Electronic Supplementary Information

Anticancer therapeutic potential of quinazoline based small molecules via global upregulation of miRNAs.

Smita Nahar[ab], Debojit Bose [b], Sumit Kumar Panja[c] Satyen Saha*[c] and Souvik Maiti*[abd]

[a]Academy of Scientific and Innovative Research (AcSIR), Anusandhan Bhawan, 2 Rafi Marg, New Delhi-110001,India
[b]CSIR-Institute of Genomics and Integrative Biology, CSIR, Mathura Road, Delhi 110 020, India. Fax: +91-11- 2766-7471; Tel: +91-11- 2766-6156; E-mail: souvik@igib.res.in.
[c]Department of Chemistry, Faculty of Science, Banaras Hindu University, Varanasi-221005, India, Fax: +91-542-2368127 ;Tel: +91-542-6702464, E-mail: ssahabhu@yahoo.com
[d]CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, CSIR, Pune 411008, India

Table of contents

Note: All the excel (xls) files are attached along with supporting information.
xls 1- miRNA expression fold change values treated with each compound in MCF-7 cell line.
xls 2- miRNA expression fold change values treated with each compound in A549 cell line
xls 3- miRNA –target list treated with each compound.
xls 4- Pathway analysis of genes predicted to be modulated by compound 1.
xls 5- Pathway analysis of genes predicted to be modulated by compound 2.
xls 6- Pathway analysis of genes predicted to be modulated by compound 3.

Experimental Section

Synthesis of quinazoline based compounds. The compounds were synthesized via multicomponent one pot green synthesis as described previously.[1] General procedure for synthesis of these quinazoline derivatives was as following: Molecular iodine (0.1 mmol) was used as catalyst in reaction of substituted 2-aminobenzophenone (2 mmol), with a substituted aromatic aldehyde (2 mmol) and ammonium acetate (5 mmol). The reaction mixture was heated at 40 ˚C for about 2.5 hrs. The reaction was monitored by regular checking of TLC. After completion of the reaction, crude mixture was washed with water (3 x 20 mL) followed by ethanol (3 x 20 mL) and dried in high vacuum to afford NMR pure product. The products were confirmed by ^{1}H and ^{13}C NMR, IR and ESI-MS.

Cell culture MCF-7 (human breast cancer cell line), A549 (human adenocarcinomic alveolar basal epithelial cell line) and Hela cells (human cervical cancer cell line) were maintained according to the guidelines provided by ATCC (www.atcc.org) and incubated at 37˚C in 5% CO_2 humidified chamber. Dulbecco's modified Eagle's Minimum Essential Medium (DMEM) High Glucose GlutaMAX™ (Life Technologies) was supplemented with heat inactivated 10% fetal bovine serum, FBS (Life Technologies) to the cells and subcultured every other day.

MiRNA expression profiling. The MCF-7 cells were cultivated in 6-well plate (3×10^5cells/well), grown to 70% confluency and treated with 20 μM of each of the quinazoline compounds (Compound 1, compound 2, compound 3) and incubated at 37 °C for 48 hrs in 5% CO_2 incubator. The media was removed and cells were washed gently with 1X PBS buffer. Total RNA was isolated using TRizol® Reagent (Invitrogen, USA). 1μg of starting RNA was used to synthesize cDNA. Universal Reverse primer was provided by Human miRNome profiler kit (SBI). MiRNA expression profiling was done

in pre-formatted qPCR array format as instructed by System Biosciences (SBI). The same protocol was followed for A549 cell line also.

Cell viability assay. MCF-7 cells were seeded in 96 well-plates (6000 cells/well), grown to 70% confluency and then treated with each of the three compounds at different concentrations ranging from 0-100 μM. After 48hrs of growth cells were treated with 1 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma-Aldrich) and incubated for 4hrs at 37 °C in CO_2 incubator. After incubation, the medium was decanted and formazan crystals were dissolved in 200 μl DMSO solution. Sample absorbance was measured at 570 nm with a reference wavelength of 630 nm. Data was normalized with the DMSO treated set. The MTT assay was also performed in two other cell lines (A549 and HeLa cell line).

Bioinformatic Analysis. The profiling expression data of miRNA revealed a list of upregulated miRNAs. (see xls1 attached with ESI†) All the upregulated miRNAs were chosen and their experimentally validated targets were listed using miRtarbase server. A list of unique mRNAs that can be targeted by any of these upregulated miRNAs were made for each molecule (see xls3 in ESI†) .Next, those unique mRNAs were searched for pathway analysis in Genecodis using panther pathway databases to find out the pathways that are getting enriched by any of the treatment.(see xls4, xls5, xls6 in ESI†).

Apoptosis Detection by TUNEL Assay. To determine the extent of apoptosis induced in response to three quinazoline compounds treatment, we performed the apoptosis assay using the DeadEnd™ Colorimetric TUNEL System kit, Promega following the instructions of the manufacturer. Briefly, MCF-7 cells were seeded on poly-lysine coated coverslips in 6-well plates (3×10^5 cells/well) and treated with each of the three compounds at a final concentration of 20 μM and grown to 48 hrs. The cells were then washed twice with 1X PBS, and fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were permeabilized with 0.2% Triton-X for 5 min at room temperature. The cells were washed with PBS, and incubated with TUNEL reaction mixture (biotinylated nucleotide mix) for 60 min at 37°C in humidified chamber. Negative control was prepared which was treated with the TUNEL label reaction mixture (without Terminal deoxynucleotidyl transferase enzyme). Endogenous peroxidase activity was quenched using 0.3% H_2O_2 for 5 min at room temperature. The cells were treated with streptavidin-HRP (1:500) and stained with DAB components. The cells were washed with deionized water several times before imaging.

