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Abstract

Semicarbazide was brought to the forefront of scientific discussions by the scientific community in the early 2000's as the substance was discovered in several food products from both synthetic and natural sources. Although semicarbazide was processed through several toxicological assays and classified as a non-genotoxic carcinogen, underwhelming amounts of toxicological data exists for the compound. The present study is one of the first *in-vitro* studies to examine the relationship between sub-pathophysiological concentrations of reactive oxygen species and the anomalous non-genotoxic carcinogenicity induced by semicarbazide through the upregulation of intracellular signalling pathways. A novel finding of the present study was where NRK cells were exposed to micromolar concentrations of semicarbazide, an inverse relationship between protein kinase C activity, and free radical concentration proportionally increases > 2-fold. It is the hypothesis of the present study that the >2-fold increase in free radical concentration and upregulation of protein kinase C from the 0.01mM treatment of semicarbazide has the capacity to alter physiological signaling mechanisms into a sub-pathophysiological state. These changes in the cellular environment could be the initiation mechanism that facilitates the manifestation of cancer in a cell population. The induced upregulation of protein kinase C activity within NRK cells by semicarbazide can be extrapolated to downstream mediators of the Ca^{2+} dependent signaling pathway and the mitogen activated protein kinase signaling pathway. The hypothesis of the present study concludes by highlighting the importance of these pathways in the manifestation of semicarbazide induced non-genotoxic carcinogenicity. In conclusion, this study classifies semicarbazide as an intracellular signaling mitogen based on its capacity to modulate protein kinase C activity and the concentrations of intracellular reactive oxygen species.

Keywords: Carcinogenicity; Cell signalling; Oxidative stress; Protein kinases; Semicarbazide.

1. Introduction

The prevalence of cancer in modern society has come to the forefront in both general and scientific conversations due to the increasing number of incidences. Cancer has been at the center of the toxicological sector for hundreds of years, dating back to the 18th century where Percival Pott correlated the formation of epidermoid carcinomas with toxin exposure (Waldron, 1983). Although the number of cancer cases has increased significantly throughout the 20th and 21st century, it must not be assumed that this type of disease is relatively new, as the development of cancer has existed since the beginning of human civilizations. The early work conducted by Rosalie and Zimmerman (2010) gave insight into the development of cancers in ancient civilizations by assessing over 10,000 Egyptian mummies. Although cancer incidences were

identified, it was highlighted that the prevalence of cancer incidences were not as abundant in ancient societies as they are now in the 21st century (2010). As of 2018, the number of cancer cases and mortality incidences have increased 33% and 24% respectfully in the UK, which corresponds to 4.2 million new cases of cancer have developed and an increase of 1.91 million deaths associated with cancer (International Association of Cancer Registries, 2018).

Cancer manifestation is based on the principle that all mammalian cells have an interconnected signaling system that dictates whether the cell undergoes proliferation, differentiation or cell death (Jiang, Nick and Sood, 2015). All cancers consist of cells that have been transformed as a result of alterations in the functionality of interconnected cellular signalling systems (Aapro, 2006). When cells undergo cancerous changes, autonomy against regulatory signals develops which subsequently facilitates the uncontrolled growth and proliferation associated with cancer (Aapro, 2006). The. current dogma states that cancer is a multi-step disease originating from a single abnormal cell with mutations in its genome sequence. Uncontrolled proliferation of these abnormal cells is followed by a second mutation leading to an aberrant stage. Persistent mutation and selective expansion of these cells induces the formation of a tumour (Demitrack & Samuelson, 2017). Vizoso et al. (2015) noted that tumours can uptake subsequent mutations, accelerating the expansion rate, which eventually allows the tumour to break through the basal membrane encapsulating tissues and metastasizes throughout the body. Initiation and progression of cancer depends on both endogenous and exogenous factors that can induce impacts independently, but also synergistically or antagonistically modulate the induction process (Vizoso et al. 2015). As carcinogens can derive from biological, chemical and physical origins, Cattley and Radinsky (2004) highlight that a single mechanism of carcinogenicity does not exist but rather multiple pathways that cross-link between cellular systems. The EFSA (2005) outlined that compounds are classified for carcinogenicity based on how the mechanism of action interacts with the genetic sequence (EFSA 2005). Compounds that directly interact with the genetic sequence are classified as "*genotoxic carcinogens*", whereas compounds that elicit genetic mutation without interacting with genetic material are referred to as "*non-genotoxic carcinogens*" but also as "*epigenetic carcinogens*" of which semicarbazide has been classified (Benigni, Bossa and Tcheremenskaia, 2013).

Non-genotoxic carcinogenicity (NGC) is the formation of cancerous cells mediated through processes that do not interact or alter the genome directly, but instead through indirect processes (Perez, et al., 2016). In the context of semicarbazide, the concept of non-genotoxic carcinogenicity came to the fore when the EFSA (2005) observed compounds resulting as negatives in carcinogenicity screening assays, such as the Ames Bacterial Reversion Mutation Assay (EFSA 2005). Benigni, Bossa and Tcheremenskaia (2013) note that NGC mechanisms of action are continuously emerging as there are a vast array of biological systems that aid in cellular maintenance; and any dysfunction to these system's components could potentially give rise to cellular dysfunction and or proliferation (Benigni, Bossa and Tcheremenskaia, 2013).

Semicarbazide (SEM; EC: 200-339-6, CAS: 57-56-7) is a urea and hydrazine derivative that was brought to the forefront of scientific conversations in the early 2000's. The European Food Safety Authority (EFSA, 2003) highlighted four suspected substances that have associations with the production of semicarbazide: (1) nitrofuran antibiotics,

(2) thermal degradation of azodicarbonamide (ADC), (3) hypochlorite sterilization and (4) a byproduct of ADC when added to flour (EFSA, 2003). Initially, it was the work carried out by EFSA (2005) in assessing for nitrofuran drugs that increased scientific interest in semicarbazide. The EFSA highlighted that the testing laboratory that identified semicarbazide's prevalence in food products concluded that the source of semicarbazide could not have originated from the hypothesized illicit administration of nitrofuran drugs (EFSA, 2005). By investigating the experimental design of the laboratory, Hoenicke and colleagues (2004) suggested that semicarbazide was synthesized as a byproduct of foods that had undergone hypochlorite treatment for sterilization. In a subsequent study by Baty and co-workers (2004), the degradation of ADC, a blowing agent used in the formation of plastic seals, was believed to pose a greater risk in the generation of semicarbazide than that of the hypochlorite theory. A more recent study by Kwon (2017) outlined that in all cases of assessment, an acid hydrolysis step was incorporated. It was concluded that the presence of semicarbazide could originate as an artefact of this method (Kwon, 2017).
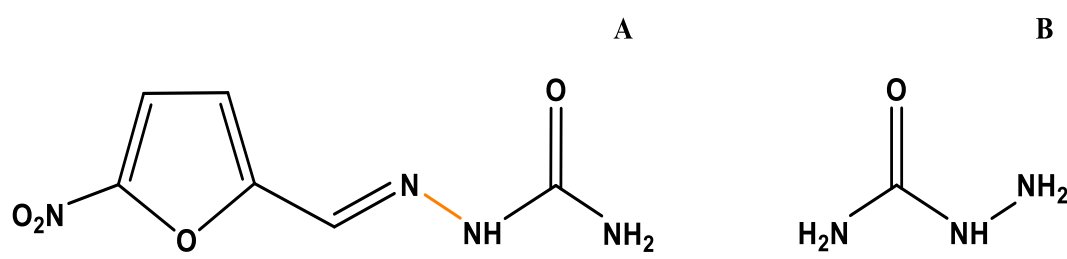

