Molecular mechanisms underlying the renoprotective effects of 1,4,7-triazacyclononane: a β -lactamase inhibitor

Nrateng Tsotetsi · Daniel G. Amoako · Anou M. Somboro · Hezekiel M. Khumalo · Rene B. Khan

Received: 10 May 2020 / Accepted: 7 September 2020 / Published online: 12 September 2020
© Springer Nature B.V. 2020

Abstract Broad-spectrum β -lactam antibiotics such as penicillin are routinely used against both Gram-negative and Gram-positive bacteria. However, bacteria that produce β -lactamase have developed resistance against these antibiotics by cleaving the β -lactam ring and rendering the antibiotic inactive. To combat this effect, 1,4,7-Triazacyclononane (TACN), a cyclic organic compound derived from cyclononanes has been shown to preserve the activity of β -lactam antibiotics by inhibiting β -lactamase. However, its cytotoxic effects require elucidation. Given that the cytotoxic target for many therapeutics is the kidney, this study investigated the effects of TACN on human embryonic kidney cells (Hek293) cells. Hek293 cells were treated with TACN (0–500 μ M) for 24 h and the cytotoxicity was assessed (MTT and LDH assay). Apoptosis was luminometrically detected by measuring phosphatidylserine externalisation and caspase activity and fluorescently detecting necrosis. DNA fragmentation was visualised using

fluorescent microscopy. Expression of the apoptosis-related protein were determined by western blot. The results generated indicate that TACN does not initiate necrosis as LDH was decreased. Likewise, decreased apoptosis was supported by the decreased phosphatidylserine, caspases, Bax, cleaved PARP, IAP and NF- κ B. However, increased DNA fragmentation was associated with increased p53. Therefore, effects of TACN at the nucleus, produced a p53 response to initiate DNA repair and did not culminate in cell death. The findings show that TACN is not cytotoxic to Hek293 cells via the apoptotic route. Since TACN did not induce cell death, its potential as a metallo- β -lactamase inhibitor (MBLI) may be exploited to counteract the effect of MBL-producing bacteria. Restoring β -lactam activity will curb the global menace of antibiotic resistance.

Keywords 1,4,7-Triazacyclononane (TACN) · Human embryonic kidney (Hek293) cells · Apoptosis · Metallo- β -lactamase inhibitor (MBLI) · Antibiotic resistance · Cytotoxicity

N. Tsotetsi · H. M. Khumalo · R. B. Khan (✉)
Discipline of Medical Biochemistry, School of Laboratory
Medicine and Medical Science, University of KwaZulu-
Natal, Durban, South Africa
e-mail: myburg@ukzn.ac.za

D. G. Amoako (✉) · A. M. Somboro
Biomedical Resource Unit, School of Laboratory
Medicine and Medical Sciences, College of Health
Sciences, University of KwaZulu-Natal, Durban, South
Africa
e-mail: amoakodg@gmail.com

Introduction

Antibiotic-resistant bacteria are becoming increasingly common and are causing a global health crisis (Blair et al. 2015; Agyepong et al. 2019; Ramsamy et al. 2020a, b). Research has therefore intensified in

an effort to find novel compounds to counteract the effect of MBL-producing bacteria by restoring β -lactam activity (Ramchuran et al. 2018; Somboro et al. 2018; Sosibo et al. 2019). Metallo- β -lactamase (MBL) is a bacterial enzyme that cleaves the β -lactam ring of broad-spectrum antibiotics such as penicillin, rendering the antibiotic inactive (Rotondo and Wright 2017; Somboro et al. 2018; Ramsamy et al. 2020a, b). However, the stability of β -lactams differs based on their inter-atomic amide bond distances (C–N), where the six-membered ring lactams C–N bond are more rigid than those with five-membered ring, suggesting that electronic factors play more of a role on reactivity of the β -lactam ring (Ibeji et al. 2019). Zhang et al. (2018) reported that enethiol containing MBL inhibitors (MBLIs) use their sulphur group to inhibit MBL from different subclasses by displacing the zinc (II) ion (Zhang et al. 2018). The fungal natural product aspergillomarasmine A has also proven to be potent against New Delhi metallo- β -lactamase (NDM-1) and MLB (Zhang et al. 2017). Recently 1,4,7 Triazacyclononane (TACN), a cyclic organic compound derived from cyclononanes (Fig. 1) has successfully inhibited MBL while preserving the activity of meropenem (MEM) (Somboro et al. 2019).

Prior studies have shown that TACN and its functionalized derivatives such as caminomethyl-TACN can be precursors of valuable bifunctional chelating agents for nuclear medicine imaging (Désogère et al. 2014). Many potential MBLIs investigated have previously shown substantial *in vitro* activity, but cannot be used clinically due to their cytotoxicity in the liver and the kidney (Yoshizumi et al. 2013; Somboro et al. 2018; Satyo et al. 2020). The cytotoxic target for many therapeutics is the kidney due to the central role in the filtration and reabsorption of substances it is a common point of attack by many therapeutics agents (Jourde-Chiche et al. 2019). A common cytotoxic effect is apoptosis followed by the death of organ (Jourde-Chiche et al. 2019). Any potential MBLI should not induce cell death by apoptosis or necrosis above physiological levels.

