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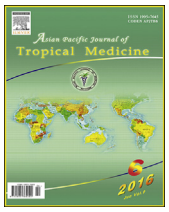

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Nevirapine induces apoptosis in liver (HepG2) cells

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ABSTRACT

Objective: To generate insights into the mechanism of NVP induced hepatotoxicity.
Methods: Liver (HepG2) cells were cultured with various concentrations of NVP. This cell line was chosen because it has low expression of cytochrome P450, allowing evaluation of the effects of NVP rather than specific metabolites. Cytotoxicity was determined using a proliferation assay and cell numbers were monitored using trypan blue exclusion assay for long term culture experiments and apoptosis induction was determined by morphological and biochemical investigation.
Results: HepG2 cells treated with the highest concentration of NVP tested (819 μM) initially showed a rounded morphology and all cells had died by week three of exposure. Nuclear condensation and fragmentation, increased Annexin V/propidium iodide staining and caspase 9 activation all supported the induction of apoptosis in HepG2 cells in response to NVP treatment.
Conclusions: There is a clear induction of apoptosis in response to NVP which suggests that NVP has significant cytotoxicity, over and above any cytotoxicity of metabolites and may contribute directly to patient hepatotoxicity.

1. Introduction

Nevirapine (NVP) is classified as a non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) and is frequently used in combination with other antiretroviral drugs for the treatment of HIV infections in resource limited settings [1]. NNRTIs bind near the catalytic site of reverse transcriptase and limit the ability of the enzyme to change conformation, resulting in increased enzyme rigidity and inhibiting the polymerization function of the enzyme [2]. However, the main drawback to the use of NVP is the high risk of hepatotoxicity, and NVP induced hepatotoxicity remains poorly understood. NVP-associated liver toxicity is believed to occur into two main ways. The first is a drug hypersensitivity reaction or immune-mediated mechanism typically occurring within the first 6–12 weeks of treatment and is associated with elevation of transaminases and skin reactions [3]. The second mechanism of NVP-related liver toxicity typically has a delayed onset occurring some 4–5 months after start of the treatment [4] and it was shown that NVP has a greater association with hepatotoxicity than a similar NNRTI, efavirenz [4].

For acute toxicity, in humans NVP undergoes cytochrome P450-mediated oxidation to 2-, 3-, 8- and 12-hydroxy-nevirapine (12-OH-NVP) which subsequently undergo glucuronidation followed by excretion [5]. Animal model studies have suggested that 12-OH-NVP (formed by oxidation of an exocyclic methyl group) can directly induce a skin rash, while the further metabolite quinone methide (either produced directly from a benzylic free radical intermediate in the generation of 12-OH-NVP or via sulfation to generate a 12-sulfoxy-NVP intermediate and subsequent desulfation) can induce both the skin rash and hepatotoxicity

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directly [6]. Other studies have suggested that the metabolite 12-sulfoxy-NVP is able to form specific covalently linked protein adducts. Using the synthetic model electrophile 12-mesyloxy-NVP, a number of sites of modification of human serum albumin and hemoglobin were identified [7], and stable hemoglobin NVP adducts were detected in 12 out of 13 samples from HIV patients undergoing antiretroviral NVP therapy [8].

While the mechanism remains incompletely known, it has been proposed that the covalent binding of NVP to liver cell proteins may induce cell death through apoptosis and necrosis [9]. This is supported by studies that show mitochondrial depolarization of lymphocytes in HIV-1 infected patients undergoing NVP treatment [10]. While studies have consistently shown a reduction in cell proliferation and modulation of the cell cycle in response to NVP treatment, some authors have suggested an increase in differentiation [11], while others have proposed that there is an increase in cell senescence [12]. Given the widespread use of NVP, and the significant risk of hepatotoxicity, this study sought to investigate the mechanism of NVP induced hepatotoxicity in long term treatment. We postulated that long term treatment causes an accumulation of NVP-free form over the ability of phase I enzymes to combat, as in patient who develop liver toxicity after long term treatment with NVP. To explore this HepG2 cells were chosen as the model cell in this study as they are highly differentiated and have many features of normal liver cells and these cells have been used by others to investigate the mechanism of NVP induced hepatotoxicity [13]. Significantly however, HepG2 cells have low levels of phase I and phase II enzymes [14] allowing dissection of the direct effect of NVP on liver cells.

