UNIVERSITY OF
FORWARD
THINKING
WESTMINSTER⌗

Withania somnifera Root Extract Enhances Chemotherapy through 'Priming'

Henley, A., Yang L., Chuang K-L., Sahuri Arisoylu, M., Wu, L-H., Bligh, S.W.A. and Bell, J.D.

RESEARCH ARTICLE

Withania somnifera Root Extract Enhances Chemotherapy through 'Priming'

Aine Brigette Henley[1¤], Ling Yang[2], Kun-Lin Chuang[2], Meliz Sahuri-Arisoylu[1], Li-Hong Wu[3], S. W. Annie Bligh[1]*, Jimmy David Bell[1]

1 Department of Life Sciences, Faculty of Science and Technology, University of Westminster, London, United Kingdom, **2** Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung, Taiwan, **3** Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, People's Republic of China

¤ Current address: Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, Sweden

* a.bligh@westminster.ac.uk
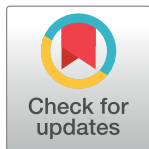
Abstract

Withania somnifera extracts are known for their anti-cancerous, anti-inflammatory and anti-oxidative properties. One of their mechanisms of actions is to modulate mitochondrial function through increasing oxidative stress. Recently 'priming' has been suggested as a potential mechanism for enhancing cancer cell death. In this study we demonstrate that 'priming', in HT-29 colon cells, with *W. somnifera* root extract increased the potency of the chemotherapeutic agent cisplatin. We have also showed the *W. somnifera* root extract enhanced mitochondrial dysfunction and that the underlying mechanism of 'priming' was selectively through increased ROS. Moreover, we showed that this effect was not seen in non-cancerous cells.

Introduction

Cancer is one of the major causes of death around the globe, despite the progress observed in surgery, radiation and chemotherapy [1–3]. Cancer progression relies on the ability of cancer cells to exploit the normal physiological processes of the host [1]. This unfortunately means that the cytotoxicity of chemotherapeutic drugs is not limited to cancer cells and can affect non-cancer cells [1, 4]. Today the most common therapeutic strategy of chemotherapy drugs is the use of two drugs in combination, however this is invariably associated with side effects which include chemoresistance [5–7]. It is therefore essential to explore and enhance current methods of chemotherapy to improve their efficacy while also reducing side effects. One such approach is 'priming', whereby cancer cells are pre-treated with a 'priming' agent (curcumin, quercetin, aspirin) prior to chemotherapy treatment [8–12]. The underlying mechanism underpinning 'priming' appears to be the enhancement of cell death through mitochondrial dysfunction [8, 9]. Mitochondrial dysfunction can alter ROS levels, ATP production and overall cell viability and is a novel key target in cancer treatment [9, 13, 14].

Withania somnifera is an Ayurvedic medicinal plant whose root and leaves extracts have been used for its antioxidant and restorative properties as well as to reduce cancer growth

[2, 6, 15]. *W. somnifera* extracts have been found to be effective in treating several types of cancer including skin, leukaemia, breast, colon and pancreas [15–21]. However, the mechanisms of action have yet to be fully elucidated, but indications of involvement in mitochondrial membrane permeability have been reported in several studies [1, 21–23]. Additionally, *W. somnifera* extracts have been shown to increase reactive oxygen species (ROS) [1, 19, 23]. The mitochondria is an important regulator of cell survival and progression and is the main source of ROS which is linked with mitochondrial function [24]. Cancer cells metabolism is known to have an altered phenotype whereby they primarily respire through lactate production in a process known as the 'Warburg Effect' [25–27]. This alteration in metabolism is a key hallmark of cancer cell progression and has been linked to an alteration in mitochondria function [9, 25]. *W. somnifera* has been reported to induce mitochondrial dysfunction in human leukaemia cells and also reduce mitochondrial function in breast cancer cells [1, 19]. Investigating this mitochondrial alteration further could highlight the value of *W. somnifera* as an anti-tumour agent.

Mitochondrial dysfunction can alter ROS levels, ATP production and overall cell viability and is a novel key target in cancer treatment [9, 13, 14]. In this study we investigated the potential of *W. somnifera* as a 'priming' agent, and showed that 'priming' with this root extract enhanced the efficacy of cisplatin through increased ROS in cancer cells while having no detectable effect on non-cancer cells.

Materials and Methods

Extraction

The extraction method was performed according to the British Pharmacopeia. *Withania somnifera* root powder (1.0 g, Lot no. 6051SS/03, Pukka, UK), was shaken with 2 mL of dilute ammonia R4. Methanol (20 mL) was added and the mixture was sonicated for 20 minutes. It was then heated on the water bath for 3 minutes and filtered. The filtrate was evaporated to dryness at 60˚C. A stock solution of 0.08335 g of dry extract /mL DMSO was prepared for biological studies.

HPTLC

The dry extract was reconstituted in methanol and filtered. The methanolic extract was then applied to a precoated silica gel 60 F_{254} high performance plate (Merck). CAMAG HPTLC System (Automatic TLC Sampler 4; ADC2 Automatic Developing Chamber; TLC Visualizer; Chromatogram Immersion Device III; TLC Plate Heater III; VisionCats software) was used. Application: 2 µL of reference and test solutions. Mobile phase: Toluene, ethyl acetate, formic acid 10:3:1 (v/v/v). Derivatization: 5% sulphuric acid methanol. Dip (time 0, speed 5), heat at 110˚C for 2 min, detection at UV 366 nm.

HPLC

The methanolic root extract (0.3 mg/mL) was analysed using Waters ACQUTTY UPLC—Synapt G2 QTOF (Waters, USA) and a BEH C18 (2.1×100 mm, 1.7 µm) column (Waters, USA) at 40˚C to provide efficiency to the peaks. The mobile phase consisted of water (A) and methanol (B), which were applied in the following gradient elution: from 60:40 (A:B) in 15 min to 20:80(A:B). The flow rate and sample volume were set to 0.3 mL/min. The sample volume was 10 µL. The ESI source was operated in positive (ESI+) ionization mode. The optimized conditions to trigger maximum response of metabolites were listed as follows: capillary voltage, +3 kV; sample cone, + 30 V; extraction cone, +4.0 V; source temperature, 120˚C;

desolvation temperature, 350°C; cone gas (nitrogen) flow, 50 L/h; and desolvation gas (nitrogen) flow, 600 L/h. Argon was used as collision gas. Leucine- enkephalin (2 ng/mL) was used as the lock mass generating a reference ion at m/z of 556.2771 by a lockspray at 5 μL/min to acquire accurate mass during analysis.

