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R-Roscovitine (Seliciclib) inhibits DNA damage-induced Cyclin A1 up-regulation and hinders non-homologous end joining: a rationale for therapeutic combinations with DNA damaging agents

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Abstract

Background

CDK-inhibitors can diminish transcriptional levels of cell cycle-related cyclins through the inhibition of E2F family members and CDK7 and 9. Cyclin A1, an E2F-independent cyclin, is strongly up-regulated under genotoxic conditions and functionally was shown to increase NHEJ activity. Cyclin A1 outcompetes with cyclin A2 for CDK2 binding, possibly redirecting its activity towards DNA repair. To see if we could therapeutically block this switch, we analyzed the effects of the CDK-inhibitor R-Roscovitine on the expression levels of cyclin A1 under genotoxic stress and observed subsequent DNA damage and repair mechanisms.

Results

We found that R-Roscovitine alone was unable to alter cyclin A1 transcriptional levels, however it was able to reduce protein expression through a proteosome-dependent mechanism. When combined with DNA damaging agents, R-Roscovitine was able to prevent the DNA damage-induced up-regulation of cyclin A1 on a transcriptional and post-transcriptional level. This, moreover resulted in a significant decrease in non-homologous end-joining (NHEJ) paired with an increase in DNA DSBs and overall DNA damage over time. Furthermore, microarray analysis demonstrated that R-Roscovitine affected DNA repair mechanisms in a more global fashion.

Conclusions

Our data reveal a new mechanism of action for R-Roscovitine on DNA repair through the inhibition of the molecular switch between cyclin A family members under genotoxic conditions resulting in reduced NHEJ capability.

Introduction

The cell cycle is comprised of a series of highly coordinated events culminating in cell growth and division. Cyclin-dependent kinases (CDK) and their cyclin counterparts strictly regulate and drive cell cycle progression and different CDK/cyclin complexes are responsible for the timely occurrence of each phase transition in order to maintain genetic integrity throughout generations. Cancer cells have been frequently found to have de-regulated CDK activity allowing them to escape the normal cell cycle and proliferate uncontrollably. For these reasons CDKs have been considered attractive targets for cancer therapy and several CDK-inhibitors have been developed and are under intense investigation[1].

R-Roscovitine (Seliciclib, CYC202; herein referred to as Roscovitine), one of the most promising members of the CDK-inhibitor family, is an orally available adenosine analogue prominently targeting CDK2 (also affecting CDKs 1, 7 and 9 at a much lower rate)[2] with a low off-target effect on other members of the human kinome[3], and a nice toxicity profile[4]. In preclinical studies Roscovitine has shown significant in vitro and in vivo antitumor activity on a wide panel of human cancers and is currently in phase II clinical trials[5]. Since preclinical experimentation, it has become evident that, CDK-inhibitors, such as Roscovitine, may actually curb the activity of DNA repair machinery [6, 7], hence becoming an attractive candidate for therapeutic association with either radiation therapy[8, 9] or genotoxic agent-based chemotherapy[10]. However, the mechanism of this inhibition is still elusive.

One of the proposed means for CDK-inhibitors to affect DNA repair is through checkpoint deregulation[11-13], but increasing evidence supports a complex network of direct interactions between individual CDKs and proteins that play a key role in DNA damage repair (DDR). It is known that different DNA repair pathways are preferentially activated at specific stages of the cell cycle possibly suggesting a functional crosstalk between CDK/cyclin complexes and DNA repair mechanisms[14]. In particular, CDK2 has been shown to interact with p53[15], BRCA1[16], BRCA2[17], and both, CDK1 and CDK2, can modulate BRCA1-BARD1 activity[13, 18]. Moreover, CDK2 knock-down cells have an attenuated capacity to repair DNA damage suggesting a pivotal role for CDK2[7] in DDR. Given the ability of CDKs to compensate for each other in vivo, overall CDK activity has been proposed to be influential in DDR regulation[19].

Cyclins, similarly to CDKs, have been correlated to DDR. Cyclin E levels are up-regulated under genotoxic stress conditions[20] and a post-translational cleavage generates an 18-amino acid peptide, which has been shown to interact with Ku70[21] promoting the release of the pro-apoptotic factor Bax from the inactivating complex Bax/Ku70. Moreover, an increasing amount of data suggests an important role in DDR for the A-type cyclins, and in particular for cyclin A1. Differing from cyclin A2, ubiquitously expressed during the S and G2/M phases of the cell cycle, cyclin A1 is a testis-specific cyclin, which interacts with CDK2 and is involved in germ cell meiosis and spermatogenesis[22]. Cyclin A1 may have a role in carcinogenesis, as it has been found to be overexpressed in myeloid leukemia and various other tumour types[22-24], however, its role in cancer is still particularly obscure. In somatic non-testicular tissues, cyclin A1 is not expressed or is expressed at very low basal levels. After genotoxic insult, cyclin A1 mRNA is up-regulated in vitro[25] and in vivo[26]. At a molecular level, human CDK2/cyclin A1 complexes interact with members of the Ku family and phosphorylate Ku70[26, 27], a pivotal player in the non-homologous end-joining (NHEJ) double strand break (DSB) repair pathway. Furthermore, under genotoxic conditions the kinase activity of CDK2/cyclin A1 complex increases, while the relative kinase activity of CDK2/cyclin A2 decreases and the CDK2/cyclin A1 complex out-competes with CDK2/cyclin A2 for Ku70 binding[27]. Although its role in DDR is not completely understood, cyclin A1 knock-out mice and Xenopus embryos exhibited a clear defect in DNA repair[26, 28].

Taken together these data support that during genotoxic stress differential transcriptional levels and activity of cyclin A family members may redirect CDK2 toward DNA repair resulting in a modulation of NHEJ. Therefore, we hypothesized that the inhibition of DNA repair mechanisms by Roscovitine may occur through a modulation of this molecular switch in cyclin A family member levels. Physiological CDK-inhibitors have been found to down-regulate cyclin expression through the inhibition of E2F-family transcription factors, which drive and regulate cell cycle-related cyclin transcription. Given that the promoter of the cyclin A1 gene, CCNA1, is different from the other cell cycle-related cyclins, not being under the regulation of E2Fs[29], here we investigated the effects of Roscovitine on cyclin A1 expression and modulation of DNA repair mechanisms. We demonstrated that Roscovitine alone is not sufficient to reduce the basal levels of cyclin A1, in contrast to cell cycle related cyclins. However, Roscovitine treatment abolished the DNA damage-induced cyclin A1 up-regulation thus reducing NHEJ and significantly hindering DNA repair over time.