Fig. 1 Chemical structure of 1,4,7 Triazacyclononane (TACN)

Apoptosis is a programmed form of cell death that is usually preferable over other forms such as necrosis and autophagy (Hassan et al. 2014). Apoptosis is triggered by cellular stress that results in DNA damage and if not repaired, the cell will undergo apoptosis. Apoptosis is characterized by morphologic changes such as chromatin condensation and nuclear fragmentation as well as biochemical changes that include activation of cysteine aspartyl-specific proteases (caspases) (Kiraz et al. 2016). Additional features that include DNA, protein and membrane surface modifications allow the apoptotic cell to be recognized and engulfed by phagocytic cells (Koff et al. 2015).

Old, injured, auto-reactive cells and cells with irreversible DNA damage are eliminated from the body of all the multicellular organisms by apoptosis (Anusha et al. 2016). A balance is sustained between the rate of cell division and cell death in multicellular organisms to ensure homeostasis (Pfeffer and Singh 2018). The Bcl-2 family proteins that play a key role in modulation of apoptosis are largely categorized into pro- and anti-apoptotic proteins. BAD, BAK, BAX, PUMA and NOXA are pro-apoptotic. Anti-apoptotic proteins include Bcl-2 family, Bcl-xL, Bcl-w and survivin and activated Nf- κ B which results in the transcription of inhibitors of apoptosis (IAP) (Kiraz et al. 2016).

Apoptosis can be initiated in two pathways, the extrinsic and intrinsic pathway together lead to activation of a caspase cascade (Baig et al. 2016). The extrinsic pathway is activated by external death signals that bind to tumour necrosis factor (TNF) family death receptors that engage with death domain-containing receptors (Ma et al. 2018). This results in activation of death effector domain-containing caspases such as caspases 8 that will initiate apoptosis and activate effector caspase 3/7. The intrinsic pathway is activated by damaged DNA or upregulated oncogenes or other stimuli such as growth factor deficiency, excess Ca^{2+} , DNA-damaging molecules, generation of oxidants and increased concentration of Bcl-2 family proteins (Redza-Dutordoir and Averill-Bates 2016). Bcl-2 are central regulators of the intrinsic pathway, which either suppress or promote changes in mitochondrial membrane permeability required the release of cytochrome c and other apoptotic proteins (Hassan et al. 2014). The intrinsic pathway is mediated by caspase 9 resulting in proteolytic cleavage and activation of caspase 3/7. Active caspase 3/7 catalyses

the specific cleavage of many key cellular proteins, for example poly-ADP-ribose polymerase (PARP) leading to cell death (Pfeffer and Singh 2018). This study aimed to investigate the cytotoxicity of TACN in Hek293 cells, elucidate the possible induction of cell death and the underlying molecular mechanisms.

Materials and methods

Materials

The Hek293 cells were purchased from Highveld Biologicals (Johannesburg, South Africa). Cell culture reagents and low melting point agarose (LMPA) were purchased from Whitehead scientific (Johannesburg SA). TACN, phosphate buffered saline (PBS), methylthiazol tetrazolium (MTT) salt, Hoechst and bichinchonic acid (BCA) were acquired from Sigma Aldrich (Johannesburg, SA). Caspases, Annexin V kit and antibodies were purchased from Anatech (Johannesburg, South Africa). Lactate dehydrogenase (LDH) and protease/phosphatase inhibitors were acquired from Roche (Johannesburg, South Africa), western blotting reagents were acquired from Bio-Rad and bovine serum albumin (BSA) was purchased from Inqaba Biotech (Johannesburg, South Africa).

Cell culture

Hek293 cells are immortalised cell line that are commonly used in biomedical research and they are advantageous over primary cells because of their ability to proliferate almost forever (Achilli et al. 2018). The Hek293 cell line was started by transferring the cryopreserved cells from the cryovial into a sterile 25 cm³ cell culture flask containing 10 mL of complete culture medium (CCM) [Dulbecco Modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 1% L-glutamine and 1% penicillin-streptomycin-fungizone]. The flasks were incubated at (37 °C, 5% CO₂). After 4 h, the CCM was changed and the flasks were maintained until 90% confluency was reached. Confluent flasks were washed once with phosphate-buffered saline (PBS). The cells were dislodged by agitation, resuspended in CCM and counted (150 µL of CCM, 50 µL of trypan blue and 50 µL of cell suspension were mixed in an Eppendorf

and 10 µL of this mixture was transferred to a haemocytometer) for use in the various assays.

Preparation of TACN treatments

The TACN stock solution (7740 µM) was prepared by dissolving 5 mg of TACN in 5 mL of CCM. The TACN stock solution was used to prepare the 12 dilutions used for the MTT assay (0–500 µM) and treatment concentrations.

MTT assay

Hek293 cells were plated into 36 wells of a 96 well plate at a density of 20,000 cells (200 µL CCM/well) and allowed to adhere overnight. From the prepared treatment serial dilutions, 300 µL was aliquoted into respective wells in triplicate and the plate was incubated at (37 °C, 5% CO₂) for 24 h. The treatment medium was discarded and replaced with 120 µL of MTT solution (4 mg MTT salt dissolved in 800 µL PBS reconstituted in 4 mL CCM). The plate was incubated (37 °C, 5% CO₂) for 4 h, the MTT salt solution was removed and 100 µL of solubilizing detergent (DMSO) was dispensed into each well. After an hour incubation, the optical density was read using the Bio-Tek µQuant spectrophotometer (USA) at 570 nm/690 nm.