FACS assay MCF-7 cells were seeded in 6-well plates at a density (7 x 10^4 cells/well) and treated with compounds at 20 μM and incubated for 48 hrs prior to harvesting. The Flow cytometry assay was performed as per manufacturer's instructions (Invitrogen). In brief, harvested cells were then washed twice with cold PBS ad incubated with annexin V- Alexa 488 (Invitrogen) and PI (50 μg/ml, Invitrogen) for 15 min in dark. The stained cells were analyzed by flow cytometry to determine the percentages of $AnnexinV^+/PI^-$ (early apoptosis), $AnnexinV^+/PI^+$ (late apoptosis) cells using FACS (BD-LSR-II).

Quantitative RT-PCR. MCF-7 cells maintained in growth medium (DMEM high-glucose, 10% FBS, without antibiotic and antimycotic*)* were seeded in 12 well plate (7×10^4 cells/well) and treated with each of the three compounds at a concentration range of 0-20 μM at 70% confluency. Cells were incubated at 37 °C humidified incubator with 5% CO_2 for 48 hrs after treatment. Following treatment, the cells were lysed using TRizol® Reagent (Invitrogen) and total RNA was extracted. On the basis of profiling data, four miRNAs from each treatment was selected for concentration dependent qRT-PCR. miR-133a, miR-141, miR-15a, miR-192 was chosen for compound 1; miR-15a, miR-192, miR-215, miR-143 was chosen for compound 2; and miR-215, miR-205, miR-141 and miR-146 was chosen for compound 3. Expression of candidate microRNAs was quantified using QuantiMir kit. (SBI, catalog number RA660A-1). Primers used for qRT-PCR is listed below:

Forward primer for miR-133a: AGCTGGTAAAATGGAACCAAAT
Forward primer for miR-15a: TAGCAGCACATAATGGTTTGTG
Forward primer for miR-141: TAACACTGTCTGGTAAAGATGG
Forward primer for miR-192: CTGACCTATGAATTGACAGCC
Forward primer for miR-215: ATGACCTATGAATTGACAGAC
Forward primer for miR-205: TCCTTCATTCCACCGGAGTCTG
Forward primer for miR-146a: TGAGAACTGAATTCCATGGGTT

Reverse primer: QuantiMir universal reverse primer.

Forward primer was purchased from Sigma and the universal reverse primer was provided in the QuantiMir kit. The reaction was conducted for 40 cycles and SYBR-green I PCR master mix (Applied biosystems) was used to quantify mature microRNA levels on Roche Lightcycler 480. U6 gene was amplified as the reference for miRNAs and the data was normalized with respect to it. Data analysis was done by using $\Delta\Delta$-C_T method.[2]

Western Blot. Total protein was extracted from treated (10 μM of compound 1, compound 2, compound 3) and untreated MCF-7cells using celLytic M buffer (Sigma- Aldrich). Cells treated with DMSO were also taken as negative control. Protein concentration for each sample was estimated using BCA™ protein assay (Pierce Chemical Co., Rockland, IL). An equivalent amount of protein from each sample was loaded on 12% SDS-PAGE. Following transfer to PVDF membrane, the blot was blocked with 5% BSA overnight at 4˚C. Next, the blot was incubated with primary antibody (1:1000) specific for Bcl-2 protein (Abcam) and PARP (Cell Signalling Technology). After washing the blot thrice with 1X TBS, it was probed with alkaline phosphatase conjugated secondary antibody (1:7000 dilution, Abcam). The blot was developed using AP developing solution (B genie) and the image was captured. β- actin was used as a loading control in both the blots.