Figure 1 Molecular structure of nitrofuran (A) and semicarbazide (B). The compounds differ through the binding of the hydrazine moiety (displayed in orange). Image has been used with the permission of Kwon (2017).

Points, Thorburns and Walker (2014) state that semicarbazide is a ubiquitous organic molecule that could derive from many sources other than nitrofuran molecules. It has been noted by Crews (2012) that traces of semicarbazide have been found in several food products including baby foods, honey, sterilised vegetables and a range of condiments. Recent studies conducted by Van Poucke et al.(2011) and McCracken et al. (2013) have outlined that semicarbazide could possibly occur naturally while investigating the presence of semicarbazide in the *Macrobrachium rosenbergii* shrimp in Bangladesh fish farms. Although semicarbazide was processed through many toxicological assays in the early 2000's, the EFSA (2005) states that semicarbazide's mechanism of carcinogenicity remains inconclusive. The lack of significant toxicological research prevents a conclusive decision on whether or not semicarbazide poses a risk to human health. In the early 2000's, essential genotoxicity assays were performed to determine the nature of semicarbazide's carcinogenicity. In the majority of assays, semicarbazide produced negative results except in the specific strains of the Ames assay where it produced positive results (2005). This weak positivity in the Ames assay led Hirakawa et al.(2003) to conclude that the mechanism of action must be non-genotoxic. The comprehensive study by Hirakawa et al. (2003) gave insight into the capacity of hydrazine containing compounds to induce cancer in laboratory animals; semicarbazide is a member of this family of compounds (2003).

Oxidative stress has been defined by Sies and Jones (2007) as *"an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox*

signaling and control and/or molecular damage" (2007, pp. 45-48). Oxidative stress can be divided into two categories based on the oxidant concentration and their function at a cellular level. At low concentrations, the term used is '*oxidative eustress*', whereby oxidants can mediate signalling and regulatory pathways. '*Oxidative distress'* is the term used when intracellular oxidants reach a higher concentration by which biomolecule damage and altered cellular signalling can be induced (Sies, 2017).
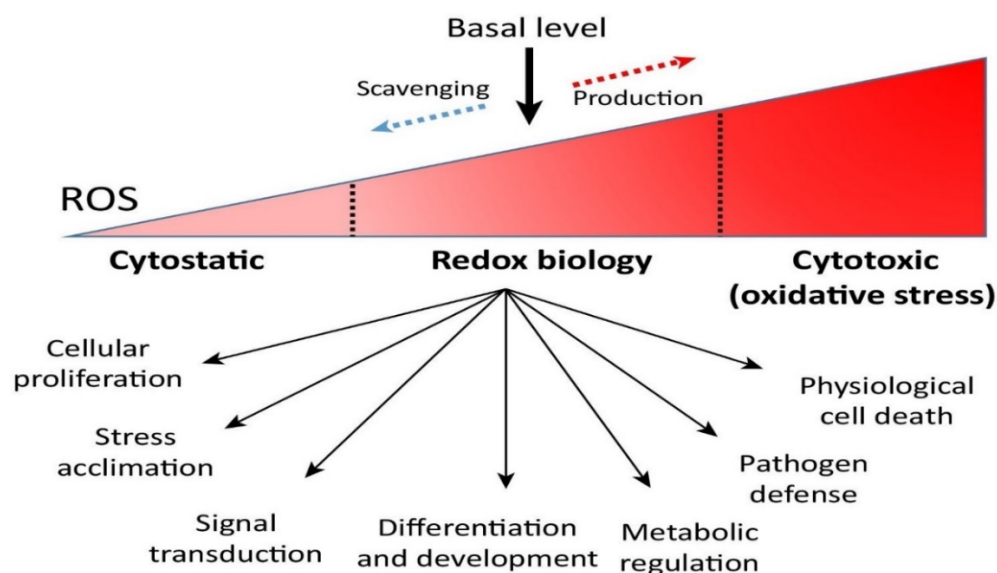
Figure 2 Graphic depiction of cellular outcomes in response to oxidative eustress and oxidative distress (used, with permission, from Mittler, 2017).

It has been noted by Burton and Jauniaux (2011) that oxidative stress is arguably the most common mechanistic characteristic in toxicology and that oxidative stress is a mechanism that applies universally. Burton and Jauniaux (2011) also note that free radicals are compounds that contain an incomplete outer electron shell by lacking one or more electrons. Free radicals are often associated with a high degree of reactivity, which derives from the number of unpaired electrons of these molecules. In order to regulate the formation of free radicals through biological systems, endogenous enzymes, antioxidants, vitamins and metals can interact and detoxify free radicals (Aruoma, 1998). Cadenas and Davies (2000) outline the generation of free radical species is not restricted to exogenous xenobiotics, as normally functioning cells also generate free radicals as by-products of cellular processes and the concentration of individual free radicals fluctuates throughout a cells lifespan. A review by Droge (2002) discusses how allosteric homeostasis of oxidative stress can elicit the activation of cellular signalling elements such as transcription factors, protein kinases and the modulation of ion channels. At higher concentrations, oxidative stress has shown to mediate lipid peroxidation, post-transcription protein modification and DNA oxidation (Droge, 2002).
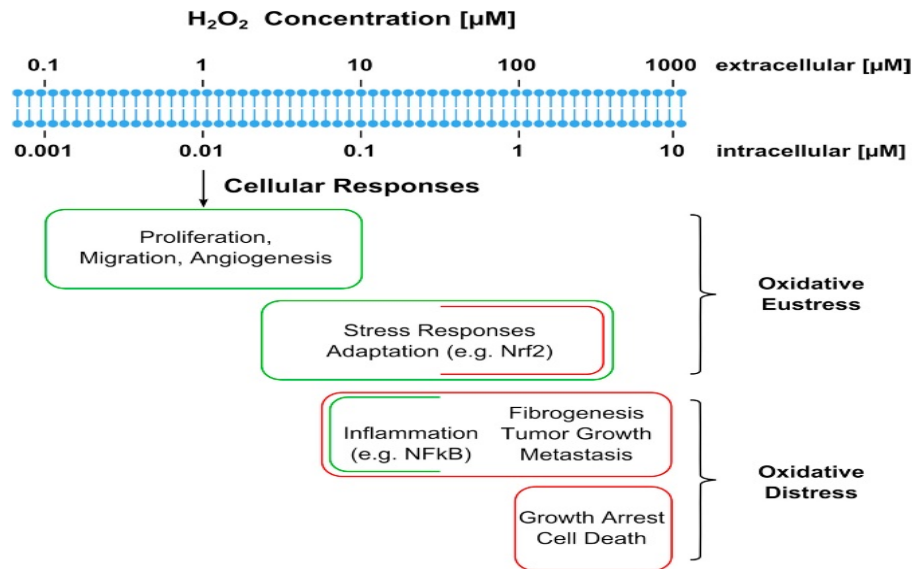
Figure 3 Graphic depiction of the effects of oxidative eustress/distress within cellular systems in response to varying concentrations of hydrogen peroxide (used, with permission, from Sies, 2017).