Cell culture

Human breast cancer cell line MDA-MB231 and HT-29 colon cancer cells were grown in DMEM (Sigma, UK) with 10% FBS (Sigma), 2% L-Glutamine (Invitrogen, UK) and 2% Penicillin/Streptomycin (P/S) solution (Invitrogen). MDA-MB231 cells were obtained from Dr. T. Kalber (Medical Research Council Clinical Sciences Centre, London) and HT-29 cells were donated by Dr. N. Haiji (Imperial College London). Human non-cancer breast epithelial MCF10A cells were grown in DMEM:F12 (Life Sciences, UK) supplemented with 5% horse serum (Sigma), 2% P/S, 20 ng/mL epidermal growth factor (Sigma), 0.5 mg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma), 10 μg/mL insulin (Sigma). MCF10A cells were donated by Dr. N. Haiji (Imperial College London). All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Treatment conditions

All cells were treated with *W. somnifera* extract (0 μg/mL -10 μg/mL) for 48 h prior to assessment. Primed cells were treated with *W. somnifera* extract (0 μg/mL -10 μg/mL) for 48 h, washed with PBS and incubated with 100 μM Cisplatin for a further 24 h. 'Priming' with quercetin was carried out as follows with 24 h of 40 μM quercetin followed by 100 μM cisplatin for 24 h [11, 28].

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma, UK). All cells were seeded in a 96-well plate at a density between 1.5–3 x10⁴ cells per well and were treated with the various treatment conditions as described above. Cell viability was assayed according to the manufacturers' protocol. Absorbance was determined at 570 nm and normalised with 690 nm background with a micro plate reader (Spectramax 340PC) after 3.25 h. The optical density was measured as a percentage of the control.

ROS assay

Cellular ROS was measured using a 2',7'–dichlorofluorescein diacetate (DCFDA) Assay (Abcam, UK). The fluorescence was detected on a fluorescent plate reader (FLUOstar Omega, BMG Labtech, UK) with an excitation of 495 nm and emission of 529 nm. Cells were seeded at 2 x10⁴ in 96-well black bottom plate. DCFDA incubation was carried out according to the protocol. Cells were treated using the various treatment conditions previously described. The optical density was measured as a percentage of the control and normalised to cell count.

ATP production, proton leak and mitochondrial respiration

ATP, proton leak and mitochondrial/non-mitochondrial respiration was measured using a Seahorse Bioanalyser (Seahorse Biosciences, USA). MDA-MB231, HT-29 and MCF10A cells were seeded 1–2.5 x10⁴ cells per well in a specific Seahorse Bioanalyser 24 well plate and treated appropriately as previously described. The protocol was carried out as specified by the

manufacturer's instructions. Oligomycin (1 µg), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (0.2 µM: MDA-MB231, 0.4 µM: MCF10A, 0.6 µM: HT-29) and antimycin/rotenone (0.25 µM) were added to the sensor plate in the appropriate dilutions directly prior to the commencement of the calibration and assay. Calculations were normalised to protein level using a standard Bradford assay (Biorad).

Assessment of drug-drug interactions

To assess the effects of the combination of *W. somnifera* and cisplatin synergy or antagonism was calculated according to the methods described by Prichard and Shipman [29]. The raw data of 5 experiments was analysed at the 95% confidence interval using the MacSynergy II software developed by Prichard and Shipman. The program calculates the synergy or antagonism of the drug interactions by calculating theoretical additive interactions from the dose-response surface.

Statistical analysis

Statistical analysis was carried out using a one-way ANOVA with Tukey correction and one sample t-test on Graphpad, Prism Software. Results are presented as mean ± SEM. Significance taken when $p < 0.05$.

Results

Cell viability assessment of *W. somnifera* root extract

The direct effect of treating cancer cells with *W. somnifera* root extract was examined using an MTT assay. Cancer and non-cancer cells were treated with a titration of 1 µg/mL, 5 µg/mL and 10 µg/mL *W. somnifera* for 48 h. A significant reduction of cell viability was observed in breast (MDA-MB231) cancer cells compared to non-treatment in a dose dependant manner ($p < 0.01$) (Fig 1A). Similar effect was observed in HT-29 colon cancer cells ($p < 0.05$) (Fig 1B). However, in non-cancer cells (MCF10A) there was no reduction in cell viability compared to non-treatment (Fig 1C).

The effect of *W. somnifera* extract on oxidative stress

Oxidative stress is an important marker of cellular response and can indicate early signs of cell death. There were no changes in ROS levels following treatment with *W. somnifera* alone in
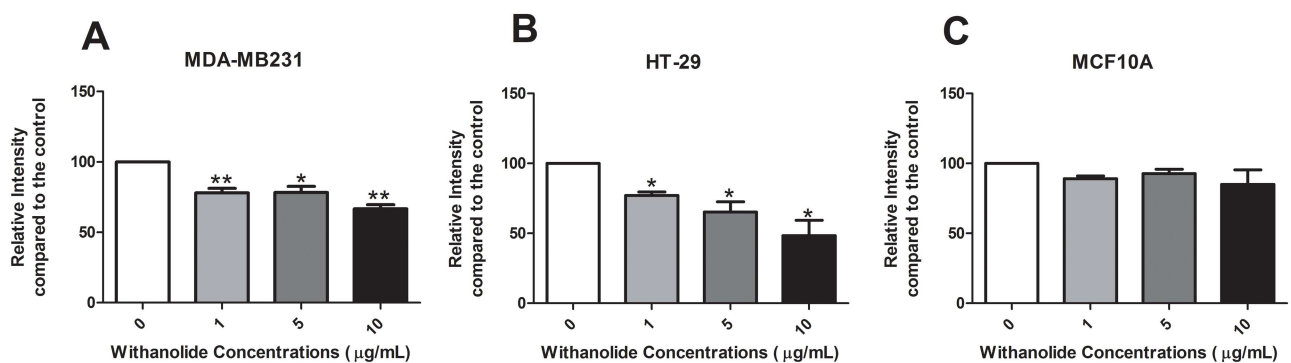

Fig 1. Assessing cell viability of *W. somnifera* root extract on cancer and non-cancer cells. Cells were treated with increasing concentrations (1 µg/mL -10 µg/mL) of *W. somnifera* root extract for 48 h in **(A)** MDA-MB231, **(B)** HT-29 and **(C)** MCF10A cells. Data represent the average of 5 independent experiments ± SEM. *p<0.05, **p<0.01 *vs* non-treatment (0).