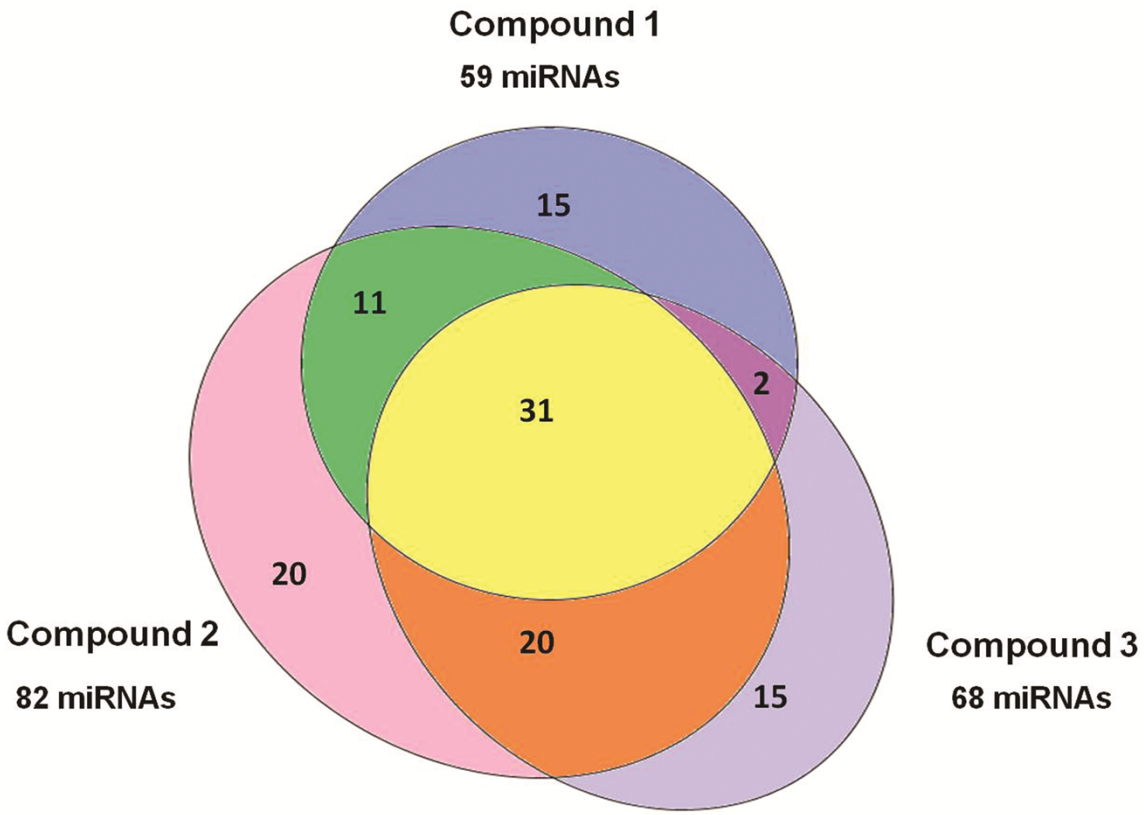

Figure S1: Venn diagram illustrating number of upregulated miRNAs by treatment of 3 compounds (compound 1, compound 2, and compound 3). 31 miRNAs were commonly upregulated by all the 3 compounds.

Table1. Analysis of miRNA expression result in MCF-7 cell line.

Compound 1	**Compound 2**	**Compound 3**
70 differentially regulated miRNA (out of ~280)	89 differentially regulated miRNA (out of ~280)	77 differentially regulated miRNA (out of ~280)
59 miRNA upregulated (84.2%)	82 miRNA upregulated (92.63%)	68 miRNA upregulated (98.31%)
50 miRNAs involved in cancer (84.7%)	76 miRNAs involved in cancer (92.68%)	51 miRNAs involved in cancer (75%)
74% are tumor suppressor miRNAs	67.1% are tumor suppressor miRNAs	58.8% are tumor suppressor miRNAs