Burton and Jauniaux (2011) highlight that the generation of superoxide anion and hydrogen peroxide has the capacity to activate upstream kinases, such as the apoptosis-regulating signal (ASK-1) by chelating to inhibitory molecules such as thioredoxin. The formulation of reactive oxygen species has become increasingly recognised as a key mechanism contributing to the toxicity associated with many exogenous xenobiotics (Boelsterli, 2007). Evidence proposed by Clement & Pervaiz (1999) states that oxidative stress could potentially trigger unscheduled cell cycle re-entry directly. The concentration of free radicals can dictate the cellular response subsequently induced, for example; hydrogen peroxide has the capacity to induce growth arrest, cell death and cell proliferation depending on the concentration generated and that which evades the chelation of cellular antioxidant elements (Clement & Pervaiz, 1999). In a study conducted by Kannan & Sushil (2000) the potential mechanism by which oxidative stress could induce cell proliferation was discussed as oxidative stimulation of mitogenic pathways.

To put the current research into context, a study conducted by Hirakawa et al.(2003) refers to the formation of reactive oxygen radicals and organic free radicals through the auto-oxidation of hydrazine derivatives and comments that free radical formation could potentially play an important role in the mutagenesis and carcinogenicity associated with hydrazine derivatives, such as semicarbazide. The hypothesis of the present study is that oxidative eustress derived from semicarbazide exposure, modulates molecular signaling pathways that precipitates in the formation of a neoplasm. To investigate this hypothesis, the present study evaluates capacity of semicarbazide to induce proliferation, fluctuation of free radicals and alterations in protein kinase activity as a means of delineating the causative agent and pathway of semicarbazide's anomalous non-genotoxic carcinogenicity.

2. Materials and Methods

Cell line selection

NRK cells (ECACC 86032002) were used for this study. This cell line was selected as cultures were in-house, metabolically stable, the cell line does not require a complex culturing media and passaging was logistically amenable as sufficient confluency is typically reached within a 48-72 hour period.

Dose determination assay

The Sulphorhodamine-B assay was conducted based on the protocol developed by Skehan et al. (1990). Cells were seeded at 2.5×10^5 cells/ml into a 96-multiwell plate. Cells were exposed to the respective concentration of SEM and aliquots were incubated overnight at 37°C. Cells were fixed with 50% v/v trichloroacetic acid for 2 hours at 4°C and subsequently washed several times with ddH_2O. Fixed cells were stained with 0.4%. v/v SRB (Sigma), washed with 1% v/v acetic acid several times and solubilised with Tris base (10mM, pH 10.5). Absorbance was measured at 540nm using a BioTek, ELx800 plate reader.

Cellular proliferation assay

Cells were seeded at 1×10^5 cells/ml into respective wells of a 96-multiwell plate. Post-incubation with the respective concentration of SEM, each well was washed with a 1X PBS solution containing 137mM NaCl, 2.7 mM KCl and 10mM phosphate buffer; followed by the addition of 10µl of MTT (5mg/ml; Sigma) for a three-hour static incubation at 37°C. Post-incubation, 100µl of neat DMSO was added to solubilise the formazan product and absorbance was read on a BioTek, ELx800 plate reader at 540nm.

Protein kinase C activity assay

The protein kinase C activity assay (Abcam) was conducted in accordance with the protocol provided by the supplier, with slight modification. The two modifications were: (1) an in-house lysis cocktail consisting of Cell lytic M (Sigma) and protease inhibitor cocktail (Pierce™ ,ThermoScientific,) was prepared in a 4:1 ratio instead of the lysis cocktail outlined by the company and (2) in order to determine protein content, the BioRad protein assay was adopted instead of the Abcam BCA assay.
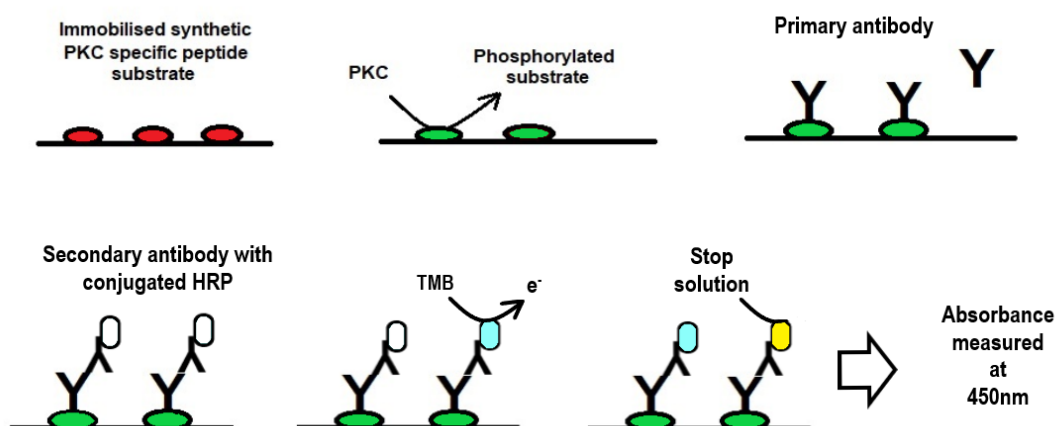
Figure 4 Schematic diagram of the Abcam protein kinase assay outlining the detection of protein kinase isoforms
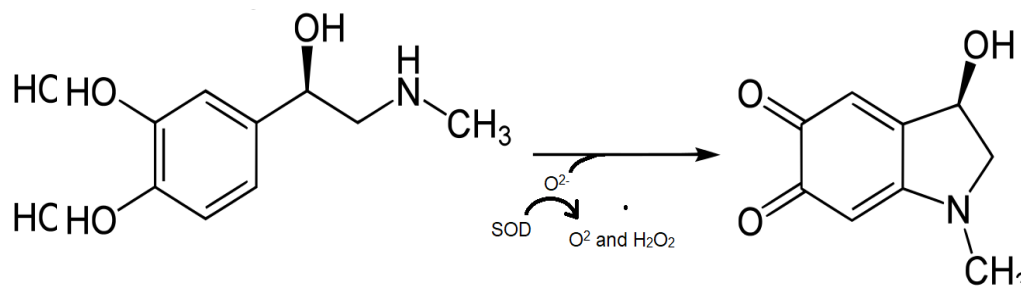
Superoxide dismutase assay – Adrenochrome method