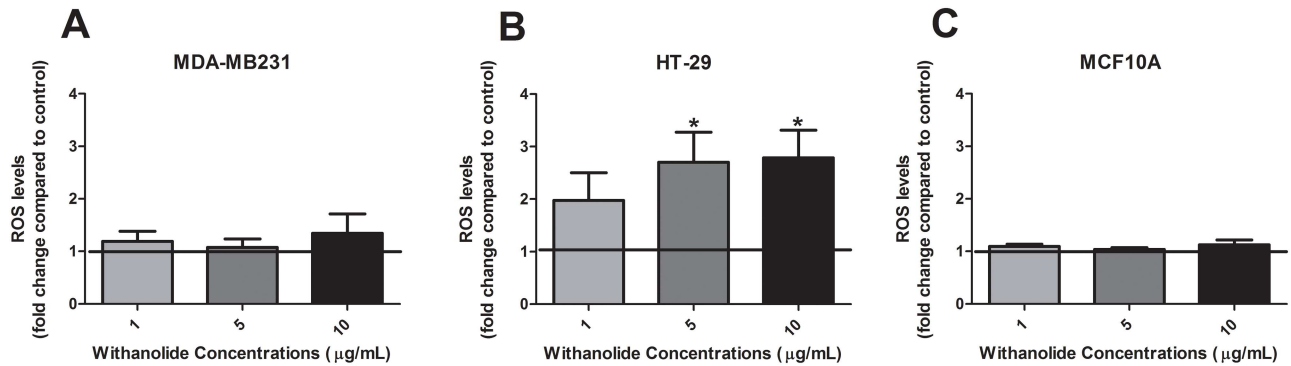doi:10.1371/journal.pone.0170917.g001

Fig 2. Oxidative stress alteration following treatment with *W. somnifera* in cancer and non-cancer cells. ROS levels were measured using a DCFDA assay following 48 h treatment with 1 μg/mL, 5 μg/mL and 10 μg/mL of *W. somnifera* root extract in **(A)** MDA-MB231, **(B)** HT-29 and **(C)** MCF10A cells. The bold line in each graph represents control treatment levels for the experiment. Data represent the average of 5 independent experiments ± SEM. *$p < 0.05$ *vs* non-treatment.

doi:10.1371/journal.pone.0170917.g002

MDA-MB231 cancer cells or MCF10A non-cancer cells (Fig 2A and 2C). However, in HT-29 cancer cells there was a significant increase in ROS levels compared to the control following 48 h of treatment with 5 μg/mL and 10 μg/mL of *W. somnifera* root extract ($p < 0.05$) (Fig 2B).

Mitochondrial function following *W. somnifera* treatment

Mitochondrial basal respiration of MDA-MB231 cells was reduced following 48 h of treatment with 10 μg/mL *W. somnifera* root extract ($p < 0.05$) (Fig 3A). The cell's ATP production levels ($p < 0.01$) and proton leak ($p < 0.05$) were also reduced (Fig 3B, 3C and 3D). However, there was no change in maximal respiration. Interestingly in HT-29 cells this reduction in mitochondrial function was more pronounced for basal respiration ($p < 0.001$), ATP production levels ($p < 0.01$), proton leak ($p < 0.01$) and maximal respiration ($p < 0.01$) (Fig 3E, 3F, 3G and 3H). The mitochondrial function in non-cancer MCF10A cells was unchanged following *W. somnifera* root extract treatment (Fig 3I, 3J, 3K and 3L).

'Priming' with *W. somnifera* root extract enhances chemotherapy

Cisplatin treatment reduced cancer cell growth in MDA-MB231 cells ($p < 0.01$), HT-29 ($p < 0.01$) and MCF10A cells ($p < 0.05$) (Fig 4). The drug combinations of cisplatin and *W. somnifera* were assessed to observe if their activities were synergistic or antagonistic following the Prichard and Shipman equation [29]. The drug-drug interactions were analysed at a 95% confidence limit and displayed as peaks above (synergy) and below (antagonism) a predicted additive plane in a 3-D graph (S3 Fig). MDA-MB231, HT-29 and MCF10A cells were antagonistic following combination of cisplatin and *W. somnifera*.

'Priming' with *W. somnifera* (treatment: 48 h prior to 100 μM cisplatin) showed there was no added effect in MDA-MB231 cells (Fig 4A). However, in HT-29 colon cancer cells 'priming' with 1 μg/mL of *W. somnifera* root extract showed an added effect compared to cisplatin treatment alone ($p < 0.05$) (Fig 4B). This enhanced effect increased with increasing *W. somnifera* root extract concentration ($p < 0.001$). There was no enhanced effect of 'priming' in non-cancer MCF10A cells (Fig 4C). Indeed, there was a slight trend to increased cell viability compared to cisplatin treatment though this did not reach significance.

As a positive control of 'priming' the cells were treated with quercetin, an agent that has been shown to enhance chemotherapy following this 'priming' method [11, 28]. Following the same incubation conditions MDA-MB231 showed a reduction in cell viability following
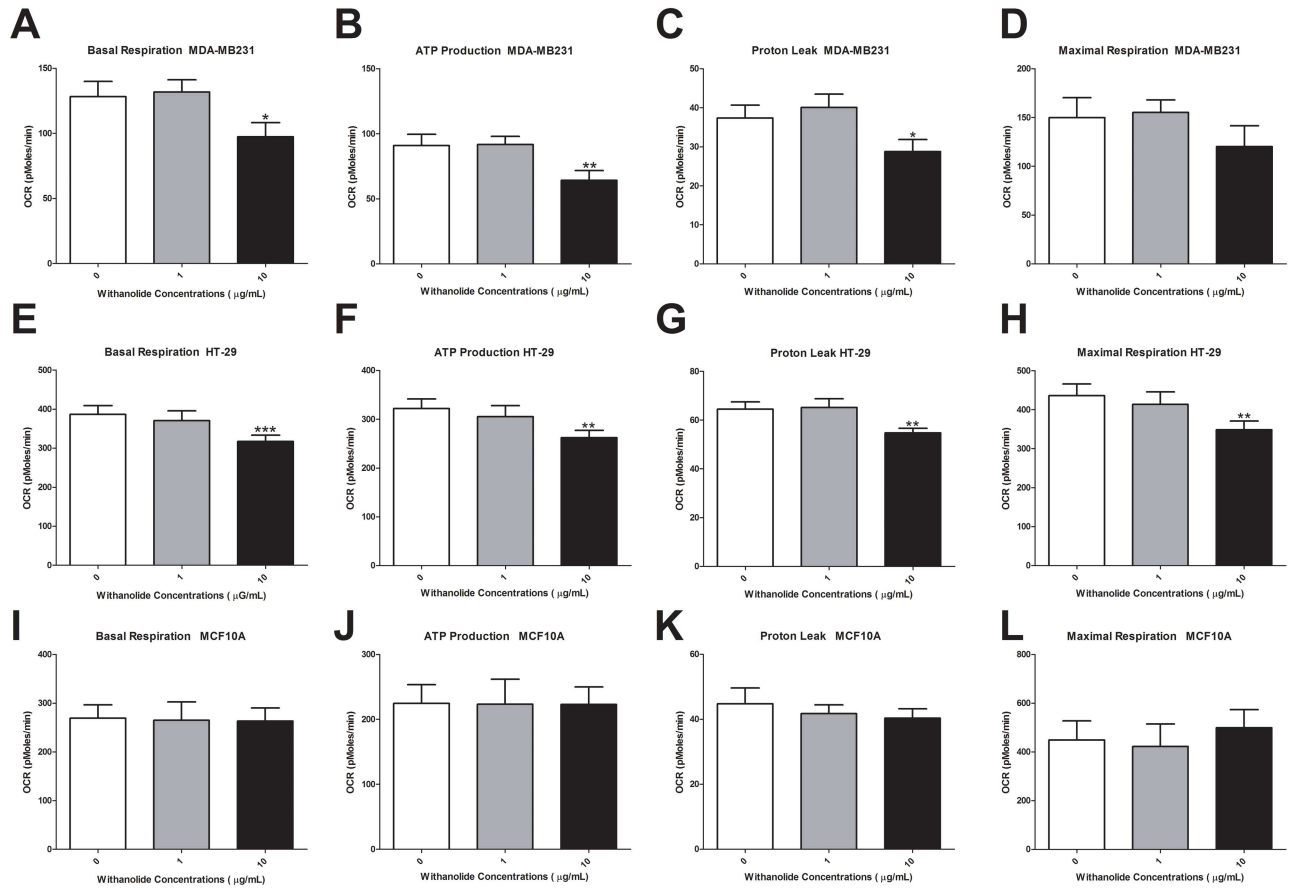
Fig 3. Mitochondrial functional analysis following 48 h of *W. somnifera* root extract treatment in MDA-MB231, HT-29 and MCF10A cells. Mitochondrial function was assessed using a Seahorse Bioanalyser following 48 h of 1 µg/mL and 10 µg/mL *W. somnifera* root extract treatment. Alterations in MDA-MB231 cancer cells **(A)** basal respiration, **(B)** ATP production, **(C)** proton leak and **(D)** maximal respiration was investigated following treatment. The same functional alterations were observed in HT-29 cells (E–H) and in MCF10A cells (I-L). Data represents the average of 3 independent experiments ± SEM. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* non-treatment.

