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Fluoxetine mediates G0/G1 arrest by inducing functional inhibition of cyclin dependent kinase subunit (CKS)1

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ABSTRACT

Fluoxetine, a well-known antidepressant used clinically for mental depression has gained attention in cancer research owing to its chemosensitizing potential in drug resistant cell lines. Some preliminary reports, however, suggested its independent cytotoxic potential which is not yet well characterized. Our aim in this study was to characterize its antiproliferative activity in tumor cells and to further elucidate the mechanism. We found that fluoxetine sensitized the effect of cyclophosphamide even in drug sensitive MDA MB 231 and SiHa cells. IC_{50} values of 28 and 32 μM were obtained for fluoxetine mediated antiproliferative response in these cells. Further, PARP and caspase 3 cleavage analyses confirmed fluoxetine mediated apoptosis at molecular level. Cell cycle analysis showed that fluoxetine arrested cells at G0/G1 phase in a time dependent manner. The application of bioinformatics tools at this juncture predicted CKS1 as one of the possible targets of fluoxetine, which is of relevance to cell cycle biology. Fluoxetine showed the potential to disrupt skp2–CKS1 assembly required for ubiquitination and proteasomal degradation of p27 and p21. Our *in vitro* results were in agreement with the predictions made *in silico*. We found that fluoxetine treatment could accumulate p27 and p21, an immediate outcome characteristic of functional inhibition of CKS1. This was accompanied by the accumulation of cyclin E, another possible target of CKS1. We observed CKS1 downregulation also upon prolonged fluoxetine treatment. Fluoxetine had downregulated cyclin A which confirmed G0/G1 arrest at the molecular level. We conclude that fluoxetine induced cell cycle arrest is CKS1 dependent.

1. Introduction

Fluoxetine hydrochloride (Prozac) is a well-known antidepressant used for clinical depression for more than two decades. It comes under the class of selective serotonin reuptake inhibitors (SSRI) and mediates its antidepressant action through inhibiting serotonin reuptake transporters in neurons [1]. Recent studies have identified its potential to reverse multidrug resistance generated by two major pump proteins MRP1 and P-glycoprotein [2]. Fluoxetine sensitized the cytotoxic potential of conventional anticancer agents in drug resistance cells and human xenograft mouse tumor models [3]. It has also been shown to induce cytotoxicity in some tumor derived cells when used alone [4,5]. But detailed mechanism of its antiproliferative action is not well characterized. Additionally, fluoxetine stimulates neuronal differentiation by selectively targeting neuronal stem cells [6].

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Cell division cycle progresses by the differential catalytic activities of cyclin–cdk complexes. Their catalytic activities are controlled by another group of proteins known as cyclin dependent kinase inhibitors (CKIs). Aberrant elevation of cyclins and downregulation of CKIs has been proven to be poor prognostic features in many cancers [7,8]. CKIs regulate the kinase activities of cyclin–cdk complexes mainly at two check points each in G1/S and G2/M transition phase. G1/S check point is controlled by INK4 and cip/kip family of CKIs of which p27kip1 and p21cip1 are well characterized [9]. Degradation of p27 and p21 is mediated by the proteasomal machinery and involves SCF skp2 ubiquitin ligase. SCF skp2 is composed of skp1, Cul1, p45skp2, and ROC1/Rbx1. This E3 ligase requires association of cyclin dependent kinase subunit 1 (CKS1) for its ubiquitin ligase activity towards p21 and Thr-187 phosphorylated p27 [10,11]. Thus, CKS1 serves as an adaptor protein which links these CKIs to skp2 for ubiquitination and further proteasomal degradation.

Our aim in this study was to characterize the antiproliferative effect of fluoxetine and to further elucidate the mechanism of action. Human breast and cervical cancer cells were used for this study. Fluoxetine sensitized the cytotoxic potential of cyclophosphamide (CPA) even in these drug sensitive cell lines. We found that fluoxetine arrested cells at G0/G1 phase of the cell division cycle in a time dependent manner. In order to identify potential molecular targets of fluoxetine, a bioinformatics based approach was employed. A structure based, molecular docking protocol was used to identify putative protein targets of fluoxetine among a subset of cell cycle-related proteins, whose three-dimensional coordinates were retrieved from the Protein Data Bank (PDB). The results from this ''reverse docking or reverse screening'' approach predicted a possible molecular interaction between the small molecule fluoxetine and the skp2-binding site located on CKS1. Such an interaction is likely to perturb and disrupt the skp1–skp2–CKS1–p27 complex, leading to reduced ubiquitination of p27 (and by extrapolation, p21 since both p27 and p21 are assumed to share the same mode of complex formation with CKS1). These predictions were consistent with our laboratory results which showed accumulation of p27 and p21 in fluoxetine treated cells. CKS1 downregulation was also observed upon prolonged exposure of fluoxetine. In addition, we have observed accumulation and downregulation of cyclin E and cyclin A.

2. Materials and methods

2.1. Materials

Human cervical cancer (SiHa) and breast cancer cells (MDA MB 231) were purchased from ATCC (USA) and maintained in DMEM containing 10% fetal bovine serum (Sigma, USA) and 1% antibiotic antimycotic cocktail (Himedia, India). Taq DNA polymerase, 10× Taq buffer and pre-mix dNTP were purchased from Biogene (USA). RNAsin, random primers, M-MLV RT 5× buffer and M-MLV RT enzyme were purchased from Promega (USA). All the other chemicals and antibodies to cyclin E and cyclin A were purchased from Sigma (USA). Antibodies to p21, PARP, caspase3 and β-actin were purchased from Cell signaling technology (USA) and antibodies to p27, CKS1 and secondary antibodies (HRP-conjugated antimouse and antirabbit) were purchased from Santa Cruz biotechnology (USA).

2.2. Cytotoxicity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was performed to assess cell viability. Briefly, cells were (7500 cells) seeded in a 96 well cluster plate and allowed to reach the exponential phase of growth. Cells were given fluoxetine treatment (10–40 μM) for 48 and 72 h and the amount of formazan crystals formed was measured after 4 h of MTT addition. The crystals were dissolved in isopropyl alcohol and OD measured at 570 nm. For chemosensitization experiments, cells were given combined treatment of CPA (2.5–10 mM) and fluoxetine (10 μM). Further, fractional inhibitory concentration (FIC) index value was calculated to assess whether the chemosensitization is due to additive or synergistic effect. The FIC of a factor is the concentration that kills when used in combination with another agent divided by the concentration that has the same effect when used alone. The FIC index for the combination of A and B is the sum of their individual FIC values. On combination treatment with two factors; additive interactions have a FIC index of 1; an FIC index <1 defines synergistic interactions; combinations with an FIC index >1 are antagonistic.