The principle of this assay is based on the auto-oxidation of adrenaline (Sigma) to adrenochrome by the free radical, superoxide anion. A decrease in the conversion of adrenaline to adrenochrome is indicative of an increase in the concentration of superoxide anion. A blank was prepared by adding 1990µl of carbonate buffer (50mM, pH 10.2;) to a 3ml cuvette. 10µl of pre-prepared adrenaline in 0.1M HCl was added to give a final concentration of 50mM, and the solution was covered and inverted.

A negative control was prepared by adding 1985µl of carbonate buffer (50mM, pH 10.2;) to a 3ml cuvette. 10µl of adrenaline (50mM) and 5 µl of the negative control lysate (SEM 0mM) was added and the solution was covered and inverted. A positive control was prepared by adding 1985µl of carbonate buffer (pH 10.2; 50mM) to a 3ml cuvette. 10µl of adrenaline in 0.1M HCl (50mM) and 5 µl of standard Superoxide Dismutase (SOD; 1mg/ml) was added and the solution was covered and inverted.

Pre-treated lysates were assessed by adding 1985µl of carbonate buffer (pH 10.2; 50mM) to a 3ml cuvette. 10µl of adrenaline in 0.1M HCl (50mM) and 5µl of the respective lysate was added and the solution was covered and inverted. The absorbance of adrenochrome was measured at 320nm on a SHIMADZU UV mini 1240 spectrophotometer. By determining the difference between the treated samples and the control respectfully, the fold difference in free radical concentration was determined.


Figure 5 Schematic diagram of adrenaline (left) oxidation via superoxide anion ($O^{2-\cdot}$) to adrenochrome (right) and enzymatic reaction of superoxide dismutase (SOD) to inhibit the radical mediated oxidation.

3. Results

Sulpharhodamine-B dose range determination assay

Table 1 Experimental data for the determination of a suitable dose range for incorporating into subsequent assays.

[SEM] (mM)	Mean Absorbance (540nm) ± S.D.	% control cell growth
0	1.25 ± 0.066	100
0.001	1.607 ± 0.246	128.56
0.01	1.798 ± 0.532	143.84
0.1	1.683 ± 0.386	134.64
1	1.599 ± 0.291	127.92
10	1.515 ± 0.011	121.2
100	1.099 ± 0.051	87.92
1000	0.7975 ± 0.088	63.8
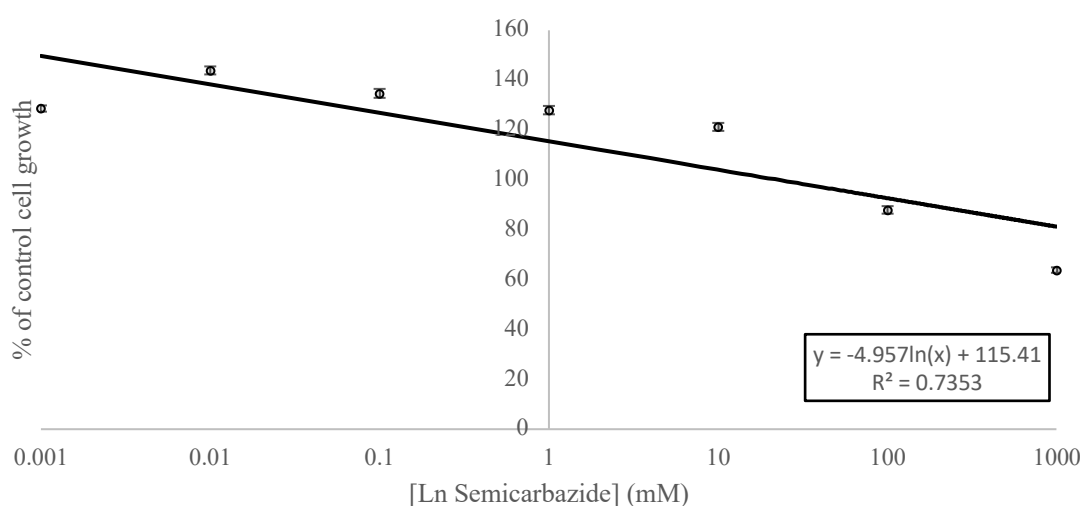
Figure 6 Dose-response analysis of cell proliferation in response to varying concentrations of semicarbazide over a 24-hour period at 37°C.

MTT cell density linearity assessment

Table 2 Experimental data for the determination of MTT cell density linearity.

Cell density/well	Mean Absorbance (540nm) ± SD
1×10^6	1.186 ± 0.109
5×10^5	0.825 ± 0.045
1×10^5	0.336 ± 0.023
5×10^4	0.315 ± 0.039
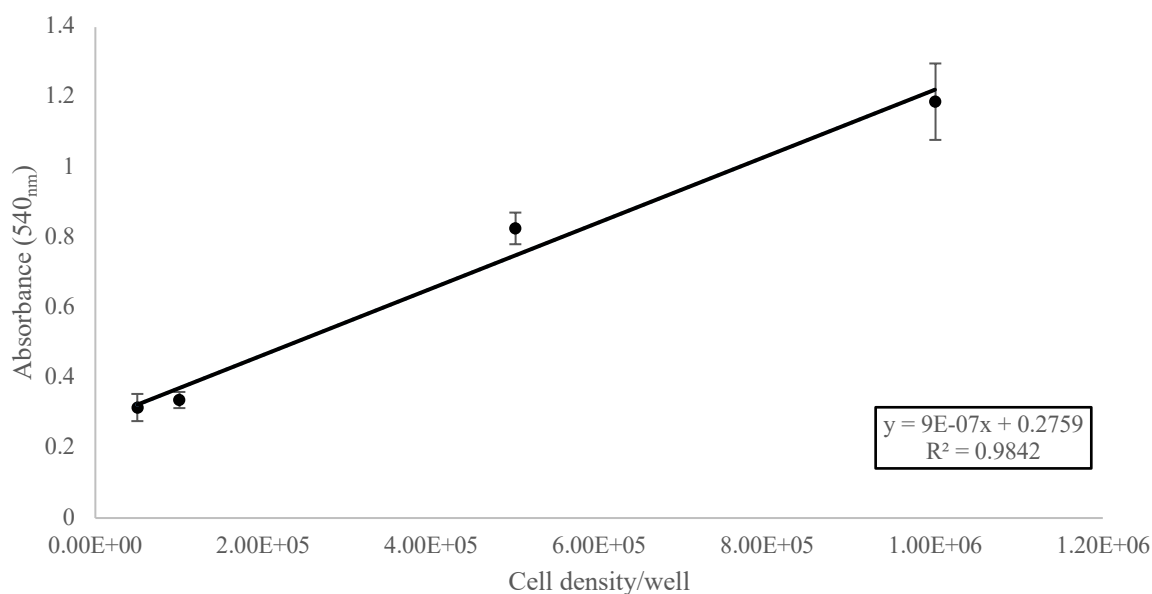
Figure 7 Dose response of MTT to several NRK cell densities ranging from $1 \times 10^6 – 5 \times 10^4$ cells/well as outlined by (Mosmann, 1983) as best practice prior to MTT assessment.

