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ARTICLE

Transactivation of P53 by cypermethrin induced miR-200 and apoptosis in neuronal cells

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Abstract:

Cypermethrin, a pyrethroid pesticide has been shown to induce neurotoxicity in adult mammals, however studies are also needed to explore its toxicity in developing brain and understand its mechanism of action in neurons. In our recently published study [1], using nerve growth factor (NGF) differentiated PC12 cells, we have identified miR-200 family as major up-regulated miRNAs, which regulate differentiation of PC12 cells in the neurons. In present study, toxicity of cypermethrin is compared between undifferentiated and neuron like differentiated PC12 cells and role of miR-200 family is studied in cypermethrin induced neuronal cell death. Our studies have shown that non-cytotoxic concentration of cypermethrin, selectively induces miR-200 family and apoptosis

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in differentiated PC12 cells, while no significant alterations were observed in undifferentiated PC12 cells. Further, our studies have demonstrated that cypermethrin induces miR-200 by inducing P53 levels in differentiated PC12 cells and we have identified a direct correlation between expression of miR-200 and levels of P53 in PC12 cells. Further, BCL2 is identified as a target protein of miR-200b/c, and down-regulation of BCL2 protein regulates cypermethrin-induced apoptosis of differentiated PC12. Rescue experiments carried out with inhibitors of miR-200 family, have further confirmed role of miR-200 family in apoptosis of differentiated PC12 cells exposed with cypermethrin. In conclusion, our studies have shown that differentiated PC12 cells are more sensitive to cypermethrin exposure than naïve and undifferentiated PC12 cells, and P53 mediated induction of miR-200 family regulates cypermethrin induced apoptosis of differentiated neuron like PC12 cells.

Keywords: MicroRNA, Apoptosis, miR-200, Cypermethrin, Neuronal Differentiation, Neurotoxicity, Oxidative stress.

A. INTRODUCTION

Differentiation and synaptogenesis of progenitor cells results in formation of mature and functional neurons in developing brain[2]. Differentiation of post mitotic neurons is a crucial event in brain development, which is regulated by several regulatory molecules like transcription factors, cell cycle regulators, surface markers, structural proteins and small regulatory RNA molecules known as microRNAs (miRNAs)[1, 3]. Pyrethroid pesticides are known toxin for both adult and developing mammals and several studies have identified a correlation between pyrethroid exposure and neurodegenerative diseases development[4-7]. Synthetic pyrethroids, such as cypermethrin, are widely used pesticide, which binds and disrupts the voltage-gated sodium channel in mammalian brain [8-10]. Cypermethrin has also been reported to modulate the levels of GABA receptors in rodent brain and induce oxidative stress mediated neuronal apoptosis and DNA damage in the brain [11]. Moreover earlier studies from our lab have shown that exposure of low levels of cypermethrin in developing (prenatal or

gestational) rats can imprint the gene expressions in brains of adult rats [12-14].

MiRNAs are regulatory RNA molecules, which have been demonstrated to regulate neurogenesis in both adult and developing brain and the expression of miRNAs reported to play significant role in neurotoxin induced apoptosis of brain cells[15-18]. Dicer (miRNA maturation enzyme) knockout studies have proved that miRNAs are essential for neuronal differentiation and maturation [1, 15, 19, 20]. Studies have also shown that miRNAs, which are specifically expressed in the brain, are involved in maintaining normal neuronal function like neurogenesis, apoptosis and cell cycle[21]. It has been reported that miRNAs regulate almost every step of neurogenesis, starting from neural stem cell proliferation to neuronal differentiation and maturation[22]. Interestingly, several studies have identified regulation of miRNAs by transcription factors or vice-versa, and a common consensus is emerging that miRNAs work in cooperation with transcription factors to fine-tune the expression of protein coding mRNAs [23-25]. Dramatic regulation reported in expression of miRNAs in patients with neurodegenerative disorders, provides opportunity to develop them as novel biomarkers of neurotoxin exposure[26].

PC12 cells, a rat pheochromocytoma derived cell line of rat adrenal medulla differentiates in neuronal cells when exposed with nerve growth factor (NGF), proved to be an excellent *in vitro* model for studying developmental neurotoxicity[27-29]. In our recently published study, dramatic up-regulation was observed in several miRNAs, when PC12 cells were differentiated with NGF[1]. MiR-200 family was identified as most dramatically up-regulated miRNAs in NGF differentiated PC12 cells[1]. Alterations in transcriptional profile by neurotoxin exposure are well reported but no previous report is available on the role and possible consequences of perturbations in miRNA expression under cypermethrin exposure [30]. In the present study, we have compared the toxicity of cypermethrin between naïve and NGF differentiated PC12 cells and investigated role of miR-200 family in cypermethrin-induced apoptosis of differentiated neuron like PC12 cells. Moreover, studies were also carried out to identify the regulator of cypermethrin-mediated induction of

miR-200 and potential target protein of miR-200 in differentiated PC12 cells.

B. Materials & Methods:

a) Chemicals: Reverse transcription (RT) kit, miRNA PCR assays, miRVana miRNA isolation kit and other reagents required for real time PCR were obtained from Life Technologies, Carlsbad, CA, USA. NM-F12 (Nutrient mixture-F12), fetal bovine serum, horse serum and antibiotic-antimycotic solution were also procured from Life Technologies, Carlsbad, CA, USA. Primary antibodies for NFL(pan) procured from Biomol International (Farmingdale, NY, USA, NA1297). Antibodies for βIII–tubulin (ab18207)) and Bad (ab31310) were procured from Abcam, Cambridge, MA, USA. Anti P53 (134100) was procured from Life Technologies, Carlsbad, CA, USA. Anti β-Actin (A1978) and BCL2 (AB1722) were obtained from Sigma-Aldrich USA, Millipore Darmstadt, Germany respectively. Secondary antibodies with Infra Red (IR) label for 700 and 800nm channel were obtained from LI-COR Biosciences, Lincoln, Nebraska USA. Apoptosis assays were performed with the FITC- Annexin V apoptosis detection kit of BD Biosciences, San Jose, CA, USA. MiRNA mimics and NTC were obtained from Dharmacon, USA. Transfection studies were carried out using Turbofect SiRNA transfection reagent from Fermentas, USA. All other regular chemicals including cypermethrin were procured from Sigma Aldrich, USA.

b) Cell culture, neuronal differentiation, chemical exposure and transfection: PC12 cells obtained from ATCC, USA were grown in nutrient mixture- F12 supplemented with 2.5 % fetal bovine serum, 12.5% horse serum, 1% antibiotic and antimycotic solution. Cells were kept in 5% CO_2 - 95% atmosphere with high humidity at 37^{o}C. Before differentiation, PC12 cells were seeded on poly-L-lysine (0.01% solution) coated surface for 12 hours. For full differentiation, PC12 cells were exposed with 50ng/ml NGF for 8 days and after every alternate day fresh culture media with NGF was added in differentiating PC12 cells.

c) Identification of non-cytotoxic dose of cypermethrin: Non-cytotoxic concentration of cypermethrin was identified in PC12 cells using 3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Neutral red uptake (NRU) and Presto Blue assay. MTT and NRU assays were carried out as described in our earlier study [31], while Presto Blue assay was carried out according to manufacturer's instructions. Cells were exposed with different concentrations (1-300μM) of cypermethrin in 96 well plates and viability was measured at 72 hours.