2.3. Isolation of primary fibroblasts from breast and cervical tumors

Primary fibroblasts were isolated from human breast and cervical tumors. Fresh tumor tissues were chopped into fine pieces followed by trypsin (0.3%) treatment, in cold conditions, under mild shaking for 16 h. The tissue mass were then kept at 37 °C in a shaking water bath for another 15 min followed by vigorous pipetting to detach individual cells. Bulk pieces were allowed to settle, collected supernatant and centrifuged (5000 rpm, 10 min) to collect the cell pellet. Fresh medium (DMEM containing 10%FBS) was added to the pellet, resuspended and seeded in culture flask for a mixed culture of fibroblasts and epithelial cells. Fibroblasts were differentially trypsinized out from the mixed culture. Pure fibroblast cultures were obtained after the tenth passage.

2.4. Apoptotic assays

For fluorescence cytochemical study, cells were seeded in a 35 mm culture dish and fluoxetine treatment was given for 24 and 48 h. Medium was removed and the cells were washed with phosphate buffered saline. The cells were then given a combined staining of acridine orange (50 μg/ml) and ethidium bromide (5 μg/ml) for 5 min at room temperature, and examined by an inverted fluorescence microscope. Similar method was followed for Hoechst staining (5 μg/ml) except for the final step in which the cells were given an incubation period of 15 min at 37 °C before visualization.

For annexin-binding assay, 10^6 cells were seeded in 60-mm culture dishes. Fluoxetine treatment was given for 24 and 48 h and the cells were harvested and stained with FITC-labeled

annexin using Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instruction, and a flow cytometric analysis was then carried out using FACS Aria (Special order system, BD, USA).

2.5. Cell cycle analysis

For flow cytometric analysis, 10^6 cells were seeded in 60-mm culture dishes. Fluoxetine treatment was given for different time intervals (12, 24 and 36 h) and the cells were harvested and fixed with 70% ethanol for 1 h. The fixed cells were then given RNase A (100 μg/ml) treatment for 1 h followed by incubation with propidium iodide (10 μg/ml), in the dark, for 15 min. Finally, DNA content of the cells was analyzed using FACS Aria (Special order system, BD, USA).

2.6. Prediction of molecular targets of fluoxetine: ''Inverse screening'' based on molecular docking

For identifying putative molecular targets of fluoxetine (of relevance to the present study looking at the molecule's effects on cell cycle-related events), a subset of the Proteins Data Bank (PDB) were screened based on molecular docking. A list of sixty proteins (for screening) was compiled that met the following criteria:

1. Only human proteins were selected.
2. Only proteins involved in processes related to and including cell cycle regulation and apoptosis were included.
3. Only proteins with X-ray crystal structures were included.
4. Finally, only proteins with explicitly defined binding sites (either from bound ligand or from other sources) were included.

Swiss-pdb viewer (Deep view 3.7) was used to perform many of the following steps [12]. The molecular docking package used for the study was GOLD (Genetic Optimization for Ligand Docking). GOLD is distributed by Cambridge Crystallographic Data Centre. GOLD employs a genetic algorithm (GA) to explore the full range of conformational flexibility of the ligand and also the rotational flexibility of selected receptor hydrogens [13,14]. The entire computational work was carried out on an Intel Pentium 4 processor, 2.8 GHz.

1. Protein (''Receptor'') input file preparation: For each of the protein–ligand complexes chosen for the study, a 'clean input file' was generated by removing water molecules, ions, ligands and subunits not involved in ligand binding from the original structure file. Hydrogen atoms were then added to the protein and the active site was inspected to make suitable corrections for tautomeric states of histidines, hydroxyl group orientations and protonation states of charged residues. Local minimization was then performed in the presence of restraints to relieve potential bad contacts, at the same time maintaining the protein conformation very close to that observed in the crystallographic model. The resulting receptor model was saved to a PDB file (compatible with GOLD input file formats).
2. Ligand input file preparation: For docking studies, ligand (fluoxetine) input structure was generated with Corina (Demonstration license from Molecular Networks GmbH) [15]. The ligand structure was saved to a PDB file. The starting conformation of fluoxetine, whose molecular structure was created by Corina, was chosen arbitrarily (following several rounds of energy minimization) since GOLD samples random conformations and fluoxetine has not been reported to undergo major flipping conformational rearrangements.
3. GOLD docking protocol: For the study, the binding pocket of each receptor was defined from the crystallographic coordinates of the ligand (residues within 3.5 Å of the ligand) or residues mapped from information extracted from other sources. Dockings were performed under 'Standard default settings' mode-number of islands was 5, population size of 100, number of operations was 100,000, a niche size of 2, and a selection pressure of 1.1.
4. The ligand–protein docked complex structural model was energy minimized using 200 cycles of steepest descent.

2.7. Immunoblot analysis

2×10^6 cells were seeded in 100-mm culture dishes and fluoxetine treatment was given for different time periods (12, 24, 36 and 48 h). Cells were then lysed and the total protein content was measured using Bradford's reagent. 50 μg of total protein was loaded for SDS-PAGE and immunoblotting was carried out using specific antibodies. Horseradish peroxidase-conjugated secondary antibodies were used followed by detection using enhanced chemiluminescence (ECL) method.

2.8. Reverse transcriptase RT-PCR

Cells were treated with fluoxetine for different time periods (12, 24 and 36 h) and total RNA was isolated using trizol reagent. cDNA was prepared and PCR amplification was done using the following sense (S) and antisense (AS) primer sequences and annealing temperatures: CKS1-S, 5′-GAATGGAGGAATCTTGGCGTTC-3′ and CKS1-AS, 5′-TCTTTGGTTTCTTGGGTAGTGGG-3′ (Ta, 55.7 °C); p27-S, 5′-ACGGGAGCCCTAGCCTGGAGC-3′ and p27-AS, 5′-TGCCCTTCTCCA CCTCTTGCC-3′ (Ta, 61.2 °C); p21-S, 5′-ACCGAGGCACTCAGAGGA-3′ and p21-AS, 5′-AGATGTAGAGCGGGCCTTT-3′ (Ta, 61.2 °C); cyclin E-S, 5′-CTCCAGGAA GAGGAAGGCAA-3′ and cyclin E-AS, 5′-TCGATTTTGGCCATTTCTTCA-3′ (Ta, 60 °C); cyclin A-S, 5′-GTCACCACATACTATGGACATG-3′ and cyclin A-AS, 5′-AAGTTTTCCTCTCAGCACTGAC-3′ (Ta, 55.7 °C). The mRNA levels were normalized to human GAPDH mRNA levels and the following primers were used: GAPDH-S, 5′-TTGGTATCGTGGAAGGACTCA-3′ and GAPDH-AS, 5′-TGTCATCATATTTGGCAGGTT-3′ (Ta, 60°). Analysis of amplified products was done on 1.5% agarose gel and visualized using Fluor-S™ Multiimager (Bio-Rad). Densitometric analysis of RT PCR products was performed by the computer software (Bio-Rad Quantity One). A 100-bp ladder was used as a size standard.