MTT cellular proliferation assay

Table 3 Experimental data for the determination of cellular proliferation in NRK cells in response to overnight exposure to semicarbazide.

[SEM] (mM)	Mean Absorbance (540nm) ± S.D.	% Proliferation	% Proliferation of control growth
0	0.346 ± 0.018	100.000	0.000
0.001	0.360 ± 0.017	103.914	3.914
0.01	0.357 ± 0.011	103.173	3.173
0.1	0.357 ± 0.025	103.131	3.131
1	0.357 ± 0.026	103.131	3.131
10	0.355 ± 0.018	102.472	2.472
100	0.349 ± 0.030	100.700	0.700
1000	0.254 ± 0.014	73.465	-26.535
[SDS] (%)			
0	0.201 ± 0.0173	100	0.000
0.05	0.183 ± 0.0135	91.418	-8.582
0.1	0.174 ± 0.0044	86.816	-13.284
0.5	0.156 ± 0.0113	77.985	-22.015
1	0.147 ± 0.0055	73.259	-26.741
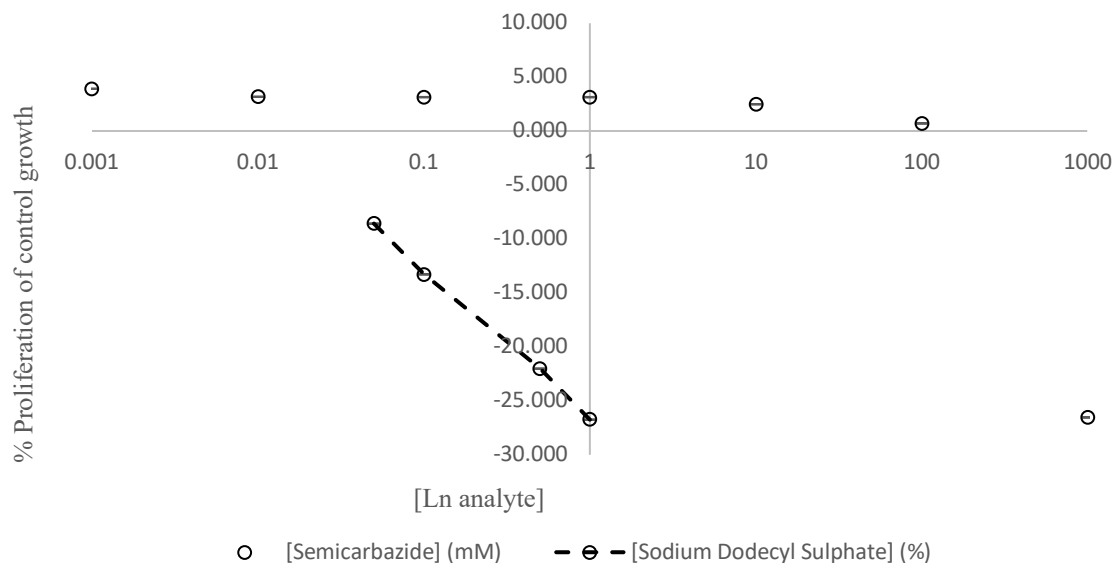
**Figure 8** Proliferation index of NRK cells exposed to varying concentrations of semicarbazide and SDS with respect to the control growth rate.

Superoxide Dismutase activity – Adrenochrome method

Table 4 Spectrophotometric measurements of adrenaline oxidation for the determination of free radical concentrations in semicarbazide pre-treated NRK monocultures. Values represent the mean absorbance at 320nm of duplicate measurements on a SHIMASZU UV mini 1240 spectrophotometer.

Time	0mM	0.01mM	0.1mM	1mM	10mM	+ Control
0	0	0	0	0	0	0
1	0.005	0.002	-0.003	0.001	0.001	0.002
2	0.015	0.007	0.002	0.005	0.002	0.003
3	0.033	0.017	0.007	0.013	0.006	0.004
4	0.064	0.032	0.018	0.024	0.015	0.005
5	0.11	0.051	0.034	0.039	0.031	0.006
6	0.175	0.074	0.054	0.058	0.052	0.008
7	0.254	0.099	0.079	0.081	0.073	0.009
8	0.346	0.128	0.107	0.106	0.098	0.011
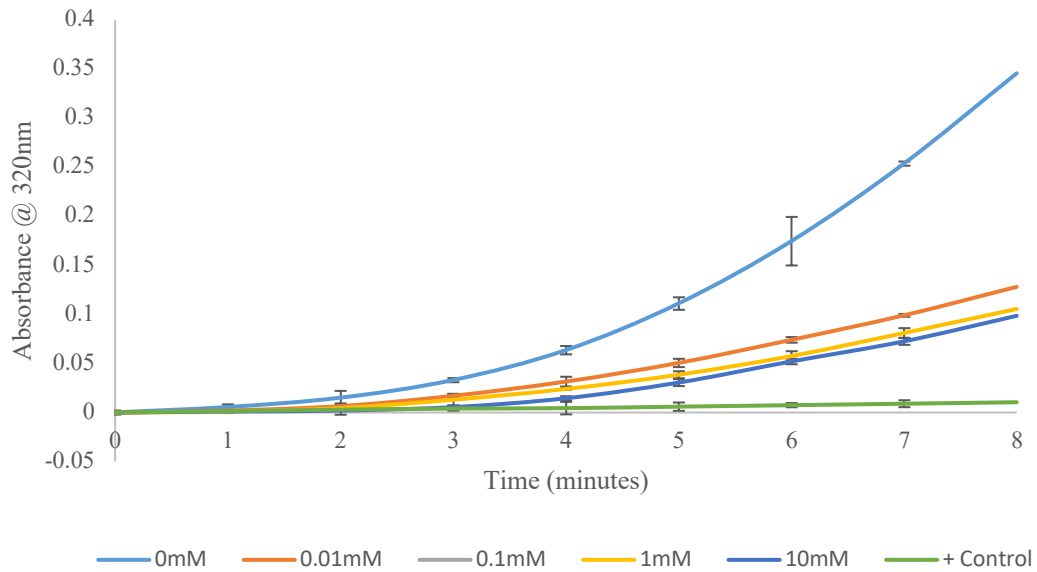
Figure 9 Graph depicting the rate of adrenaline auto-oxidation in semicarbazide pre-treated NRK cell monocultures as an endpoint of the adrenochrome method.