d) Isolation of total RNA and real time PCR of miRNAs and mRNAs: The total RNA, which also contained small RNAs was isolated using miRVana miRNA isolation kit as described by the manufacturer. Reverse transcription of miRNAs or mRNAs and their real time PCR was carried out as described in our earlier studies [1, 16]. For miRNAs, primers were procured from Life Technologies. Sequence of primers used for SYBR Green based real time PCR of different genes are following: BCL2: Forward: 5'-CTGAGTACCTGAACCGGCATC-3', Reverse: 5'- GAGCAGCGTCTTCAGAGACAG-3'; BAD: Forward: 5'- GAGCGATGAATTTGAGGGTT-3', Reverse: 5'- GATCCCACCAGG ACTGGATAA-3'; BAX: Forward: 5'-GGCGAATTGGAGATGAACTG -3', Reverse: 5'-CCCCAGTTGAAGTTGCCAT-3'; P53: Forward: 5'-GCACAAACACGAACCTCAAAG-3', Reverse: 5'-TCCGACTATACCACTATCCACTAC-3'; β-actin: Forward: 5'-GGAAATCGTGCGTGACATTAAAG-3', Reverse: 5'-CGGCAGTGGCCATCTCTT-3'. βIII-tubulin: Forward: 5'-GGCCTTTGGACACCTATTCAG-3', Reverse: 5'-TCTCACATTCTTTCCTCACGAC-3'. Details of NFL-M primers are mentioned in our earlier study[1].

e) Western blotting: The total cell lysates were prepared using Cell Lytic M cell lysis reagent of Sigma, USA supplemented with a protease inhibitor cocktail and DTT. Western blotting was performed as described in our earlier studies [1]. In brief, protein samples were subjected to SDS–PAGE (8% acrylamide stacking gel and 12 or 15% acrylamide separating gel). After electrophoresis, proteins were transferred to PVDF membrane with low background and incubated in blocking solution of LI-COR Biosciences for 1 hour at room temperature. The membranes were incubated overnight at 4°C with primary antibody (1:1000

dilutions) and transferred to room temperature for 30 minutes. After washing, membranes were incubated in IR dye conjugated secondary antibody (1:5000 dilutions) for 1 hour. After 5 washings of 5 minutes each, membranes were scanned using LI-COR ODYSSEY CLx machine.

f) Immunocytochemistry: For immunocytochemistry, PC12 cells were seeded in PLL coated chamber slides (20,000/well in each well of 4 well slides) and exposed with NGF for 8 days. After differentiation, cells were fixed with 3.7% paraformaldehyde. After 20 minutes of fixation, cells were washed with PBS and exposed to 0.5% H_2O_2 in methanol for 1 hour. After completion of 1 hour, cells were again washed with PBS, and incubated in blocking solution containing 0.2% triton X-100 in 0.1% bovine serum albumin for 1 hour. After blocking, cells were incubated with primary antibody (Pan-NFL and βIII-tubulin) at 1: 200 dilution (2 hours at room temperature) followed by washing with PBS and transferred to secondary antibody (1:500 dilution for 2 hours at room temperature) labelled with Alexafluor-488. After final washing with PBS, DAPI (nuclear stain) with antifade was added on cells and images were taken using fluorescent microscope.

g) Flow cytometric assays: Apoptosis and MMP was measured using flow cytometry as described in our publication [16] using FITC labeled Annexin-V for apoptosis and JC-1 dye for MMP loss.

h) Statistical Analysis: All the experiments were carried out in triplicates. The student's t-test was employed to calculate the statistical significance. $P < 0.05$ in student's t-test was considered as significant.

C. RESULTS

a) NGF induced differentiation of PC12 cells and identification of non-cytotoxic concentrations of cypermethrin in PC12 cells: Morphological studies revealed that exposure of NGF (50ng/ml) to PLL adhered PC12 cells induced differentiation (Fig. 1a). Immunocytochemical studies with markers of neurons, like NFL or βIII-tubulin has shown the formation of new neurites in differentiating PC12 cells and fully mature neurons were formed by day 8 of NGF exposure (Fig. 1a-d). RT-PCR and western blotting studies carried out in

undifferentiated and differentiated PC12 cells has also shown a time dependent increase in mRNA and protein expression of NFL and βIII-tubulin with several fold increase occurring in the expression of these neuronal markers on day 8 when compared to undifferentiated PC12 cells (Fig. 1a-d).

Viability of PC12 cells exposed to cypermethrin for 72 hours was studied using MTT, NRU and Presto Blue assay with the concentrations ranging from 1-300μM to identify the non-cytotoxic concentration of cypermethrin for further experiments (Fig. 1e). Exposure of PC12 cells with cypermethrin did not produce any significant alterations in either MTT or NRU or Presto Blue assay up to 25 μM. However, a dose dependent decrease in cell viability was observed in all the assays when the concentration of cypermethrin was increased from 50 to 300μM. As more than 50% viability was observed at 100μM and no marked change was observed in the morphology of PC12 cells, at this concentration. This concentration of cypermethrin has also been used earlier in *in vitro* studies dealing with the effect of cypermethrin on apoptosis in cultured astrocytes [32].

b) Comparative effect of cypermethrin exposure in undifferentiated and differentiated PC12 cells: Both undifferentiated and differentiated PC12 cells were exposed with identified non-cytotoxic concentrations of cypermethrin (100μM) for 3 days and apoptosis along with different regulatory genes of apoptosis were measured in PC12 cells (Fig. 2). Flow-cytometric analysis has shown that, exposure of cypermethrin significantly induces apoptosis and MMP loss in differentiated PC12 cells, while no significant alterations were observed in undifferentiated PC12 cells (Fig.2a&b). In differentiated PC12 cells, cypermethrin induced mRNA and protein levels of pro-apoptotic genes Bad and Bax, while no significant alterations were observed in undifferentiated PC12 cells (Fig.2c-e). Conversely, cypermethrin has significantly down-regulated BCL2 mRNA and protein levels in differentiated PC12 cells, while no significant alterations were observed in undifferentiated PC12 cells (Fig. 2 c-e).

c) Cypermethrin selectively induced the expression of miR-200 family in differentiated

PC12 cells: To further understand the role of miR-200 family in differentiated PC12 cells, we have studied the regulation of miR-200 family by cypermethrin (100μM) or MPP^+(known neurotoxin, which induces PD like symptoms) in undifferentiated and differentiated PC12 cells (Fig. 3a). Both, cypermethrin and MPP^+ significantly ($p<0.05$) induced the expression of miR-200a/b/c in differentiated PC12 cells, while no significant alteration was observed by these neurotoxins in undifferentiated PC12 cells (Fig. 3a). In differentiated PC12 cells, MPP^+ produced a maximum increase in miR-200b (8.97 folds), while cypermethrin produced maximum increase in miR-200a (9.5 folds) (Fig. 3a).

d) P53 regulates induction of miR-200 family in cypermethrin exposed differentiated PC12 cells: Several studies have shown P53 dependent neuronal cell death as one of the major pathway of neuronal apoptosis in response to neurotoxins [33-36]. We have studied the effect of cypermethrin on levels of P53 proteins in undifferentiated or differentiated PC12 cells. Western blotting of P53 has shown a significant increase in mRNA and protein levels of P53, when differentiated PC12 cells were exposed with cypermethrin (Fig. 3b-d). Similarities observed between induction pattern of miR-200 family and P53 protein indicated possible role of P53 in increased expression of miR-200 family. To explore role of P53 in miR-200 induction, PC12 cells were exposed with CP-31398, a chemical which stabilizes the active conformation of P53 and promotes P53 dependent apoptosis of cells [37], and expression of miR-200a/b/c were measured by real time PCR (Fig. 4c). Immunoblotting studies have shown significant increase in P53 levels in PC12 cells exposed with either 10μg/ml or 15μg/ml CP-31398 (Fig.4a&b). Exposure of CP-31398 (15μg/ml) significantly induced expression of all studied miR-200s (Fig.4c) in PC12 cells. Maximum increase was observed in miR-200a (12.29 folds), followed by miR-200c (10.50) and miR-200b (5.85) by exposure of CP-31398 (Fig.4c). Western blotting of P53 in NGF differentiating PC12 cells have shown consistent increase in P53 levels with NGF induced differentiation of PC12 cells (Fig.4d&e). Induction pattern of P53 protein matches with induction of

miR-200 expression in differentiating PC12 cells, as reported in our recently published study [1].