3. Results

3.1. *Fluoxetine is cytotoxic to tumor cell lines and tumor derived primary fibroblasts*

Previous literatures have reported the chemosensitization potential of fluoxetine in different drug resistant cell lines [2,3]. In order to check similar possibilities in drug sensitive systems, we performed a chemosensitization assay using CPA sensitive SiHa and MDA MB 231 cells. The assay was based on the ability of viable cells to reduce MTT to formazan crystals. Fluoxetine, at a lower dose of 10 μM, sensitized CPA induced cytotoxicity even in these drug sensitive cells (Fig. 1). Fractional inhibitory concentration (FIC) index value showed that the combination had synergistic effect. Further, cell viability assays were performed to check the possible independent cytotoxic potential of fluoxetine. The concentrations kept were in the range of 10–40 μM. Fluoxetine reduced viability of both MDA MB 231 and SiHa cells in a dose and time dependent manner. The IC_{50} values obtained at 48 h were 28 and 32 μM, respectively. Fibroblasts, being the central component that constitutes tumor microenvironment and associated with cancer cells at all stages of cancer progression, represent an important target for cancer therapies [16]. So we have included fibroblasts also in this preliminary cytotoxicity study. Primary fibroblasts were isolated from fresh human tumors of cervix and breast origin. Fluoxetine was more efficacious, for its cytotoxic potential, towards these primary cells compared to their epithelial counterparts, with IC_{50} values ranged from 15 to 17 μM. These results showed that fluoxetine has independent cytotoxic actions on human breast and cervical cancer cells as well as primary fibroblasts.

3.2. *Fluoxetine induced loss of viability is mediated through apoptotic pathways*

To further characterize the cytotoxic potential, cells were exposed to fluoxetine for 24 and 48 h at a fixed dose of 30 μM

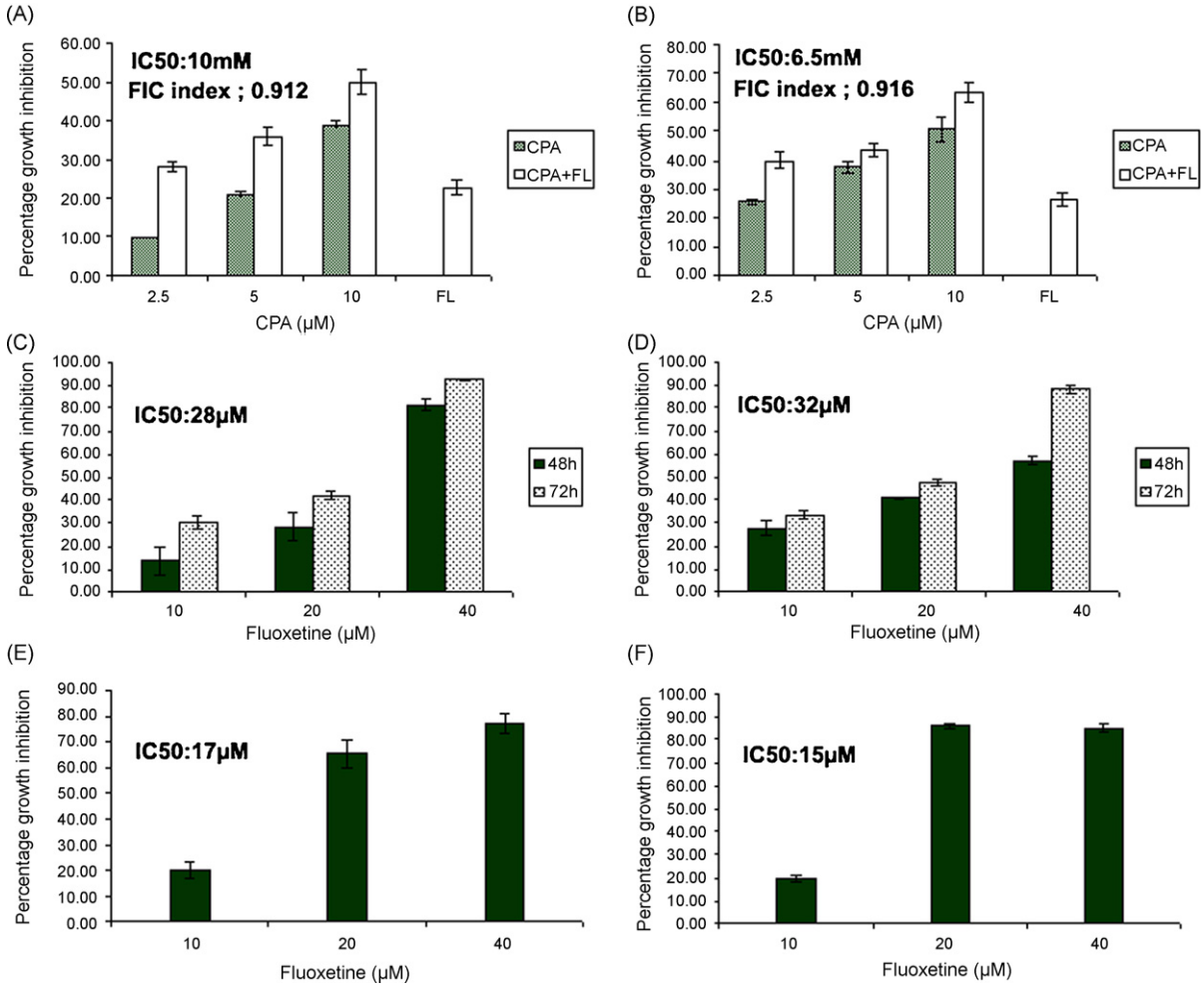

Fig. 1 – Fluoxetine (FL) is cytotoxic to tumor cell lines and tumor derived fibroblasts. Combined treatment of CPA (2.5, 5 and 10 mM) and fluoxetine (10 μM) for 48 h sensitizes the cytotoxic potential of CPA in MDA MB 231 (A) and SiHa (B) cells. IC_{50} values of CPA in combination with fluoxetine and FIC index for a combination of CPA (2.5 mM) and fluoxetine (10 μM) are shown. A dose range of 10–40 μM of fluoxetine promotes a time dependent cytotoxic response in MDA MB 231 (C) and SiHa cells (D) at 48 and 72 h. IC_{50} values at 48 h fluoxetine treatment are shown here. Fluoxetine induces a dose dependent cytotoxic response in primary fibroblasts from breast (E) and cervical tumor (F) at 48 h.