Methods

Cell Culture and Serum Starvation

The following solid cancer human cell lines were purchased from and authenticated by American Type Culture Collection (ATCC; Manassas, VA) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air, within the appropriate medium according to supplier recommendations supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA) and 100U of Penicillin and 100μg/ml of Streptomycin (Sigma-Aldrich; St. Louis, MO): NSCLC cell lines A549 and H23, breast cancer cell lines MCF-7 and MDA-MB-231, prostate cancer cell lines LNCAP and DU145, and the adenovirus transformed human embryonic kidney epithelial cells HEK293FT. Cells were regularly sub-cultured according to ATCC recommendations with a 0.25% trypsin-EDTA solution (Sigma). To obtain synchronous populations of cells, confluent plates of A549 cells were incubated in media supplemented with 0.1% (v/v) heat-inactivated fetal bovine serum for 96 hours. Cells were then sub-cultured into serum-containing medium and time points were taken every four hours.

Drugs, irradiations and treatments

Doxorubicin was obtained from BioMol International (Plymouth Meeting, PA). Lyopholized drug was re-suspended into a 1:1 mixture of dimethyl sulfoxide (DMSO; Fisher Scientific; Pittsburgh, PA) and MilliQ filtered H_2O (Millipore; Bellerica, MA) to a concentration of 4.31 mM, aliquoted for use and stored at -20°C. Roscovitine was obtained from Signa Gen Laboratories (Gaithersburg, MD). Lyophilized drug was re-suspended into DMSO to a concentration of 14.1 mM, aliquoted and stored at -20°C until use. Fresh dilutions from the stock solutions were prepared for each treatment. Taxol was obtained from USB Corporation (Cleveland, OH). Lyophilized drug was re-suspended into DMSO to a concentration of 5.86 mM, aliquoted and stored at -20°C until use. MG-132 (Z-Leu-Leu-Leu-al) was obtained from Sigma. Lyophilized drug was re-suspended into DMSO to a concentration of 10mg/ml, aliquoted and stored at -20°C until use. Irradiations were performed in an AECL Gamma Cell 40, Cs-137 irradiator at a dose rate of 1 Gy/minute for respective doses. In treatments throughout this article the control samples refer to cells treated with an equal concentration (v/v) of DMSO as in the highest drug concentration used per experiment.

Western Blot Analysis and SDS-PAGE

Equal amounts (50-100 μg) of whole cell lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Whatman Inc., Piscataway, NJ) by wet electrophoretic transfer. Non-specific binding sites were blocked for 1 hour at room temperature with 3% non fat dry milk (NFM) in tris-buffered saline containing 0.01% Tween-20 (TBS-T) and probed with the following primary antibodies in 3% NFM in TBS-T overnight at 4°C; rabbit anti-cyclin A1 (sc-15383; Santa Cruz Biotechnology Inc.; Santa Cruz, CA), mouse anti-cyclin A2 (CY-A1; Sigma), mouse anti-cdc2 (A17; Abcam, Cambridge, MA), rabbit anti-CDK2 (sc-163; Santa Cruz), rabbit anti-p53 (sc-6243; Santa Cruz), mouse anti-Hsp70 (sc-24; Santa Cruz), mouse anti-p130/Rb2 full length (610262; BD Biosciences, San Jose, CA), rabbit anti-serine 952 phosphorylated p130/Rb2 (sc-16298; Santa Cruz), rabbit anti-serine-2 phosphorylated RNA polymerase II (A300-654A; Bethyl Laboratories Inc., Montgomery, TX), rabbit anti-serine-5 phosphorylated RNA polymerase II (A300-655A; Bethyl), mouse anti-α-tubulin (sc-58666; Santa Cruz), and mouse anti-ser139 phosphorylated histone γH2AX (Millipore cat. #05636; lot# DAM1567248). Membranes were washed for 15 minutes in TBS-T and then incubated for 1 hour with either goat anti-mouse (31432; Pierce; Rockford, IL) or mouse anti-rabbit (31464; Pierce) horseradish peroxidase conjugated IgG at a dilution of 1:10,000 in 3% NFM in TBS-T. This was followed by 15 minutes of wash in TBS-T and enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. All western blot images included in article are representative of at least three consecutive independent experiments.

Immunostaining

Following respective drug treatments, cells grown directly on sterilized glass coverslips were fixed and permeabilized for 10 minutes in 70% cold methanol (MeOH), immunostained (for Cyclin A1 and γH2AX) and analyzed as previously described[39].

Flow cytometry

Cells (1 x 106) were collected, after respective drug treatments, washed, resuspended in 1ml of PBS and fixed and permeabilized for at least 10 minutes in 70% cold ethanol. After fixation cells were pelleted, washed 3 times with PBS, re-suspended into a primary antibody solution (10 μg/ml antibody diluted in PBS) and incubated on ice for 15 minutes. Cells were then pelleted, washed 3 times with PBS, re-suspended into FITC-

conjugated secondary antibody solution (10 μg/ml) and incubated for 15 minutes on ice protected from the light. Cells were washed 3 times in PBS and re-suspended in propidium iodide staining solution, 10 μg/ml propidium iodide (from stock of 0.5 mg/ml in 0.38 mM sodium citrate pH7.0) and 25 μg/ml DNase-free RNase A (from stock of 10mg/ml RNase A in 10 mM Tris pH 7.5 and 15 mM NaCl) diluted in PBS. Cells were incubated at 37°C for a minimum of 30 minutes protected from light and analyzed immediately by flow cytometry utilizing an Epics XL-MCL BeckmanCoulter (The Wistar Institute, Philadelphia, PA). Graphs represent average fluorescence intensity or average percentage of cells found in cell cycle phase over three consecutive independent experiments.

Reverse Transcriptase-PCR and Real time (RT-PCR)

Total RNA from cell lines was extracted using the High Pure RNA Isolation Kit (Roche) following the manufacturer's instruction. cDNA was synthesized from 1 μg of total RNA by using random hexamers as primers and moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) according the manufacturer's protocol in a final volume of 20 μl. As a control for genomic contamination a reverse transcription (RT) reaction was carried out without the addition of the reverse transcriptase (RT-). After cDNA synthesis, samples were diluted 1:10 and 4 μl was used in each real time polymerase chain reaction (real time PCR). cDNA was amplified using species specific intragenic primers for CCNA1, CCNA2, CCNB1, CCND3, CCNE1, TP53 and GAPDH genes (Additional File 5). Real time PCR was carried out utilizing SybrGreen Master Mix (Roche, Basel, Switzerland) following the manufacturer's instructions in a final reaction volume of 10 μl. Reactions were performed on a LightCycler 480 II (Roche Diagnostics, Indianapolis, IN) with an initial denaturation of 5 minutes at 95°C; 45 cycles of 10 seconds at 95°C, 20 seconds at 60°C, and 10 seconds at 72°C where fluorescence was acquired. Each sample was run in triplicate and data was analyzed using the comparative Ct method with GAPDH as the endogenous control and control cells as the reference sample in each experiment. Final data points represent the average fold change respect to control ($2^{-\Delta\Delta Ct}$) or expression levels respect to GAPDH ($2^{-\Delta Ct}$) of at least three consecutive independent experiments.