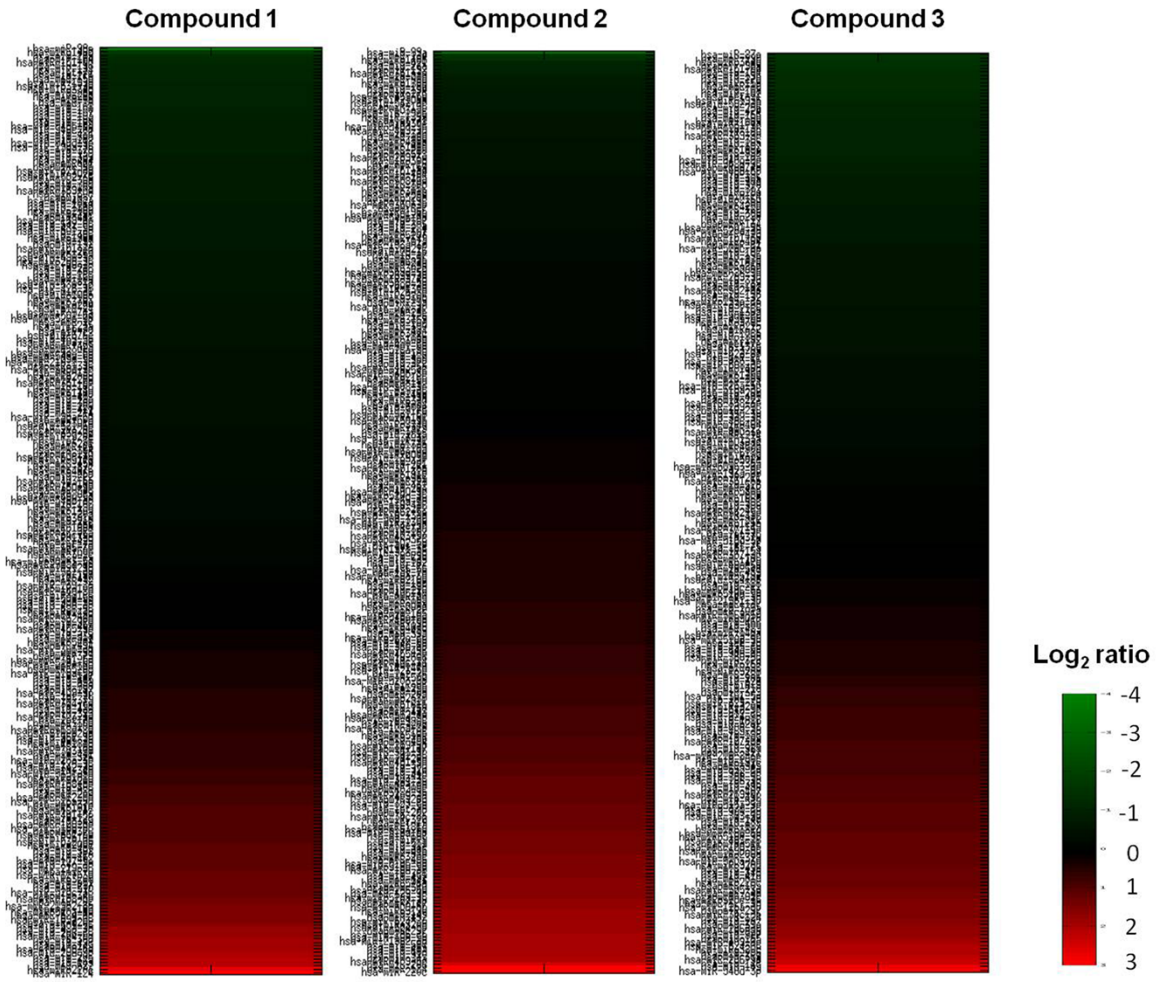

Figure S2: miRNA expression heat map of A549 cell line upon administration of compound 1, 2 and 3. The expression profile revealed ~ 276 miRNAs to be expressed (within $81<c_t<30$), and among the differentially expressed miRNAs (taking 2 fold as cut- off), 60% miRNAs are upregulated in compound 1 treated cells. In case of compound 2 and 3, 91.3% and 69.01% miRNAs were upregulated respectively. Red color indicates upregulated miRNA; green indicates downregulated and black indicates no significant change in the expression of treated vs vehicle control.

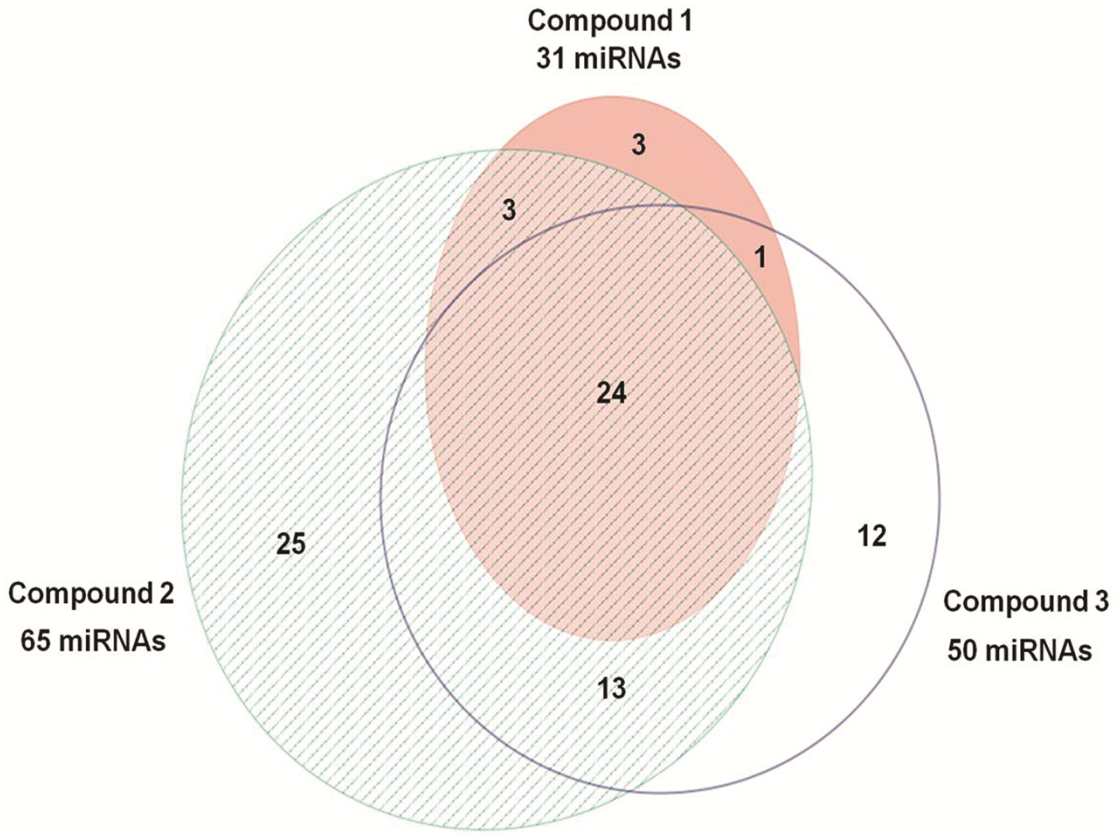

Figure S3: Venn diagram illustrating number of upregulated miRNAs in A549 cell line by treatment of 3 compounds (compound 1, compound 2, and compound 3). 24 miRNAs were commonly upregulated by all the 3 compounds.