e) Expression of BCL2 is regulated by miR-200b/c in PC12 cells: Screening of 3'-UTR of BCL2 gene has shown presence of binding site for miR-200b/c in its 3'-UTR (Fig.5a). Moreover, in our earlier results (Fig. 2), we have found significant down-regulation in BCL2 mRNA and protein levels, when differentiated PC12 cells were exposed to cypermethrin (Fig. 2c-e), so transfected PC12 cells with mimics of miR-200b/c to identify the direct effect of miR-200b/c on the regulation of BCL2 (Fig. 5b-d). Over-expression of miR-200b or miR-200c significantly inhibited the mRNA and protein expression of BCL2 in PC12 cells (Fig. 5b-d).

f) Inhibition of miR-200 family rescues cypermethrin-induced apoptosis of differentiated PC12 cells: For rescue experiments, PC12 cells differentiated with NGF (X 5days) was transfected with equimolar mixture of miR-200a/b/c inhibitors or NTC (Fig. 6). Exposure of cypermethrin (100μM) significantly induced apoptosis in NTC transfected PC12 cells, while no alterations was observed in PC12 cells transfected with inhibitors of miR-200 family (Fig. 6).

D. Discussion:

In our recently published study, we have identified critical role of miR-200 family in NGF induced differentiation of PC12 cells [1]. The present study demonstrates a new role of miR-200 family in the cypermethrin-induced death of differentiated and mature neurons. Our studies have shown that PC12 cells, which are differentiated with NGF, prior to cypermethrin exposure, are more sensitive to cypermethrin than naïve or undifferentiated PC12 cells. Earlier studies from our lab and elsewhere have shown the neurotoxic potential of cypermethrin in mammals [10, 12, 13, 32]. Moreover, earlier studies from our lab have also shown that developmental exposure of cypermethrin imprints the expression of xenobiotic metabolizing CYP450 genes in adult brain [12, 13]. The damaging effect of cypermethrin and bifenthrin, another pyrethroid on cell morphology in SH-SY5Y and neurites outgrowth during PC12 cell differentiation has also been demonstrated in earlier studies [38, 39].

As miR-200 family plays a significant role in controlling NGF induced differentiation of PC12 cells, we have extended our studies to investigate the role of miR-200 in neurotoxin induced neuronal cell death. The selective and significant increase observed in the expression of miR-200 family by cypermethrin or MPP^+ in differentiated PC12 cells indicated towards a new role of miR-200 in neuronal cells. Earlier studies of Magenta et al., (2011) have shown that oxidative stress induced apoptosis and senescence is regulated by miR-200c mediated down-regulation of ZEB1 protein[40]. Moreover, a recent study has also shown a role of miR-200 family in oxidative stress induced death of hippocampal cells[41]. Pyrethroids are known to induce oxidative stress in experimental animals as well as in *in vitro* conditions[42, 43]. Both, earlier studies[40, 41] and selective induction of miR-200 in differentiated neurons by cypermethrin, observed in present study indicated towards involvement of miR-200 in neuronal apoptosis.

Transactivation of P53 protein has been reported to induce the expression of miR-200 family in cancer cells [44]. P53 is known transcription factor, which regulates neuronal cell death in response to different neurotoxins [33], so we have carried out studies to identify any possible role of P53 in cypermethrin mediated induction of miR-200 and apoptosis of differentiated PC12 cells by cypermethrin exposure. Similar induction pattern observed between miR-200 family [1] and P53 levels observed in present study in NGF differentiated PC12 cells or cypermethrin exposed differentiated PC12 cells indicated linkage between P53 levels and miR-200 induction. Significant induction observed in expression of miR-200 by P53 stabilizer (CP-31398) confirmed that expression of miR-200 is regulated by P53 in cypermethrin exposed neuronal cells. A recently published study has also shown that in liver cells, P53-dependent expression of miR-200a-3p regulates apoptosis by inhibiting a p38/P53/miR-200 feedback loop [45]. In present study, consistent increase observed in levels of P53 in differentiating PC12 cells, correlates with consistent increase observed in the expression of miR-200 family in differentiating PC12 cells as reported in our earlier studies [1].

In our recently published study, we have identified SOX2 and KLF4 (transcription factors) as target of miR-200b in differentiating PC12 cells. In

differentiating PC12 cells, exposure of NGF dramatically down-regulates expression of SOX2 and KLF4 [1], which indicates the probability of a new target proteins for miR-200 in cypermethrin exposed differentiated PC12 cells. The reverse pattern observed between expression of BCL2 and miR-200 family raised possibility of BCL2 regulation by miR-200 in cypermethrin exposed differentiated PC12 cells. Earlier studies from our lab and elsewhere have shown that BCL2 protein is regulated by several miRNAs in different kind of cells[16, 18, 46-49] and 3'-UTR of rat BCL2 harbors targeting site for miR-200b/c. Over-expression of miR-200b/c in PC12 cells significantly down-regulated BCL2 mRNA and proteins, which shows that at least in part miR-200 mediated apoptosis of differentiated PC12 cells are regulated by BCL2 protein. Rescue experiments done with inhibitors of miR-200 family confirms role of miR-200 in cypermethrin-induced apoptosis of differentiated PC12 cells.

In conclusion, the present study has demonstrated that cypermethrin is more toxic to neuron like differentiated PC12 cells than undifferentiated PC12 cells and P53 mediated transactivation of miR-200 family regulates cypermethrin induced apoptosis of differentiated neurons.

Abbreviations:

DF: Differentiated, FBS: fetal bovine serum, FITC: fluorescein isothiocyanate, IDV: integrated density value; miRNA: microRNA, mRNA: messenger RNA, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, NFL: Neurofilament, NGF: nerve growth factor, PLL: Poly-L-Lysine, PCR: polymerase chain reaction, RQ: relative quantification, RT: reverse transcription, UD: undifferentiated, NRU: neutral red uptake, IR: infra red, PBS: phosphate buffer saline, DAPI: 4',6-diamidino-2-phenylindole, MPP^+: 1-methyl-4-phenylpyridinium, PVDF: Polyvinylidene fluoride.

Conflict of interest

The authors declare no conflict of interest.

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Legends to figure:

Figure. 1: NGF induced differentiation of PC12 cells and identification of non-cytotoxic dosages of cypermethrin. Expression of βIII-tubulin and NFL was detected in PC12 cells exposed with NGF for 0, 4 or 8 days using immunocytochemistry (a), western blotting (b&c), and real time PCR (d). Viability of PC12 cells exposed with different concentrations of cypermethrin for 72 hours using MTT, NRU and PrestoBlue methods (e). Values showing $p<0.05$ are statistically significant and marked with (*). (RQ: relative quatification; IDV: Integrated density value).