Alkaline Comet Assay

After appropriate drug treatments, cells were harvested and analyzed utilizing the alkaline comet assay as previously described[40],[41]. Briefly, cells were mixed in a suspension of low melting point agarose

and spread on agarose-coated slides. Once the agarose solidified, slides were incubated in lysis buffer followed by electrophoresis to allow migration of DNA and detection of DNA damage. Cells were then stained with 1 μg/mL ethidium bromide and analyzed using the fluorescence microscope Olympus BX40 (Melville, NY) with a Spot-RT digital camera and software (Webster, NY). At least 200 cells were evaluated per experimental point. Visual scoring of comet images using fluorescence microscopy was performed according to Norbury[42]. Briefly, each nucleus is assigned a score from 0-4 depending on the relative intensity of DNA fluorescence in the tail (0 = no damage, 4 = >80% of DNA found in the tail) and the final score is calculated as the average DNA damage found in all cells counted from three consecutive independent experiments. Statistical analysis was carried out using a standard student's t test.

Transient transfections

The human cyclin A1 IMAGE clone 5172478 (GenBank:BC036346.1) was purchased from ATCC (MGC-34627) transformed into DH5α heat-shock competent E. coli cells and grown in on LB agar plates or broth with 100 μg/ml Ampicillin (Fisher) at 37°C. Plasmid DNA was extracted using the Genopure Plasmid Midi Kit (Roche) following manufacturer's instructions then verified by restriction enzyme digestion and gel electrophoresis. HEK293FT cells were transiently transfected using a 6:2 ratio of Fugene HD (Roche) and plasmid DNA (2 μg) following manufacturer's protocol. Enhanced yellow fluorescent protein (pEYFP) plasmid DNA was utilized as a control for transfection efficiency at the same concentration. Cells were analyzed after 36 hours of transfection by western blot and fluorescence microscopy to confirm expression of transfected protein and then utilized in experiments as described.

In vitro NHEJ assay

The in vitro NHEJ assay was performed on respectively treated cell lysates as previously described[43] utilizing 120 μg of protein extract and 60 μg of purified BamHI (Roche) digested pCI-neo plasmid DNA (Promega). A reaction including the incubation of 20 μM Wortmannin with whole cellular lysate for 15 minutes on ice before the addition of digested plasmid DNA was included as a negative control for NHEJ activity in each experiment. After incubation samples were diluted 1:10, phenol chloroform 25:24:1 (Fisher) extracted, and ethanol precipitated overnight at 4°C. DNA was resuspended into 20 μl of Tris-EDTA buffer and 1 μl was utilized in each real time PCR reaction. To detect plasmid re-ligation one set of primers amplified an intact region of the plasmid to

act as the endogenous control, while a second set of primers bound both up-stream and down-stream of the enzymatic cut site. Samples were run in triplicate with each primer pair following the real-time PCR protocol described above. Final results represent the average fold change ($2^{-\Delta\Delta Ct}$) in re-ligation respect to control, over three consecutive independent experiments.

Microarray Analysis

Total RNA was isolated by Trizol (Invitrogen). Fifteen µg of total RNA was converted to cDNA by using Superscripts reverse transcriptase (Invitrogen), and T7-oligo-d(T)24 (Geneset) as a primer. Second-strand synthesis was performed using T4 DNA polymerase and E.Coli DNA ligase and them blunt ended by T4 polynucleotide kynase. cDNA was purified by phenol-chloroform extraction using phase lock gels (Brinkmann). Them cDNAs were in vitro transcribed for 16 hours at 37°C by using the IVT Labelling Kit (Affymetrix) to produce biotinylated cRNA. Labelled cRNA was isolated by using the RNeasy Mini Kit column (QIAGEN). Purified cRNA was fragmented to 200-30 mer using a fragmentation buffer. The quality of total RNA, cDNA synthesis, cRNA amplification and cRNA fragmentation was monitored by capillary electrophoresis (Bioanalizer 2100, Agilent Technologies). Fifteen micrograms of fragmented cRNA was hybridised for 16 hours at 45°C with constant rotation, using a human oligonucleotide array U133 Plus 2.0 (Genechip, Affymetrix, Santa Clara, CA). After hybridisation, chips were processed by using the Affymetrix GeneChip Fluidic Station 450 (protocol EukGE-WS2v5_450). Staining was made with streptavidin-conjugated phycoerythrin (SAPE)(Molecular Probes), followed by amplification with a biotinylated anti-streptavidin antibody (Vector Laboratories), and by a second round of SAPE. Chips were scanned using a GeneChip Scanner 3000 G7 (Affymetrix) enabled for High-Resolution Scanning. Images were extracted with the GeneChip Operating Software (Affymetrix GCOS v1.4). Quality control of microarray chips was performed using the AffyQCReport software[44]. A comparable quality between microarrays was demanded for all microarrays within each experiment.

Microarray Statistical Analysis

The background subtraction and normalization of probe set intensities was performed using the method of Robust Multiarray Analysis (RMA) described by Irizarry et al.[45]. To identify differentially expressed genes, gene expression intensity was compared using a moderated t test and a Bayes smoothing approach developed for a low number of replicates[46].

To correct for the effect of multiple testing, the false discovery rate, was estimated from p values derived from the moderated t test statistics[47]. The analysis was performed using the affylmGUI Graphical User Interface for the limma microarray package[48].

Results

DNA damage induces a switch in the respective levels of A-family cyclins

To determine the effects of DNA damage on Cyclin A1 expression in unsynchronized human non small cell lung cancer (NSCLC) cell line, A549, we treated cells with isoeffective doses of Doxorubicin or Taxol (at IC50 and IC90 respectively). Doxorubicin is an anthracycline antibiotic, which intercalates the DNA inhibiting the progression of Topoisomerase II resulting in DNA DSBs, Taxol is a "spindle poison" that binds the β-tubulin subunit and stabilizes microtubules interfering with their physiological dynamic and ultimately leading to mitotic catastrophe. Immunofluorescence staining of phosphorylated histone γH2AX (herein referred to as γH2AX) foci a marker of DNA DSBs, confirmed that Taxol does not induce a significant level of DNA DSBs in comparison to Doxorubicin treatment (data not shown). Comparably, through reverse-transcription real-time PCR we found that treatment with Doxorubicin for 24 hours induced an up-regulation of Cyclin A1 mRNA, approximately 50- and 200-fold when treated with 750nM (IC50) and 2.5μM (IC90) respectively. Whereas in cells treated with isoeffective doses of Taxol (25 nM, IC50 and 50 nM, IC90), Cyclin A1 mRNA expression was only slightly up-regulated with no significant differences between the two dose levels (Figure 1).