The cell viability was calculated [$(\text{Absorbance of treated cells})/(\text{Absorbance of control cells}) \times 100$] and a concentration dependant response curve was plotted using GraphPad Prism v5.0 software. A 50% maximum inhibitory concentration (IC₅₀) was generated and was divided by 2 to get an extrapolated IC₂₅ that was used for all following assays.

Preparation of cells for luminometry and the comet assay

Three confluent flasks of Hek293 cells were treated with 294 µM and 588 µM TACN for 24 hours; untreated cells served as the control. The treatment medium was removed and reserved for use in the LDH assay; the cells were washed once with PBS and resuspended in 2 mL PBS. The cells were counted so that 20,000 cells (in 50 µL PBS) were dispensed into opaque white plates in triplicate for each luminometric treatment, and 40,000 cells (in 50 µL PBS) into eppendorf for the comet assay.

Annexin V and necrosis assay

According to NanoLuc[®] Binary Technology, the Real-Time-Glo[™] annexin V apoptosis and necrosis assay (JA1011) NanoBit[®] substrate containing 1000× complementary subunits of NanoBit luciferase (annexin V-LgBit and annexin V-SmBiT) and calcium, was added to 2 mL of CCM and vortexed immediately. Thereafter, 1000× Necrosis detection reagent was added and vortexed to ensure homogeneity. The annexin/necrosis reagent (25 µL) was pipetted into their respective wells containing treated cells, incubated for 30 minutes (dark, room temperature). Luminescence for annexin V add fluorescence (blue filter) for necrosis was read using the GLOMAX multi + detection system (Modulus[™] microplate luminometer (Turner BioSystems, Sunnyvale, California, USA).

Caspases

The activity of initiator caspases 8 (G8200) and 9 (G8210), and effector caspase 3/7 (G8090) were detected using the Promega caspase-Glo[®] assay. Caspase-Glo detection reagent was reconstituted as per manufacturer's instructions and 25 µL of the reagent was pipetted into each well containing treated cells. Subsequently, the plate was incubated at room temperature for 30 minutes. The activity of caspases was detected using the Modulus[™] microplate luminometer (Turner BioSystems, Sunnyvale, California, USA).

LDH assay

The LDH reagent was prepared as per manufacturer's instructions (Roche, 04744926001). Briefly, 100 µL of the treatment medium was plated in duplicate into a 96 well plate. The plate was incubated for 30 min at room temperature in the dark, then 25 µL of stop solution was added the plate was read at 490/600 nm using Bio-Tek µQuant spectrophotometer (USA).

SCGE

The treated Hek293 cells were encapsulated in low melting point agarose (LMPA) gels on frosted end microscopic slides (duplicate) as follows. The first layer was made by pipetting 800 µL of 2% LMPA

towards the frosted end of microscopic slide, overlaying it with a coverslip and allowing the gel to solidify at 4 °C for 10 minutes. The coverslip was removed and the second layer (1% LMPA (800 µL) with 2 µL of gel red and cell suspension of 40,000 cells) was prepared. This was pipetted on top of the first solid layer (400 µL per slide), immobilised with a coverslip solidified at 4 °C for 10 min. The coverslip was removed. Finally, the third layer comprising 400 µL of 1% LMPA was pipetted onto the second solidified layer; a coverslip was placed over it and solidification proceeded at 4 °C for 10 min. The coverslips were removed from all the slides and the slides were submerged in a freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA 1% Triton X, 1 M Tris and 10% DMSO). After 1 h incubation in lysing solution (dark, 4 °C), the solution was removed. Slides were placed side by side in an electrophoresis tank filled with alkaline electrophoresis buffer (300 Mm NaOH and 1 mM Na2EDTA with the pH of 13 to equilibrate) for 20 min of equilibration. Thereafter, a voltage of 25 V (current of 300 mA) was applied for 35 minutes at room temperature using Bio-Rad compact power supplier (Hercules California, USA). The slides were washed three times (5 minutes each with neutralising buffer (0.4 M Tris, pH 7.4). The slides were viewed using the Olympus IX5I inverted fluorescence microscope (filter 4 equipped with 510–560 nm excitation and 590 nm emission) and LS Software imaging system to capture 50 comets and measure the length in µM.

SDS-PAGE and Western Blotting

Confluent flasks of Hek293 cells were treated with TACN as described previously. After 24 h, crude protein was isolated from the washed (PBS) treated cells by adding 200 µL of cytobuster supplemented with protease and phosphatase inhibitors to each flask. The flasks placed on ice for 15 minutes. The cells were removed from the flasks by scraping and transferred into 1.5 mL microcentrifuge tubes. Cell debris was pelleted by centrifugation at 2000×g, 4 °C for 10 min. The crude protein supernatant was transferred into new 1.5 mL tubes, and the protein concentration was determined using the bicinchoninic acid assay.