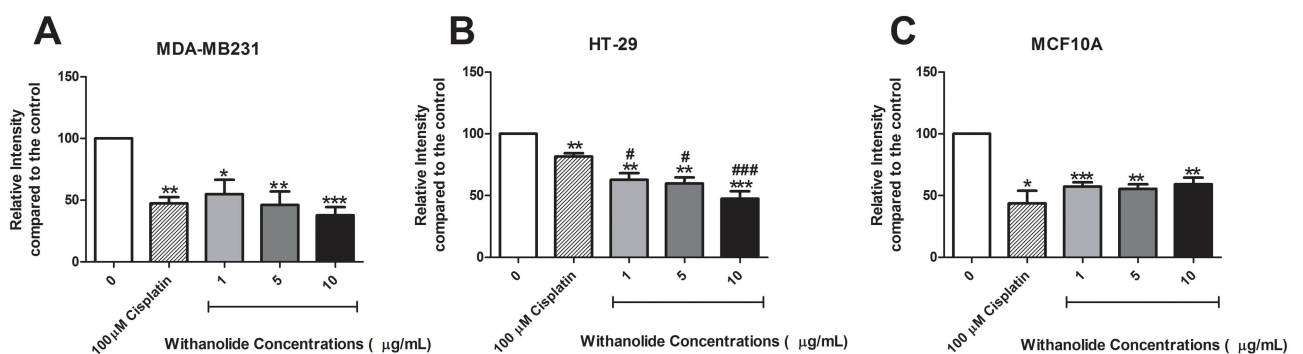doi:10.1371/journal.pone.0170917.g003

Fig 4. The effect of 'priming' with *W. somnifera* root extract prior to cisplatin treatment on cell viability. Cell viability was assessed following 'priming' with *W. somnifera* root extract (1 µg/mL, 5 µg/mL and 10 µg/mL) for 48 h prior to 100 µM cisplatin treatment. **(A)** MDA-MB231, **(B)** HT-29 and **(C)** MCF10A cell viability following 'priming' with *W. somnifera* root extract and 100 µM cisplatin treatment alone. Data represents the average of 5 independent experiments ± SEM. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* non-treatment, # $p<0.05$, ### $p<0.001$ *vs* cisplatin.
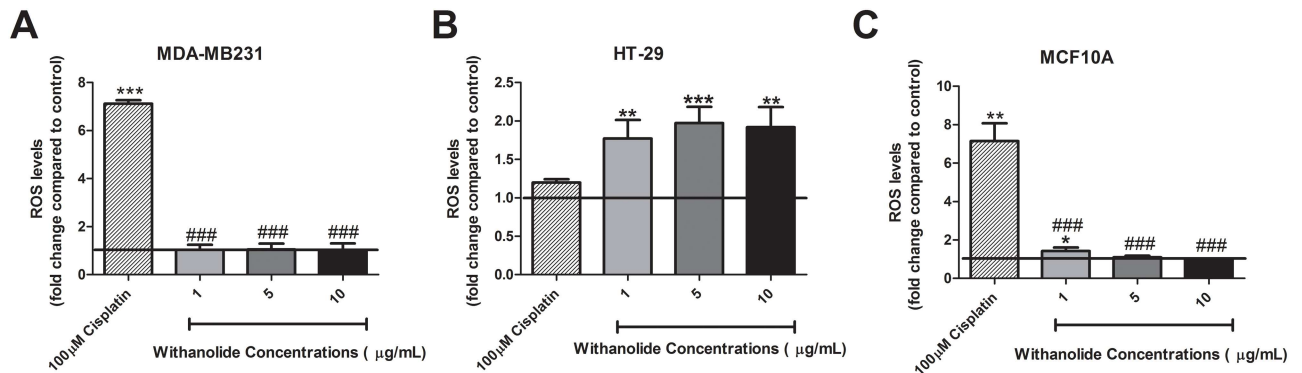
doi:10.1371/journal.pone.0170917.g004

Fig 5. Oxidative stress response following 'priming' with *W. somnifera* root extract. The alteration to oxidative stress levels was assessed following 'priming' with *W. somnifera* from 1 μg/mL to 10 μg/mL 48 h prior to 100 μM cisplatin treatment in **(A)** MDA-MB231, **(B)** HT-29 and **(C)** MCF10A cells. The bold line in each graph represents control treatment levels for the experiment Data represents the average of 5 independent experiments ± SEM. *p<0.05, **p<0.01, ***p<0.001 *vs* non-treatment, # p<0.05, ## p<0.01, ### p<0.001 *vs* cisplatin.

doi:10.1371/journal.pone.0170917.g005

'priming' with quercetin though this was not enhanced compared to cisplatin treated alone (Fig A in S4 Fig). This effect was also observed with HT-29 and MCF10A cells (Figs B and C in S4 Fig).