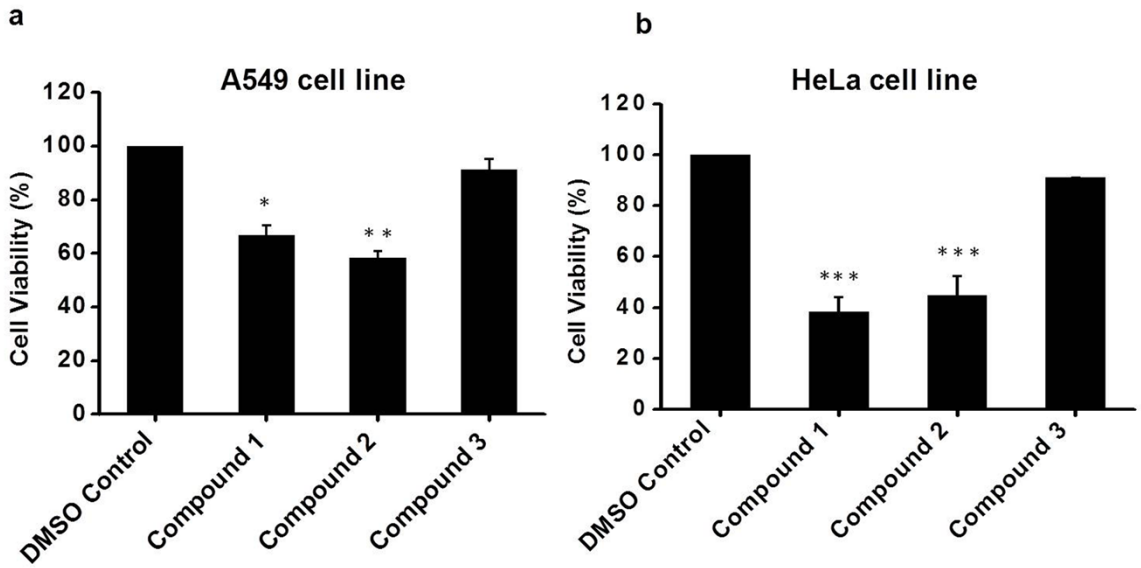

Figure S4. Evaluation of cell viability in a) A549 cell line and b) HeLa cell line upon treatment of compound 1, 2 and 3 at 20 μM after 72 hrs. Error bars represent means of 3 replicates ± S.E. Statistical significance calculated using Student's *t* test with * pvalue < 0.0001, ** p-value <0.00001, *** p-value <0.000001

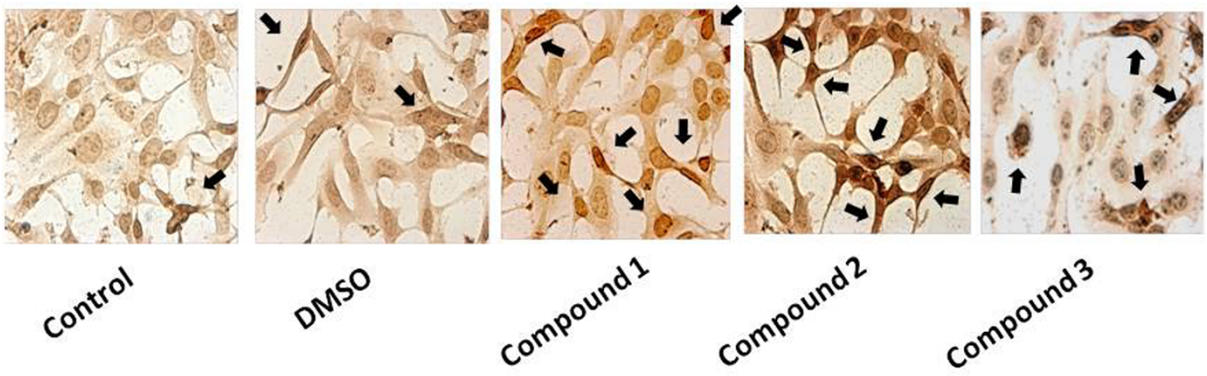

Figure S5. Apoptotic cells (as indicated by black arrows) increased by treatment of each of the three compounds as demonstrated by TUNEL assay.

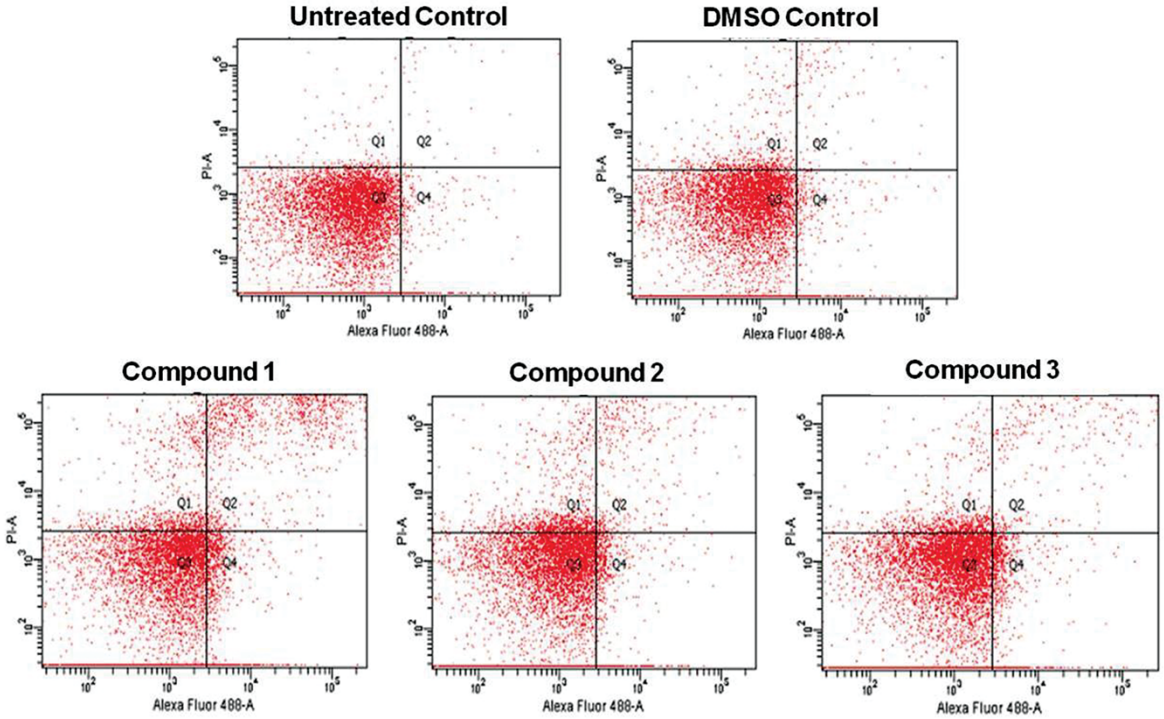

Figure S6. Assessment of apoptosis after treatment of quinazoline compounds (compound 1, 2 and 3) was measured by FACS assay with Annexin V- PI staining compared to the untreated and vehicle (DMSO) control.