Fig. 2: Effect of cypermethrin in apoptosis of undifferentiated (UD) PC12 cells and differentiated (DF) PC12 cells. Effect of cypermethrin on apoptosis of UD and DF PC12 cells was measured using Annexin-PI method (a), MMP loss using JC-1 dye (b), real time PCR (c) and western blotting (d&e) of apoptosis related gene. Both UD and DF PC12 cells are exposed with non-cytotoxic concentration of cypermethrin (100μm) for 72 hours. Values showing $p<0.05$ are statistically significant and marked with (*). (RQ: relative quantification; IDV: Integrated density value; 1: undifferentiated control; 2: Undifferentiated PC12 cells exposed with cypermethrin for 72 hours; 3:

Differentiated control; 4: Differentiated PC12 cells exposed with cypermethrin for 72 hours).

Fig. 3: Regulation of miR-200 family and P53 by cypermethrin in PC12 cells. Real time PCR of miR-200a/b/c (a) in undifferentiated (UD) or differentiated (DF) PC12 cells exposed with cypermethrin (Cyper: 100μM) or MPP^+ (100μM). Real time PCR (b) and western blotting (c&d) of P53 in UD or DF PC 12 cells exposed with cypermethrin (100μm) for 72 hours. Values showing $p<0.05$ are statistically significant and marked with (*). (IDV: Integrated density value; 1: undifferentiated control; 2: Undifferentiated PC12 cells exposed with cypermethrin for 72 hours; 3: Differentiated control; 4: Differentiated PC12 cells exposed with cypermethrin for 72 hours).

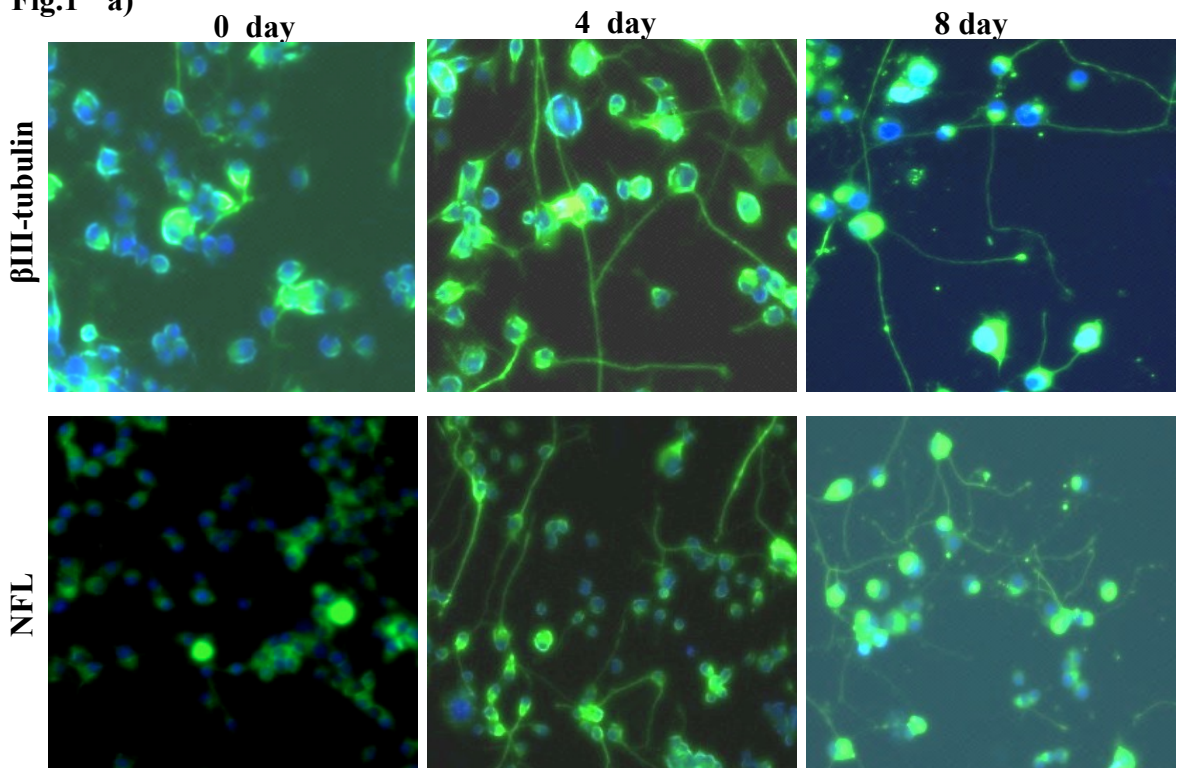
Fig. 4: Role of P53 in regulation of miR-200 miRNAs. Western blotting of P53 (a&b) and real time PCR of miR-200a/b/c (c) in PC12 cells exposed with CP-31398 for 12 hours. Western blotting of P53 in NGF differentiated PC 12 cells (d&e). Values showing $p<0.05$ are statistically significant and marked with (*). CP-31398 is P53 stabilizer. (IDV: Integrated density value).

Fig. 5: Increased miR-200 expression regulates BCL2 mRNA and protein levels. Scanning of 3'-UTR of rat BCL2 gene (a), real time PCR (b) and western blotting (c&d) of BCL2 in PC12 cells transfected with mimics of miR-200b or miR-200c. Values showing $p<0.05$ are statistically significant and marked with (*). (IDV: Integrated density value).

Fig. 6: Effect of miR-200 inhibition on cypermethrin induced apoptosis of differentiated PC12 cells. Apoptosis was measured using flow cytometry based annexin-PI method. PC12 cells are exposed with NGF for 8 days in four groups. After 5 days of NGF exposure two groups of cells were transfected with NTC and two groups with inhibitors of miR-200a/b/c. After 24 hours of transfection, one set of both NTC and miR-200 inhibitor transfected cells were exposed with cypermethrin (100μM). After 48 hours of cypermethrin exposure cells were processed for apoptosis assay.

Fig. 7: Schematic presentation of role of miR-200 family in cypermethrin-induced apoptosis of NGF differentiated PC12 cells. Double green upward arrows indicate induction, while double red arrow indicates down-regulation in gene expression.

Fig.1 a)



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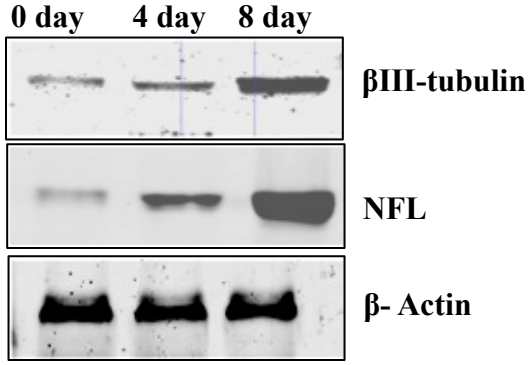

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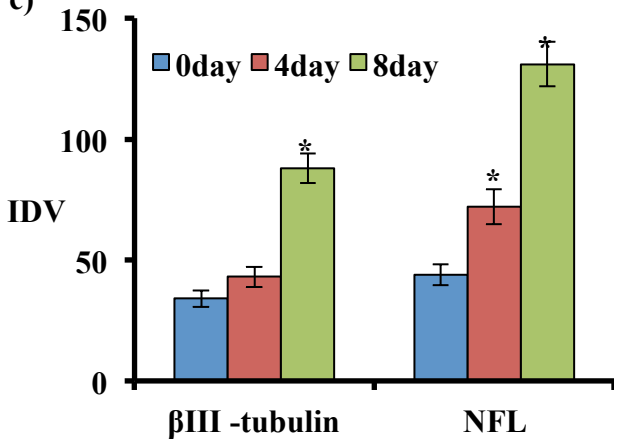