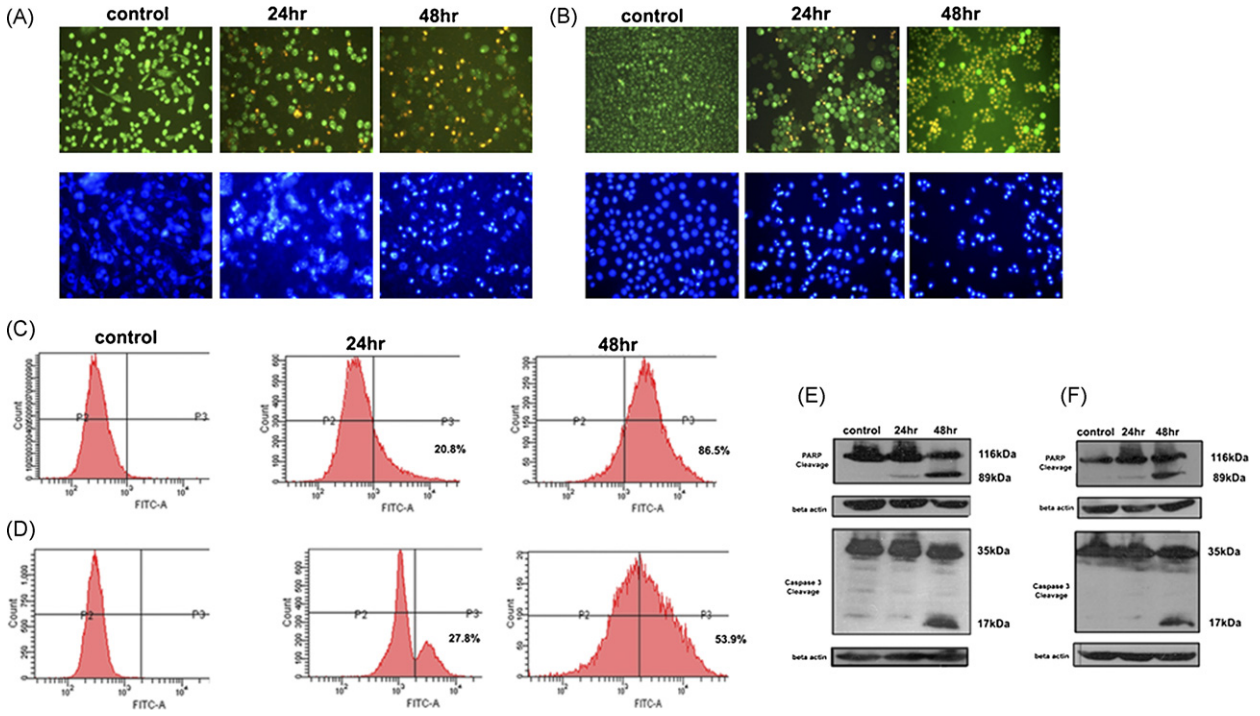

Fig. 2 – Characterization of cytotoxic actions of fluoxetine. The fluorescence cytochemistry is carried out after exposing MDA MB 231 (A) and SiHa (B) cells to 30 μM fluoxetine for 24 and 48 h. Upper panel represents combined staining of acridine orange and ethidium bromide and lower panel represents Hoechst staining. Chromatin condensation is visualized using fluorescence microscope. Ethidium bromide is selectively taken up by apoptotic cells and stained the condensed nuclei whereas the control cells have only taken up acridine orange. In Hoechst staining, apoptotic cells are observed to carry peripherally clumped or condensed chromatin. FITC-conjugated annexin binding to phosphatidyl serine, exposed to the outer leaflet, is measured by FACS analysis in MDA MB 231 (C) and SiHa (D) cells. Apoptotic population (P3) shows a right shift in the FACS histogram plotted against cell count *vs.* FITC staining. Effect of fluoxetine on PARP and caspase 3 cleavages are shown in MDA MB 231 (E) and SiHa (F) cells.

and fluorescence cytochemical study was carried out. Cytochemical staining using acridine orange and ethidium bromide revealed characteristic chromatin condensation, a hallmark of apoptosis, in fluoxetine treated cells. Ethidium bromide was selectively taken up by apoptotic cells and stained the condensed nuclei where as the control cells had only taken up acridine orange (Fig. 2). Chromatin condensation was further confirmed using Hoechst dye in which apoptotic cells observed to carry peripherally clumped or condensed chromatin. Chromatin condensation was observed to increase in a time dependent manner with more number of cells susceptible at 48 h.

Translocation of phosphatidyl serine (PS) from inner lipid bilayer to cell surface is a characteristic feature in the early phase of apoptosis. Translocation of PS rendered easy access to FITC-conjugated annexin and the resulted fluorescence was measured by flow cytometry to detect apoptotic population, which had a right shift in the FACS histogram due to the fluorescence emitted by them. The histogram showed an increase in percentage apoptotic population to 20.8 and 27.8 in MDA MB 231 and SiHa cells respectively at 24 h, whereas the increase was 86.5 and 53.9 at 48 h fluoxetine treatment. We have performed PARP and caspase3 cleavage analyses to characterize apoptotic cell death at the molecular level. Fluoxetine induced PARP cleavage in both the cell lines, which was prominent at 48 h compared to earlier time point of 24 h. Similar pattern was observed for caspase3 also with no cleavage observed at 24 h. These results showed that fluoxetine induces apoptosis in a time dependent manner with majority of cells undergo apoptotic cell death at 48 h.