2. Materials and methods

2.1. Cultivation of HepG2, NVP treatment and cell viability assays

The HepG2 cell line was acquired from ATCC (ATCC-Number HB-8065) and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a tissue culture flask at 37 °C in 5% CO_2. NVP (Sigma, St. Louis, MO) was made up as a 0.273 M stock in DMSO. To determine the IC_{50} for NVP cells were incubated with various concentrations of NVP for 24 h before analysis of cell viability using the CellTiter 96® Aqueous One Solution Cell Proliferation assay (MTS, Promega, WI) according to the manufacturer's recommendations. For long term culture experiments cells were incubated in the presence or absence (control) of NVP with replacement of the medium twice a week. Once a week the cells were trypsinized, counted by trypan blue dye exclusion assay and further cultured as a 1:3 dilution. All experiments were conducted independently in triplicate.

2.2. Confocal microscopy

HepG2 cells were grown to 80% confluence on 12-mm coverslips under standard conditions for 24 h. The cells culture medium was removed and then washed with PBS. The cells were incubated with and without NVP (3, 30.3, 91 and 819 μM) with media replacement (with or without NVP as appropriate) on day 4 of culture. The coverslips were collected at day 7. Moreover, the floating (detached) cells were collected on days 4 and 7 by centrifugation using cytospin column as described elsewhere [15]. The cells were fixed with 100% ice-cold methanol at room temperature for 15 min followed by washing two times with PBS. The cells were permeabilized with 0.3% Triton X-100/PBS for 10 min in humidified chamber and then washed twice with 0.03% Triton X-100/PBS at room temperature with gentle agitation. The cells were incubated with a 1:250 dilution of DAPI diluted in PBS at room temperature and dark in humidified chamber for 1 h follow by washing six times with 0.03% Triton X-100/PBS. The coverslips were mounted onto glass slides using Prolong® Gold antifade reagent (Invitrogen) before visualization under an Olympus FluoView 1000 (Olympus Corporation, Shinjuku-ku, Tokyo) with a 60× objective lens and equipped with Olympus FluoView Software v. 1.6.

2.3. Western blot analysis

HepG2 cells were cultured under standard conditions with or without nevirapine as appropriate. On day 7 treatment, the culture medium was discarded and the cells were washed with cooled PBS and lysed with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). The cell lysates were incubated on ice for 30 min with periodic mixing and sonicated twice at 4 °C for 5 min before centrifugation at 10000 ×g for 10 min after which supernatants were collected. Proteins were quantified by the Bradford assay and stored at −80 °C until use. A total of 20 μg of total proteins were separated by electrophoresis through a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting. The membrane was incubated with a 1:1000 dilution of an anti-human caspase 9 rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA) followed by a 1:8000 dilution of a HRP-conjugated goat anti-rabbit IgG polyclonal antibody (Thermo Fisher Scientific Inc., Rockford IL). The membrane was subsequently re-probed with a 1:1000 dilution of an anti-human GAPDH mouse monoclonal antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) followed by a 1:5000 dilution of a HRP-conjugated goat anti mouse IgG polyclonal antibody (Sigma–Aldrich Co. Saint-Louis, MO). Signal was recorded by an Azure Biosystems c400 quantitative western blot imaging system (Azure Biosystems, Dublin, CA).

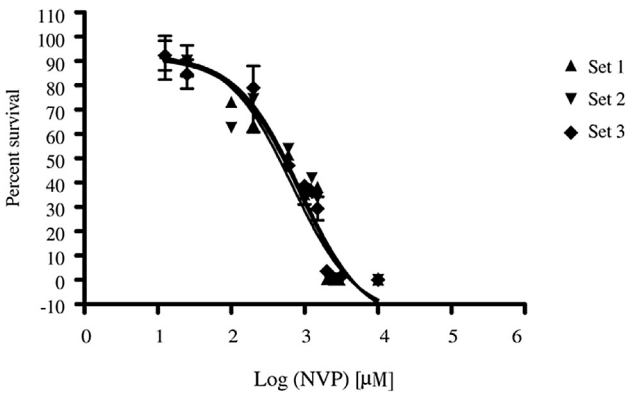

Figure 1. Cytotoxicity of nevirapine to HepG2 cells as assessed by MTS assays after 24 h of exposure. Experiment was conducted independently in triplicate. Error bars show S.D.