Table 5 Semi-quantification of free radical concentration through the determination of the fold change with respect to the varying concentrations of semicarbazide.

[SEM] (mM)	ΔAbsorbance/min	Fold-change
0	0.0483	0.000 ± 0.00092
0.01	0.0182	2.654 ± 0.00007
0.1	0.0156	3.096 ± 0.00021
1	0.0151	3.199 ± 0.00021
10	0.0141	3.426 ± 0.000028

$$y = 0.1034\ln(x) + 3.1953$$
$$R^2 = 0.926$$
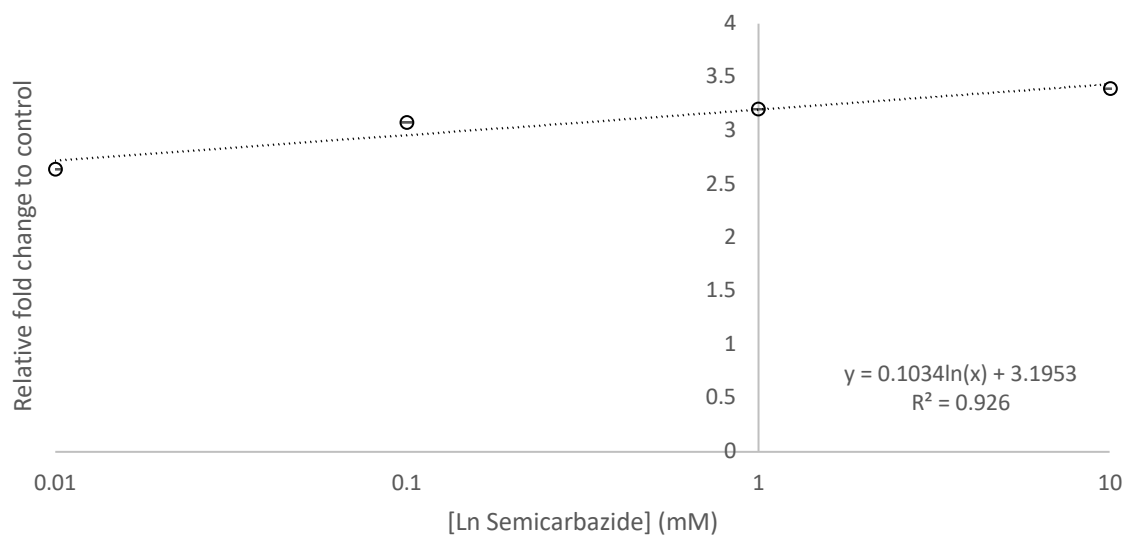
Figure 10 Free radical concentration fold change with respect to semicarbazide concentrations in the adrenochrome/SOD assay.

Table 6 Exemplary quantification of free radical concentration in response to semicarbazide exposure. Quantification was achieved by integrating the molar extinction co-efficient from Bors, Saran and Lengfelder (1978) into the Beer-Lambert equation with the present studies data.

[Semicarbazide] (mM)	[O_2^- and H_2O_2] (μM)
0	15.17
0.01	40.26
0.1	46.96
1	48.52
10	51.97

$$Absorbance = \varepsilon c l$$

$$0.346 = 2.28 x 10^4 \ M^{-1} cm^{-1} * c * 1 cm$$

$$\frac{0.346}{2.28 x 10^4 \ M^{-1} cm^{-1} * 1} = 1.517 x 10^{-5} M \ or \ 15.17 \mu M$$

BioRad™ Protein determination assay for the PKC assay

Table 7 Experimental data for the determination of protein concentration in semicarbazide treated cell lysate.

[BSA] (mg/ml)	Mean Absorbance (630nm) ± S.D.
0	0.000 ± 0.003
0.2	0.323 ± 0.003
0.4	0.483 ± 0.027
0.6	0.612 ± 0.031
0.8	0.791 ± 0.087
1	0.768 ± 0.045
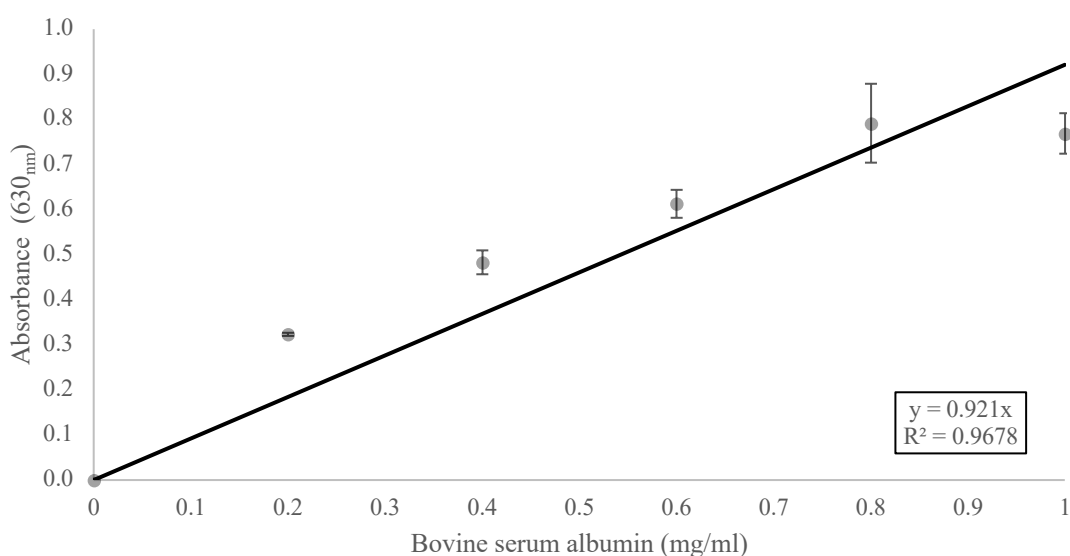
Figure 11 Protein standard curve for the standardization of cell lysates prior to conducting the protein kinase C enzyme linked immunosorbent assay kit.

Table 8 Protein concentration for standardization prior to PKC activity assessment in each cell lysate sample.

| SEM treated (mM)	Mean absorbance (540nm) ± S.D.	[Protein] (mg/ml)
0	0.511 ± 0.029	0.218
0.01	0.478 ± 0.007	0.175
0.1	0.493 ± 0.013	0.194
1	0.504 ± 0.014	0.208
10	0.481 ± 0.035	0.179

Protein kinase C kinase activity assay

Table 9 Spectrophotometric measurements for the construction of an active PKC standard curve to facilitate quantification of active PKC in cell lysates.