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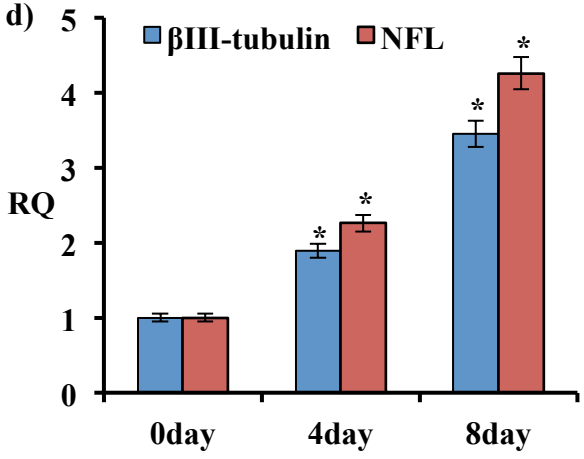

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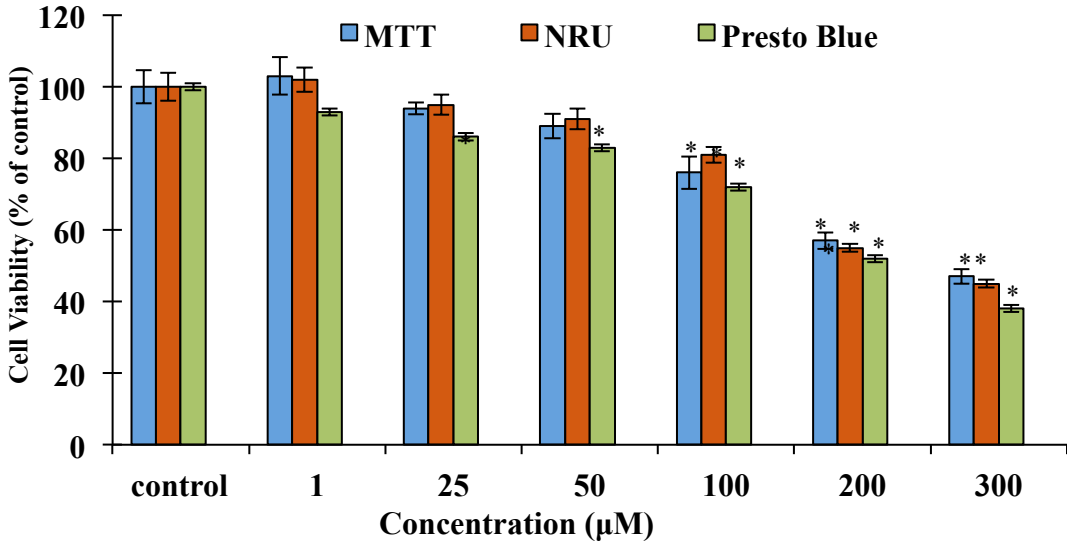
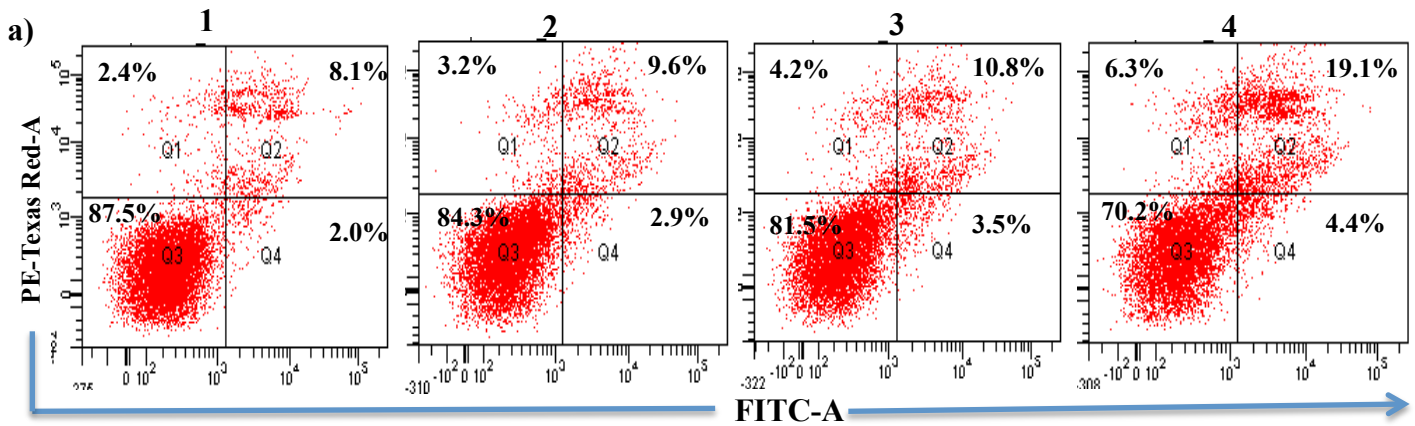
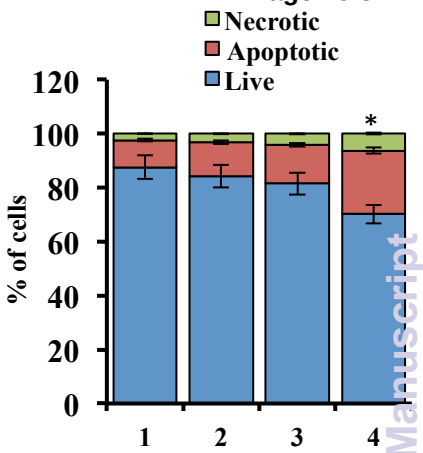
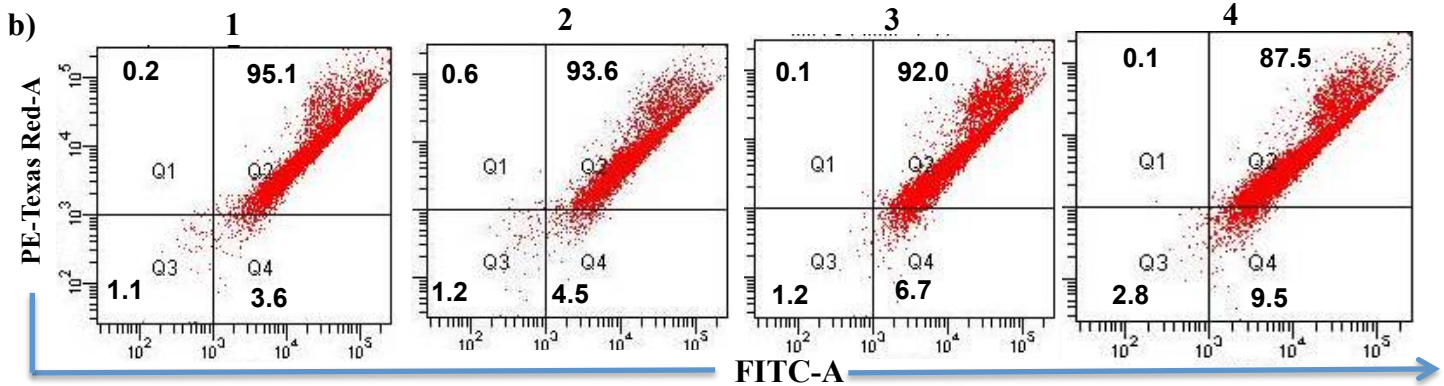
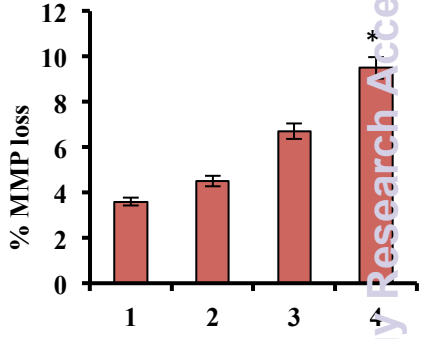
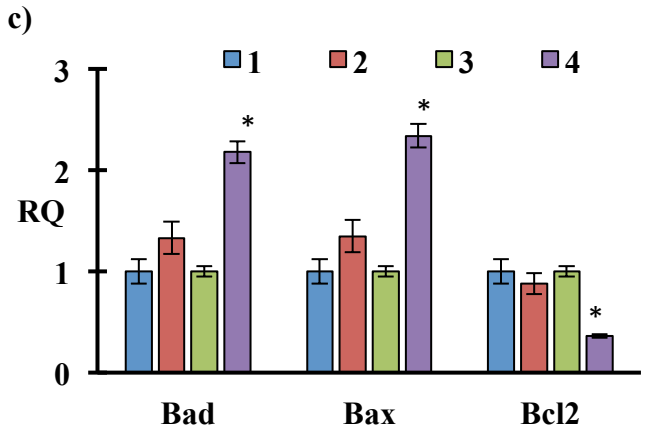