3.3. *Fluoxetine induced loss of viability is preceded by G0/G1 arrest in the cell division cycle*

Apoptotic assays demonstrated that a minor population of cells only undergoes apoptotic cell death at 24 h fluoxetine treatment and majority of the total population remains viable during the time. Therefore, we have performed cell cycle analysis to check the possibility of fluoxetine to act as a cytostatic agent at early time periods. Fluoxetine treatment induced a time dependent G0/G1 arrest in both the cell lines. It arrested G0/G1 population from 75.1 to 94.4% and 69 to 93.5% in MDA MB 231 and SiHa cells respectively (Fig. 3). Cell cycle arrest was initiated between 12 and 24 h with more percentage of cells underwent arrest at 36 h in MDA MB 231 cells. However, in SiHa cells, growth arrest observed even at 12 h of fluoxetine treatment. Increase in G0/G1 population was associated with simultaneous reduction in S

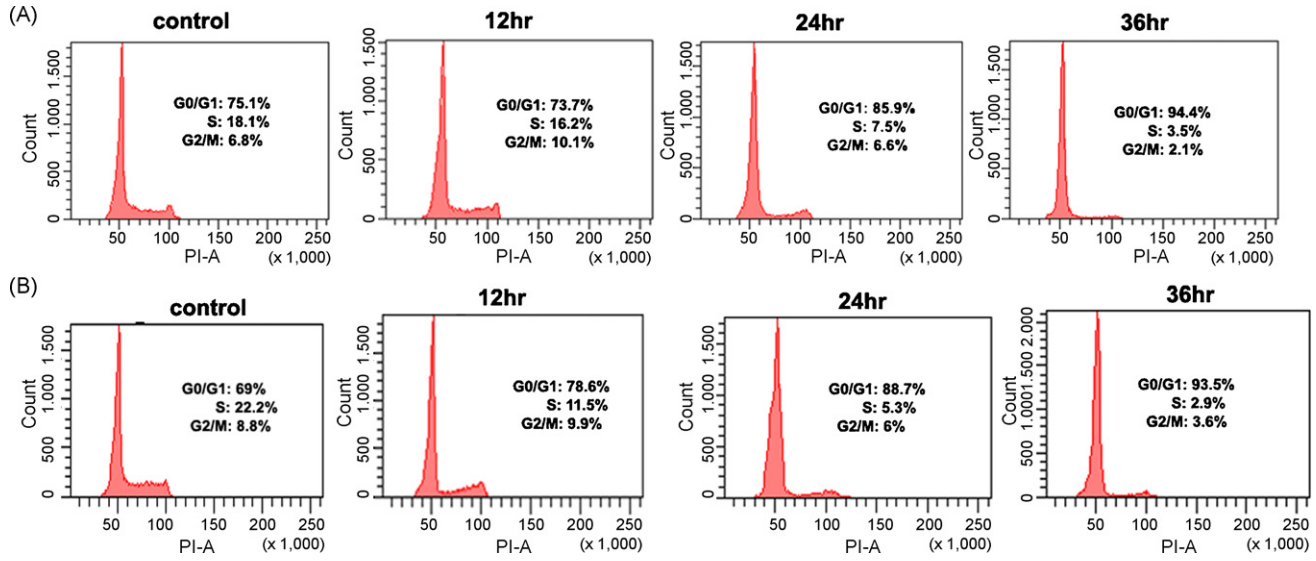

Fig. 3 – Effects of 30 μM of fluoxetine treatment on cell division cycle of asynchronously growing SiHa and MDA MB 231 cells, assessed by FACS analysis. Representative histogram of 3 independent experiments, plotting cell count *vs.* DNA content, after 12, 24 and 36 h of fluoxetine treatment in MDA MB 231 (A) and SiHa (B) cells.

phase population. These results showed that fluoxetine induced apoptotic cell death might be attributed to cell cycle arrest at G0/G1 phase.

3.4. *Fluoxetine "docks" well onto the skp2-binding surface of human cyclin dependent kinase subunit 1 (CKS1)*

Molecular docking of the top ranking "hits" (proteins to which fluoxetine docked well), strongly suggest CKS1 as a possible molecular target of fluoxetine. Fluoxetine docked well into the skp2-binding site of human CKS1 protein (1DKT). The amino acids in the skp2-binding site of CKS1 that made contact with fluoxetine were identified to be His 36, Glu 42 and Glu 40. GoldScore which is a measure of the predicted affinity of the small molecule for the receptor, calculated by GOLD for the fluoxetine–CKS1 complex, was 51.00 (Fig. 4). There was large contribution to the GOLD score from external hydrogen bonding.

3.5. *Fluoxetine treatment accumulates p27 and p21 and prolonged exposure leads to downregulation of CKS1*

Human CKS1 has been identified to be essential for SCFskp2 E3 ligase mediated turn over of both p27 and p21 [10,11]. CKS1 binds with skp2 and alters its confirmation to enhance its accessibility to these CKIs for their proteasome dependent degradation. Bioinformatics tools predicted that fluoxetine could bind the skp2-binding site of human CKS1, which can lead to the functional inhibition of CKS1. Therefore, we have performed immunoblot analyses of p27 and p21 expression as an indirect way to measure the possible functional inhibition of CKS1 by fluoxetine. The assay was performed following 12, 24 and 36 h of fluoxetine treatment. Our results favored the bioinformatics prediction by revealing p27 and p21 accumulation in a time dependent manner (Fig. 5). p27 was found to accumulate at an early time period of 12 h in both the cell lines where as accumulation of p21 was observed to begin at 12 and 24 h in SiHa and MDA MB 231 cells respectively. Accumulation of CKIs thus correlated well with the G0/G1 arrest observed. There was not much change observed in CKS1 expression at early time points of 12 and 24 h. However, the expression was found to decline, which was more prominent in SiHa cells, at a late time interval of 36 h. These results showed that fluoxetine mediated G0/G1 arrest are mediated possibly through the functional inhibition of CKS1. Thus, fluoxetine can be considered as a functional antagonist to CKS1.

3.6. *Fluoxetine treatment accumulates cyclin E and downregulates cyclin A*

Cyclin E–cdk2 and cyclin A–cdk2 complex is mainly involved in the G1/S transition phase of cell division cycle. Cyclin E accumulation has been previously reported in CKS1$^{-/-}$ mouse cell line [11]. So, we have analyzed the expression levels of these two cyclins to investigate the additional molecular alterations involved with fluoxetine mediated cell cycle arrest. We found cyclin E accumulation, following fluoxetine treatment, in a manner similar to p27 and p21 (Fig. 6). Cyclin E, being a possible substrate of CKS1 mediated ubiquitination, thus reinforced the finding of CKS1 functional inhibition by fluoxetine. On contrast, we observed cyclin A downregulation in fluoxetine treated cells which was more prominent at later time points of 24 h and 36 h. Cyclin A downregulation, a characteristic feature of G1 arrest [9] due to the reduced transcriptional activity of E_2F, thus confirmed the effect of fluoxetine on cell division cycle at the molecular level.