[Active PKC] (ng/µl)	Absorbance (450nm)
0	0.000
0.625	0.542
1.25	0.570
2.5	0.916
5	2.107
10	3.489

$y = 0.3661x$
$R^2 = 0.988$
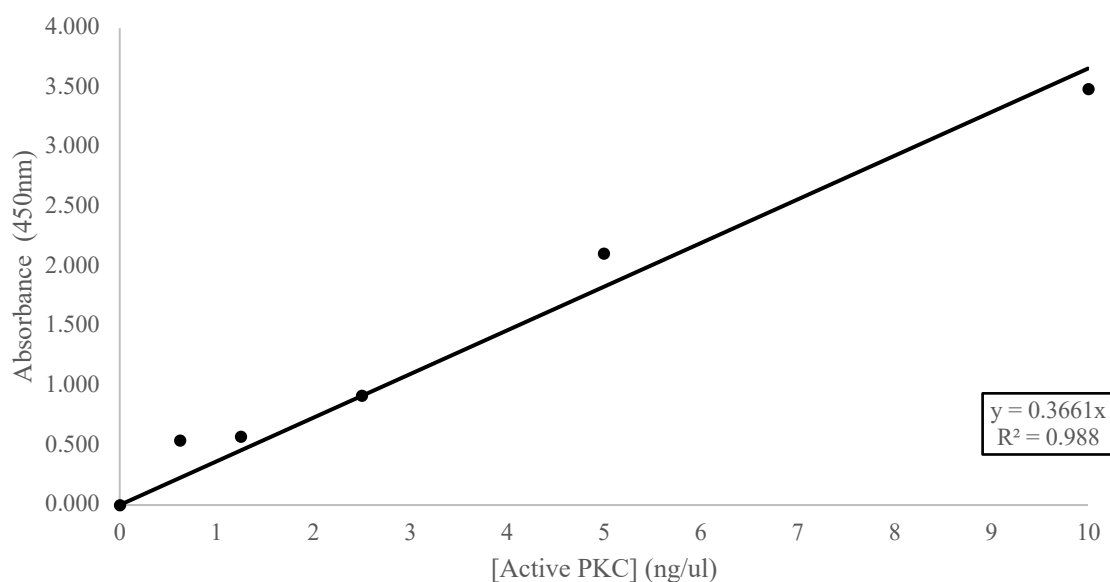
Figure 12 Protein kinase C standard curve for the determination of protein kinase activity in semicarbazide treated cell lysates. Graph was constructed based on single measurements due to the small aliquot of active PKC available.

Table 10 Experimental data for the determination of protein kinase C concentration/lysate and activity in semicarbazide pre-treated NRK cell lysates.

[SEM] (mM)	Mean absorbance (450nm) ± S.D.	[PKC]/lysate (ng/µl)	Relative PKC activity
0	0.24 ± 0.006	0.535	0.000
0.01	0.615 ± 0.023	1.632	2.146
0.1	0.522 ± 0.014	1.360	1.611
1	0.517 ± 0.116	1.345	1.583
10	0.407 ± 0.187	1.023	0.957
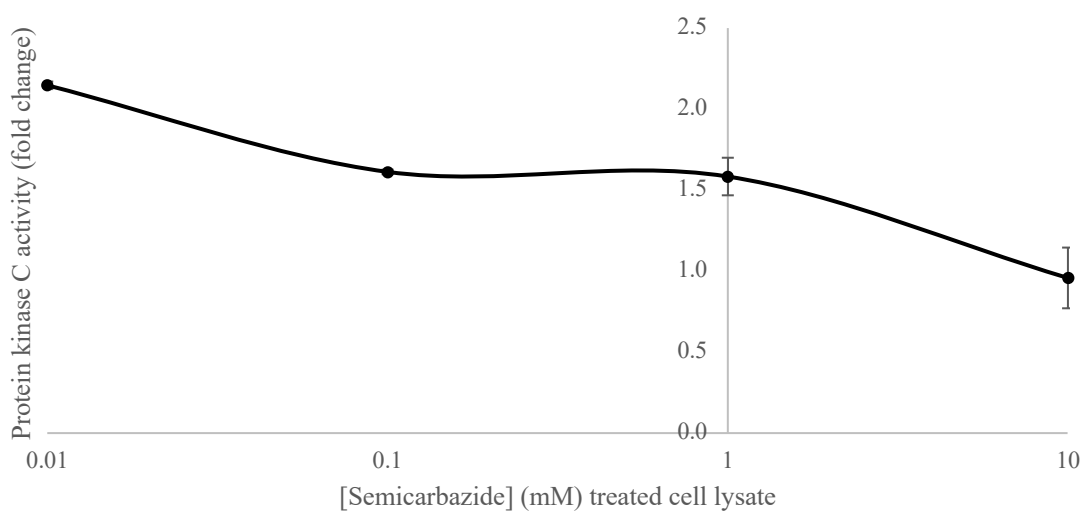
Figure 13 Protein kinase C activity in cell lysates derived from cell monocultures pre-treated with varying concentrations of semicarbazide with respect to the control.

4. Discussion

In order to determine the appropriate concentrations of semicarbazide to use in subsequent experiments. The results outlined in Figure 6 determines two parameters: (1) a dose range of semicarbazide concentrations to be assessed in subsequent assays and (2) facilitates a preliminary assessment of semicarbazide induced cellular proliferation after a 24-hour exposure. Interestingly, Figure 6 indicates that cellular proliferation is dose-dependent with respect to the concentration of semicarbazide. However, at the lowest concentration (0.001mM) of semicarbazide cellular proliferation does not retain linearity but rather achieves peak proliferation at the 0.01mM concentration. A dose range of 0.001mM – 100mM could have been implemented into subsequent assays but the author decided to assess the full range of concentrations through the MTT assay to examine the correlation between the two assay's cellular responses.

The experimental data outlined in Table 3 shows that semicarbazide induces an increase in cell density in a dose dependant manner. Cell density is shown to increase 3.914% at 0.001mM and decreases proportionally as the concentration of semicarbazide increases. At the highest concentration, 1000Mm, the viability of the cell population declined to 73.465% in the MTT assay which correspond with the SRB assay results shown in Table 3 SEM did not elicit a great enough cytotoxicity response in order to

determine an LD_{50}. By assessing the results from both the SRB and MTT assays, the author concluded that a dose-range of 0.01mM to 10mM should be implemented in subsequent assays.