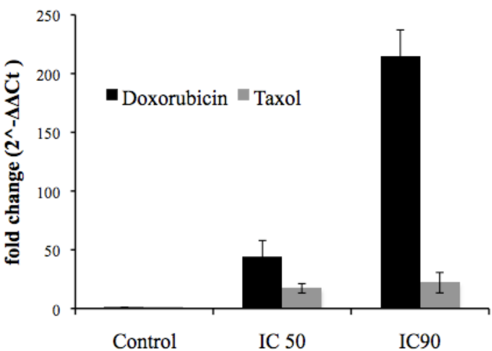


Fig.1 Relative expression levels respect to GAPDH (2^-ΔCt) of cyclin A1 (*CCNA1*) mRNA in A549 NSCLC after 24 hours of treatment with isoeffective doses of Doxorubicin (750 nM and 2,5 μM) and Taxol (25nM and 50 nM).

Furthermore, mRNA levels of both members of the cyclin A family after treatment with increasing doses of Doxorubicin (from 250 nM up to 5 μM) were compared. We found that cyclin A1 up-regulation is dose dependent with a plateau that is reached around 2.5 μM (IC90). On the contrary, Doxorubicin treatment caused a down-regulation of cyclin A2 mRNA levels with a nadir that is reached at the dose of 750 nM (IC50) followed by a relative increase close to basal levels (that are not reached) at a dose of 2.5 μM (IC90) and further followed by a constant decline at higherdoses (Figure 2).

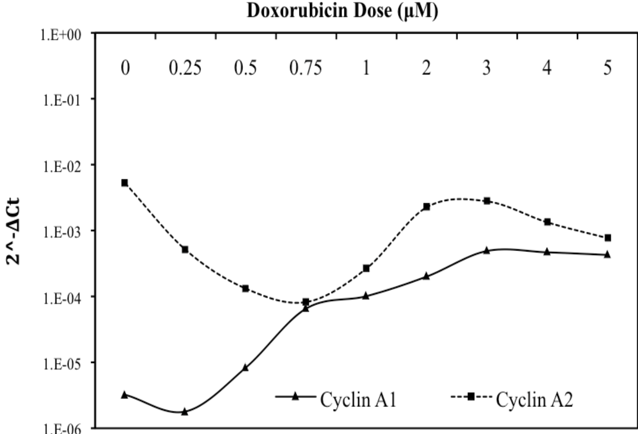


Fig.2 Relative expression levels respect to GAPDH (2^-ΔCt) of cyclin A1 (*CCNA1*) vs. cyclin A2 (*CCNA2*) mRNA after 24 hours of treatment with increasing doses of Doxorubicin (250 nM to 5 μM).

These finding were congruent with protein levels of both cyclins A1 and A2 (Figure 3).

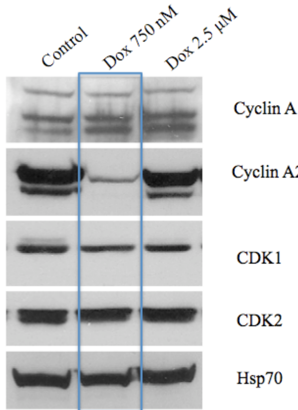


Fig.3 Western blot analysis of cyclin A1, cyclin A2, CDK1 and CDK2 expression levels with Hsp70 as a loading control after 24 hours of treatment with Doxorubicin (Dox 750 nM and 2.5 µM)

The cyclin A1 antibody we utilized detected two bands, which both augmented upon treatment. The upper band we hypothesized to be a phosphorylated or hyper-phosphorylated form of cyclin A1, which was barely detectable when phosphatase inhibitors were excluded from the lysis buffer. The lower band a hypo-phosphorylated or non-phosphorylated form, which was detectable when cell lysis was performed with or without phosphatase inhibitors (Figure 4).

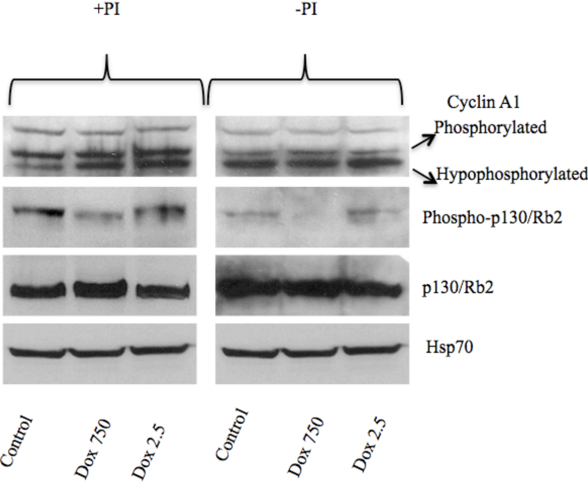


Fig. 4 Western blot analysis of cyclin A1 protein expression with and without the inclusion of phosphatase inhibitors in lysis Phosphatase inhibitor activity was confirmed by probing for phosphorylated p130/Rb2 in comparison to full-length p130/Rb2. After 24 hours of Doxorubicin treatment (750 nM and 2.5 μM), cyclin A1 protein levels clearly augment in cells lysed with the inclusion of phosphatase inhibitors, whereas the increase is not as notable in cells lysed without the inclusion of phosphatase inhibitors.

Relative quantification of bands showed that Doxorubicin, while inducing a slight increase in the hyper-phosphorylated form of cyclin A1, induced a marked dose-dependent increase in the hypo-phosphorylated form (Figure 5).

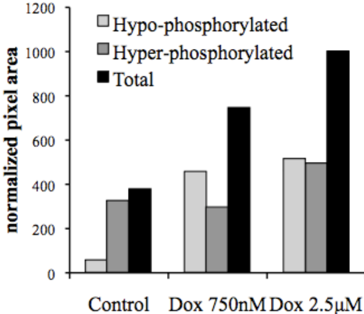


Fig. 5 Quantification of cyclin A1 expression levels as normalized pixel area respect to Hsp70.

These finding were also noted in A549 cells 1 hour after gamma-irradiation (Figure 6).

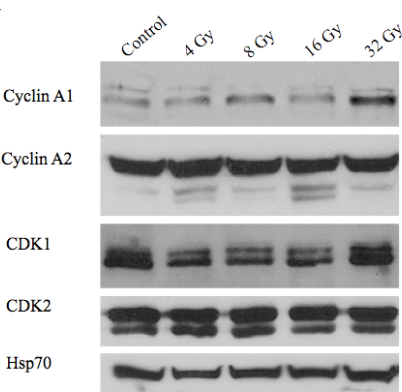


Fig.6 Western blot analysis of protein expression 1 hour after administration of increasing doses of γ-irradiation (4 Gy to 32 Gy)

To ensure that the increase in cyclin A1 expression observed was not a result of cell cycle redistribution, we analyzed the expression of cyclin A family members during the synchronous cell cycle in the A549 NSCLC cell line. We observed that unlike cyclin A2, which, as expected, was expressed during the S and G2/M phases, cyclin A1 remained fairly constant throughout the cell cycle (Figure 7).