ROS is key in establishing the effect of 'priming' with *W. somnifera* root extract

When treated with cisplatin there was an increase in ROS levels in MDA-MB231 (p<0.001) HT-29 (p<0.01) and MCF10A (p<0.01) cells (Fig 5).

Interestingly 'priming' with *W. somnifera* reduced the effect cisplatin had on ROS levels in MDA-MB231 cancer cells (p<0.001), bringing it to a sustainable level (Fig 5A). However, in HT-29 cells an increase in ROS levels were observed following 'priming' compared to cisplatin treatment alone (p<0.05). In non-cancer MCF10A cells following 'priming', a reduction of ROS levels was observed compared to cisplatin treatment (p<0.001) (Fig 5C).

'Priming' with *W. somnifera* root extract increases mitochondrial dysfunction

There was no effect of cisplatin treatment on mitochondrial function in MDA-MB231 cells only a slight trend to decrease in basal respiration, ATP production and maximal respiration (Fig 6A, 6B, 6C and 6D). However, the HT-29 cells basal mitochondrial respiration was decreased following cisplatin treatment (p<0.001) along with ATP production levels (p<0.001), proton leak (p<0.001) and maximal respiration (p<0.001) (Fig 6F, 6G, 6H and 6I). Interestingly MCF10A cells also had a reduction in basal respiration following cisplatin treatment (p<0.001) (Fig 6K). There was also a reduced change in proton leak (p<0.001) and maximal respiration (p<0.05) (Fig 6M and 6N), but not in ATP levels.

'Priming' in MDA-MB231 reduced basal respiration rate (p<0.001), ATP production (p<0.001), proton leak (p<0.001) and maximal respiration rate (p<0.001) (Fig 6A, 6B, 6C and 6D). Similarly, HT-29 cells following 'priming' decreased basal respiration (p<0.001), ATP production levels (p<0.001), proton leak (p<0.001) and maximal respiration (p<0.001) (Fig 6F, 6G, 6H and 6I). However, the reduction observed following 'priming' in HT-29 cells was not as reduced as cisplatin treatment alone and the differences observed were significantly different; basal respiration (p<0.001), ATP production (p<0.001), proton leak (p<0.001) and
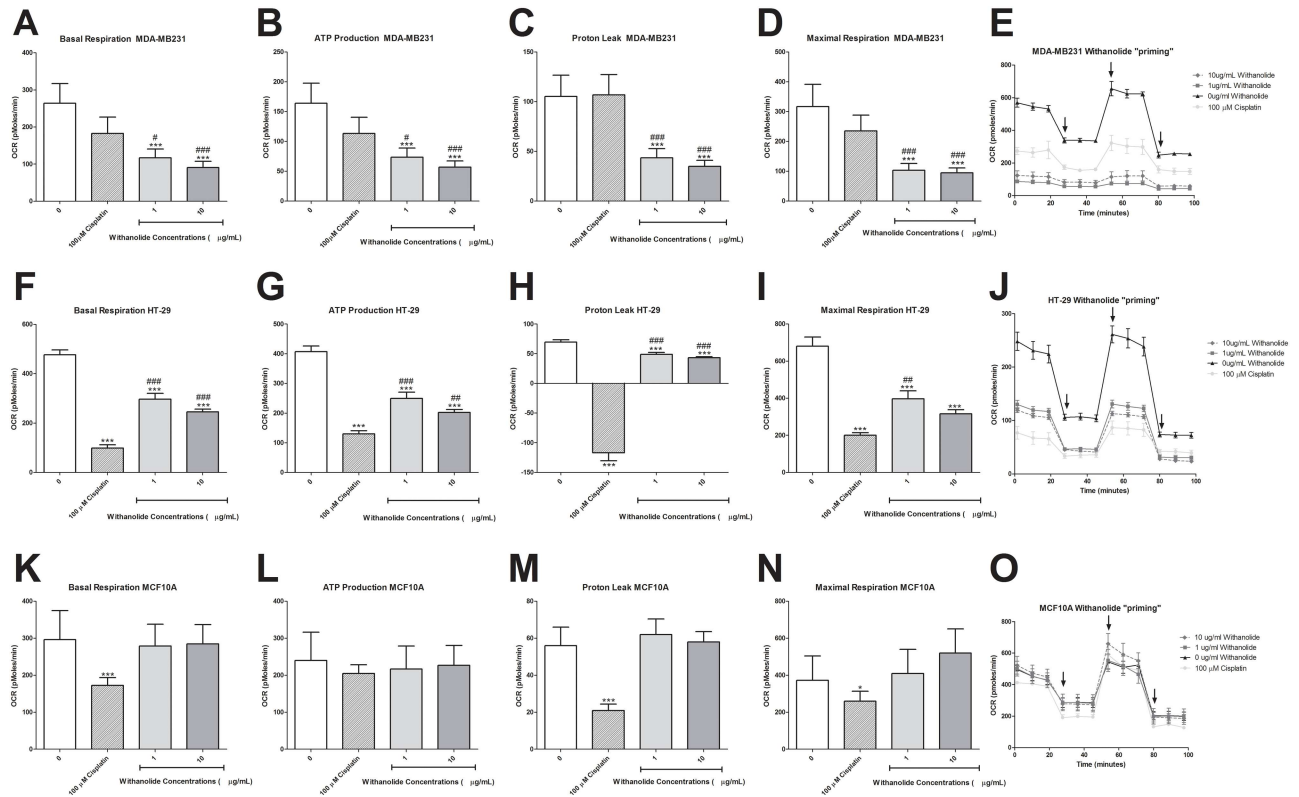
Fig 6. 'Priming' with *W. somnifera* root extract alters mitochondrial function. Mitochondrial function was analysed using the Seahorse Bioanalyser to observe if there was any change following 'priming'. MDA-MB231 **(A)** basal respiration, **(B)** ATP production, **(C)** proton leak and **(D)** maximal respiration was analysed following 'priming' with *W. somnifera* root extract. **(E)** A representative graph of the experiment shows the experimental procedure and injection set up with the first arrow representing oligomycin, second arrow FCCP and third arrow representing antimycin and rotenone. HT-29 cells **(F-J)** and MCF10A cells **(K-O)** were also assessed. Data represents the average of 3 independent experiments ± SEM. *p<0.05, ** p<0.01, *** p<0.001 *vs* non-treatment, # p<0.05, ## p<0.01, ### p<0.001 *vs* cisplatin.

maximal respiration (p<0.01). 'priming' reversed the effects of cisplatin on MCF10A cells mitochondrial function (Fig 6K, 6L, 6M and 6N).