Briefly, prepared BSA standards (0–1 mg/mL) and sample were transferred in duplicate (25 µL per well) into a 96 well plate. The BCA assay working solution

(4 μL CuSO_4 , 198 μL BCA) was pipetted into each well (200 μL per well) and incubated the plate in the dark at 37 °C for 30 min. The optical density was measured on Bio-Tek spectrophotometer at 562 nm. The optical density of the BSA standards were used to construct a standard curve and the concentration of isolated proteins was calculated from the equation generated. The concentrations of protein samples were then standardized to 1.4 mg/mL (200 μL). Standardized samples were prepared for running by diluting them in 50 μL Laemmli sample buffer (SDS, β -mercaptoethanol, glycerol, 1M Tris and bromophenol blue) and the samples were boiled for 5 min. A 1.5 mm polyacrylamide gel comprising 10% resolving [distilled water, 1.5 M Tris (pH 6.8), 10% SDS, Bis/Acrylamide, 10% PSA and TEMED] and 4% stacking [distilled water, 0.5 M.

Tris (pH 6.8), 10% SDS, Bis/Acrylamide, 10% PSA and TEMED] were prepared. The prepared gels were transferred to the electrode assembly and placed in a tank filled with 1 $\times$ running buffer (distilled water, Tris, glycine, SDS).

The standardised denatured samples (25 μL) were loaded into the gels, the apparatus was sealed and a Bio-Rad compact power supplier (Hercules California USA) was used to apply a voltage of 150 V until bromophenol blue dye reaches the bottom of the gel. The electrophoresed gels were placed in transfer buffer (Tris, glycine and methanol) for 10 min so that the gels and proteins can equilibrate, transfer membrane (nitrocellulose) and fibre pads were also soaked in transfer buffer. A gel sandwich was made by placing a fibre pad, nitrocellulose membrane, equilibrated SDS-PAGE gel and another fibre pad into the Trans-blot Turbo cassette (Bio-Rad, Hercules California USA). A current (2.5 A, 25 V) was applied for 30 min to facilitate protein transfer.

Following transfer, the membrane was placed in 5 mL of blocking solution [5% BSA in TTBS (Tris pH 7.5 NaCl, tween)] and incubated for two hours on shaker. Each membrane was incubated in their appropriate primary antibody (1:1000 in 5% BSA/TTBS) for an hour on the shaker, then overnight at 4 °C. Primary antibodies included mouse (48,818), rabbit (5023). Following overnight incubation, the membranes were put on shaker for an hour allowing the membrane to reach the room temperature. The membranes were washed 5 times (10 min each) with TTBS to remove the primary antibodies. The membranes

were probed with their respective HRP-conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG, 1:2500 in 5% BSA/TTBS) for 2 h on the shaker. The membranes were washed with TTBS 5 times (10 min each).

The membranes were rinsed with deionized water, the probed with chemiluminescence reagent (1:1000) and viewed using the Bio-Rad Chemidoc XRS. Images were captured and analysed using image lab software 6.0.1 windows. The expression of p53, PARP, Bax, NF- κ B and IAP proteins was normalised using the house-keeping protein, β -actin. Briefly, the membranes were stripped upon addition of 10 mL water hydrogen peroxide and incubated at 37 °C for 30 min.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, USA). The statistical significances were determined by unpaired Students t-test with Welch's correction and ANOVA [results reported as mean $\pm$ standard deviation (SD)] and a 95% confidence interval with a *p* value of less than 0.05.

Results

MTT assay

The MTT assay was used to measure TACN toxicity in Hek293 cells. The dose response curve (Fig. 2a, b) showed the highest cell viability of 105% at 25 μM and the lowest cell viability of about 60% at 250 μM . A concentration of 588 μM reduced the viability of Hek293 cells by 50%; therefore, this IC₅₀ concentration was used for all following assays. The cell viability decreased in dose-dependent manner from 50 to 250 μM (Fig. 2b), after which the response remained at approximately 1020%.

Caspases

The activity of caspases was assessed using luminometry (Fig. 3). The activity of caspase 8 (Fig. 3a) showed 2.7- and 2.18-fold (*p* $\leq$ 0.0278) decrease at IC₂₅ and IC₅₀ treatments respectively, relative to the

Fig. 2 Represent the effect of TACN on Hek-293 cell viability. TACN induced a decrease in the viability of Hek-293 cells following a 24 h treatment

Fig. 3 Graphical presentation of TACN influence on caspase activity after 24 h exposure. Both (a) caspase-8 and (b) caspase-9 were significantly reduced as compare to the control with the

$p < 0.0169$ and $p < 0.0001$ respectively. Caspase 3/7 were non-significantly reduced relative to the control, the p value 0.0186

control ($6,126,000 \pm 64,660$ RLU). Caspase 9 (Fig. 3b) showed a significant 4.37-fold decrease in IC₂₅ treatment ($p \leq 0.0278$), whilst IC₅₀ showed 2.1-fold decrease ($p \leq 0.0278$) in comparison to the control ($10,050,000 \pm 50,000$ RLU). However, the activity of effector caspases 3/7 (Fig. 3c) was also reduced by 3.41-fold and 6.40-fold at IC₂₅ and IC₅₀ respectively when compared to the control ($213,400 \pm 18,200$ RLU).

Annexin V

The ability of annexin V to bind to externalised phosphatidylserine (PS) was assessed via luminometry. In comparison to the control ($155,000 \pm 33,260$ RLU) (Fig. 4), the binding affinity of annexin V PS was reduced by 3.71-fold at IC₂₅ while a reduction of 1.46-fold was observed at IC₅₀.

