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ORIGINAL ARTICLE

Evidence for Serpentine as a novel antioxidant by a redox sensitive HABP1 overexpressing cell line by inhibiting its nuclear translocation of NF-κB

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Abstract

Herbal antioxidants are gradually gaining importance as dietary supplements considering the growing implications of oxidative stress in most degenerative diseases and aging. Thus, continuous attempts are made to search for novel herbal molecules with antioxidative properties, using chemical methods predominantly with the need arising for cell based assays. We have generated a stable cell line F-HABP07, by constitutively overexpressing human Hyaluronan Binding Protein1 (HABP1) in murine fibroblasts which accumulates in the mitochondria leading to excess ROS generation without any external stimuli. In the present study, we demonstrated the nuclear translocation of p65 subunit of NF-κB in F-HABP07 cells, an important signature of ROS induced signalling cascade providing us an opportunity to use it as a screening system for ROS scavengers. Using known antioxidants on our designer cell line, we have demonstrated a dose dependant reduction in ROS generation and observed inhibition of p65 subunit of NF-κB nuclear translocation, increase in glutathione content and down-regulation of apoptotic marker Bax establishing its antioxidant biosensing capacity. With the help of this cell line, we for the first time demonstrated serpentine, one of the active components from the roots of *Rauwolfia serpentina* (a traditional medicinal plant), to be a novel non-cytotoxic antioxidant. The authenticity of this cell line screening system based discovery was validated using standard chemical assays thus, opening up new therapeutic avenues for this herbal compound and the use of this designer cell line.

Keywords: *Reactive Oxygen Species(ROS), HABPI, Designer cell line, Serpentine, NF-kappa B, Oxidative stress*

Introduction

Generation of reactive oxygen species is an inevitable consequence of aerobic metabolism occurring in mitochondria which is balanced by the cellular antioxidative system. Oxidative stress results from an imbalance between the formation and neutralization of these free radicals. Growing evidences indicate that overproduction of ROS is integral to aging and in the development of many neurodegenerative and cardiovascular diseases [1] alongwith inflammation and cancer. Thus, there is considerable interest in identification of new compounds capable of ROS scavenging activity. Studies suggest that regular moderate intake of antioxidants are associated with a reduced risk of neurodegenerative diseases [2]. Thus, the dietary supplementation of antioxidants from natural sources are in demand for disease preventive measure and maintenance of good health [3,4,5].

Presently, the antioxidative capacity of a compound is predominantly tested by chemical assays. These assays do not reflect the cellular physiological conditions and are unable to consider the bioavailability, metabolism and toxicity of the compound. Therefore, there's an increasing demand for the development of

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a simple, sensitive and precise cell based model system reflecting the *in vivo* conditions, for rapid screening of antioxidants.

In our laboratory, we have developed a stable transfectant F-HABP07, by overexpressing human Hyaluronic acid binding protein 1 (HABP1) in murine fibroblast F111 cell line [6]. The mitochondrial accumulation of this protein resulted in growth retardation, mitochondrial dysfunction, excess ROS generation and apoptosis induction in the cell line. Taking account of all the characteristics generated, this cell line appeared to be suitable as a screening tool for compounds with probable antioxidative properties.

In this paper, we initially examined the subcellular localization of p65 subunit of NF-kappaB in F-HABP07 cell line as it is the hallmark of oxidant induced stress. NF-kappaB is an important mediator in the pathophysiology of diseased states characterized by elevated levels of cytokines and ROS, such as sepsis and inflammation [7]. Antioxidant compounds inhibit NF-kappaB translocation with consequent reduction in cell damage [8,9]. Using known antioxidants in the medium, we confirmed the reduction in oxidants generated and the reversal of ROS induced nuclear translocation of p65 subunit of NF-kappaB and Bax expression. As a part of the study, this cell line was utilized to identify a novel antioxidant serpentine, the active principle found in the root of *Rauwolfia serpentina* and *Catharanthus roseus*, which is commonly used in traditional medicinal system for several diseases, although its activity was unknown.

Materials and methods

Dulbecco's modified Eagle's medium, Fetal Bovine Serum and antibiotics were from Invitrogen Co. (USA). All chemicals including Superoxide dismutase (SOD) and catalase activity kit were from Sigma Aldrich Chemicals Pvt. Ltd. (USA) unless mentioned otherwise. Antibodies were from Santa Cruz Biotechnology Inc., (USA) and Cell Signalling Tech. (USA). 5, 6-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular Probes Inc. (Eugene, OR). Serpentine (CID number 73391, SID number 215313) was isolated in > 98% purity with possible contamination of ajmalicine in our lab.

Cell culture and treatments

Cell lines were maintained in Dulbecco's Modified Egale Medium (DMEM), 10% Fetal Bovine serum (FBS) in humidified CO_2 (5%) at 37°C. F111, a murine fibroblast cell line is transfected with human HABP1 (96% homology with murine c-DNA) to generate a stable cell line referred to as F-HABP07 [6]. The cells in culture are passaged after every 48 h and grown in 10% FBS and the media changed after every 24–36 h when the cells are in log phase [6]. For experimental set up, cells were seeded in required numbers and allowed to stick to substratum for 6 h after which a media change was given. Then, the treatments were given according to the required concentration by directly adding to the culture at 36 h of growth without changing the media and the cells were harvested for the various assays accordingly at 60 h.

Serpentine was dissolved in 50 % DMSO at 1M concentration initially which was further diluted 1000 times with serum free media to form the working stock solution from which the final treatment was given to the culture. As a vehicle control to the experiment, the same dilution of DMSO without serpentine was used in the culture.