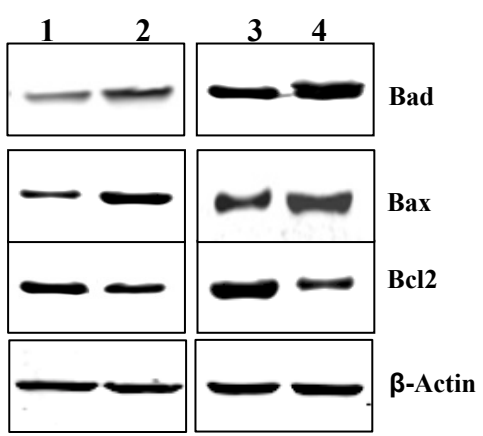












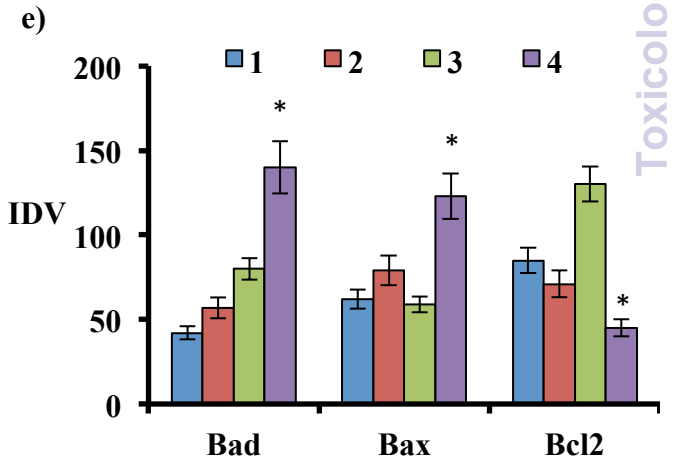
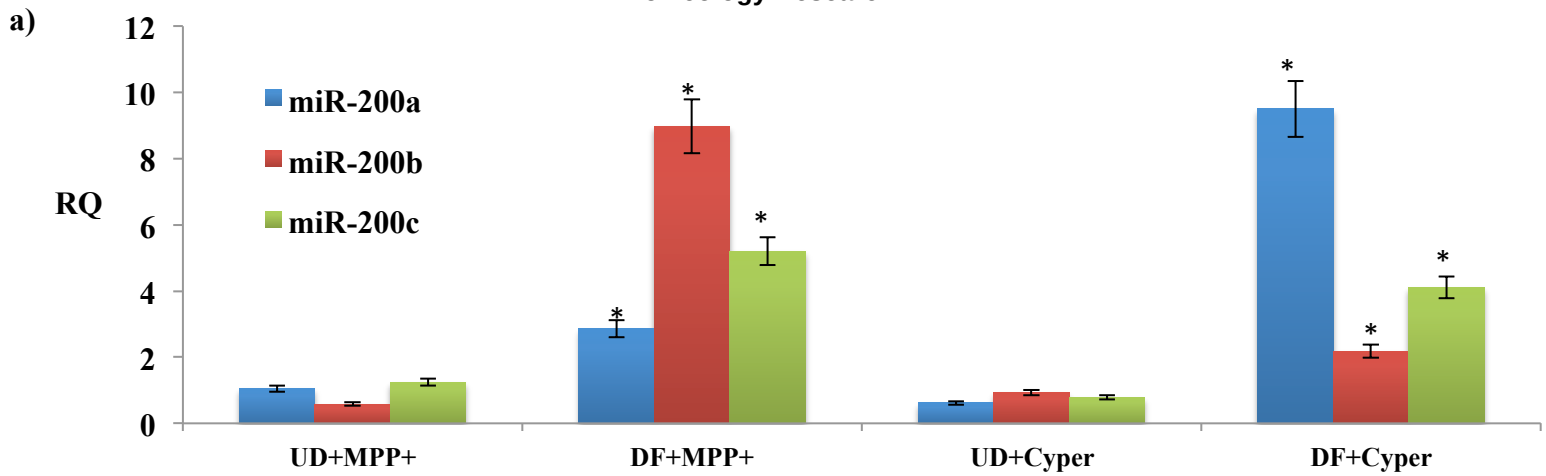
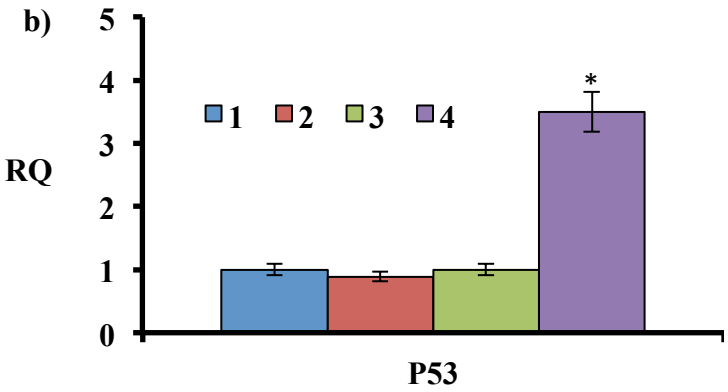
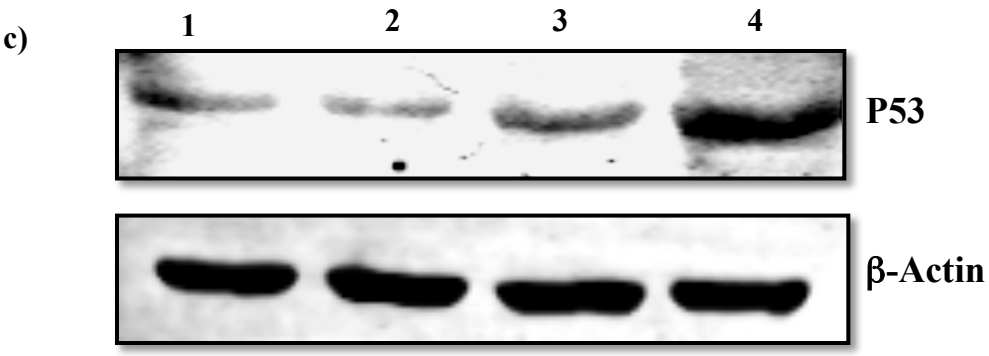