3.7. *Fluoxetine treatment leads to fluctuations in p27, p21, CKS1, cyclin E and cyclin A mRNA levels*

We have performed reverse transcriptase RT PCR to investigate the effect of fluoxetine on p27, p21, CKS1, cyclin E and

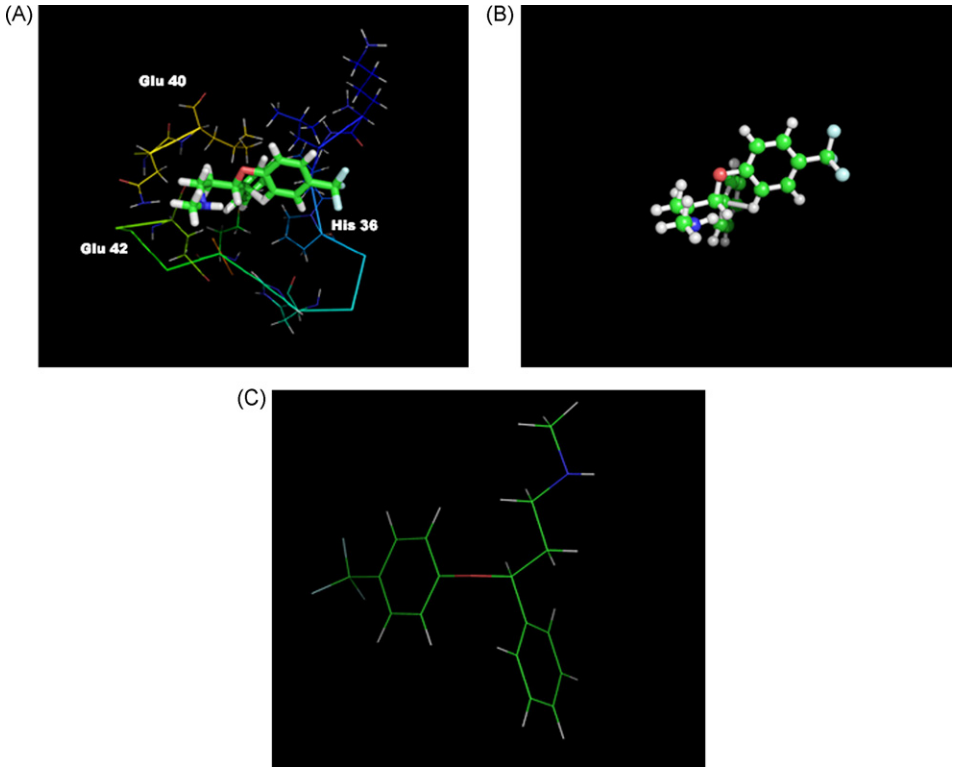

Fig. 4 – Fluoxetine molecule docked into the skp2-binding site on human CKS1. The amino acids making contact with fluoxetine (His 36, Glu 42 and Glu 40) are shown as thin lines (blue cyan, green and yellow colored backbone for the different amino acids) while fluoxetine molecule is depicted in green (A). Another view of the pose of fluoxetine molecule that docked well into the skp2-binding site of human CKS1 (B). Molecular structure of fluoxetine is also shown here (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cyclin A mRNA levels (Fig. 7). Our results showed slight fluctuations in p27 levels in SiHa cells all through fluoxetine treatment whereas a gradual decline in its levels occurred in MDA MB 231 cells at 24 and 36 h preceding an initial slight increase at 12 h post-treatment period. However, a similar pattern of lowering of p27 levels, compared to respective controls, was identified at 36 h irrespective of the cell line used. We have observed fluctuations in p21 levels also throughout the treatment periods in both the cell lines. Nonetheless, there was not much deviation from the control except at 24 h time period in SiHa cells where a marked reduction has observed in its levels. CKS1 and cyclin A levels were augmented initially at 12 h in both the cell lines which was followed by reduction in those levels compared to the respective controls at further treatment periods. Similar pattern was observed for cyclin E also except in MDA MB 231 cells where slight fluctuations in its levels occurred throughout the time periods but always maintained at a lower level compared to the control. Thus, the mRNA analysis does not show a definite and uniform pattern of kinetics except for CKS1 and cyclin A. Moreover, downregulation of these two proteins at later treatment periods are well correlated with its mRNA levels. Together these results indicate that the fluctuations observed in p27, p21 and cyclin E mRNA levels

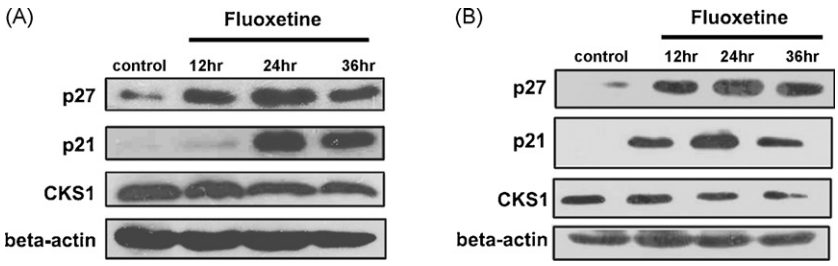

Fig. 5 – Effect of fluoxetine treatment on p27, p21 and CKS1 expression in MDA MB 231 (A) and SiHa (B) cells. Both p27 and p21 are accumulated in a time dependent manner. CKS1 expression decreased at 36 h fluoxetine treatment. β-Actin was used to verify equal gel loading.

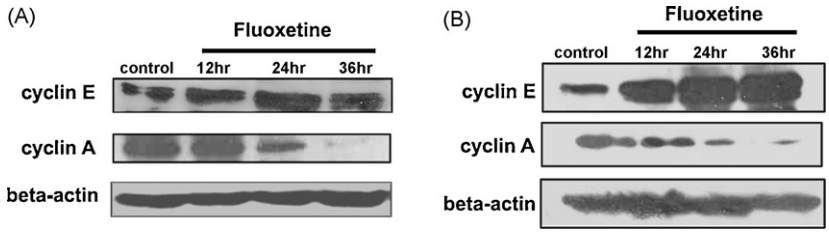

Fig. 6 – Effect of fluoxetine treatment on cyclin E and cyclin A expression in MDA MB 231 (A) and SiHa (B) cells. Cyclin E is accumulated in a time dependent manner whereas cyclin A is downregulated. β-Actin was used to verify equal gel loading.