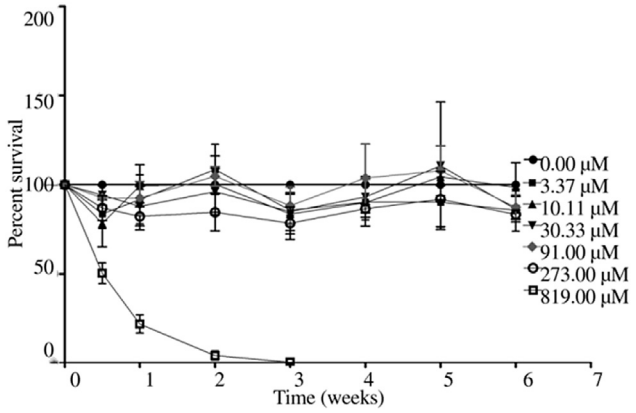

Figure 2. The 6-week cytotoxicity profile of nevirapine in HepG2 cells. The cells were treated with different concentration of the compound and are counted once a week by means of the trypan blue dye exclusion assay. The control cells were treated with 0.3% DMSO. The results are expressed as a percentage of the control.

2.4. Annexin V/propidium iodide staining

Control and nevirapine treated cells were collected on day 7 and washed with ice-cold PBS and resuspended in binding buffer (BD, Franklin Lakes, NJ), followed by double staining with the addition of 50 μg/mL FITC-conjugated Annexin V and 20 μg/mL propidium iodide. After 15 min, the cells were analyzed by flow cytometry on a FACSCalibur cytometer (BD Biosciences, San Jose, CA) using CELLQuest™ software.

2.5. Data analysis

IC_{50} curves were generated using the program GraphPad Prism version 5.0. Percent survival (Y axis) versus log concentration of nevirapine (X axis) was plotted. The IC_{50} was calculated by the software. Each experiment was done independently in triplicate with duplicate analysis. The graphs of 6-week cytotoxicity profiles of NVP in HepG2 cells were plotted using GraphPad Prism version 5.0. Percent survival (Y axis) was calculated in percent of the control versus time (X axis) was plotted.

3. Results

3.1. IC_{50} of NVP in HepG2 cells

To determine the IC_{50} of NVP, 4×10^3 HepG2 cells were incubated in wells of a 96 well plate with 50–3 000 μM NVP for 24 h under standard conditions after which cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay. Experiment was undertaken independently