Extraction and purification of serpentine from plant source

The roots of *C. roseus* were collected from the research farm of CIMAP, Lucknow. The methanol extract of the dried root powder was evaporated under vacuum at 40 C and then exhaustively extracted with 2% HCl solution. The acidic extract was defatted with petroleum ether, basified with Na_2CO_3 up to pH-9, extracted thrice with chloroform, which afforded alkaloid extract at pH-9. The remaining basic aqueous layer was further basified with 2% NaOH solution up to pH-14 and then extracted with *n*-BuOH (saturated with water), which afforded alkaloid extract at pH-14. The *n*-BuOH extract at pH-14 was subjected over Flash Chromatographic separation using silica. Elution of the flash was carried out with chloroform and methanol in various proportions and the fractions were pooled on the basis of their TLC profile visualized under UV at 254 and 365nm. Fractions 153–197 eluted with $CHCl_3$: MeOH (94: 6 to 93:7) contained an alkaloid whose ^{1}H, ^{13}C NMR spectroscopic data is given below.

Yellow powder (120.0 mg), ^{1}H NMR (300 MHz, CD_3OD): δ 8.45 (1H, *d*, J = 6.6 Hz, H-6), 8.31 (2H, *m*, H-5 & H-9), 7.76 (1H, *m*, H-11), 7.72 (1H, *d*, J = 8.4 Hz, H-12), 7.71 (1H, *s*, H-17), 7.43 (1H, *m*, H-10), 4.75 (1H, *m*, H-19), 3.82 (3H, *s*, H-23), 1.44 (3H, *d*, J = 6.8 Hz, H-18). ^{13}C NMR (75 MHz, CD_3OD): C-2 (135.8) , C-3 (141.2), C-5 (134.2), C-6 (116.8), C-7 (132.6), C-8 (121.3), C-9 (124.0), C-10 (123.2), C-11 (132.9), C-12 (113.9), C-13 (145.4), C-14 (31.9), C-15 (26.0), C-16 (107.2), C-17 (156.3), C-18 (14.2), C-19 (72.9), C-20 (38.5), C-21 (57.6), C-22(168.4), C-23 (51.8). It has been confirmed as serpentine since its ^{1}H, ^{13}C NMR data was comparable with serpentine previously isolated from *Rauwolfia serpentina* [10].

Assay of intracellular ROS in cell lines

Intracellular oxidants cause H_2DCFDA oxidation, yielding fluorescent product 2, 7-dichlorofluorescein

(DCF). Cells were incubated with 10μM H_2DCFDA under various conditions for 10 min in dark. The media was removed, cells lysed using cold RIPA buffer and centrifuged quickly to remove debris. The fluorescence in the supernatant was measured spectrofluorometrically (Perkin Elmer LS 55). Data was normalized against normoxic untreated control. The fluorometric measurements were expressed as fluorescence intensity arbitrary unit [F.I. (A.U.)] with respect to total mg of protein. The data was normalized against basal fluorescence.

Preparation of nuclear and cytoplasmic cell extract

Harvested cells were suspended in Buffer A (20mM HEPES, pH 8.0; 20% glycerol; 1.5mM $MgCl_2$; 10 mM NaCl; 0.2mM EDTA; 0.1% Triton X-100; 0.02 mM PMSF) and incubated on ice for 30 min with occasional shaking. Centrifuged at 2500 rpm for 15 min. Collected the supernatant containing cytosolic fraction. Resuspended the pellet in Buffer B (20 mM HEPES, pH 8.0; 20% glycerol; 1.5 mM MgCl2; 10 mM NaCl; 0.2mM EDTA; 0.1% SDS; 0.02 mM PMSF). Incubated at room temp for 15 min. Vigorous vortexing for 1 min, centrifuged at 14,000 rpm for 10 min at 4^0C. The supernatant collected contained nuclear fraction.

Methylthiazol Tetrazolium (MTT) assay for cell viability

MTT assay is a standard colorimetric assay to measure cytotoxicity. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. After the respective cell treatments (as already mentioned in Cell Culture and Treatment section), MTT (5 mg/ml) was added to the cells at 60 h, then incubated for 3-4 h. Thereafter DMSO was added to solubilize the formazan crystals and absorbance measured at 570nm. Cell viability was calculated in percentage against control.

Glutathione assay

Cells were suspended in 0.5 ml cold sulphosalicylic acid containing 0.5 mM EDTA. Incubated on ice for 1 h, then centrifuged at 14,000 rpm for 15 min at 4°C. 50 μL of supernatant added to 1 ml PBS containing 60 μg DTNB, 20 μg NADPH and 1unit of Glutathione Reductase. Reaction rate was monitored by measuring absorbance at 412 nm over period of time. Amount of glutathione calculated from standard curve made by using purified glutathione serial dilution [11].

Superoxide radical scavenging assay

Absorbance of reaction mixture containing varying concentration of test samples and PMS (0.1 mM), NADH (1 mM) and NBT (1 mM) in phosphate buffer (0.1M, pH 7.4) measured at 560 nM. Scavenging efficiency is calculated as percentage inhibition of test samples against control [12].

Hydrogen peroxide scavenging activity

A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentrations of test samples were added to 0.6 ml of H_2O_2 solution, incubated at RT for 10 min. Absorbance taken at 230 nm.

Superoxide dismutase and catalase assay

Cells treated at 36 h and harvested at 60 h. Processed according to manufacturer's protocol.