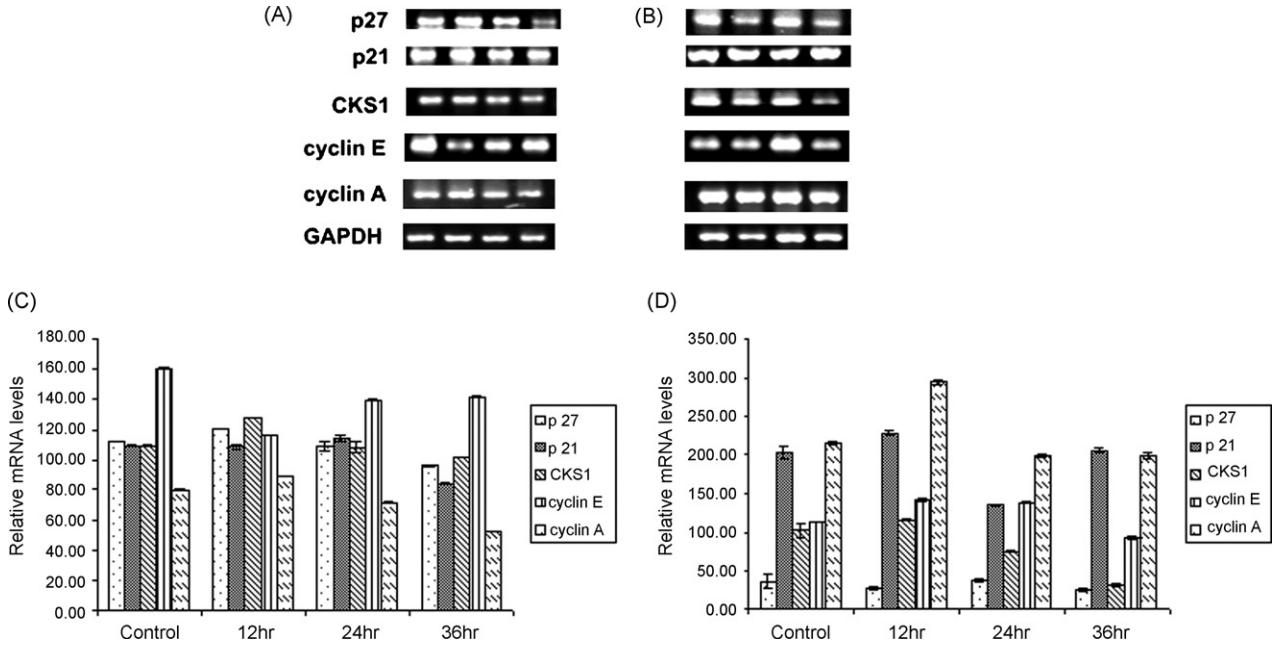

Fig. 7 – Effect of fluoxetine on p27, p21, CKS1, cyclin E and cyclin A mRNA levels in MDA MB 231 (A and C) and SiHa (B and D) cells. Values are normalized to GAPDH mRNA levels. Bar graph shows mean value ± S.E. from three independent experiments.

might be a result of cells' self-regulatory mechanism to respond to the deviation occurred in the normal proteolytic turn over of these proteins.

4. Discussion

Fluoxetine has been introduced as an antidepressant drug and to date is at a stage where the drug is recommended to replace hormonal therapy to treat hot flashes in women with a history of breast cancer [17]. It gained attention in cancer research due to its chemosensitizing effects in drug resistant cells by virtue of its ability to block multidrug extrusion (MDR) pump which is highly expressed in those cells [2,3]. As a preliminary study, we have investigated its chemosensitization potential in drug sensitive cells which do not possess drug induced over-expression of MDR pumps. We have selected CPA as the conventional drug candidate, since it is currently used clinically for breast cancer treatment in combination with other drugs. We observed that fluoxetine sensitized the effect of CPA in those cells also. An IC_{50} value of 10 mM or more has been reported for CPA in different drug sensitive cell lines [18,19]. We have got similar IC_{50} value for CPA in SiHa cells, and upon combined treatment with fluoxetine, the IC_{50} value has been reduced to 6.5 mM. We have also shown that fluoxetine could sensitize the effect of CPA in MDA MB 231, the cell type in which CPA resistance has not been reported previously. Moreover, few preliminary studies reported its independent cytotoxic potential in some tumor derived cells [4,5]. These findings have strongly indicated a separate mode of action accounting for a major role in fluoxetine mediated chemo-sensitizing effect.

Our MTT assay results showed a dose and time dependent cytotoxic effect of fluoxetine in MDA MB 231 and SiHa cells. Its cytotoxic effect was consistent in primary fibroblast cultures of breast and cervical tumors. Previous literatures reported that plasma concentrations of fluoxetine reach not more than 1 μM after 30 days of its normal therapeutic dosing at 40 mg/day. But its high lipophilic nature helps it to accumulate in tissues [20]. 13 μM was reported as the average concentration of fluoxetine in brain for 12 subjects whose intake was between 10 and 40 mg of fluoxetine per day [21]. It has been

reported as a safe drug even when taken at 30 times its normal prescribed therapeutic dose and elicits serious adverse effects only at concentrations higher than 75 times of its normal dose [20]. This safety profile might be due to the proper functioning of MAO and COMPT enzymes in efficiently removing the excess serotonin released following high dose fluoxetine treatment. Therefore, we have taken 30 μM, which is near its IC_{50} value in both the cell lines, for our further experiments. We have observed that a majority of cell population undergo apoptosis following 48 h fluoxetine treatment. Fluoxetine has been shown to induce apoptosis previously in biopsy like Burkitt lymphoma cells, but spares human peripheral blood mononuclear cells (HPBMC) and normal resting B cells [20]. These reports are in agreement with our findings on its apoptotic potential in abnormally proliferating cells under *in vitro* conditions. The sparing effect of fluoxetine to normal cells might be the reason for its high safety profile to normal tissues even at its overdose.

We observed that fluoxetine treatment arrested cells at G0/G1 phase. Previous literatures suggested that the outcome of G1 arrest could be either cell differentiation or apoptosis [5,22,23,24], based on the conditions favored. Thus, apoptotic changes we observed in the cells might be attributed to the fate of cell cycle arrest. Fluoxetine has been previously reported to stimulate neuronal cell differentiation [6]. Lack of differentiation factors in our *in vitro* system might have stimulated the cells to undergo apoptosis.