Discussion

This study has shown that 'priming' with *W. somnifera* root extract leads to enhanced cell death in HT-29 colon cancer cells but not in MDA-MB231 cells or MCF10A non-cancer cells. We propose that the 'priming' effect of *W. somnifera* root extract was exclusive to HT-29 cancer cells due to increased ROS production and reduced mitochondrial function.

W. somnifera has many positive medicinal properties which include antitumour, antioxidative and anti-inflammatory effects [1, 19, 20, 30]. The efficacy of Withaferin A in *W. somnifera* has been shown to have an effect on killing breast cancer cells from 1 μM to 5 μM (17,29). Interestingly, in this study using a lower dose of 1 μg/mL *W. somnifera* root extract for 48 h showed a reduction in breast cancer cells and colon cancer cells but had no effect on non-cancer cells. This suggests that lowering the concentration but prolonging the treatment period could have an even greater effect on cancer cell inhibition without effecting normal cells. It has been reported that using 2.5 μM Withaferin A for 6 h can increase oxidative stress in MDA-MB231 cells [19]. However, using *W. somnifera* root extract for 48 h had no effect on oxidative stress in MDA-MB231 cells. There was an increase in oxidative stress in HT-29 cells

which proposes that breast cancer cells are able to protect themselves from *W. somnifera* treatment over a prolonged period.

Although the use of chemotherapeutic agents in killing cancer cells is a very effective treatment, it can lead to several side effects which include chemoresistance [31–33]. An emerging new method of cancer treatment is 'priming' which sensitizes cells to chemotherapy treatment [8–10, 28]. This technique has most potential when looking at aggressive tumours such as triple negative breast cancer, MDA-MB231, which have the worst outcome after chemotherapy than any other breast cancer subtype [34, 35]. Triple negative breast cancers account for 20–25% of all breast cancer cases [36]. The response of this subgroup of breast cancer to treatment relies heavily on chemotherapy as they do not respond to endocrine therapy or trastuzumab [35, 36]. Another tumour that is difficult to treat is the clinically diverse colon cancer which is difficult to treat due to its heterogeneity [37]. Along with its heterogeneity is its poor response to drug treatments which could be in part due to underlying molecular changes altering its genetic stability [38, 39] One such study identified, BRAF inhibition by the small-molecule drug PLX4032 is extremely effective in melanoma treatment but colon cancer cells with the same BRAF lesions do not respond to the same drug [39]. This poor response to drug treatments highlights the importance of developing novel therapeutic treatments. Using this new approach, we showed that 'priming' with *W. somnifera* root extract enhanced the therapeutic effect of cisplatin in HT-29 cells. Furthermore, we showed that there was no added effect in non-cancer cells.

A key characteristic of cancer cells is an alteration in metabolism to a process known as the 'Warburg Effect' [27, 40]. In this process, cancer cells respiration is primarily anaerobic being driven through lactate production rather than through the aerobic citric acid cycle. One key mechanism of 'priming' is mitochondrial dysfunction whereby mitochondrial respiration is reduced due to increased VDAC1 activity and increased oxidative stress [4, 8, 9, 41, 42]. ROS are important constitutively expressed regulators that control both cell survival and cell death [24, 43, 44]. The main source of cellular ROS is from the mitochondria which is also a prime target when excessive ROS is expressed [19, 24, 43, 45, 46]. Uncontrolled levels of ROS can increase cell death through mitochondrial dysfunction [24, 43, 45, 46]. 'Priming' with *W. somnifera* root extract in HT-29 cells increased ROS levels compared to control while also enhancing cell death compared to cisplatin treatment alone. Furthermore, in HT-29 cells there was a reduction in basal mitochondrial respiration, ATP production and maximal respiration suggesting reduced mitochondrial function. The interaction between ATP production and ROS is tightly linked and abnormal activity can lead to TCA cycle damage [14, 23].This mitochondrial profile was replicated with MDA-MB231 cells however there was no change in ROS suggesting that even though the mitochondrial function is reduced there was no added oxidative stress enhancing the effect of cisplatin. There is no added generation of ROS from the mitochondria in the MDA-MB231 cells following 'priming' which is key in opening the mitochondrial membrane and activating cell death [14, 47]. More importantly there was no evidence of added cell death with non-cancer cells, no added ROS concentrations and no alteration to mitochondrial respiration. There was a slight trend to increase in cell viability and maximal respiration rate suggesting the non-cancer cells were increasing their cell growth in response to 'priming' with *W. somnifera* root extract.

Interestingly, both MDA-MB231 and HT-29 cells have mutant p53 (42). However, the mutation present in HT-29 cells, R273H, has been identified to increase drug resistance, proliferation and avoidance of cell death (43). This dominant-negative mutation is one of the most prevalent p53 mutations and is not evident in MDA-MB231 cells (44). This mutation can mask wild-type p53 in the cell to abrogate its function which is crucial in oxidative phosphorylation (27, T44). The reduction in mitochondrial function following 'priming' in

MDA-MB231 cells was more enhanced than cisplatin alone. This effect was not as pronounced in HT-29 cells which could be due to the restoration of the wild-type p53, leading to increased cell death from 'priming' (45). This could explain the difference in mitochondrial function observed between MDA-MB231 and HT-29 cells whereby an effect in cell death was observed in the latter cell type.

To conclude, *W. somnifera* root extract enhances the effect of cisplatin in killing HT-29 cells through increasing ROS and mitochondrial dysfunction. This effect appears to be mainly through increased oxidative stress as there was no 'priming' effect in MDA-MB231 cancer cells. It has been suggested that mitochondria are the gatekeepers to chemotherapy and are ideal therapeutic targets for cancer therapy, therefore the impact of *W. somnifera* in enhancing chemotherapy through mitochondrial dysfunction may prove an important new approach. Further studies are required to examine the molecular mechanism of 'priming' with *W. somnifera* root extract.