Results

Nuclear localization of p65 subunit of NF-kappaB and change in glutathione level indicates oxidative stress in F-HABP07

F-HABP07, a stable cell line constitutively overexpressing HABP1 [6] is already established to generate excess ROS [13]. The nuclear translocation of the p65 subunit of NF-κB was examined in F-HABP07 since it is an important event subsequent to ROS generation [14]. The p65 subunit of NF-kappaB was clearly demonstrated to be predominantly localized in the nucleus by immunocytochemistry (Figure 1A) thereby confirming the presence of redox signal in F-HABP07 cells.

Glutathione maintains one of the most important antioxidative systems of the cellular machinery. It was observed that the glutathione content almost doubled in F-HABP07 ($p < 0.005$ compared to F111) as compared to the normal F111 and vector-transfected control, FpCDNA01 (Figure 1B).

Superoxide dismutase (SOD) and catalase are important antioxidant enzymes but, surprisingly, there was an insignificant change in the activity of either enzymes in F-HABP07 as compared to untransfected F111 and vector-transfected control, FpCDNA01 (Figure 1C and D).

F-HABP07 cell line as an antioxidant biosensing tool

The state of oxidative stress in F-HABP07 prompted us to examine whether this cell line could be used to detect antioxidants by utilizing its unique characteristics like ROS generation, NF-κB p65 subunit nuclear translocation, Bax expression and glutathione level. Known antioxidants were treated to this cell line to ascertain its biosensing capacity. Besides establishing the antioxidant detecting ability of F-HABP07

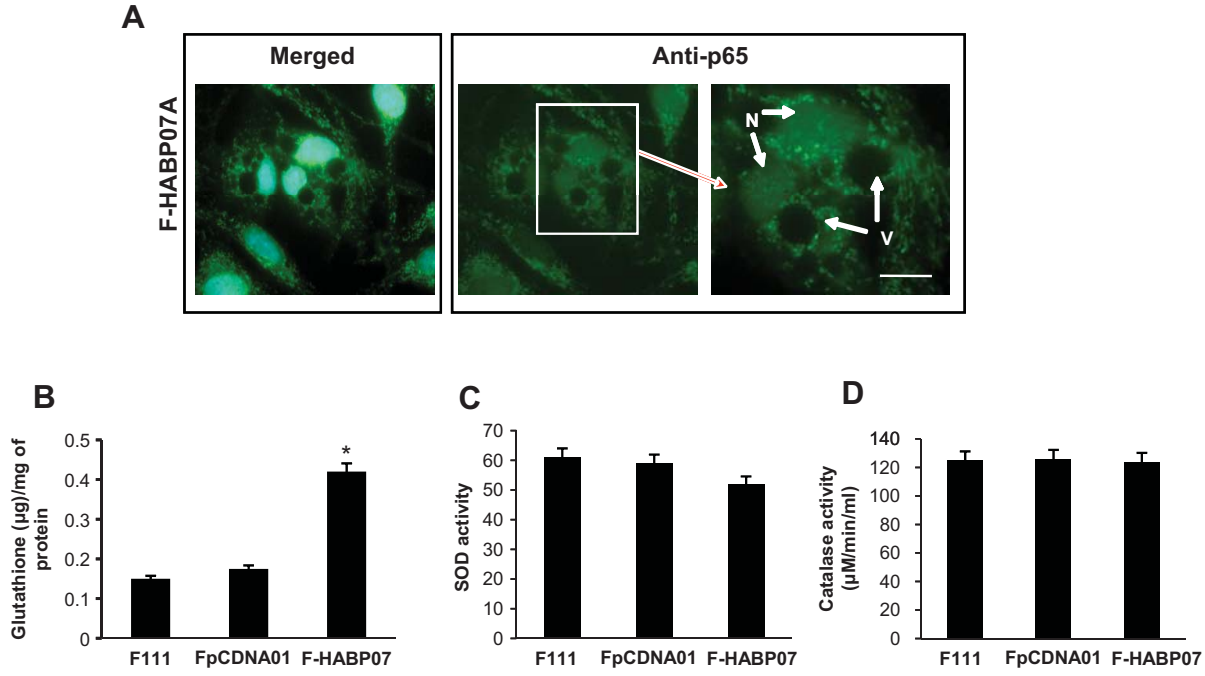

Figure 1. *Confirmation of oxidative stress induced by HABP1 expression in fibroblast F-HABP07 by nuclear translocation of p65 subunit of NF-κB* (A): F-HABP07 cells fixed after 60 h of growth without any treatment were immunodetected by polyclonal anti-p65 antibody to analyze its localization pattern. Merged denotes the immunostaining with DAPI showing the nucleus. All bars represent 10 µm. Here N denotes nucleus whereas V for vacuoles. *Change in the endogenous antioxidant system of ROS generating F-HABP07 cell line:* F111, FpCDNA01 and F-HABP07 cells are harvested at 60 h of growth and processed respectively for glutathione (B), SOD (C) and catalase (D) assay. Data showed a double increase in glutathione level of F-HABP07 (B) against normal fibroblast (F111) (* denotes $p < 0.005$ against normal in F-HABP07). Quantitative estimation of SOD (C) and Catalase (D) activity showed insignificant change in F-HABP07 as compared to F111 and FpCDNA01 cells. All data representative of mean value of ($n = 3$) experiments with ± SE.

using chemicals with known antioxidant activity, an herbal compound serpentine with unknown activity, was also treated to this cell line and compared to evaluate for its probable free radical quenching effect.