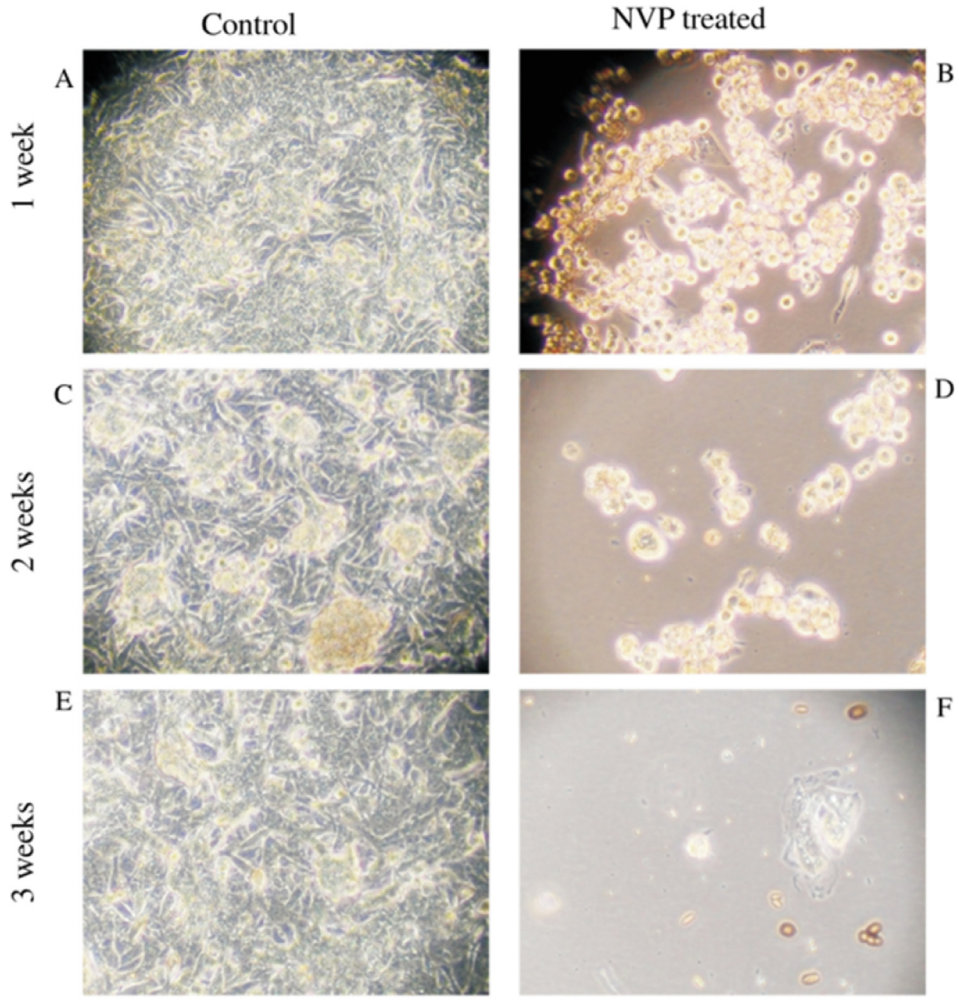

Figure 3. Cell morphology of control (0.3 μM DMSO) and treated (819 μM NVP) HepG2 cells over three weeks of treatment as observed under an Olympus light microscope with magnification ×200.