Supporting Information

S1 Fig. HPTLC. HPTLC image graphs of the extract samples. Graph A is measured at the wavelength 366 nm and B is the same graph under white RT light. BP means the extraction method is followed by British Pharmacopoeia. Two replications of each extract samples. The Rf value represents 0.09 (Withaferin A), 0.014 (Standard/Withanolide A) 0.24 (Withanolide B),0.6 (β-sitosteol).
(PDF)

S2 Fig. HPLC-MS/MS data. (A) Ashwagandha root extract total ion chromatogram, **(B)** Ashwagandha root extract 471 $[M+H]^+$ ion chromatogram, **(C)** Withanolide, peak 1 mass spectrum, **(D)** Withanolide, peak 2 mass spectrum, **(E)** Withanolide, peak 3 mass spectrum.
(PDF)

S3 Fig. Evaluating the drug-drug interactions upon combining treatments of *W. somnifera* and cisplatin. The interactions of *W. somnifera* and cisplatin was assessed by the methods described in Prichard and Shipman (29). Cell viability was examined following treatment with *W. somnifera* root extract (0 µg/mL -10 µg/mL) in combination with cisplatin (0 µM -150 µM). **(A)** MDA-MB231, **(B)** HT-29 **(C)** MCF10A 3D models and data table sets represent the antagonist effect of the drug-drug interactions. Data represents the average of 5 independent experiments.
(PDF)

S4 Fig. The Effect of 'priming' with Quercetin prior to cisplatin treatment on cell viability. Cell viability was examined following treatment with quercetin (40 µM), cisplatin (100 µM) and 'priming' with quercetin prior to cisplatin treatment. **(A)** MDA-MB231, **(B)** HT-29 **(C)** MCF10A cell viability following treatments. Data represents the average of 3 independent experiments ± SEM. ** $p<0.01$ *vs* non-treatment, ¢ $p<0.05$ *vs* Quercetin.
(PDF)

Author Contributions

Conceptualization: ABH SWAB JDB.

Data curation: ABH LY KLC MSA LW SWAB JDB.

Formal analysis: ABH LY KLC MSA LW.

Funding acquisition: SWAB JDB.

Investigation: ABH LY KLC MSA LW.

Methodology: ABH LY KLC LW.

Project administration: ABH SWAB JDB.

Resources: ABH LY KLC LW MSA SWAB JDB.

Software: ABH LW.

Supervision: ABH SWAB LW JDB.

Validation: ABH LY KLC MSA LW.

Visualization: ABH SWAB JDB.

Writing – original draft: ABH SWAB JDB.

Writing – review & editing: ABH LY KLC LW MSA SWAB JDB.

References

1. Malik F, Kumar A, Bhushan S, Mondhe DM, Pal HC, Sharma R, et al. Immune modulation and apoptosis induction: Two sides of antitumoural activity of a standardised herbal formulation of Withania somnifera. Eur J Cancer. 2009; 45(8):1494–509. doi: 10.1016/j.ejca.2009.01.034 PMID: 19269163

2. Jayaprakasam B, Zhang Y, Seeram NP, Nair MG. Growth inhibition of human tumor cell lines by withanolides from Withania somnifera leaves. Life Sci. 2003; 74(1):125–32. PMID: 14575818

3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016; 66(1):7–30. doi: 10.3322/caac.21332 PMID: 26742998

4. Galluzzi L, Larochette N, Zamzami N, Kroemer G. Mitochondria as therapeutic targets for cancer chemotherapy. Oncogene. 2006; 25(34):4812–30. Epub 2006/08/08. doi: 10.1038/sj.onc.1209598 PMID: 16892093

5. Rajeswaran A, Trojan A, Burnand B, Giannelli M. Efficacy and side effects of cisplatin- and carboplatin-based doublet chemotherapeutic regimens versus non-platinum-based doublet chemotherapeutic regimens as first line treatment of metastatic non-small cell lung carcinoma: A systematic review of randomized controlled trials. Lung Cancer. 2008; 59(1):1–11. doi: 10.1016/j.lungcan.2007.07.012 PMID: 17720276

6. Biswal BM, Sulaiman SA, Ismail HC, Zakaria H, Musa KI. Effect of Withania somnifera (Ashwagandha) on the development of chemotherapy-induced fatigue and quality of life in breast cancer patients. Integr Cancer Ther. 2013; 12(4):312–22. doi: 10.1177/1534735412464551 PMID: 23142798

7. Florea AM, Busselberg D. Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. Cancers (Basel). 2011; 3(1):1351–71. Epub 2011/01/01.

8. Chonghaile TN, Sarosiek KA, Vo TT, Ryan JA, Tammareddi A, Moore VD, et al. Pretreatment Mitochondrial Priming Correlates with Clinical Response to Cytotoxic Chemotherapy. Science. 2011; 334 (6059):1129–33. doi: 10.1126/science.1206727 PMID: 22033517

9. Sarosiek KA, Ni Chonghaile T, Letai A. Mitochondria: gatekeepers of response to chemotherapy. Trends Cell Biol. 2013; 23(12):612–9. doi: 10.1016/j.tcb.2013.08.003 PMID: 24060597

10. Kuhar M, Sen S, Singh N. Role of mitochondria in quercetin-enhanced chemotherapeutic response in human non-small cell lung carcinoma H-520 cells. Anticancer Res. 2006; 26(2A):1297–303. PMID: 16619537

11. Kuhar M, Imran S, Singh N. Curcumin and Quercetin Combined with Cisplatin to Induce Apoptosis in Human Laryngeal Carcinoma Hep-2 Cells through the Mitochondrial Pathway. Journal of Cancer Molecules. 2007; 3(4):121–8.

12. Kumar A, Singh SM. Priming effect of aspirin for tumor cells to augment cytotoxic action of cisplatin against tumor cells: implication of altered constitution of tumor microenvironment, expression of cell

cycle, apoptosis, and survival regulatory molecules. Mol Cell Biochem. 2012; 371(1–2):43–54. doi: 10.1007/s11010-012-1421-9 PMID: 22893064

13. Jiang K, Wang W, Jin X, Wang Z, Ji Z, Meng G. Silibinin, a natural flavonoid, induces autophagy via ROS-dependent mitochondrial dysfunction and loss of ATP involving BNIP3 in human MCF7 breast cancer cells. Oncol Rep. 2015; 33(6):2711–8. doi: 10.3892/or.2015.3915 PMID: 25891311

14. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol. 2004; 287(4):C817–33. Epub 2004/09/10. doi: 10.1152/ajpcell.00139.2004 PMID: 15355853

15. Muralikrishnan G, Dinda AK, Shakeel F. Immunomodulatory effects of Withania somnifera on azoxymethane induced experimental colon cancer in mice. Immunol Invest. 2010; 39(7):688–98. doi: 10.3109/08820139.2010.487083 PMID: 20840055

16. Yadav B, Bajaj A, Saxena M, Saxena AK. In Vitro Anticancer Activity of the Root, Stem and Leaves of Withania Somnifera against Various Human Cancer Cell Lines. Indian J Pharm Sci. 2010; 72(5):659–63. doi: 10.4103/0250-474X.78543 PMID: 21695006

17. Yu Y, Hamza A, Zhang T, Gu M, Zou P, Newman B, et al. Withaferin A targets heat shock protein 90 in pancreatic cancer cells. Biochem Pharmacol. 2010; 79(4):542–51. doi: 10.1016/j.bcp.2009.09.017 PMID: 19769945