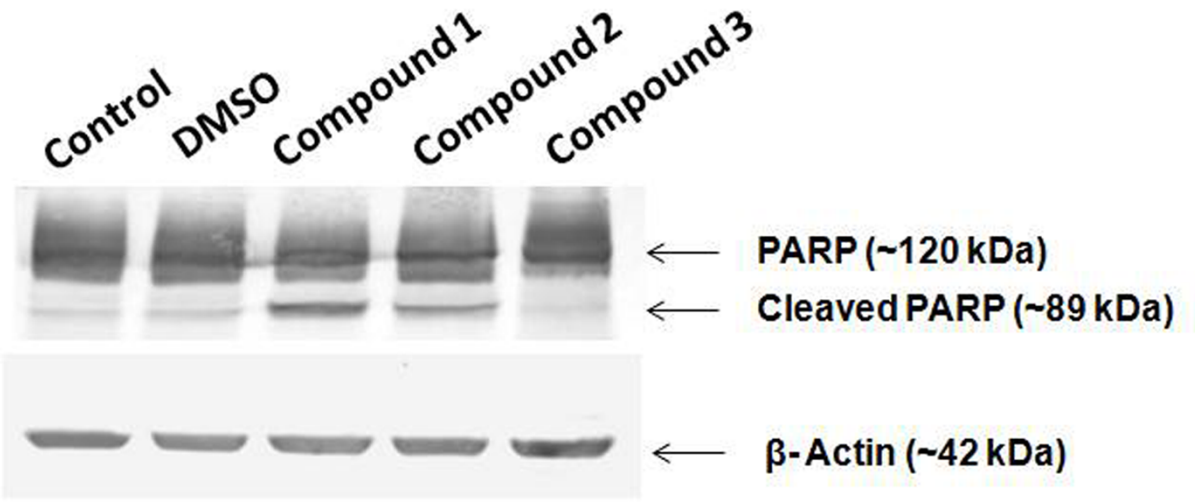

Figure S7. Increased PARP cleavage activity upon treatment with 3 compounds. Compound 3 exhibits less PARP cleavage as compared to compound 1 and 2 suggesting its less evident role in apoptosis.