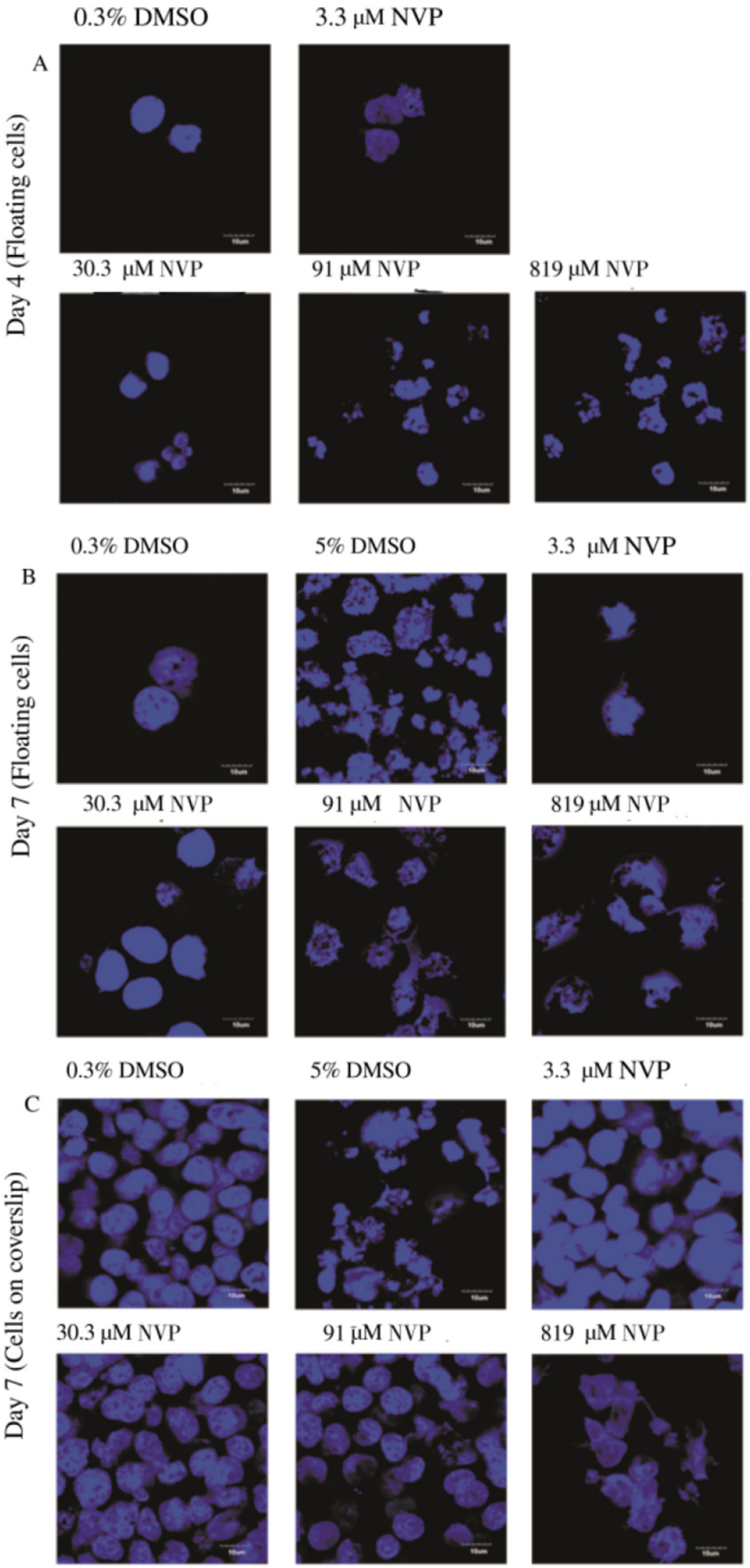

Figure 4. Nuclear morphology of control (0.3% and 5% DMSO) and NVP treated (at concentration indicated) HepG2 cells.
(A) Floating cells collected on day 4 of treatment (B) floating cells collected on day 7 of treatment and (C) attached cells collected on day 7 of treatment. Cells were stained with DAPI and observed under an Olympus FluoView 1000 confocal microscope at magnification ×60.