Fig.3







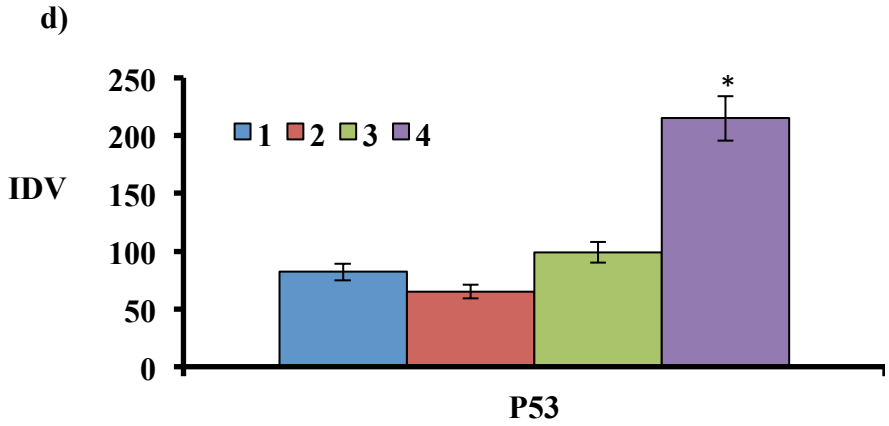


Fig. 4

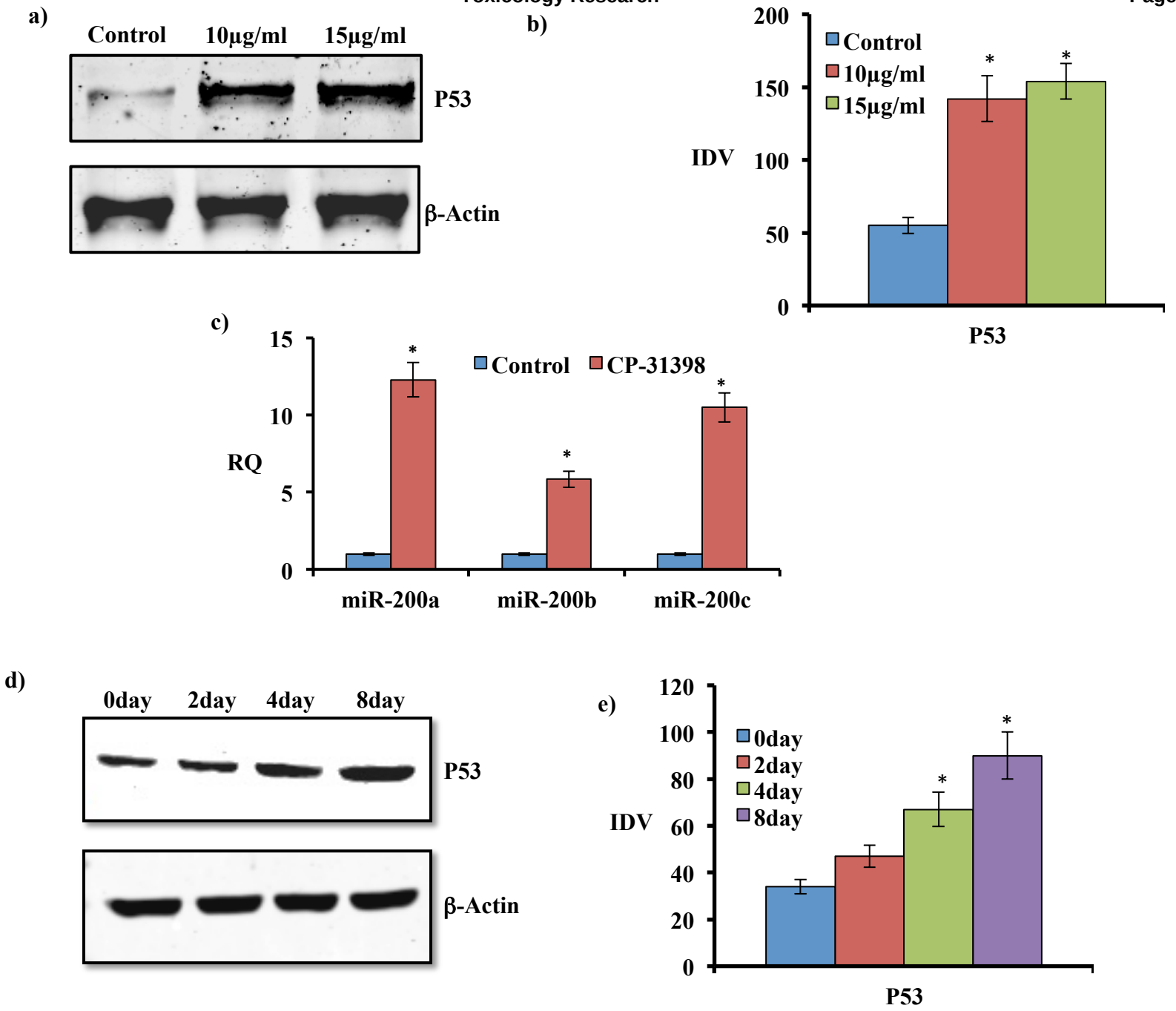
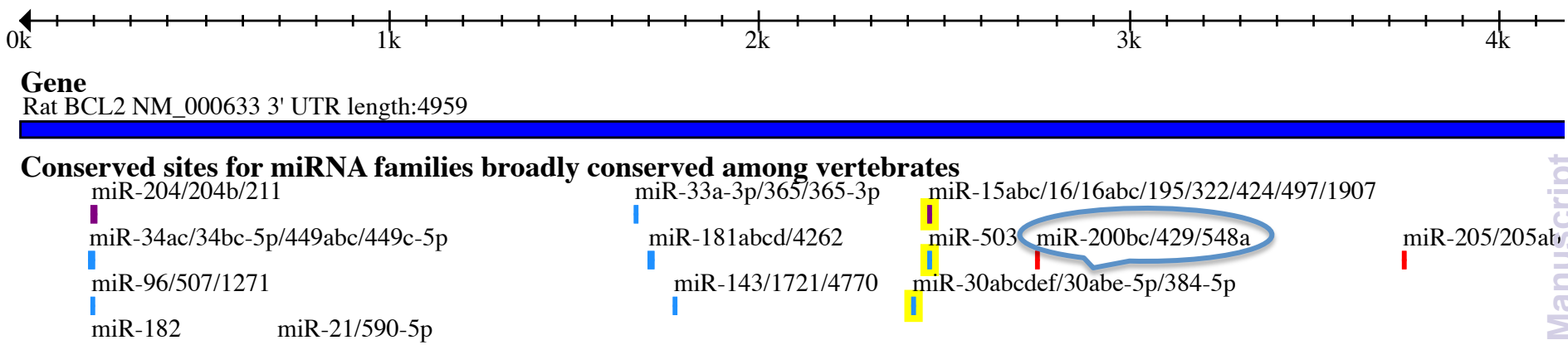


Fig. 5. a)