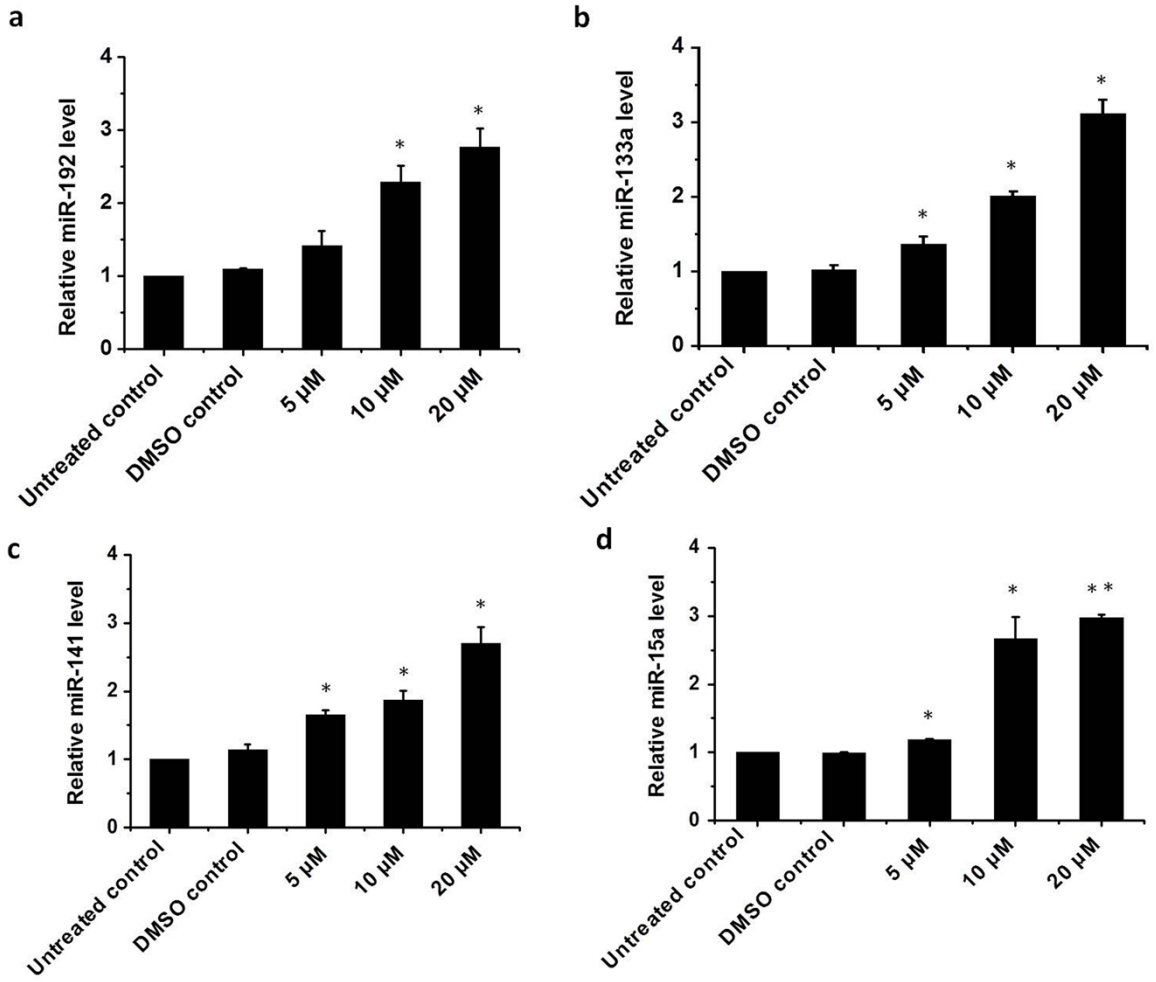

Figure S8. Relative levels of miRNAs quantitated by qRT-PCR. Dose dependent increment in four candidate miRNAs a) miR-192 b) miR-133a c) miR-141 d) miR-15a upon treatment with compound 1. The error bars represent the means of 3 replicates with ± SD. The asterisk (*) indicates significant difference between treated and control as calculated by student's *t* test. (* p-value < 0.05, ** p-value <0.001).

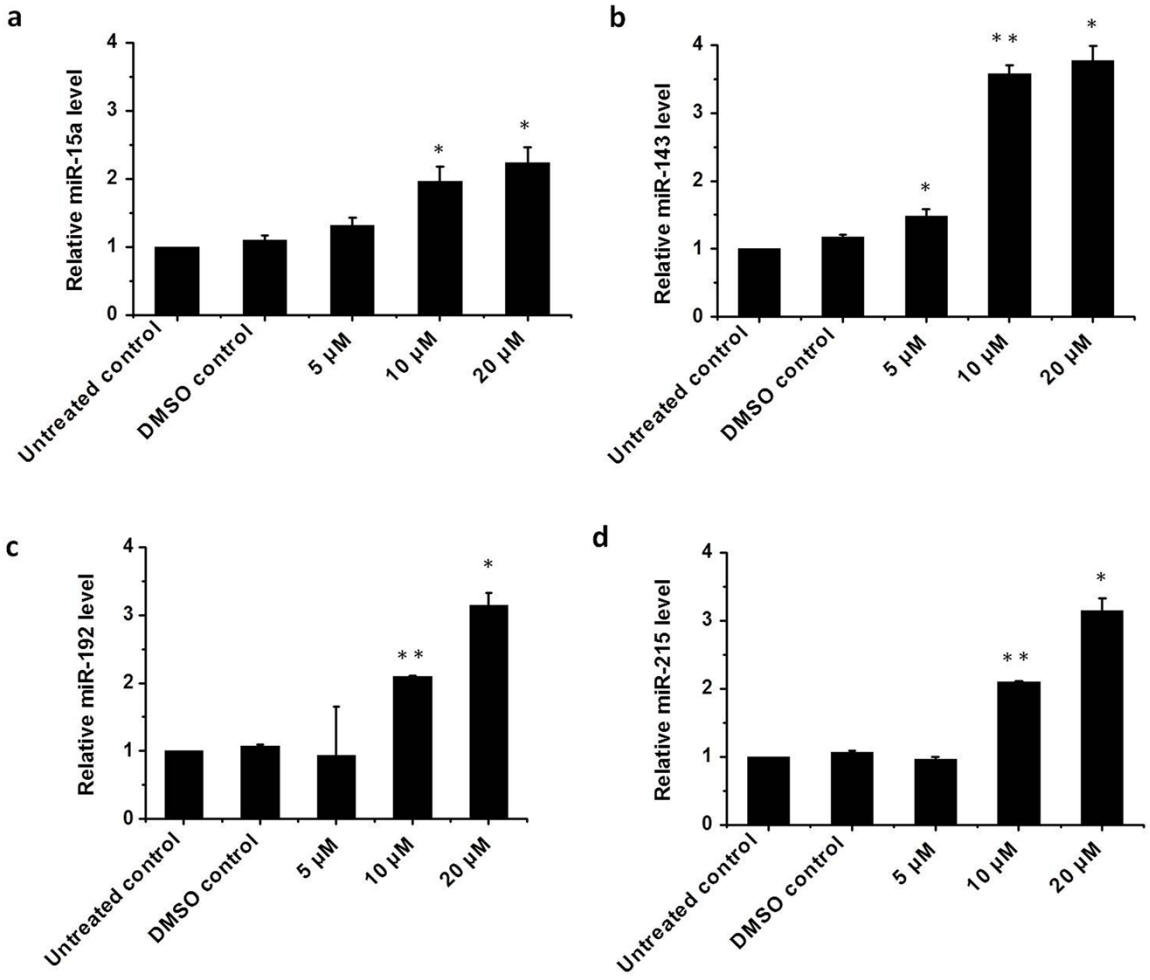

Figure S9. Real time RT-PCR quantification of miRNA levels. Dose dependent increase in four candidate miRNAs a) miR-15a, b) miR-143, c) miR-192, d) miR-215 upon treatment with compound 2. The error bars represent the means of 3 replicates with ± SD. The asterisk (*) indicates significant difference between treated and control as calculated by student's *t* test. (* p-value < 0.05, ** p-value <0.001).