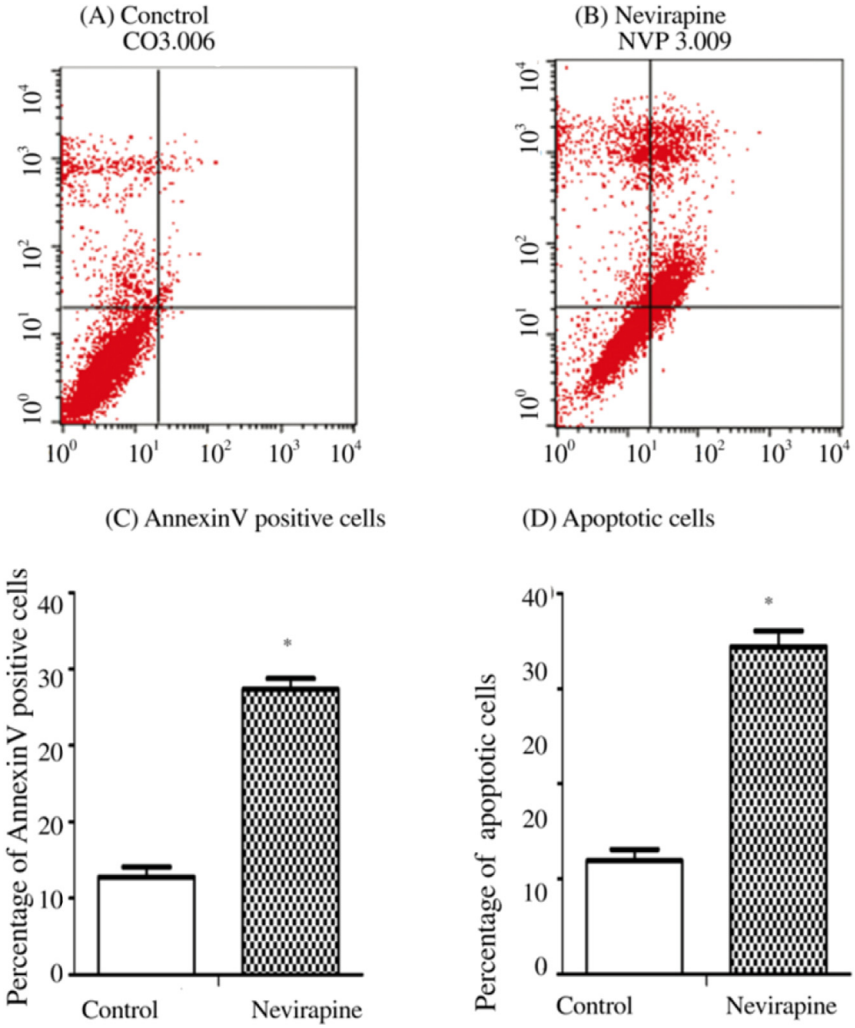

Figure 5. Analysis of Annexin V/propidium iodide staining of control and NVP treated HepG2 cells. Cells were not treated (control) or treated with NVP (819 μM) for one week before analysis by flow cytometery after staining with Annexin V and propidium iodide.
(A, B) representative scatter plots for untreated (control) and treated (NVP) cells. (C, D) tabulation of (C) Annexin V positive and (D) apoptotic cells from three independent experiments. CO: Control, NVP: nevirapine treated cells. * $P < 0.05$.

in triplicate. The results showed that NVP inhibited HepG2 cell viability in a dose-dependent manner (Figure 1), and the IC_{50} of NVP at 24 h was calculated as 818.975 ± 88.99 μM, a value lower than that previously reported for 24 h, but in very close agreement with the value of 814 μM reported at 48 h in a previous study [13].

3.2. Long term culture of HepG2 cells with NVP

To determine the long term effects of NVP treatment, HepG2 cells were plated at a density of 4×10^6 cells in T25-cm^2 flasks and after 16–18 h the complete medium was removed. The control flasks were refilled with complete medium containing 0.3% DMSO whereas the remaining flasks were filled complete medium containing NVP at six different concentrations (819, 273, 91, 30.3, 10.1 and 3.3 μM). Cells were cultured for 6 weeks with replacement of the medium every 3days and subculture every 7 days. Cell numbers were counted weekly using the trypan blue exclusion assay. Results, calculated as a percentage of control cells are shown in Figure 2. Cells treated with highest concentration of NVP (819 μM) initially showed a rounded cell morphology with cells detaching from week one and by week three, all cells had died (Figure 3). Some growth

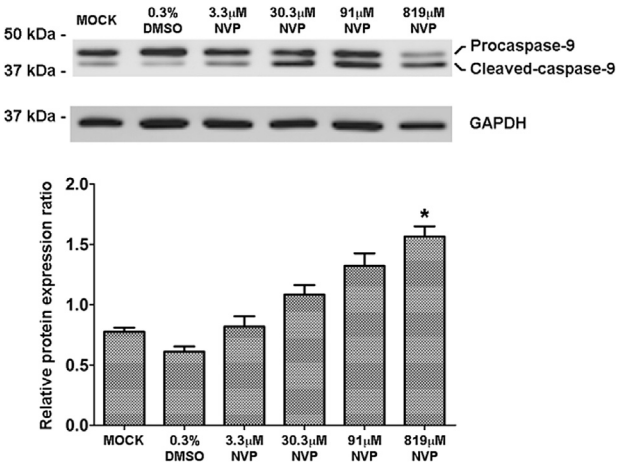

Figure 6. Western blot analysis of (pro)caspase 9 activation.
HepG2 cells were treated with a range of concentrations of NVP as indicated or not treated (mock) or treated with vehicle only (0.3% DMSO) and on day 7 post treatment cells were investigated for (pro)caspase 9 expression by western blot. Signal for cleaved (active) caspase 9 was quantitated by Image J densitometry and plotted for three independent experiments. *denotes statistically significant difference, $P < 0.05$.

inhibition was observed at 273 μM, but growth was approximately normal at lower conditions, and no changes in cell morphology was seen when cells were cultured at 273 μM or lower concentrations.