Fig. 4 Externalisation of phosphatidylserine in Hek239 cells after 24 h exposure to TACN, the binding affinity of annexin V to PS it was non-significantly reduced

LDH and necrotic cells

The amount of lactate dehydrogenase (LDH) present was assessed using the colorimetric assay (Fig. 5a). In

Fig. 5 The amount of lactate released in Hek293 cells and the number of necrotic cells induced after being exposed to TACN for 24 h. LDH was significantly reduced with the p value of 0.0001 and necrosis is non-significantly with p value of 0.3006

relation to the control (0.0855 ± 0.003753 RLU), the IC₂₅ showed 1.49-fold decrease ($p \leq 0.02$), while the IC₅₀ showed 3.35-fold decrease ($p \leq 0.0039$). The number of necrotic cells was evaluated fluorescence (Fig. 5b). When compared to the control (8020 ± 118.1 RLU), the IC₂₅ showed a non-significant 0.82-fold increase while the IC₅₀ did not vary significantly from the control (1.01-fold decrease).

SCGE

The DNA damage was detected fluorescently using the Olympus inverted microscope. Longer comet tail length is associated with higher DNA fragmentation (panel a–c). The comet tail length (Fig. 6d) showed a

significantly by 1.46-fold increase ($p \leq 0.0001$) at IC₂₅ while at IC₅₀ the length was significantly increased by 1.21-fold, ($p \leq 0.0001$) when compared to the control (11.57 ± 0.3042 μm).

Western blots

To explore the possible mechanism of TACN induced apoptosis in Hek293 cells, the expression of proapoptotic (p53 and BAX) and antiapoptotic (IAP, NF-κB) and Parp proteins were evaluated by western blot analysis and normalised against β-actin. The expression of p53 (Fig. 7a) was significantly upregulated by 1.73-fold at IC₂₅, with $p < 0.0001$ while at IC₅₀ it was significantly reduced by 1.13-fold with $p \leq 0.0002$ relative to control (0.7192 ± 0.01118). The expression of BAX (Fig. 7b) was significantly downregulated at IC₂₅ and IC₅₀ by 0.65-fold with $p \leq 0.0030$ and 0.82-fold $p \leq 0.0208$ respectively relative to control (0.9330 ± 0.03597). NF-κβ (Fig. 7c) expression was significantly decreased at IC₂₅ by 0.45-fold ($p \leq 0.0013$) but at IC₅₀ there was insignificantly increase by 1.89-fold ($p \leq 0.0374$) in relation to the control (0.2581 ± 0.00455). transcription of IAP (Fig. 7d) was significantly decreased at IC₂₅ and IC₅₀ by 0.74-fold ($p \leq 0.0001$) and 0.67-fold ($p < 0.0001$) relative to the control (1.293 ± 0.02393). the late marker of apoptosis PARP (Fig. 7e) was significantly decreased at both lower IC₂₅ and higher IC₅₀ doses by 0.47-fold ($p < 0.0001$) and 0.49-fold ($p < 0.0001$) relative to control (1.239 ± 0.01144).

Discussion

TACN is the derivate of cyclononanes that has been reported to preserve the activity of β-lactam antibiotics by inhibiting the clinical carbapenem-resistant Enterobacteriaceae (Somboro et al. 2019). However, the kidney is a main target for therapeutic agents' toxicity therefore biochemical effects of TACN on Hek293 cells require elucidation. It was hypothesised that TACN induces cytotoxic effects on Hek293 kidney cells after 24-h exposure. The Hek293 cells were treated with a range of TACN concentrations (Fig. 2). Sequential increase in TACN treatment resulted in a dose-dependent decrease in cell viability.

Fig. 6 Fragmentation of DNA in Hek293 cells after 24-h exposure to TACN, panel A-C shows migration of DNA from nucleus in control, IC₂₅ and IC₅₀. Representing the degree of DNA fragmentation induced by TACN this is an indication of DNA fragmentation, the longer the comet, the higher the extent of the damage in the cell. Graph D shows the significantly increase in length relative to the control with *p* value is 0.0001

This was observed by the decrease in the conversion of MTT salt to its purple formazan product.

Decreased viability is an indication of reduced metabolic activity of cells which is dependent on succinate dehydrogenase (SDH) also known as complex II of electron transport chain (ETC) in the mitochondria (Lussey-Lepoutre et al. 2015). This enzyme catalyses the transfer of electrons from flavin adenine dinucleotide hydrogen2 (FADH₂) to 3Fe-S groups, the heme prosthetic group and finally to ubiquinone to form ubiquinol (QH₂) (Bezawork-Geleta et al. 2017). Since TACN is an iron chelator (Wang et al. 2018) it will impair electron transfer at complex II by chelating the iron molecules in Fe-S and heme groups hence decreasing the capacity of complex II to provide electrons for the production of ATP and resulting in decreased cell viability (Fig. 2). The IC₅₀ calculated (Fig. 2a) was slightly higher than that reported for HepG2 cells (433 μM) (Somboro et al. 2019) this difference may be attributed to metabolic differences between the cell lines.

Dysregulation of SDH results in the accumulation of succinate which has been shown to be a carcinogenic initiator and most carcinogens act by inhibiting apoptosis (Dalla Pozza et al. 2020). To check for the of apoptotic cells, the ability of Annexin V binding to phosphatidylserine (the first marker for apoptosis) was analysed at IC₂₅ and IC₅₀. However, it was observed that TACN did not cause externalisation of phosphatidylserine at these concentrations (Fig. 4). To further understand the mechanism of cell death, activity of initiator caspases-8/-9 and executioner caspase3/7 were investigated.

