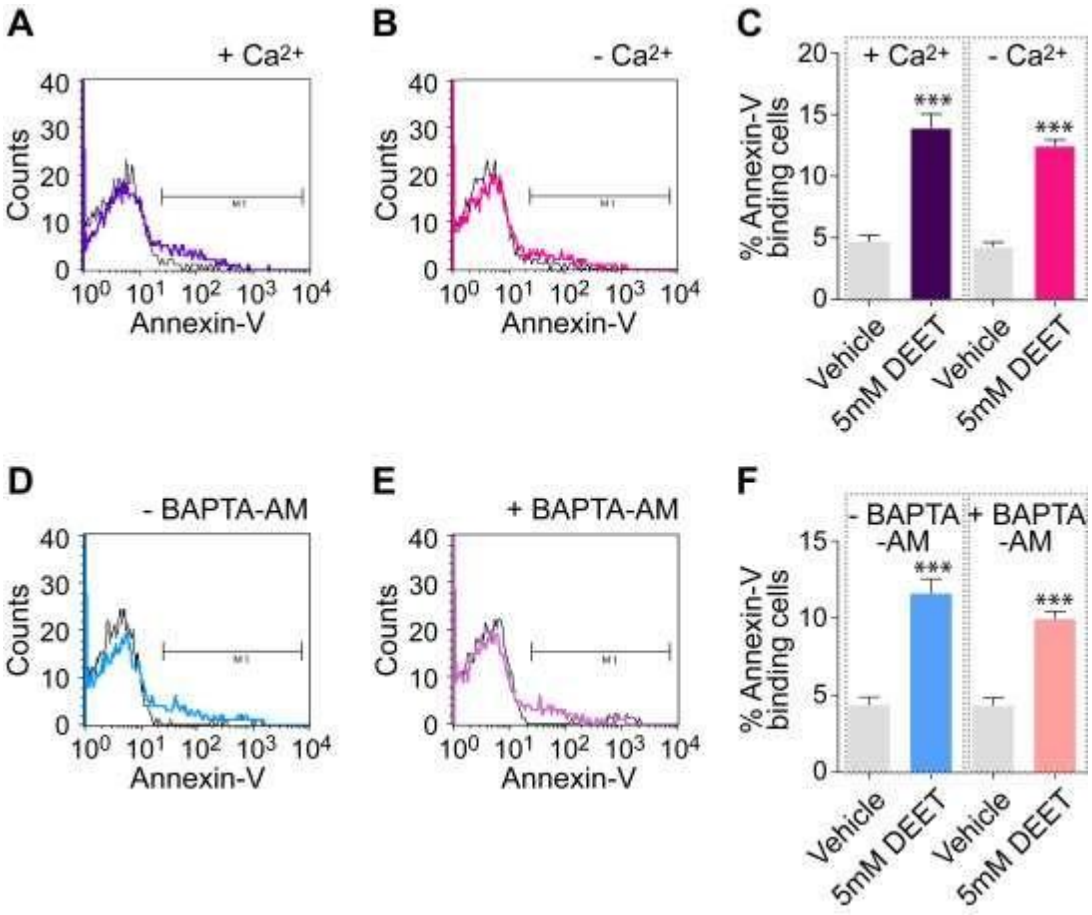
A
+ Ca2+
Counts
Annexin-V
B
- Ca2+
C
+ Ca2+
- Ca2+
% Annexin-V binding cells
***
Vehicle
5mM DEET
D
- BAPTA-AM
E
+ BAPTA-AM
F
- BAPTA -AM
+ BAPTA -AM