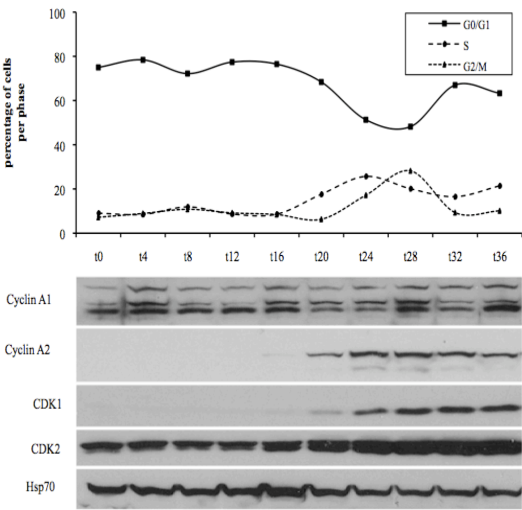


Fig. 7 Flow cytometry cell cycle analysis with corresponding western blot showing cyclin A1, cyclin A2, CDK1 and CDK2 expression levels over the course of the synchronous cell cycle induced by serum starvation.

Cell cycle analysis by flow cytometry was also performed on asynchronous A549 cells treated for 24 hours with Doxorubicin (750 nM and 2.5 μM) in comparison to untreated controls, and as expected Doxorubicin treatment resulted in an activation of DNA damage cell cycle checkpoints at G1-S and G2-M phase transitions (Figure 8). Cells treated with 750 nM Doxorubicin exhibited a decrease in the percentage of cells in S phase, which is duly noted by the observed decrease in cyclin A2 expression levels. However, treatment with 2.5 μM Doxorubicin resulted in a relative increase in the percentage of cells in S phase, which mirrors the increase in cyclin A2 expression at higher doses of Doxorubicin as seen by western blot.

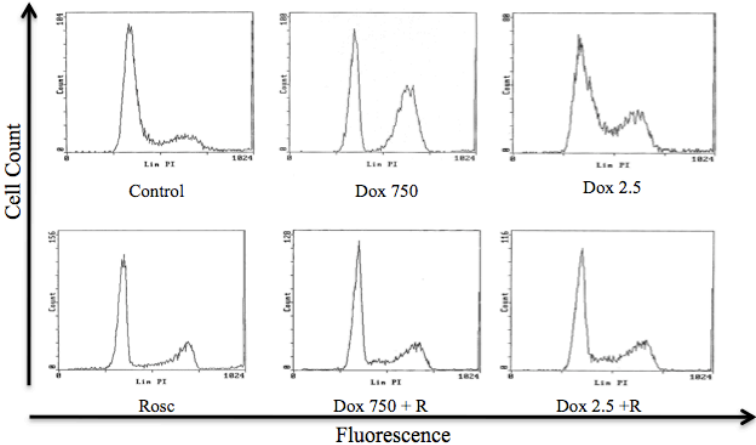


Fig.8 Flow cytometry analysis of cell cycle breakdown in A549 cells treated for 24 hours with respective treatments of Doxorubicin (750 nM or 2.5 µM) or 20 µM Roscovitine alone or in combination

These data confirm that cyclin A1 is strongly induced under DNA damaging conditions and also supports a DNA damage-induced molecular switch between cyclin A2 and cyclin A1 during genotoxic stress.

Cyclin A1 localizes to the nucleus during genotoxic conditions and its overexpression increases in vitro NHEJ activity.

To determine if cyclin A1 up-regulation under DNA damaging conditions was specific to a sub-population or was found in all cells we performed flow cytometry analysis of Doxorubicin treated A549 cells. Cyclin A1 up-regulation was observed in all cells, further confirming that this was independent of the cell cycle (Figure 9).

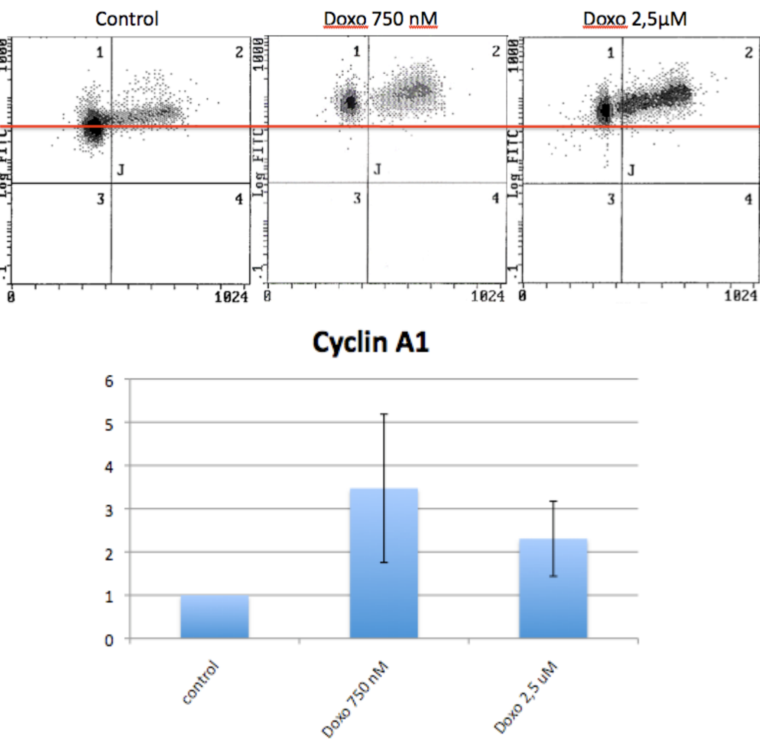


Fig. 9

We also analyzed Doxorubicin treated A549 cells by immunofluorescence staining and microscopy noting not only a dose-dependent increase in fluorescent signal but also a nuclear localization of cyclin A1 protein at higher doses of Doxorubicin (2.5 µM) treatment (Figure 10). The nuclear localization and the dose-dependent increase in cyclin A1 expression could speak further towards a specific role for cyclin A1 in DNA repair mechanisms.