18. Hahm ER, Lee J, Huang Y, Singh SV. Withaferin a suppresses estrogen receptor-α expression in human breast cancer cells. Mol Carcinog. 2011; 50(8):614–24. doi: 10.1002/mc.20760 PMID: 21432907

19. Hahm ER, Moura MB, Kelley EE, Van Houten B, Shiva S, Singh SV. Withaferin A-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species. PLoS One. 2011; 6(8):e23354. doi: 10.1371/journal.pone.0023354 PMID: 21853114

20. Halder B, Singh S, Thakur SS. Withania somnifera Root Extract Has Potent Cytotoxic Effect against Human Malignant Melanoma Cells. PLoS One. 2015; 10(9):e0137498. doi: 10.1371/journal.pone.0137498 PMID: 26334881

21. Li X, Zhu F, Jiang J, Sun C, Wang X, Shen M, et al. Synergistic antitumor activity of withaferin A combined with oxaliplatin triggers reactive oxygen species-mediated inactivation of the PI3K/AKT pathway in human pancreatic cancer cells. Cancer Lett. 2015; 357(1):219–30. doi: 10.1016/j.canlet.2014.11.026 PMID: 25444914

22. Oh JH, Lee TJ, Kim SH, Choi YH, Lee SH, Lee JM, et al. Induction of apoptosis by withaferin A in human leukemia U937 cells through down-regulation of Akt phosphorylation. Apoptosis. 2008; 13(12):1494–504. doi: 10.1007/s10495-008-0273-y PMID: 19002588

23. Malik F, Kumar A, Bhushan S, Khan S, Bhatia A, Suri KA, et al. Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic cell death of human myeloid leukemia HL-60 cells by a dietary compound withaferin A with concomitant protection by N-acetyl cysteine. Apoptosis. 2007; 12(11):2115–33. doi: 10.1007/s10495-007-0129-x PMID: 17874299

24. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. Mol Cell. 2012; 48(2):158–67. doi: 10.1016/j.molcel.2012.09.025 PMID: 23102266

25. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5):646–74. doi: 10.1016/j.cell.2011.02.013 PMID: 21376230

26. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. Science. 2009; 324(5930):1029–33. doi: 10.1126/science.1160809 PMID: 19460998

27. Warburg O, Wind F, Negelein E. THE METABOLISM OF TUMORS IN THE BODY. J Gen Physiol. 1927; 8(6):519–30. PMID: 19872213

28. Sharma H, Sen S, Singh N. Molecular pathways in the chemosensitization of cisplatin by quercetin in human head and neck cancer. Cancer Biol Ther. 2005; 4(9):949–55. Epub 2005/08/06. PMID: 16082193

29. Prichard MN, Shipman C Jr. A three-dimensional model to analyze drug-drug interactions. Antiviral Res. 1990; 14(4–5):181–205. PMID: 2088205

30. Kumar P, Kumar A. Possible neuroprotective effect of Withania somnifera root extract against 3-nitropropionic acid-induced behavioral, biochemical, and mitochondrial dysfunction in an animal model of Huntington's disease. J Med Food. 2009; 12(3):591–600. doi: 10.1089/jmf.2008.0028 PMID: 19627208

31. Arisan ED, Kutuk O, Tezil T, Bodur C, Telci D, Basaga H. Small inhibitor of Bcl-2, HA14-1, selectively enhanced the apoptotic effect of cisplatin by modulating Bcl-2 family members in MDA-MB-231 breast cancer cells. Breast Cancer Res Treat. 2010; 119(2):271–81. Epub 2009/02/25. doi: 10.1007/s10549-009-0343-z PMID: 19238538

32. Caley A, Jones R. The principles of cancer treatment by chemotherapy. Surgery (Oxford). 2012; 30 (4):186–90. Epub 27 March 2012.

33. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, et al. Molecular mechanisms of cisplatin resistance. Oncogene. 2012; 31(15):1869–83. doi: 10.1038/onc.2011.384 PMID: 21892204

34. Pille JY, Denoyelle C, Varet J, Bertrand JR, Soria J, Opolon P, et al. Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. Mol Ther. 2005; 11(2):267–74. doi: 10.1016/j.ymthe.2004.08.029 PMID: 15668138

35. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med. 2010; 363 (20):1938–48. doi: 10.1056/NEJMra1001389 PMID: 21067385

36. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol. 2008; 26(8):1275–81. doi: 10.1200/JCO.2007.14.4147 PMID: 18250347

37. De Sousa EMF, Wang X, Jansen M, Fessler E, Trinh A, de Rooij LP, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nat Med. 2013; 19(5):614–8. doi: 10.1038/nm.3174 PMID: 23584090

38. Muller MF, Ibrahim AE, Arends MJ. Molecular pathological classification of colorectal cancer. Virchows Arch. 2016; 469(2):125–34. doi: 10.1007/s00428-016-1956-3 PMID: 27325016

39. Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature. 2012; 483 (7387):100–3. doi: 10.1038/nature10868 PMID: 22281684

40. Samudio I, Fiegl M, Andreeff M. Mitochondrial uncoupling and the Warburg effect: molecular basis for the reprogramming of cancer cell metabolism. Cancer Res. 2009; 69(6):2163–6. Epub 2009/03/05. doi: 10.1158/0008-5472.CAN-08-3722 PMID: 19258498

41. Huang H, Hu X, Eno CO, Zhao G, Li C, White C. An interaction between Bcl-xL and the voltage-dependent anion channel (VDAC) promotes mitochondrial Ca2+ uptake. J Biol Chem. 2013; 288(27):19870–81. Epub 2013/05/31. doi: 10.1074/jbc.M112.448290 PMID: 23720737

42. Zhang X, Fryknäs M, Hernlund E, Fayad W, De Milito A, Olofsson MH, et al. Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments. Nat Commun. 2014; 5.

43. Mei Y, Thompson MD, Cohen RA, Tong X. Autophagy and oxidative stress in cardiovascular diseases. Biochim Biophys Acta. 2014.

44. Gupta SC, Hevia D, Patchva S, Park B, Koh W, Aggarwal BB. Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy. Antioxid Redox Signal. 2012; 16(11):1295–322. doi: 10.1089/ars.2011.4414 PMID: 22117137

45. Ferber EC, Peck B, Delpuech O, Bell GP, East P, Schulze A. FOXO3a regulates reactive oxygen metabolism by inhibiting mitochondrial gene expression. Cell Death Differ. 2012; 19(6):968–79. doi: 10.1038/cdd.2011.179 PMID: 22139133

46. Lee J, Giordano S, Zhang J. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. Biochem J. 2012; 441(2):523–40. doi: 10.1042/BJ20111451 PMID: 22187934

47. Tiwari BS, Belenghi B, Levine A. Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. Plant Physiol. 2002; 128(4):1271–81. doi: 10.1104/pp.010999 PMID: 11950976