Figure 6

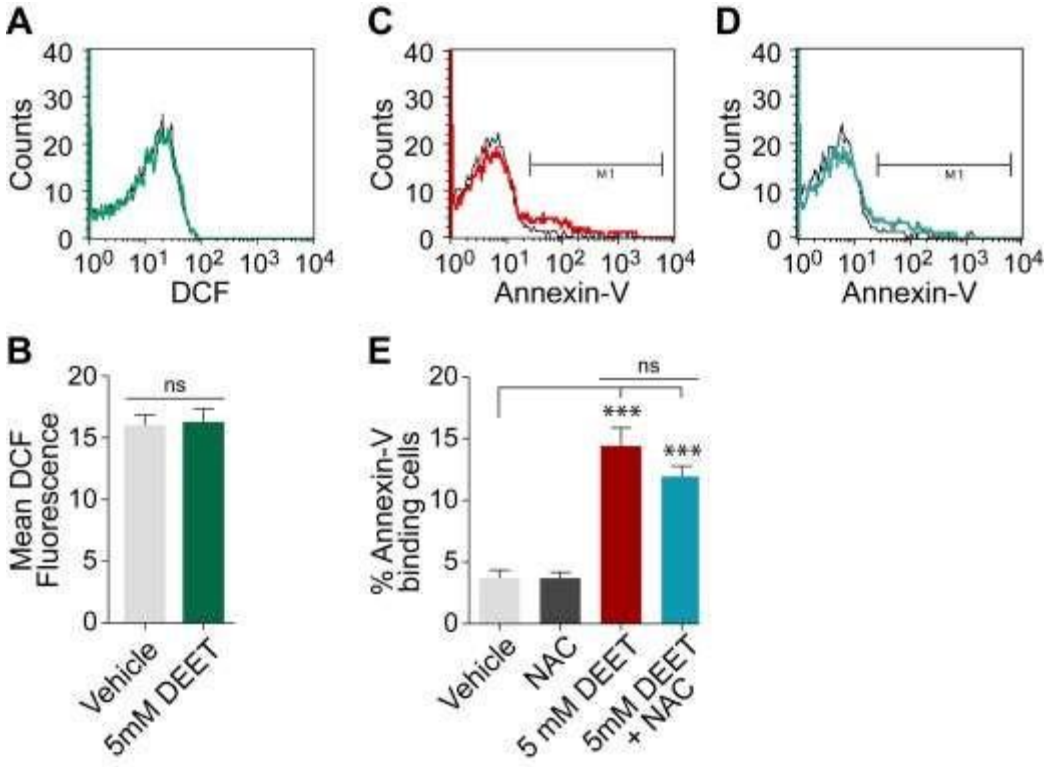
A
Counts
DCF
C
Annexin-V
M1
D
Annexin-V
M1
B
Mean DCF Fluorescence
ns
Vehicle
5mM DEET
E
% Annexin-V binding cells
ns
***
***
Vehicle
NAC
5 mM DEET
5mM DEET + NAC

Figure 7

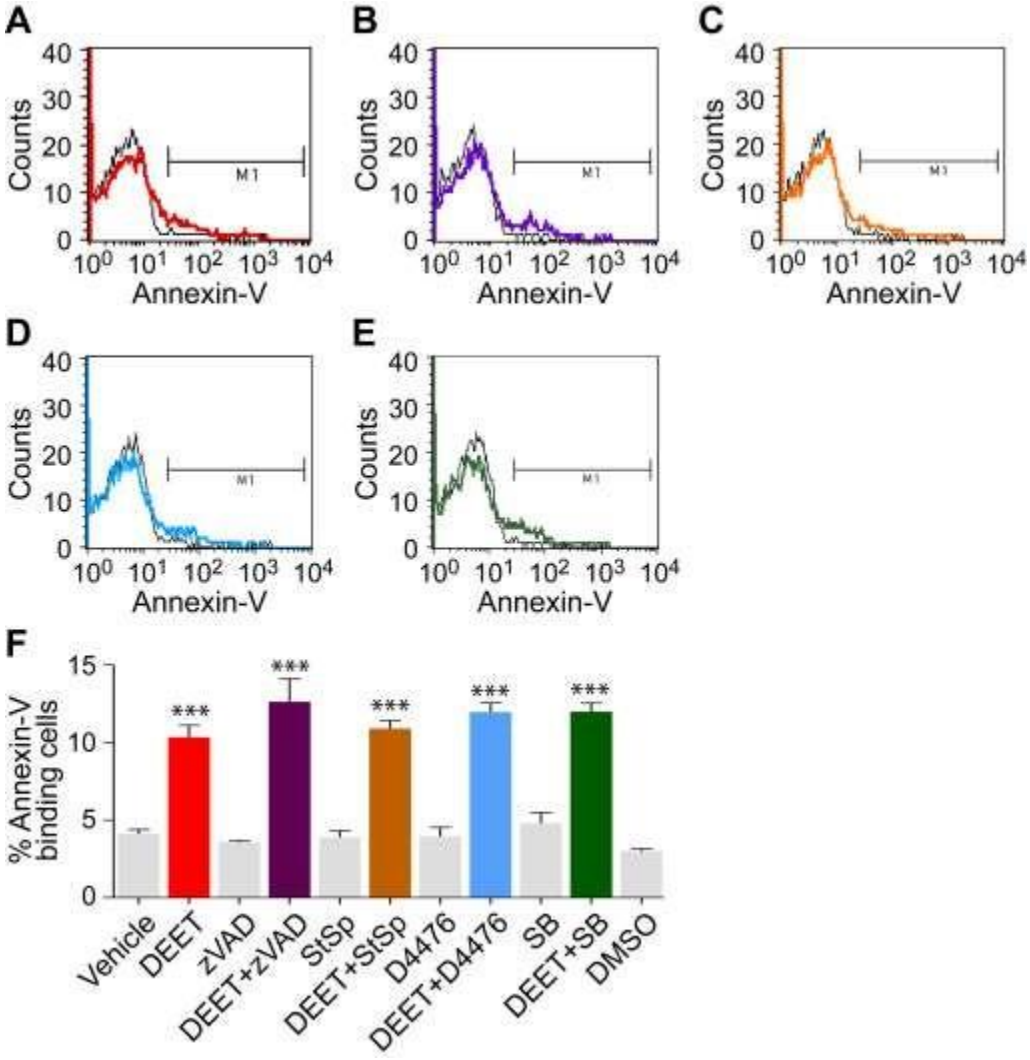
A
B
C
D
E
F
Counts
Annexin-V
M1
% Annexin-V binding cells
***
Vehicle
DEET
zVAD
DEET+zVAD
StSp
DEET+StSp
D4476
DEET+D4476
SB
DEET+SB
DMSO