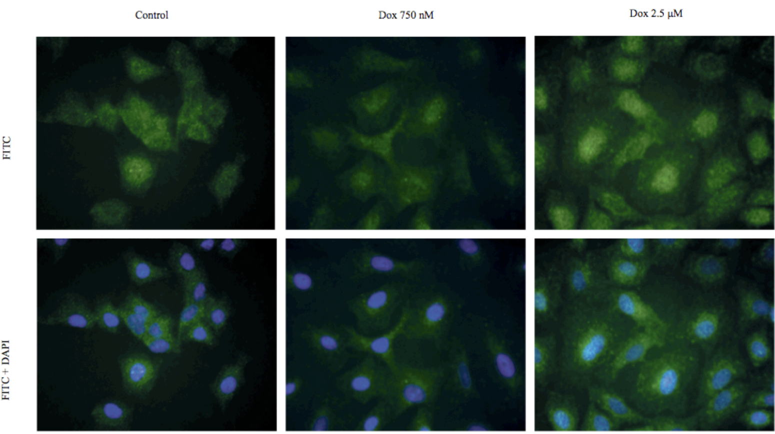


Fig. 10 Immuno-fluorescence analysis by fluorescent microscopy of cyclin A1 localization in A549 cells after treatment with Doxorubicin (750 nM and 2.5 µM). Upper panels show FITC-stained cyclin A1 expression (green) and lower panels show FITC and DAPI (blue) merge at 400x magnification.

To address the role of cyclin A1 in DNA DSB repair mechanisms, we used an in vitro plasmid re-ligation assay based on the ability of the whole cellular extract to re-join a linearized plasmid. Wortmannin, a known inhibitor of DNA dependent protein kinase (DNA PK), was used as a control to demonstrate the dependency of re-ligation upon NHEJ.

Quantification of plasmid re-ligation was performed by real-time PCR utilizing primers, which bound both upstream and downstream of the enzymatic cut site, amplifying only upon re-ligation of plasmid DNA, and values were normalized on the quantity of plasmid in each reaction

by primers which bound an intact region of plasmid DNA. We analyzed the NHEJ capability of HEK293FT cells (utilized for their optimal transfection efficiency), transiently transfected to overexpress cyclin A1 or enhanced yellow fluorescent protein (YFP, negative control). In cells overexpressing cyclin A1 there was a significant increase (approximately 6-fold) in NHEJ activity respect to YFP controls (Figure 11).

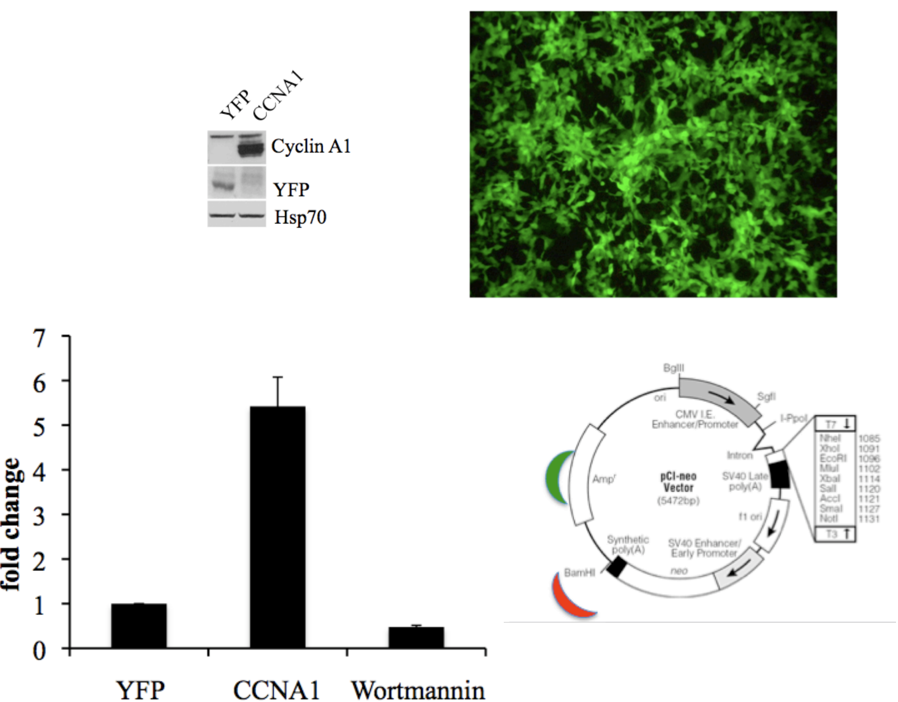


Fig. 11 Fold change, respect to YFP, of *in vitro* NHEJ pC-neo plasmid re-ligation activity as quantified by real time PCR in HEK293FT cells transiently transfected with *YFP* (control) or cyclin A1 (*CCNA1*) and respective immunofluorescence, western blot and ponceau S staining verifying overexpression respect to Hsp70.

Roscovitine, at doses primarily inhibiting CDK2, but not CDK7 or 9 prevents DNA damage-induced cyclin A1 transcriptional up-regulation and increases protein degradation.

Roscovitine, being a CDK2 inhibitor, can depress E2F-dependent transcription by blocking the phosphorylation of Rb-family proteins. Cyclin A1 expression is not E2F-dependent[28], therefore we investigated the effects of Roscovitine on cyclin A1 basal expression and eventually on the DNA damage-induced up-regulation. First we analyzed the mRNA expression levels of cyclins A1, A2, B, D, and E after 24 hours of incubation with increasing doses (up to 60 μM) of Roscovitine. We found that all cyclin mRNA expression levels were greatly reduced respect to untreated controls (Figure 12), except for cyclin A1, whose basal levels were substantially lower than the other cyclins and were not down-regulated but remained fairly constant upon Roscovitine treatment consistent with its E2F-independent transcriptional regulation (Figure 12).

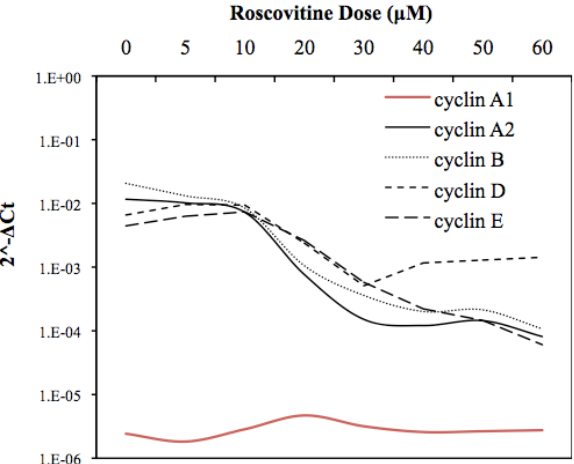


Fig. 12 Expression levels respect to GAPDH ($2^{\wedge-\Delta Ct}$), in mRNA of cyclin A1, cyclin A2, cyclin B, cyclin D and cyclin E after 24 hours of treatment with increasing doses of Roscovitine (5-60 μM)

Therefore, we treated A549 cells for 24 hours with increasing doses of Doxorubicin (as previously stated) alone or in combination with a fixed dose of 20 µM Roscovitine. We chose to use the dose of 20 µM as it was experimentally proven to preferentially inhibit CDK2 resulting in a hypo-phosphorylation of p130/Rb2, while it is the highest dose with a limited effect on CDK7 and CDK9, as shown by the phosphorylation of the C-terminal domain (CTD) of RNA Polymerase II on serine 5 and 2 respectively (Figure 13).