Reduction in ROS generation from HABP1 overexpressing cells on treatment with known antioxidants and serpentine. F-HABP07 cell line was treated with 10 µM of known antioxidants like PDTC, NAC, curcumin and tiron at 36 h of growth and ROS was assayed at 60 h of growth using H_2DCFDA. Fluorescence microscopy studies showed that these chemicals visibly reduced ROS generation in F-HABP07 cells (Figure 2A). The effect of these chemical and herbal products on ROS generation at different concentrations was also studied (Figure 2B). PDTC reduced more than 50 % of ROS generation at 5 µM and continued to do so till 20 µM at which it is maximally active. Similar is the case of curcumin treatment where its increasing doses decreased ROS generation till 20 µM after which the effect was lost. NAC was effective in curbing ROS from10 µM treatment but it was most effective at 50 µM. 5 µM Tiron treatment reduced more than 50% of ROS generation and further doses maintained the reduction.

Serpentine, a compound with hitherto unknown antioxidative properties was also observed to reduce ROS (Figure 2B) from 5 µM onwards and significantly maintained a linear of inhibiting ROS generation even upto a high concentration of 100µM thus substantiating its effectivity as a ROS quencher over a wide range of concentrations.

Reversal of ROS induced oxidative stress in F-HABP07 on treatment with known antioxidants and serpentine. One of the major signal transduction pathways that is activated in response to oxidant stress is that of the nuclear transcription factor NF-kappaB, which is crucial for cell survival, cell proliferation and immune responses via expression of its target genes [15]. NF-kappaB exists in the cytosol as a pre-formed trimeric complex. Oxidants trigger the breaking up of this complex and the p65 subunit of NF-kappaB translocates to the nucleus. Thus, we treated F-HABP07 cell line with the known antioxidants and serpentine followed by separation into cytosolic and nuclear fractions and thereafter its immunoblot was performed using anti-p65 (Figure 3A). Densitometric analysis depicting the ratio of the corresponding nuclear to cytoplasmic expression of the p65 subunit is represented in Figure 3B. As expected on the basis of immunocytochemistry result, a 2 fold increase in the influx of NF-kappaB p65 in the nucleus of F-HABP07 was observed as compared to F111 and FpCDNA01. Treatment with antioxidants and serpentine drastically reversed this phenomenon to almost similar levels of normal and vector control cell lines