G1–S progression is regulated by the controlled expression and activity of different cyclins (cyclin D, E and A), cdks (cdk 4, 6 and 2), CKIs (INK4 and cip/kip family of proteins), Rb protein and E_2F transcription factor [8]. Recent investigations have identified the important functional role of CKS1 in regulating the G1/S transition phase of mammalian cell division cycle. It has been demonstrated that degradation of p27 and p21, the regulatory CKIs at G1/S checkpoint, occurs only in the presence of functionally active CKS1 [10,25,11] which helps in the substrate recognition process of SCF skp2 E3 ubiquitin ligase [10,11,26]. $CKS^{-/-}$ mouse embryonic fibroblasts have been shown to accumulate p27 [10]. An inverse relationship between CKS1 and p27 has already been reported in different cancers [27–29]. CKS1 knock out mouse has a small phenotype which highlights its overall importance on cell proliferation [10]. Interestingly, our bioinformatics predictions strongly suggest that CKS1 is a molecular target of fluoxetine. Our laboratory results correlated well with the prediction made using bioinformatics tools. We observed p27 and p21 accumulation following fluoxetine treatment. The accumulation was correlated well with the G0/G1 arrest observed in our experiments. Moreover, prolonged treatment of fluoxetine to the cells had downregulated CKS1.

Three independent binding sites have been recognized on CKS1 for cdk2, skp2 and anionic groups [30]. The adaptor theory proposes that CKS1 binds the cyclin E–cdk2–p27 complex and brings the substrate in proximity of skp2 [30,31] whereas the allosteric model proposes that CSK1 binds to skp2 and stimulates a conformational change in skp2 and thus allows phosphorylated substrates to bind with skp2 [10]. However, both these models favor the binding of CKS1 to skp2 for its functional recognition. We have considered all the three identified binding sites on CKS1 as possible binding sites for the ligand (fluoxetine) and molecular docking calculations were performed. However, fluoxetine docked well only into the skp2-binding site on CKS1 with a Gold Score of 51.00, reflecting its predicted high affinity for the binding site. Also, further analysis of the docked complex supports this prediction since fluoxetine assumes a thermodynamically favorable conformation in the docked pose.

Thus, our observations on fluoxetine induced p27 and p21 accumulation might be attributed to functional inhibition of CKS1 by masking its skp2-binding site which is required for CKS1–skp2 interaction. This ultimately might have led to lack of conformational changes in skp2 which is a prerequisite for its assembly with the CKIs for their ubiquitination. Moreover, there was no correlation observed between accumulation of these proteins and the respective mRNA levels. Though slight fluctuations observed, both p27 and p21 mRNA levels were comparatively of similar or lower in range compared to controls at most of the treatment periods. This confirms that accumulation of these proteins is resulted from the defects in its protein degradation machinery rather than of its transcriptional elevation. Further, the accumulated CKIs might have performed their functions of inhibiting the kinase activities of cyclin–cdks complexes to stimulate G0/G1 arrest. We observed no significant change in CKS1 expression at early intervals of 12 and 24 h, whereas both p27 and p21 were found to accumulate during the period. Thus, fluoxetine may be considered as a functional antagonist to CKS1, without imparting a direct effect on its degradation. However, CKS1 expression has declined at a later time period of 36 h which correlated well with its mRNA levels. Continuous functional inhibition of CKS1 protein ultimately might have resulted in switching off its transcription at further levels. Previous reports on receptor downregulation (e.g. adenosine A1 and serotonin receptor) following continuous antagonists treatment (e.g. caffeine and setoperone) support this hypothesis [32,33]. Previous literatures on CKS1 kinetics showed both its mRNA and protein accumulation at G1/S boundary [34]. Its expression was found to be more stable in cells arrested at G1/S boundary compared to asynchronous and G2/M blocked cells [35]. Thus, our results on CKS1 downregulation at G1/S boundary can be suggested as a deviation from its natural kinetics and imply that CKS1 has a direct involvement in fluoxetine mediated G0/G1 arrest.

Similar to p27 and p21, our observations on cyclin E mRNA levels were not correlated well with its gradual protein accumulation suggesting abnormalities in its protein turn over. Cyclin E is degraded by ubiquitin mediated proteolysis which involves cul3 and SCF dependent pathways of ubiquitination. SCF dependent ubiquitination requires autophosphorylation of cyclin E at Thr-380, whereas phosphorylation is not a prerequisite for cul3 dependent pathway [36–38]. In support with the views from previous literatures [10], loss of kinase activity of cyclin E due to its functional inhibition by accumulated p27 can prevent its autophosphorylation and further degradation. Thus, accumulation of cyclin E might be an indirect effect of p27 accumulation. The possibility of a direct involvement of CKS1 on cyclin E degradation also cannot be ruled out. Nakayama et al has demonstrated that $skp2^{-/-}$ cells accumulate cyclin E and p27 [39], indicating the possibility of cyclin E to act as a substrate of SCFskp2 like p27

and p21. As in the case of p27 and p21, cyclin E degradation may also require functionally active CKS1. CKS1$^{-/-}$ mice has been reported to accumulate cyclin E [10]. But specific studies on cyclin E degradation, focusing the involvement of CKS1, are required to confirm the hypothesis.

An initial increase in cyclin A mRNA, which paralleled CKS1, might be due to cells' defense mechanism to counteract stopping signals induced by the external agent, fluoxetine. However, the mRNA levels were found to be decreased which was correlated well with its protein expression at later post-treatment periods. Thus, our observation on cyclin A protein downregulation might be due to decrease in the transcriptional activity of E_2F, a regulator of cyclin A, which is an immediate downstream event of G1 arrest [9]. Combined reduction of CKS1 and cyclin A has also been reported previously in oncostatin M treated cells [40].

Different strategies can be used to modulate cell cycle regulators for better therapeutic intervention. We report for the first time that fluoxetine mediated G1 arrest is CKS1 dependent. Aberrant elevation of CKS1 is a proven risk factor for cancer progression and the effect of fluoxetine to stop its functions at this stage seems promising. Thus, fluoxetine can be considered as a 'lead' molecule for targeting CKS1 for future anticancer research. Its high lipophilic nature and selectivity towards abnormally proliferating cells may help targeting tumor cells under *in vivo* conditions. A recent study has reported its selective antiproliferative effect to undifferentiated neural cells and modulates the levels of differentiation markers in differentiated cells [41]. This may give some clarity for its safe profile in normal systems even at an overdose. Its antidepressant effect, which can positively affect human immune system and thereby enhance drug efficacy, combined with chemosensitizing potential added up with this new finding strongly encourage to recommend its candidature as an anticancer agent. Clinical and epidemiological studies analyzed so far have been focused on an association of antidepressant intake and tumor incidence and declared fluoxetine, a safe medication [42]. Further, retrospective analysis of the records of patients', who were under the combined treatment for depression and cancer, may give some information on how it behaves in the real life.

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