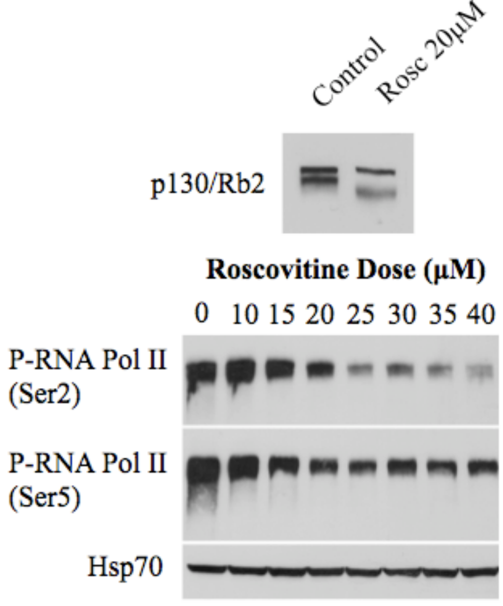


Fig. 13 (Upper blot) Western blot analysis of inhibitory activity of Roscovitine (Rosc) against CKD2 phosphorylation of p130/Rb2 as shown by a shift in p130/Rb2 band height from hyper-phosphorylated in control cells to hypo-phosphorylated in Roscovitine treated cells, upper band is non-specific. (Lower blot) Western blot analysis of Roscovitine inhibition of CDK7 and CDK9 phosphorylation of the C-terminal domain (CTD) of RNA polymerase II, on serine 5 and serine 2 respectively, in cells treated for 24 hours with increasing doses of Roscovitine (10-40 µM)

Roscovitine was able to completely abolish the Doxorubicin-induced cyclin A1 mRNA and protein up-regulation (Figure 3C&D) suggesting that a residual CDK2 activity is required for cyclin A1 up-regulation.

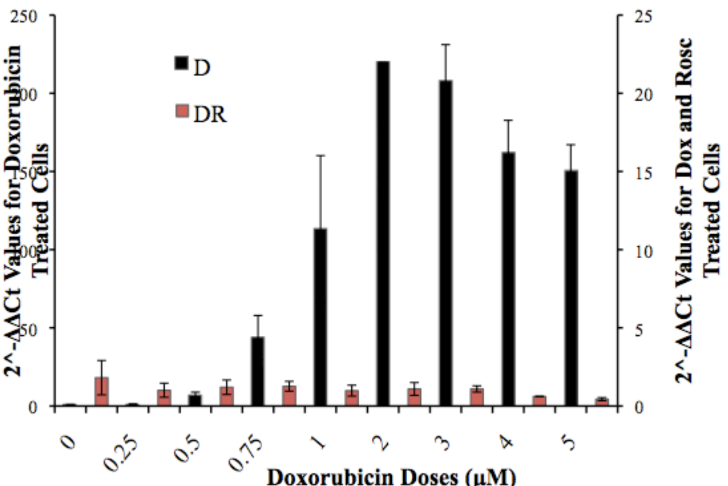


Fig. 14 Fold change, respect to control ($2^{\wedge-\Delta\Delta Ct}$), of cyclin A1 mRNA expression levels in cells treated with either increasing doses of Doxorubicin alone (250 nM to 5 μM) or increasing doses of Doxorubicin in combination with 20 μM Roscovitine for 24 hours. Note that black bars represent Doxorubicin only treated cells and correspond to the vertical axis on the left-hand side of the graph, while grey bars represent Doxorubicin and Roscovitine treated cells and correspond to the vertical axis on the right-hand side of the graph

Furthermore, co-administration of Doxorubicin and Roscovitine resulted in a change in cyclins A2, B, D and E mRNA expression levels, respect to Doxorubicin treatment alone (data not shown). In particular, cyclin A2 mRNA levels demonstrated an attenuated variation during combination

treatments, which is consistent with the cell cycle distribution as observed by flow cytometry (Figure 8).

At the protein level, the combination of Roscovitine with Doxorubicin resulted in an inversion of the Doxorubicin-induced molecular switch between cyclin A1 and cyclin A2 (Figure 15).

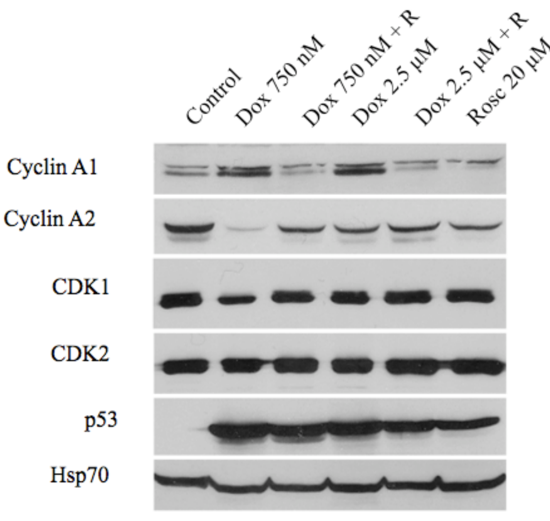


Fig. 15 Western blot analysis of cyclin A1, cyclin A2, CDK1 and CDK2 protein expression in cells treated for 24 hours with either Doxorubicin (750 nM or 2.5 μM) alone, 20 μM Roscovitine alone, or in combination (Dox 750 nM/2.5 μM + R). p53 protein expression was included as a control for drug treatments.

Unlike cyclin A1 mRNA levels, treatment with Roscovitine alone resulted in a decrease in cyclin A1 protein expression over time (Figure 16), suggesting that, aside from transcriptional regulation, Roscovitine may also regulate cyclin A1 on a post-transcriptional level. To confirm this hypothesis we treated A549 cells with Doxorubicin and Roscovitine respectively as well as 10 μM of the proteosome inhibitor MG-132. Inclusion of MG-132 significantly prevented the downregulation of

cyclin A1 protein levels after treatment with 20 μM Roscovitine (Figure 16).