For caspase dependent apoptosis to be initiated, caspases 8 and 9 (initiators) need to be activated (Koff et al. 2015). However, the IC₂₅ and IC₅₀ demonstrated a significant decrease in initiator caspases 8 (Fig. 3a). Failure to activate caspase 8 prevents caspase 3/7 activation via the extrinsic pathway. In turn inactivated caspase 8 will not cleave BH3 Interacting Domain Death Agonist (BID) that engage with Bax and induce mitochondrial outer membrane permeability (ADAMS and CORY 2007; Koff et al. 2015).

Fig. 7 The levels of apoptosis related proteins in the Hek293 cells induced by TACN after 24 h and they were determined by western blot. The proteins bands were quantified and statistically analysed. β-actin was used as housekeeping protein

Therefore, cytochrome c will not be available to recruit APAF-1 and pro-caspase 9 to form an apoptosome and activates initiator caspase 9. Thus, caspase 9 activity was decreased (Fig. 3b). Active caspase 9 ultimately activates executioner caspases 3/7 via the intrinsic pathways but this does not occur as noted by the decreased caspase 3/7 activity (Fig. 3c) implying that there was no apoptosis initiated.

The role effector caspases 3/7 in apoptosis is to initiate the hallmarks of late apoptosis, including DNA fragmentation, chromatin condensation and cell shrinkage (Kiraz et al. 2016). In the absence of active caspase 3/7, the cleavage of poly (ADP-ribose) polymerase (PARP), a 116 kDa nuclear enzyme involved in DNA repair will not occur, thus the cell will unlikely dismantle (Catri et al. 2014). This is consistent with the downregulation of PARP at both treatment concentrations (Fig. 7e). Cleaved PARP is a prominent late marker of apoptosis and is cleaved at the aspartate-glutamine-valine-aspartate (DEVD) site between Asp214 and Gly215. The cleavage of PARP generates two fragments of 24 kDa and 85 kDa (Devnarain et al. 2017; Tiloke et al. 2019). The 24 kDa fragment contains the DNA-binding region as well as the double zinc-fingers while the 85 kDa fragment possesses the catalytic and auto-modification domain. Cleavage of PARP is known to stop repair of single or double-stranded DNA breaks by preventing the recruitment of the enzyme to sites of DNA damage, which severely compromises cell survival of cells leading to death.

The inhibitors of apoptosis proteins (IAP) binds to active caspases, preventing apoptosis from being executed (Kiraz et al. 2016). The down regulation of IAP (Fig. 7d) at IC₂₅ and IC₅₀ further confirms that apoptosis was not initiated. This is supported by the significant decreased NF- κ B protein. Activated, NF- κ B regulates the gene transcription and generation of anti-apoptotic proteins including IAP (Liu et al. 2019).

It is important to note that necrosis was also not induced following TACN treatment. Necrosis is characterised by the rupture of plasma cell membrane (Brauchle et al. 2015) and release all of cell's content including lactate into the surroundings because of the leaky plasma membrane (Kumar et al. 2018). A significant decrease in the amount of LDH released (Fig. 5a) by Hek293 cells suggests that TACN treatment did not cause cell death by inducing necrosis as is evident by the decrease in necrosis (Fig. 5b).

Despite the lack of evidence for apoptosis and necrosis occurring, the comet assay revealed that DNA fragmentation was present (Fig. 7a–d). The increased DNA damage observed (Fig. 7d) is proposed to be triggered by inactivated SDH (complex II) due to metal chelating properties of TACN. SDH contains a heme b prosthetic group in its anchor domain that is essential for the structural integrity and function of the complex (Sulkowski et al. 2018). Therefore, impaired SDH leads to accumulation of tricarboxylic acid cycle (TCA cycle) intermediate succinate (Dalla Pozza et al. 2020). Succinate is associated with elevated DNA double strand breaks by suppressing the homologous recombination (HR) DNA-repair pathway (Sulkowski et al. 2018). HR is essential for the firmness of DNA double-strand breaks (DSBs) and for the preservation of genomic integrity (Sulkowski et al. 2018). The defect in homologous recombination will fail to mend the damaged DNA. This correlates with the significant increase in IC₂₅ and IC₅₀ of p53 (Fig. 7a). p53 an active protein that is upregulated by mdm-2 oncoprotein when there is cellular stress and DNA damage (Hafner et al. 2019). It has multiple functions such as stopping the progression of cell cycle; and initiating DNA repair or apoptosis.

Conclusion

Overall TACN induced cell death in Hek293 cells that was independent of apoptosis, since the most prominent indicators of apoptosis were downregulated. Likewise, necrosis was not induced. The DNA damage suggests an electron dependent process, that recruited p53 to repair the damaged DNA. Therefore, it may be safe to use TACN as an inhibitor with β -lactam antibiotics to combat the activity of β -lactamase and preserve the activity β -lactams. However, further studies are still required to evaluate the initiation of DNA repair using in vivo animal models.

Acknowledgements The authors are grateful for the financial assistance from the College of Health Sciences, University of KwaZulu-Natal, South Africa.