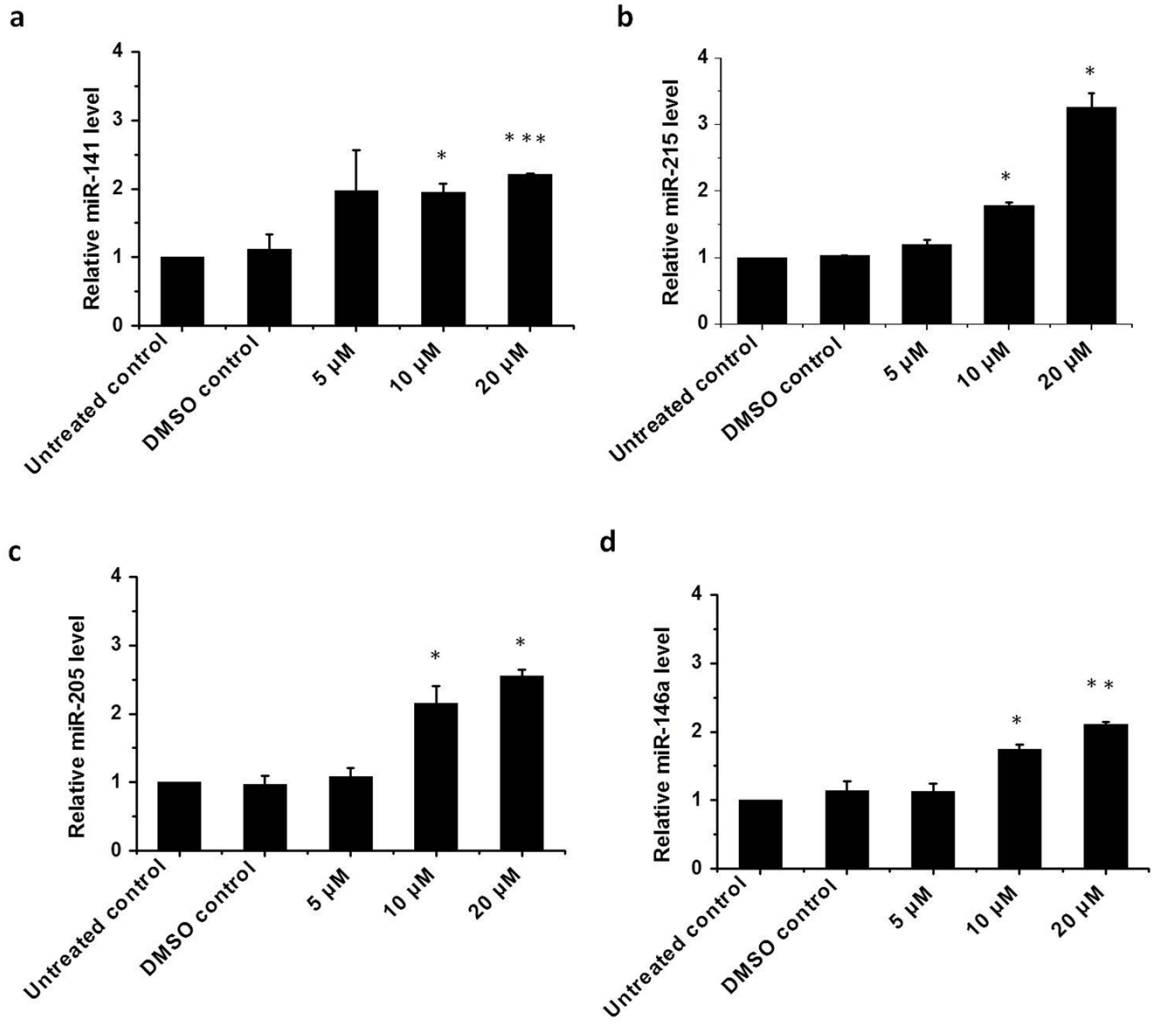

Figure S10. Real time RT-PCR quantification of miRNA levels. Dose dependent increase in four candidate miRNAs a) miR-141, b) miR-215, c) miR-205, d) miR-146a upon treatment with compound 3. The error bars represent the means of 3 replicates with ± SD. The asterisk (*) indicates significant difference between treated and control as calculated by student's *t* test. (* p-value < 0.05, ** p-value <0.001, *** p-value <0.0001).

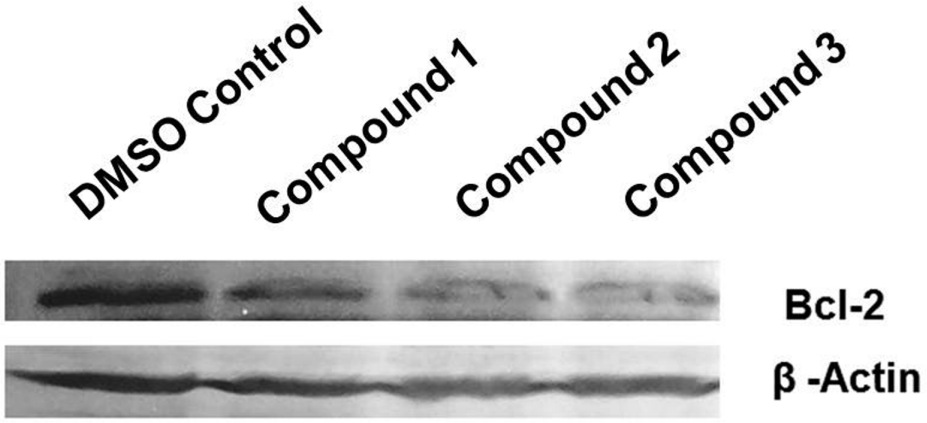

Figure S11. Western blot analysis. Levels of Bcl-2 protein were decreased upon treatment with compound 1, 2 and 3 as compared to DMSO control. β-Actin was used as a loading control.

References

1. S. K. Panja, N. Dwivedi and S. Saha, *Tetrahedron Letters*, 2012, **53**, 6167-6172.
2. K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402-408.