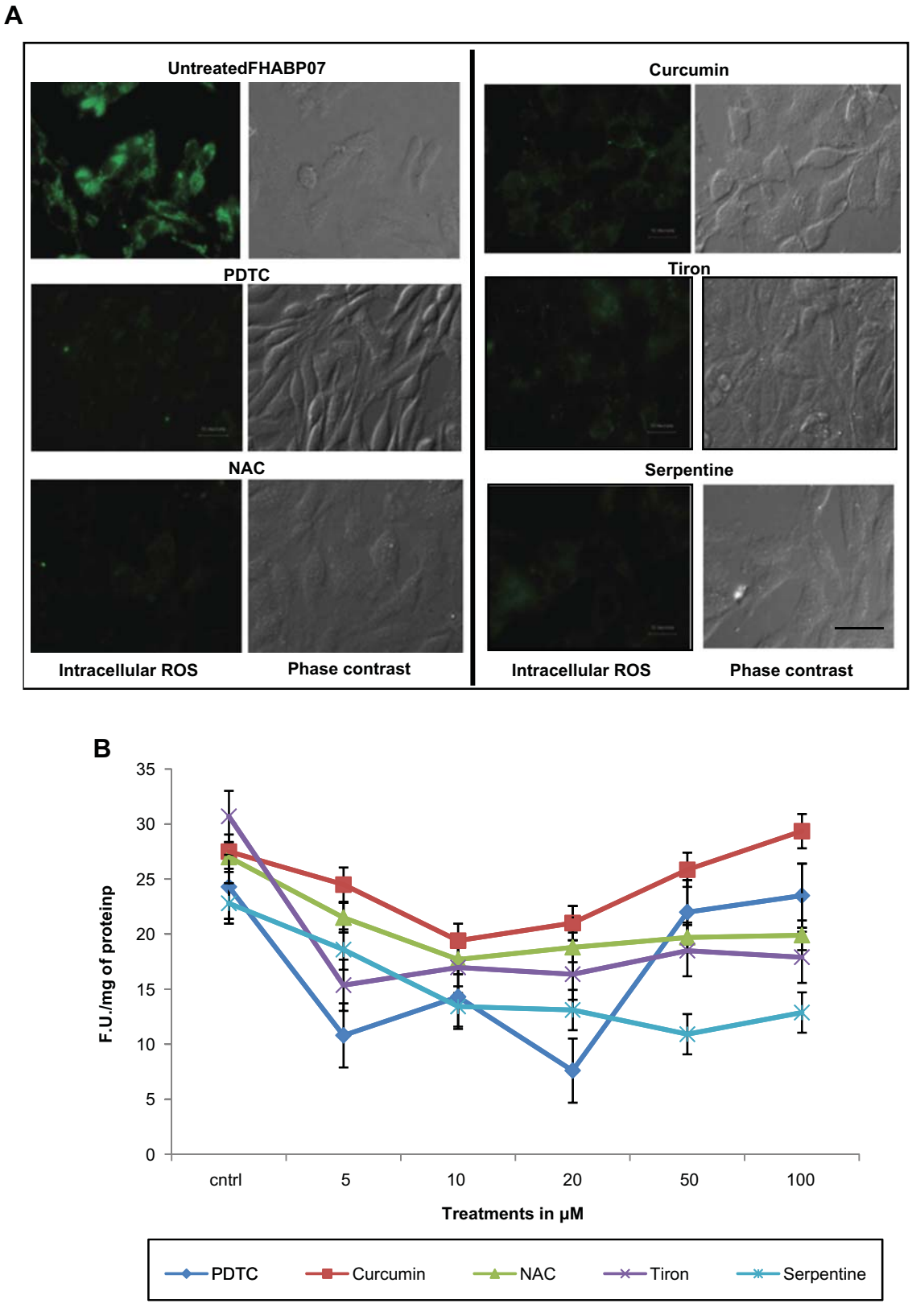

Figure 2. *Reduction in ROS generation on antioxidant treatment*: A: Detection of intracellular oxidant by H_2DCFDA fluorescence: ROS generation in untreated and treated F-HABP07 with 10 μM of different chemicals [PDTC, NAC, curcumin, tiron, serpentine] was visualized by fluorescence microscopy. Corresponding phase contrast images confirmed the presence of cells. All bars represent 10 μm. B: Dose dependant effect of various antioxidants: ROS generation in cells treated with different concentrations of known antioxidants [PDTC, curcumin, NAC, tiron] and serpentine was quantified spectrophotometrically. All data representative of mean value of (n = 8) experiments with ± SE.