Data availability statement The generated data used to support the findings of this study are included within the article.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest exists.

Ethical approval Ethical approval was obtained from the Biomedical Research Ethics Administration under the Ref No: BE365/19.

References

- Achilli C, Ciana A, Minetti G (2018) Immortalized HEK 293 kidney cell lines as models of renal cells: friends or foes? *J Controv Biomed Res* 4:6–9. <https://doi.org/10.15586/jcbmr.2018.26>
- Adams J, Cory S (2007) Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Curr Opin Immunol* 19:488–496. <https://doi.org/10.1016/j.coi.2007.05.004>
- Agepong N, Govinden U, Owusu-Ofori A et al (2019) Genomic characterization of multidrug-resistant ESBL-producing *Klebsiella pneumoniae* isolated from a Ghanaian teaching hospital. *Int J Infect Dis* 85:117–123. <https://doi.org/10.1016/j.ijid.2019.05.025>
- Anusha S, Mohan CD, Ananda H et al (2016) Adamantyl-tethered-biphenylic compounds induce apoptosis in cancer cells by targeting Bcl homologs. *Bioorg Med Chem Lett* 26:1056–1060. <https://doi.org/10.1016/j.bmcl.2015.12.026>
- Baig S, Seevasant I, Mohamad J et al (2016) Potential of apoptotic pathway-targeted cancer therapeutic research: where do we stand? *Cell Death Dis* 7:e2058. <https://doi.org/10.1038/cddis.2015.275>
- Bezawork-Geleta A, Rohlena J, Dong L et al (2017) Mitochondrial complex II: at the crossroads. *Trends Biochem Sci* 42:312–325. <https://doi.org/10.1016/j.tibs.2017.01.003>
- Blair JMA, Webber MA, Baylay AJ et al (2015) Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 13:42–51. <https://doi.org/10.1038/nrmicro3380>
- Brauchle E, Thude S, Brucker SY, Schenke-Layland K (2015) Cell death stages in single apoptotic and necrotic cells monitored by Raman microspectroscopy. *Sci Rep* 4:4698. <https://doi.org/10.1038/srep04698>
- Castri P, Lee Y, Ponzio T et al (2014) Poly(ADP-ribose) polymerase-1 and its cleavage products differentially modulate cellular protection through NF- κ B-dependent signaling. *Biochim Biophys Acta - Mol Cell Res* 1843:640–651. <https://doi.org/10.1016/j.bbamcr.2013.12.005>
- Dalla Pozza E, Dando I, Pacchiana R et al (2020) Regulation of succinate dehydrogenase and role of succinate in cancer. *Semin Cell Dev Biol* 98:4–14. <https://doi.org/10.1016/j.semdb.2019.04.013>
- Désogère P, Rousselin Y, Poty S et al (2014) Efficient synthesis of 1,4,7-triazacyclononane and 1,4,7-triazacyclononane-based bifunctional chelators for bioconjugation. *Eur J Org Chem* 2014:7831–7838. <https://doi.org/10.1002/ejoc.201402708>
- Devnarain N, Tiloke C, Nagiah S, Chuturgoon AA (2017) Fusaric acid induces oxidative stress and apoptosis in human cancerous oesophageal SNO cells. *Toxicol* 126:4–11. <https://doi.org/10.1016/j.toxicol.2016.12.006>
- Hafner A, Bulyk ML, Jambhekar A, Lahav G (2019) The multiple mechanisms that regulate p53 activity and cell fate. *Nat Rev Mol Cell Biol* 20:199–210. <https://doi.org/10.1038/s41580-019-0110-x>
- Hassan M, Watari H, AbuAlmaaty A et al (2014) Apoptosis and molecular targeting therapy in cancer. *Biomed Res Int* 2014:1–23. <https://doi.org/10.1155/2014/150845>
- Ibeji CU, Lawal MM, Tolufashe GF et al (2019) The driving force for the acylation of β -lactam antibiotics by L,d-transpeptidase 2: quantum mechanics/molecular mechanics (QM/MM) study. *ChemPhysChem* 20:1126–1134. <https://doi.org/10.1002/cphc.201900173>
- Jourde-Chiche N, Fakhouri F, Dou L et al (2019) Endothelium structure and function in kidney health and disease. *Nat Rev Nephrol* 15:87–108. <https://doi.org/10.1038/s41581-018-0098-z>
- Kiraz Y, Adan A, Kartal Yandim M, Baran Y (2016) Major apoptotic mechanisms and genes involved in apoptosis. *Tumor Biol* 37:8471–8486. <https://doi.org/10.1007/s13277-016-5035-9>
- Koff J, Ramachandiran S, Bernal-Mizrachi L (2015) A time to kill: targeting apoptosis in cancer. *Int J Mol Sci* 16:2942–2955. <https://doi.org/10.3390/ijms16022942>
- Kumar P, Nagarajan A, Uchil PD (2018) Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harb Protocol*. <https://doi.org/10.1101/pdb.prot095497>
- Liu L, Ahn KS, Shanmugam MK et al (2019) Oleuropein induces apoptosis via abrogating NF- κ B activation cascade in estrogen receptor-negative breast cancer cells. *J Cell Biochem* 120:4504–4513. <https://doi.org/10.1002/jcb.27738>
- Lussey-Lepoutre C, Hollinshead KER, Ludwig C et al (2015) Loss of succinate dehydrogenase activity results in dependency on pyruvate carboxylation for cellular anabolism. *Nat Commun* 6:8784. <https://doi.org/10.1038/ncomms9784>
- Ma Z-J, Lu L, Yang J-J et al (2018) Lariciresinol induces apoptosis in HepG2 cells via mitochondrial-mediated apoptosis pathway. *Eur J Pharmacol* 821:1–10. <https://doi.org/10.1016/j.ejphar.2017.12.027>
- Pfeffer CM, Singh ATK (2018) Apoptosis: a target for anti-cancer therapy. *Int J Mol Sci* 19:448. <https://doi.org/10.3390/ijms19020448>
- Ramchuran EJ, Somboro AM, Monaim SAHA et al (2018) In vitro antibacterial activity of teixobactin derivatives on clinically relevant bacterial isolates. *Front Microbiol* 9:1–10. <https://doi.org/10.3389/fmicb.2018.01535>
- Ramsamy Y, Mlisana KP, Allam M et al (2020) Genomic analysis of carbapenemase-producing extensively drug-resistant *Klebsiella pneumoniae* isolates reveals the horizontal spread of p18-43_01 Plasmid Encoding bla NDM-1 in South Africa. *Microorganisms* 8:137
- Ramsamy Y, Mlisana KP, Amoako DG et al (2020) Pathogenic analysis of a novel extensively drug-resistant *Citrobacter freundii* isolate carrying a blaNDM-1 Carbapenemase in South Africa. *Pathogens* 9:89. <https://doi.org/10.3390/pathogens9020089>
- Redza-Dutordoir M, Averill-Bates DA (2016) Activation of apoptosis signalling pathways by reactive oxygen species.