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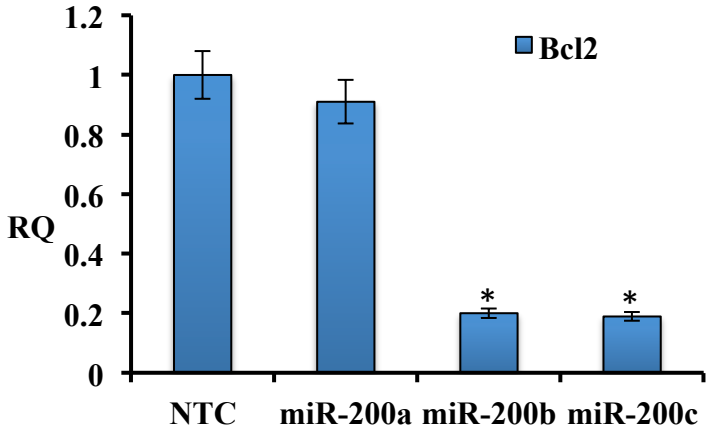

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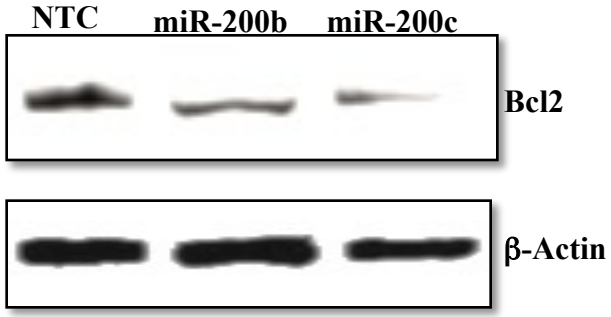

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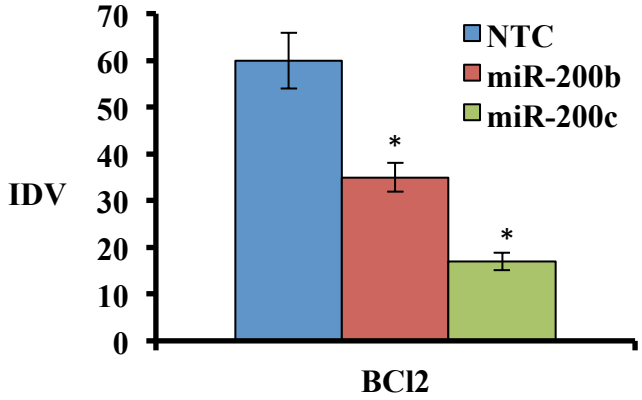
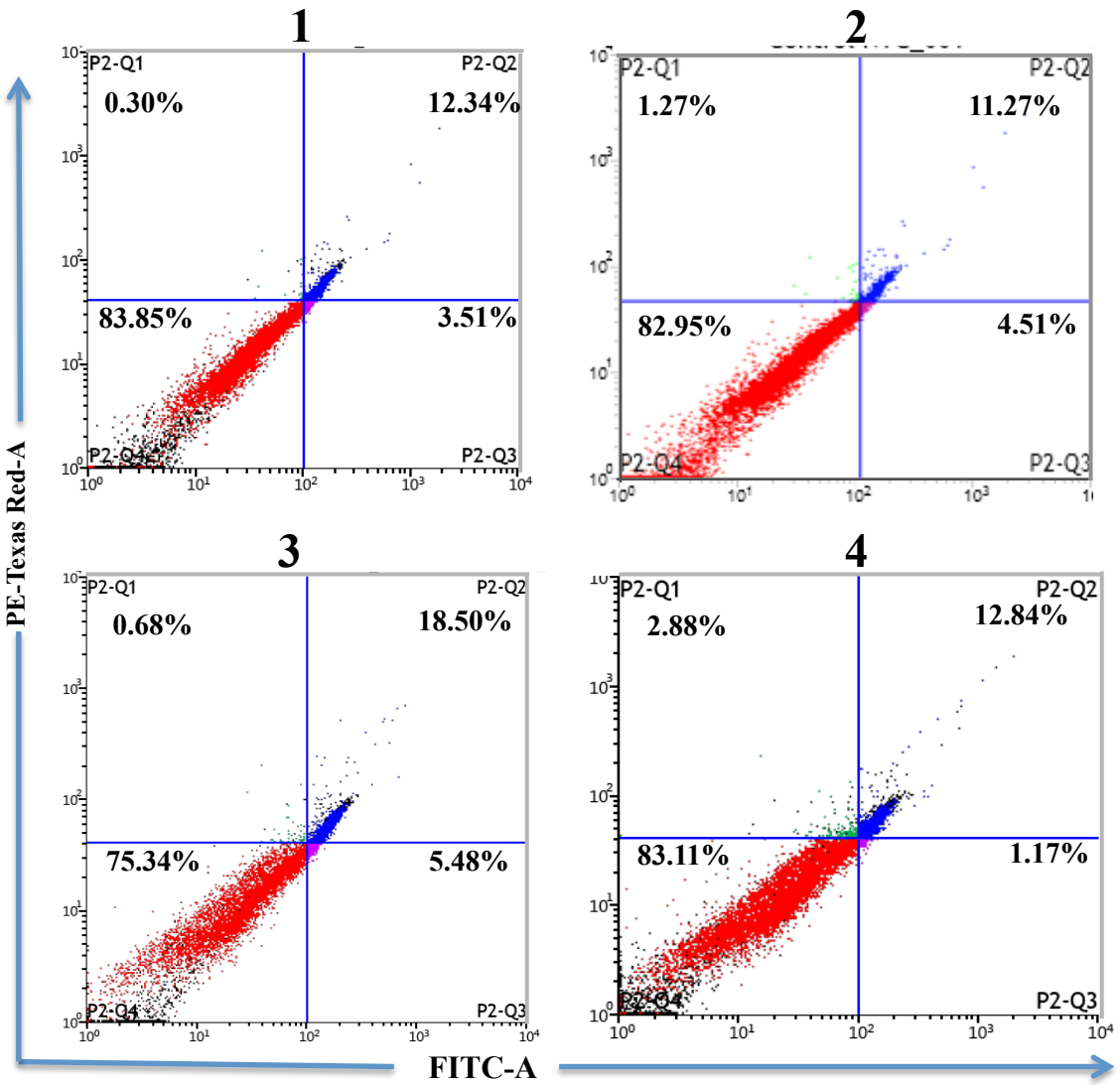
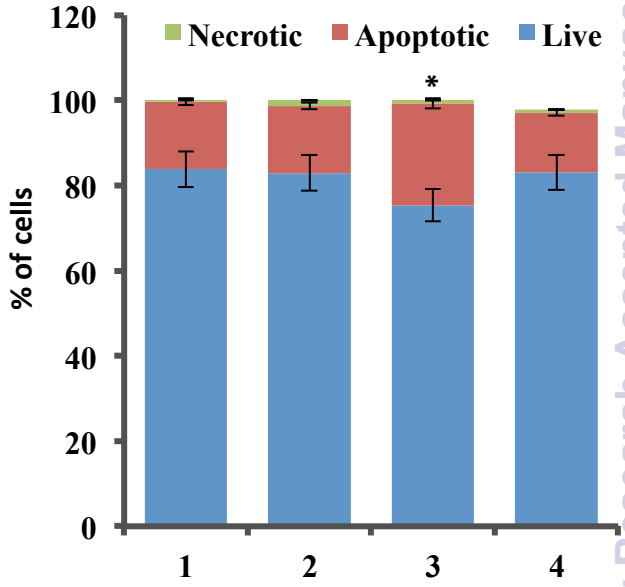


Fig. 6.





1- NGF+NTC
2- NGF+anti-miR-200a/b/c
3- NGF+NTC+Cypermethrin
4- NGF+anti-miR-200+Cypermethrin

Fig. 7.