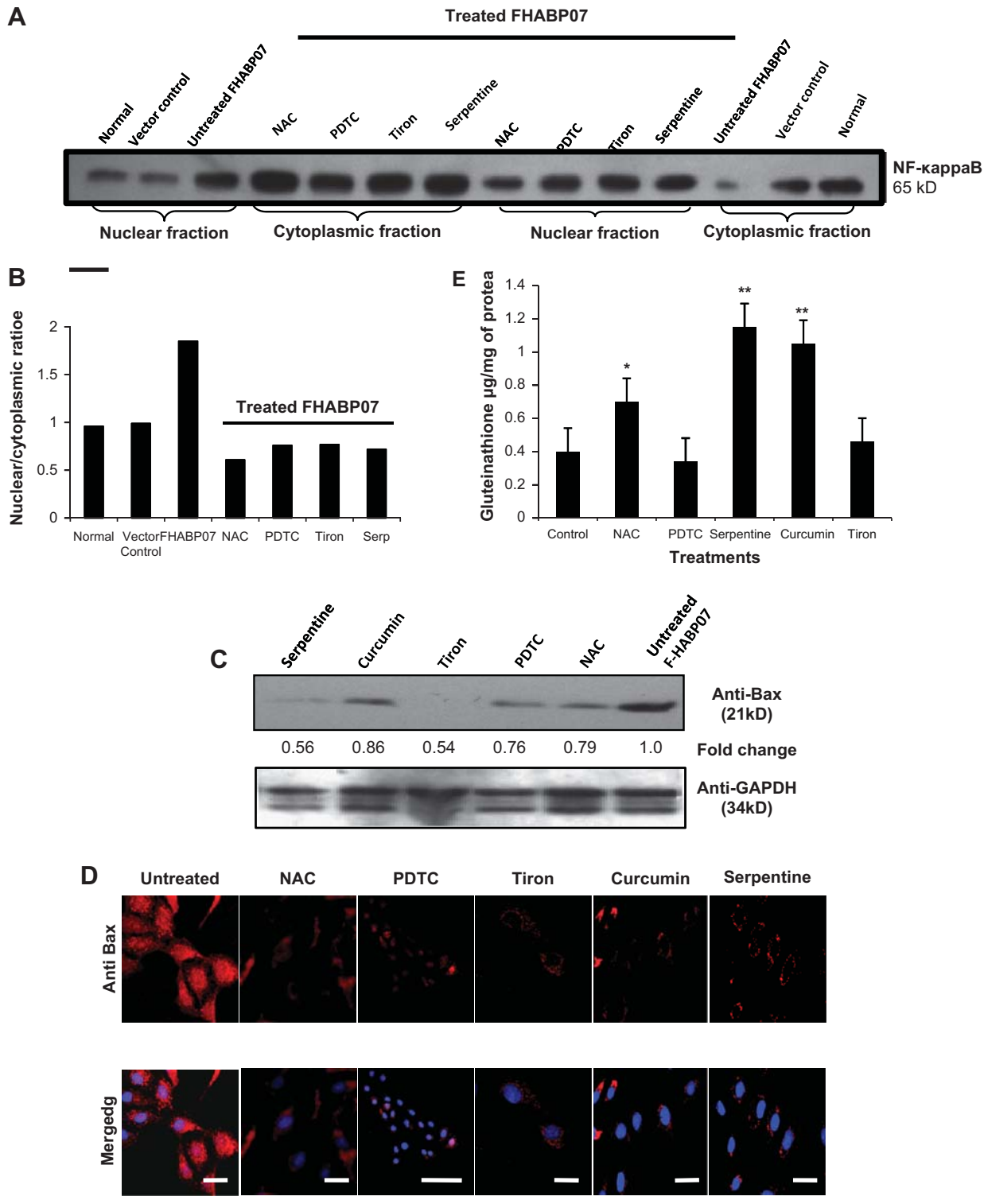

Figure 3. Change in cellular parameters on antioxidant treatment in F-HABP07 *Inhibition of nuclear translocation of p65 subunit of NF-κB on antioxidant treatment*: Increased nuclear localization of p65 subunit of NF-κB in F-HABP07 against F111 and vector transfected FpCDNA01. The fractionated cell lysates on treatment with known antioxidants and serpentine showed higher concentration of cytoplasmic p65 in antioxidant treated samples compared to untreated F-HABP07 in immunoblot analysis with anti p65 antibody (A). The densitometry analysis depicting the nuclear to cytoplasmic ratio of p65 expression from the immunoblot as a bar graph clearly demonstrated that all the antioxidants were able to block the nuclear translocation of the p65 subunit of NF-κB in F-HABP07 to the similar level to the parent cell line (B). *Reversal of apoptosis induction on antioxidant treatment:* Immunoblot analysis (C) and immunocytochemistry studies (D) revealed the downregulation of Bax expression in antioxidant and serpentine treated F-HABP07 cells represented as fold decrease. GAPDH used as loading control. *Change in glutathione level by antioxidants treatment* (E): Untreated and treated samples of F-HABP07 cells harvested and processed for glutathione assay. NAC treatment almost doubles the glutathione amount in F-HABP07 cells (as * denotes $p < 0.005$ compared to F-HABP07) while its treatment with curcumin and serpentine quantified a significantly higher rate of glutathione expression (as ** denotes $p < 0.0001$ compared to F-HABP07). All data representative of mean value of ($n = 3$) experiments with ± SE.

and an increase in amount of NF-κB p65 subunit in the cytoplasmic fractions as compared to the nuclear fraction was observed.

The expression profile of Bax, a prominent proapoptotic protein [16] was studied as a marker for programmed cell death. A marked decrease in Bax expression levels, indicating abolition of apoptosis induction in F-HABP07 cells, on antioxidant and serpentine treatment was observed by both immunoblotting (Figure 3C) and immunocytochemistry studies (Figure 3D).

The effect of the known antioxidants and serpentine treatment on the glutathione levels of F-HABP07 cells was studied. NAC treatment doubled the glutathione levels inside the cell ($p < 0.005$ compared to F-HABP07) while curcumin treatment significantly increased the level by three folds against F-HABP07 (Figure 3E). Interestingly, serpentine treatment also significantly elevated the glutathione content in F-HABP07 cells by almost three folds ($p < 0.001$ compared to untreated F-HABP07). But surprisingly, not much change in the cellular SOD and catalase enzyme activity was observed in F-HABP07 cells on treatment with the known antioxidants as well as serpentine (data not shown) indicating that antioxidant activity of serpentine was not mediated by the activation of these antioxidant enzymes.

Serpentine as a non-cytotoxic antioxidant

As per the conventional methods, the scavenging activity of serpentine was determined by superoxide (Figure 4A) and hydrogen peroxide quenching chemical assays (Figure 4B) using ascorbic acid, a well known antioxidant, as a control. The pattern of dose dependant free radical scavenging activity of serpentine was similar to ascorbic acid confirming the former's antioxidant competence as already observed in cellular physiological conditions.

The comparative scavenging potential of serpentine with the known antioxidants was analysed by:

Cell based methods: Equal number of F-HABP07 cells were seeded and treated with 10 μM concentrations of different antioxidants and serpentine. The scavenging potential was quantified as the decrease in ROS generation and increase in cell survivability. Serpentine was observed to be biologically more efficient in decreasing ROS (Figure 4C) in F-HABP07 cells as compared to other well known antioxidants like PDTC, curcumin and NAC. The total cell number of F-HABP07 at 60hrs was taken as 100% and thereafter an increase in % viable cells was evident on antioxidant treatment (Figure 4D). Serpentine was significantly effective in increasing cell survivality of F-HABP07 cells in comparison to other known antioxidants.

Chemical assays: In presence of chemically generated superoxide anion and hydrogen peroxide, the direct scavenging activity of different antioxidants and serpentine at same concentration (1μg/ml) was measured. It was evident that the direct quenching activity of serpentine is comparable to the rest of known chemical and herbal antioxidants (Figure 4E).

The discovery of the hereto unknown antioxidative potential of serpentine prompted us to examine its cytotoxicity in few varied cell lines viz. normal monkey kidney cell line (CV1), human hepatic cancer cell line (HepG2), retinal ganglion cells (RGC-5) and normal murine fibroblast cells (F111). Interestingly, serpentine does not generate any deleterious effect on the growth of any of the cell lines as indicated by insignificant change in cell survivability (Figure 4F).

Discussion

In view of the growing demand for antioxidants as a disease preventive measure, we, for the first time, report the utility of a designer cell line F-HABP07, developed in our laboratory, as a biosensor and screening system for antioxidants and identified serpentine, an active plant component originally isolated from *Rauwolfia serpentina*, as a novel antioxidant. The following observations were conclusive in substantiating our finding. First, the ROS generated on the overexpression of HABP1 in the cell line was significantly reduced with the several known antioxidants and serpentine with equal effectiveness. Secondly, this cell line was observed to be associated with the nuclear translocation of p65 subunit of NF-kappaB, one of the most crucial ROS mediated signalling event. However, treatment with known antioxidants as well as with serpentine increased the p65 subunit content in the cytoplasmic fraction compared to nuclear fraction demonstrating the inhibition of its nuclear translocation. And thirdly, the induction of apoptosis by ROS in F-HABP07 as reflected by Bax expression was significantly checked by the known antioxidants and also with serpentine.

The significance of this fibroblast cell line, F-HABP07 is that the excess ROS generated on ectopic expression of HABP1 is independent of any foreign stimuli. There is a gradual increase in endogenous oxidant generation, without any external stimuli, beginning from 36 h of growth but reaching the crescendo at 60 h of growth only after which it dwindles out and the cells show subsequent induction of apoptosis.