Figure 8

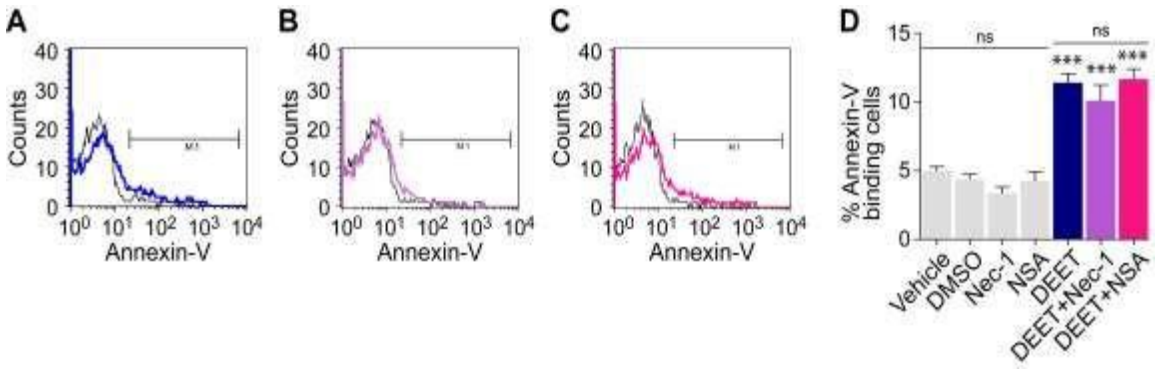
A
B
C
D
Counts
Annexin-V
% Annexin-V binding cells
ns
***
Vehicle
DMSO
Nec-1
NSA
DEET
DEET+Nec-1
DEET+NSA

Figure 9

CHAPTER VI: SUMMARY

The studies presented herein collectively support the following conclusions: (1) TCS is an effective antileukemic agent, evident by its pro-apoptotic properties demonstrated against BL cells *in vitro*; (2) TCS has a potent hemolytic and eryptotic potential against human RBCs; and (3) TCS is susceptible to inactivation by noncharged detergents often present in drug formulae as excipients.

In chapter II, TCS was demonstrated to induce apoptosis in blood cancer cells, which is a very common action mechanism of chemotherapeutic drugs. At the molecular level, TCS disrupts the cell membrane permeability, leads to cytosolic Ca^{2+} overload, which in turn causes loss of membrane asymmetry, activates caspase, and induces systematic DNA fragmentation. In parallel, TCS interferes with antioxidant enzyme expression leading to excessive ROS accumulation and oxidative stress. This redox imbalance similarly stimulates caspase activity and JNK signaling; two pathways that were shown to be essential for TCS-induced apoptosis in Burkitt lymphoma cells. Of particular interest is the ability of TCS to favorably modulate the transcriptional regulation of survival genes (e.g., *BCL2*) that are common pharmaceutical targets in existing chemotherapies.

Our study on TCS influence on RBC survival identified the antimicrobial as both pro-hemolytic and pro-eryptotic. Mechanistically, TCS was found to inflict direct membrane damage resulting in profound hemolysis, and cause a breakdown of membrane asymmetry culminating in phosphatidylserine externalization. As is the case with lymphoma cells, TCS increased cytoplasmic Ca^{2+} levels, but, conversely, was not pro-oxidative in erythrocytes. Employing a small-molecule inhibitor screening approach, we

were able to discern the indispensable roles of p38 MAPK and RIP1 as essential requirements for TCS-induced eryptosis. These findings suggest that caution with TCS use must be exercised given its potential contribution to the development of chemotherapy-induced anemia in cancer patients. Chapter IV unequivocally showed that nonionic detergents act as antagonists of TCS *in vivo* most likely through micellar solubilization. This is significant as noncharged detergents are incorporated as excipients in drug formulations which must be taken into consideration for drug development.

Building on present observations, future efforts must be directed toward identifying whether or not TCS effects described herein and elsewhere are reciprocated in highly relevant animal models. Equally important is the identification of molecular and cellular mechanisms modulated by TCS in human-based systems (reviewed in chapter I). The advent of nanotechnology and monoclonal antibody-based therapies, along with the discovery of miRNA regulators similarly paves the way to numerous opportunities to further exploit TCS for therapeutic or prophylactic purposes.