3.3. Induction of apoptosis by NVP

The rounding and detachment of the cells treated with the highest level of NVP is a classic hallmark of the induction of cellular apoptosis [16]. To further investigate this, cells were again treated, or not treated for one week with NVP at a range of concentrations. On day 4 any detached cells were collected and on day 7 both detached and attached cells were collected. Cells were stained with DAPI, and investigated under a confocal microscope. Results (Figure 4) show clear chromosome condensation and nuclear fragmentation in floating cells from day 4 on. Nuclear fragmentation can be seen in floating cells from day 4 at concentrations as low as 30.3 μM, and evidence of nuclear fragmentation is abundant at the highest concentration examined (819 μM). On day 7 some evidence of nuclear condensation in detached cells can be seen even with the lowest concentration examined (3.3 μM, Figure 4). Day 7 attached cells show evidence of altered nuclear morphology in cells treated at the highest concentration (819 μM) and in cells treated with 5% DMSO as a positive control.

To further characterize the induction of apoptosis in response to NVP treatment, cells were treated with the highest concentration of NVP (819 μM) or not treated for 1week, after which the cells were collected and assayed by staining with Annexin V/propidium iodide and assayed by flow cytometry. Results (Figure 5) show the clear induction of apoptosis, with a high proportion of cells showing positive staining with both Annexin V and propidium iodide (Figure 6).

Cellular rounding and detachment, nuclear condensation and fragmentation and positive Annexin V/PI staining are all considered classic markers of the induction of apoptosis [17]. To provide further evidence of the induction of apoptosis, cells were again treated with a range of NVP concentration or not treated, and cells examined for activation of caspase 9 by western blotting. Results show an increase in the presence of the active, cleaved form of caspase 9. The reduction of procaspase 9 and increase of cleaved caspase 9 were both statistically significant at the highest concentration used (819 μM), but the results were broadly consistent with a dose-dependent increase in caspase 9 processing.

4. Discussion

NVP is a widely used antiretroviral drug in resource poor setting, but the mechanism by which NVP induces hepatotoxicity remains to be clearly established, and moreover how NVP can induce both an early [3] and a late [4] reaction similarly remains unexplained. Current models suggest that the early reaction is at least in part an idiosyncratic drug reaction mediated by an immune response against intermediate metabolites of NVP [18]. By utilizing HepG2 cells, which have low expression of phase I and phase II enzymes we have been able to produce the first evidence that NVP in itself, and not its reactive metabolites, may contribute directly towards long term treatment associated hepatotoxicity through the induction of apoptosis.

The proposal that NVP induces specific effects is supported by a previous study that showed that liver cells treated with NVP undergo either G2/M or G1/G0 phase arrest. Using HepG2 and THLE2 cells Fang and Beland [13] proposed that the stage of cell arrest was correlated with the basal level of p53 in the cell line investigated, and that THLE2 cells which show high levels of p53 arrested in G1/G0, while in their hands HepG2 showed low levels of p53 and arrested in G2/M. However, we have previously demonstrated robust levels of p53 in HepG2 [17], and while Fang and Beland show no increase of p53 protein level or activating phosphorylation [13], other authors have shown significantly increased expression levels of p53 and p21 in Hela cells [12]. While cell cycle arrest is a common occurrence after DNA damage there is no evidence to suggest that NVP is directly genotoxic [19].

In their study, Fang and Beland [13] proposed that apoptosis was not the cause of the cell deficit seen in HepG2 cells treated with NVP. However, in this study we have clearly shown through multiple morphologic and biochemical methods that NVP induces apoptosis in HepG2 cells. Apoptosis in mammalian cells is induced through one of two main pathways, and extrinsic signaling mechanism governed by the activation of a death receptor and subsequent activation of caspase 8, and an intrinsic pathway governed by mitochondria and the activation of caspase 9 [20]. As shown here, caspase 9 is activated in the apoptotic process, showing that this is an intrinsic activation of apoptosis.

Of particular concern is the evidence that shows that even low levels of NVP treatment were associated with the induction of apoptosis. Raised levels of cleaved caspase 9 were seen at concentrations as low as 30.3 μM, and evidence of nuclear condensation can be seen with treatment after 7 days with as little as 3.3 μM. These results suggest that there is no clear threshold below which NVP has no effect. This would be consistent with reports of hepatotoxicity in HIV/HCV patients with blood levels of NVP as low as 22.5 μM [21]. Overall, our results suggest that NVP induced hepatotoxicity may results from direct consequences of NVP administration, and not only through idiopathic effects as has been suggested by others [18]. Further studies will help elucidate the mechanism of NVP induced hepatotoxicity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

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