The experimental data in Figure 9 depicts the inhibition of adrenaline oxidation to adrenochrome through a reaction between superoxide dismutase and the free radical, superoxide anion (O_2^-). The 0mM represents the basal activity of adrenochrome conversion via superoxide anion in NRK cell populations. The basal concentration of superoxide anion and hydrogen peroxide remains unclear as scientific cohorts have outlined that these concentrations vary between cell lines and are susceptible to several exogenous parameters. As the basal concentration could not be determined for the present study, the fold difference of SOD activity between the control and the treated samples provides an insight into the changing free radical concentration in response to semicarbazide treatment.

In Figure 9, cell lysates that were pre-treated with increasing concentrations of semicarbazide resulted in a proportional increase in superoxide dismutase activity. The fold change in superoxide dismutase activity in response to SEM exposure is depicted in Figure 10. By assessing the data from the SOD and PKC assay, it was determined that there is an inverse relationship between ROS concentration and PKC activity. It is the author's hypothesis that the 2.654-fold increase in free radical concentration from the 0.01mM concentration has the capacity to alter physiological signaling mechanisms into a sub-pathophysiological state. These changes in the cellular environment could be the initiation mechanism that facilitates the manifestation of cancer in a cell population. The production of secondary signalling molecules such as the membrane associated phospholipase C (PLC), inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) allows for critical cleavage processes for the activation of PKC. Reactive oxygen species such as superoxide anion and hydrogen peroxide are the major radical regulators of protein kinases in the mitogen activated kinase pathway as outlined by (Burton & Jauniaux, 2011).

A study conducted by Abe, *et al.* (1998) demonstrated that hydrogen peroxide concentrations as low as 25µM were sufficient to increase ERK activity via successive activation of PKC, Raf-1 and MEK1 (1998). The early work of Bors, Saran and Lengfelder (1978) focused on the kinetics of adrenaline autoxidation, through which the molar extinction co-efficient for adrenochrome at pH 10.2 and detection at 310nm was determined to be 2.28×10^4 $M^{-1}cm^{-1}$. Incorporation of the molar extinction co-efficient determined by Bors, Saran and Lengfelder (1978) into the present study facilitates an exemplary quantification of free radical concentrations as shown in Table 6 (1978).

The protein kinase C activity assay was conducted in order to determine whether semicarbazide causes an alteration to intracellular signalling/communication pathways. The preparation of the active protein kinase C standard curve was conducted with great precision as a correlation coefficient of 0.9741 was achieved. This value can be attributed to the application of good housekeeping techniques when manipulating the small aliquots of the positive control.

It was determined from Table 10 and Figure 13 that pre-treated NRK monocultures exposed to varying concentrations of semicarbazide expressed a 0.957 – 2.146-fold increase in protein kinase C activity with respect to the basal activity in the negative

control. The degree of protein kinase C upregulation as shown in Table 3.8 can have downstream effects on intracellular signalling pathways. Protein kinase C has been linked with the activation of ras and raf in the MAPK which allows for the transmission to downstream mediators such as mitogen activated protein kinase kinases (MEK), extracellular signal-regulated kinases (ERK) and proto-oncogenes such as *c-fos* and *c-Myc*.

Protein kinase C is an upstream element of ELK-1 in the calcium signalling pathway. ELK-1 is an upstream transcription activator for the serum response element of the *c-fos* proto-oncogene. Besnard et al. (2011) gave insight into how the activation of ELK-1 induces a serum response factor (SRF) to bind to the *c-fos* promoter, which facilitates ELK-1 binding to the *c-fos* promotor. The binding of ELK-1 to SRF occurs due to a protein-protein interaction between the β-domain of ELK-1 and SRF at the protein-DNA region on the α-domain (Besnard et al., 2011).

Regardless of the pathway that is upregulated by protein kinase C, Gruda, et al.(1994) notes that *c-fos* is one of the key downstream proto-oncogenes that is susceptible to increased expression mediated by PKC via the overexpression of Raf, MEK, ERK and ELK-1 (Gruda et al., 1994). The most recent studies in relation to semicarbazide's mechanism of action was conducted by Yu, et al. (2017) and Yue, et al. (2018) using male and female *Danio rerio* respectfully. It was concluded that semicarbazide has the capacity to disrupt the reproductive system in males, but an anti-estrogenic effect was exerted in female subjects. From the results presented in the present study, it can be hypothesised that the results from Yu, et al. (2017) and Yue, et al.(2018) may be mediated through the modulation of protein kinase C. A study by Brandon, *et al.*(2002) demonstrated that the receptor for activated C kinase (RACK-1) and protein kinase C has the capacity to bind to specific moieties on the GABAα receptor. The interaction of RACK-1 and the GABAα receptor, allows for the enhancement of PKC mediated phosphorylation of critical amino acids which subsequently induces PKC dependant modulation of the GABAα receptor. It can be hypothesised that the upregulation of PKC mediated by free radicals could be the initiating step in the manifestation of varied cellular responses to semicarbazide (2002).

5. Conclusion

The present study is one of the first *in-vitro* studies to examine the relationship between sub-pathophysiological concentrations of reactive oxygen species and the anomalous non-genotoxic carcinogenicity induced by semicarbazide through the upregulation of intracellular signalling pathways. A novel finding of the present study was where NRK cells were exposed to µM concentrations of semicarbazide, an inverse relationship between protein kinase C activity and free radical concentration increases > 2-fold. The results of the protein kinase assay gave insight into the relationship between semicarbazide, free radicals and protein kinase C activity. By assessing the data from both the free radical assessment and protein kinase C activity, an inverse relationship was established. The inverse relationship may indicate that the upregulation of protein kinase C activity is dependent on specific threshold of free radicals. It can also be hypothesised that protein kinase C and free radicals may act in a synergistic manner to upregulate downstream mediators of the calcium dependant signalling pathway and the mitogen activation protein kinase pathway. In conclusion, this study classifies semicarbazide's mechanism of non-genotoxic carcinogenicity as mitogenic of

intracellular signaling, based on its capacity to modulate intracellular free radical concentrations and protein kinase C activity.

6. Future Work

This study has shown that semicarbazide's adverse effects may alter the holistic nature of an organism as different responses were observed at varying concentrations. The following are an array of experimental concepts that could provide greater insight into the mechanistic actions of semicarbazide. 1) The upregulation of free radicals in response to semicarbazide exposure accentuates the requirement for a mitochondrial assessment of semicarbazide. This form of study could delineate the source of semicarbazide derived free radicals and weather mitochondrial function is altered as a response. 2) Analytical evaluation of the cell lysate after semicarbazide treatment could highlight a change in the cellular dynamic. This would allow qualitative observations into the holistic effects that non-genotoxins may play within the cellular microenvironment. 3) An evaluation of lipid peroxidation as the attributing factor associated with the loss of viability highlighted in the present study could be beneficial. This study could provide a greater understanding of free radical pathogenesis in response to semicarbazide.

7. Acknowledgements

8. References

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