In this communication, we observed the nuclear translocation of p65 subunit of NF-κB and altered glutathione level in F-HABP07 cells establishing the state of oxidative stress in this cell line induced by HABP1 overexpression. The reduction of oxidant generation, followed by inhibition of nuclear translocation of p65 subunit of NF-κB, with subsequent blockage of apoptosis induction as examined by Bax expression on the supplementation of known antioxidants in this cell line confirms the antioxidant biosensing capacity of

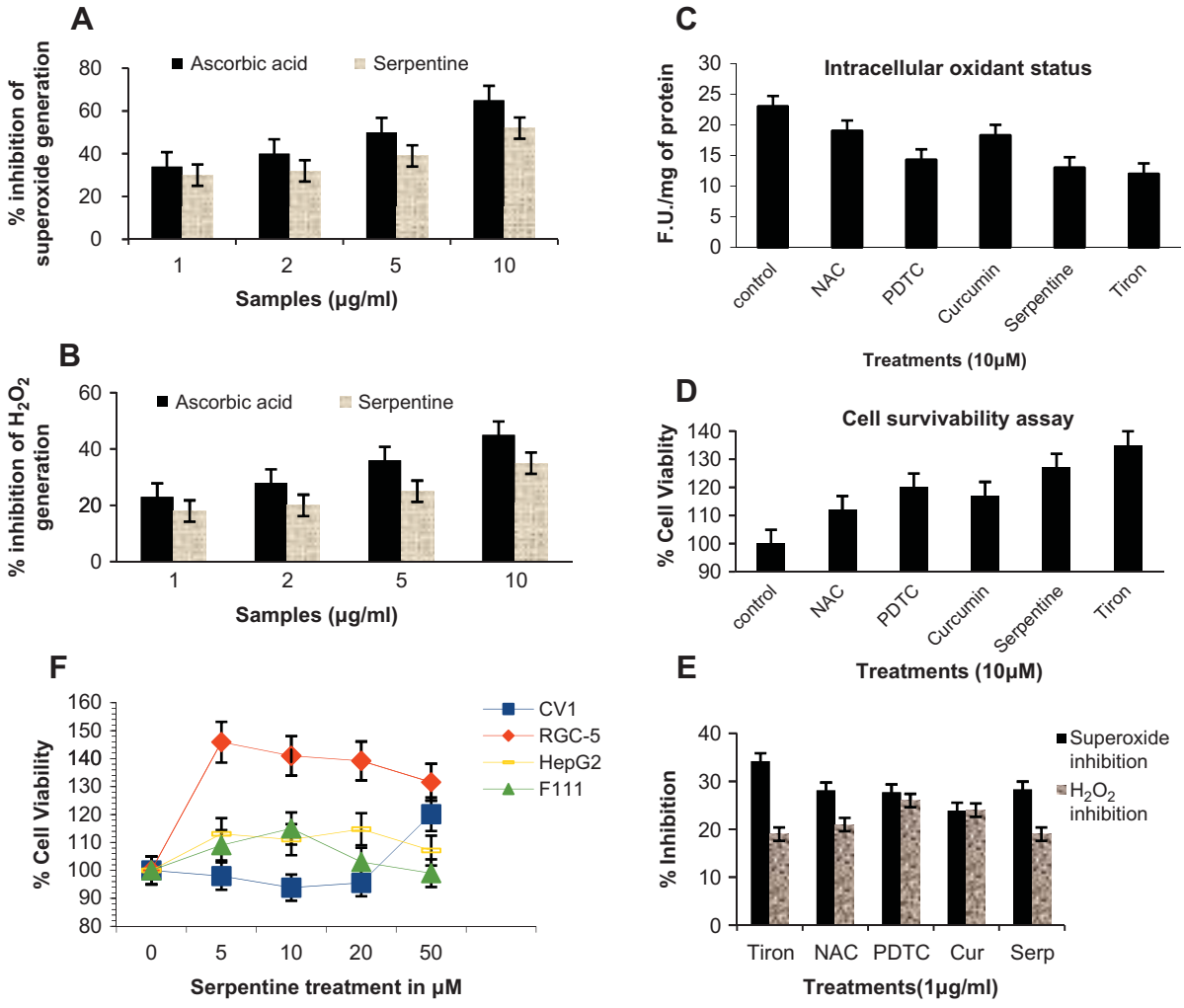

Figure 4. *Antioxidant capacity of serpentine: Chemical assays corroborate serpentine as an antioxidant:* Different concentrations' of serpentine were assayed for superoxide anion scavenging activity (A) and hydrogen peroxide scavenging activity (B) in comparison to well known antioxidant ascorbic acid *in vitro.* All data representative of mean value of (n = 5) experiments with ± SE. *A comparative analysis of antioxidant potency:* Cell line based assay: The effect of the various chemicals on the general physiology of the designer cell line was assayed. Cells grown for 36 h and treated with 10µM PDTC, NAC, curcumin, tiron and serpentine were harvested at 60 h of growth and processed for either ROS generation (C) or cell survivability (D). Chemical assay based: chemical assays measuring the direct superoxide and hydrogen peroxide quenching activity of the different chemical and herbal antioxidants at same concentrations (1µg/ml) was carried out (E). All data representative of mean value of (n = 3) experiments with ± SE. *No cytotoxicity observed on serpentine treatment* (F): Different cell lines viz. CV1, RGC-5, HepG2 and F111 were treated with different concentrations of serpentine and assayed for cell viability using MTT assay. Insignificant change in cell viability was detected. All data representative of mean value of (n = 5) experiments with ± SE.

F-HABP07. Using this cell based screening tool, serpentine was shown to be comparable in its biological activity with the other known antioxidants. For the first time, we have demonstrated that besides measuring ROS reducing activity of a compound, one of the added advantages of this cell line is that it can be used to study and compare the changes in the cellular parameters brought about by the different chemicals.