- Biochim Biophys Acta - Mol Cell Res 1863:2977–2992. <https://doi.org/10.1016/j.bbamcr.2016.09.012>
- Rotondo CM, Wright GD (2017) Inhibitors of metallo- β -lactamases. *Curr Opin Microbiol* 39:96–105. <https://doi.org/10.1016/j.mib.2017.10.026>
- Satyo L, Amoako DG, Somboro AM et al (2020) Molecular insights into Di(2-Picolyl) amine-induced cytotoxicity and apoptosis in human kidney (HEK293) cells. *Int J Toxicol* 39:341–351. <https://doi.org/10.1177/1091581820921180>
- Somboro AM, Osei Sekyere J, Amoako DG et al (2018) Diversity and proliferation of metallo- β -lactamases: a clarion call for clinically effective metallo- β -lactamase inhibitors. *Appl Environ Microbiol* 84:1–20. <https://doi.org/10.1128/AEM.00698-18>
- Somboro AM, Amoako DG, Osei Sekyere J et al (2019) 1,4,7-Triazacyclononane restores the activity of β -lactam antibiotics against metallo- β -lactamase-producing enterobacteriaceae: exploration of potential metallo- β -lactamase inhibitors. *Appl Environ Microbiol* 85:e02077–e02018. <https://doi.org/10.1128/AEM.02077-18>
- Sosibo SC, Somboro AM, Amoako DG et al (2019) Impact of pyridyl moieties on the inhibitory properties of prominent acyclic metal chelators against metallo- β -lactamase-producing enterobacteriaceae: investigating the molecular basis of acyclic metal chelators' activity. *Microb Drug Resist* 25:439–449. <https://doi.org/10.1089/mdr.2018.0272>
- Sulkowski PL, Sundaram RK, Oeck S et al (2018) Krebs-cycle-deficient hereditary cancer syndromes are defined by defects in homologous-recombination DNA repair. *Nat Genet* 50:1086–1092. <https://doi.org/10.1038/s41588-018-0170-4>
- Tiloke C, Phulukdaree A, Gengan RM, Chuturgoon AA (2019) Moringa oleifera aqueous leaf extract induces cell-cycle arrest and apoptosis in human liver hepatocellular carcinoma cells. *Nutr Cancer* 71:1165–1174. <https://doi.org/10.1080/01635581.2019.1597136>
- Wang S, Gai Y, Zhang S et al (2018) Synthesis and evaluation of a class of 1,4,7-triazacyclononane derivatives as iron depletion antitumor agents. *Bioorg Med Chem Lett* 28:117–121. <https://doi.org/10.1016/j.bmcl.2017.11.048>
- Yoshizumi A, Ishii Y, Kimura S et al (2013) Efficacies of calcium-EDTA in combination with imipenem in a murine model of sepsis caused by Escherichia coli with NDM-1 β -lactamase. *J Infect Chemother* 19:992–995. <https://doi.org/10.1007/s10156-012-0528-y>
- Zhang J, Wang S, Wei Q et al (2017) Synthesis and biological evaluation of Aspergillomarasmine A derivatives as novel NDM-1 inhibitor to overcome antibiotics resistance. *Bioorg Med Chem* 25:5133–5141. <https://doi.org/10.1016/j.bmc.2017.07.025>
- Zhang D, Markoulides MS, Stepanovs D et al (2018) Structure activity relationship studies on rhodanines and derived enethiol inhibitors of metallo- β -lactamases. *Bioorg Med Chem* 26:2928–2936. <https://doi.org/10.1016/j.bmc.2018.02.043>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.