The supplementation of various known antioxidants like PDTC, NAC, curcumin and tiron in F-HABP07 at different concentrations produced a variable dose dependant effect on ROS generation. Though all the chemicals reduced ROS generation but at higher concentrations (50 µM and above), PDTC and curcumin lost their scavenging potential. This observation in accordance with previous reports stating that both PDTC and curcumin show anti- and pro-oxidative characteristics [17,18] established the efficacy of our cell line. The ROS quenching activity of serpentine was effective even in low concentrations ranging from 1 µM to 10 µM and significantly, its scavenging activity was not lost even at high concentrations (50–100 µM) with no apparent toxicity.

Increased nuclear localization of p65 subunit of NF-κB in F-HABP07 compared to its normal counterpart F111 confirmed the state of its endogenous oxidative stress, since it is already a well established fact that oxidative stress induces the nuclear translocation of p65 subunit subsequently activating the transcription of its downstream genes [19]. The treatment with different antioxidants inhibited the nuclear translocation of the p65 subunit thus releasing the

stress from the cells. Similar observation was made on treating the cells with serpentine confirms its antioxidative nature beyond doubt.

Quenching of cellular ROS by its endogenous antioxidative system like glutathione, SOD and catalase maintains cellular homeostasis. An insignificant change in the activity of SOD and catalase enzymes in F-HABP07 cells as compared to untransfected F111 and vector transfected control FpCDNA01 showed that the oxidant generation in this cell line is not controlled by SOD or catalase. Thus, our observation of unaltered SOD and catalase activities in this cell line with the supplementation of known antioxidants and serpentine was justified. An increase in the glutathione pool of F-HABP07 cells as compared to F111 and FpCDNA01, hinted towards its fight back mechanism under oxidative stress. A substantial upregulation in glutathione levels on treatment with NAC, curcumin and serpentine was observed while there was no change on PDTC and tiron treatments. This observation concurs with previous reports stating that curcumin enhances the transcription of genes encoding glutamate cysteine ligase [20,21] while NAC directly elevates intracellular cysteine levels which ultimately boost glutathione content. With respect to the increase in glutathione levels on treatment with serpentine, we speculate that the antioxidant activity of this compound is through the glutathione system. The disparity in change in endogenous antioxidant levels on treatment with various antioxidants prove that they all act through different pathways to reach to the final objective of quenching ROS in the cell. This knowledge may be utilized in future to study the several routes of oxidative stress and its prevention.

Continuous oxidative stress in F-HABP07 cells leads to subsequent apoptosis induction after 60 h of growth [6]. The present study demonstrated the blockage of apoptosis induction in F-HABP07 cells on treatment with known antioxidants and serpentine as reflected in the marked reduction of Bax expression, a prominent proapoptotic member of the Bcl-2 family. Thus, it can be safely said that because of the reduction of ROS generation on treatment with chemicals with scavenging potential, the cells are prevented from undergoing apoptosis.

Serpentine was originally isolated from *Rauwolfia serpentina* but is also known to be present in *Catharanthus roseus* of the Apocynaceae family and in Strychnos camptoneura. *C. roseus* produces widely used alkaloids such as the anticancer drugs vinblastine and vincristine, as well as the antihypertensive compounds ajmalicine and serpentine. Siddiqui and Siddiqui [22] first isolated a series of crystalline alkaloids, one of them being serpentine, from the roots of *Rauwolfia serpentina*. The plant extract is known to be used against snake bites, malaria, insomnia, insanity, hypertension and high blood pressure [23,24,25]. Serpentine has been shown to possess antitumor activity [26] and is a selective destroyer of cancer cells in mice [27]. Dassonneville et al [28] have shown that though serpentine binds to DNA and inhibits topoisomerase II activity at 50–100 μM concentration, it is non cytotoxic to B16 melanoma cells. It has tenfold reduced DNA binding affinity as compared to cryptolepine due the presence of a bulky sidechain to the pyridoindole chromophore which might be hindering the intercalation of serpentine with the DNA [28].

The present study helped to establish serpentine as an antioxidant for the first time. It is noteworthy that our study showed serpentine to be more effective in reducing ROS generation and restoring cell viability than the well known antioxidants reinventing its therapeutic potential. Absence of any deleterious effect on the different cell lines on serpentine treatment reveals it to be a non-cytotoxic compound enhancing its utility. Standard chemical assays corroborated the antioxidant nature of serpentine as revealed by our designer cell. The hydrogen peroxide and superoxide anion radical scavenging activity of serpentine reflected a pattern similar to ascorbic acid, a well known natural antioxidant supporting its scavenging activity. Interestingly, activity of all the compounds in the cell line was more pronounced even at low concentrations as compared to their chemically measured scavenging activity. This highlighted the fact that the antioxidants may behave differently in the cellular milieu. Also, the chemical assays cannot be considered as the exact measure of a compounds' antioxidant potential as some compounds may not directly quench the ROS but may act via the activation of cellular antioxidant machinery which can be identified through a cell line based tool only. Our present observation may be exploited in future for the human benefit.

Conclusion

In conclusion, our study presents a unique model system capable of screening novel compounds with probable antioxidative characteristics in physiological conditions. This cell line provides us the opportunity to analyse how the antioxidants would function *in vivo* and their cytotoxicity can be measured which is not possible using basic chemical assays. Utilizing this designer cell line, we have unveiled a novel antioxidant serpentine which is comparable in its effectivity with other well known antioxidants. This study opens up avenues for easy and convenient cell based antioxidant detection and also highlights novel therapeutic role for serpentine.

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Declaration of interest

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