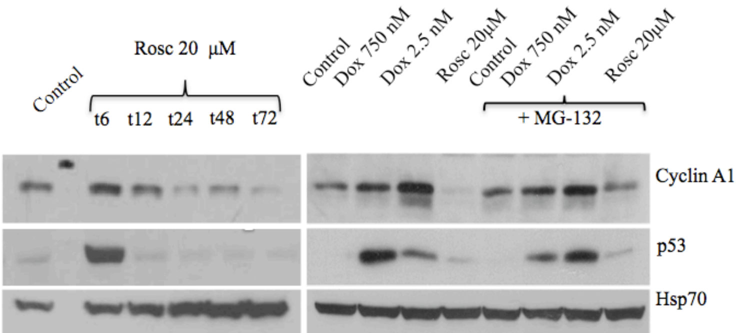
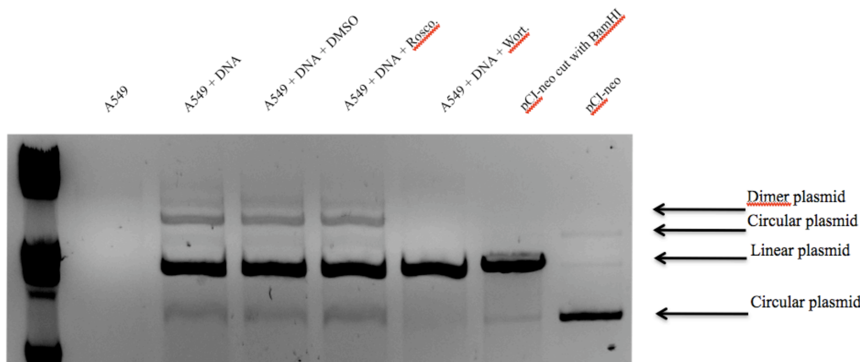


Fig. 16 Post-translational inhibition of cyclin A1 protein levels over time. (Left-side blot) cyclin A1 and p53 protein expression in cells treated for increasing amounts of time (6-72 hours) with 20 μM Roscovitine. (Right-side blot) cyclin A1 and p53 expression in cells treated for 24 hours with either Doxorubicin (750 nM and 2.5 μM) or 20 μM Roscovitine alone or in combination with 10 μM of the proteosome inhibitor MG-132.

The transcriptional and post-transcriptional regulation of cyclin A1 by Roscovitine was confirmed in a panel of NSCLC (A549 and H23), breast (MCF-7 and MDA-MB-231) and prostate cancer (LNCAP and DU145) cell lines (data not shown).

Combined treatment with Roscovitine and Doxorubicin results in a downregulation of NHEJ capability.

Cyclin A1 knock-out MEFs have shown a reduced NHEJ capability [26]. To determine if Roscovitine may have a comparable affect on NHEJ mechanisms, we incubated untreated A549 cell lysates with 20 μM Roscovitine, DMSO, or Wortmannin for 15 minutes prior to incubation with linearized plasmid. While Wortmannin was able to almost completely inhibit NHEJ activity, DMSO had no effect and Roscovitine resulted in an approximate 25% diminution in plasmid re-ligation, which can be accounted for by a direct inhibition of CDK activity and eventual off-target effects of the drug (Figure 17).



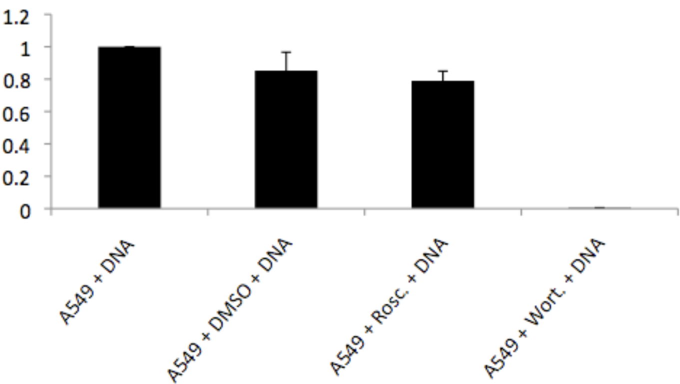
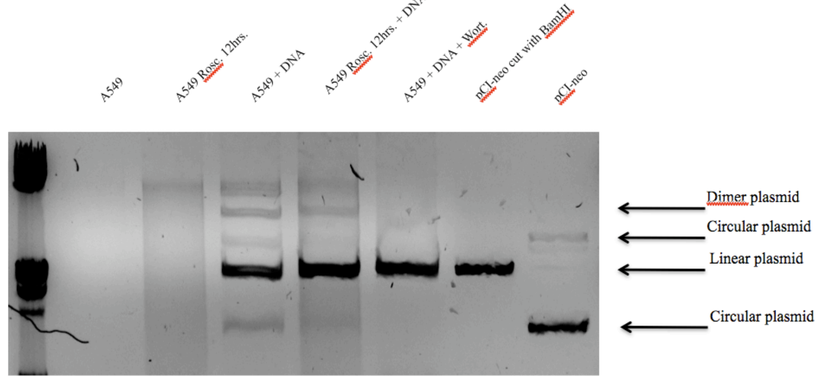


Fig. 17 Analysis by real time PCR of NHEJ plasmid re-ligation activity of untreated A549 cell lysate with the addition of 20 µM Roscovitine, DMSO or Wortmannin and correspective DNA fragments as resolved on agarose gel

However, when lysates from A549 cells treated for 12 hours with 20 µM Roscovitine were assayed for NHEJ capability, they demonstrated an approximate 45% reduction in plasmid re-ligation (Figure 18) as a result of an additional biological mechanism.



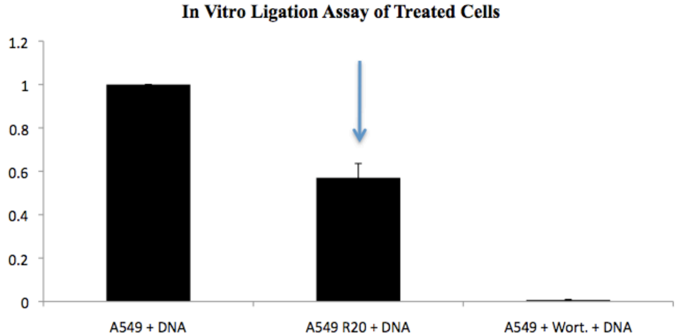


Fig. 18 Analysis by real time PCR of NHEJ plasmid re-ligation activity in A549 cells treated for 12 hours with either 1 µM Doxorubicin or 20 µM Roscovitine alone or in combination. Wortmannin was added to untreated cell lysate as a negative control for NHEJ activity *in vitro*. Correspective DNA fragments as resolved on agarose gel

Roscovitine enhances Doxorubicin-induced DSBs and delays DNA damage repair over time.

To determine if the inhibition of NHEJ activity led to an overall increase in DNA DSBs we analyzed the quantity of phosphorylated γH2AX by western blot (Figure 19). After six hours of incubation with respective drug treatments, we removed the drug-containing medium and analyzed A549 cells for γH2AX phosphorylation immediately following the six hour treatment (t0), then six (t6) and 24 (t24) hours after drug removal with respect to control cells. Doxorubicin treatment induced an activation of γH2AX, which was significantly augmented following combined treatment with Roscovitine over time (Figure 19), even though Roscovitine alone did not significantly activate γH2AX as shown by western blot and immunofluorescence staining (Figure 19 and 20).

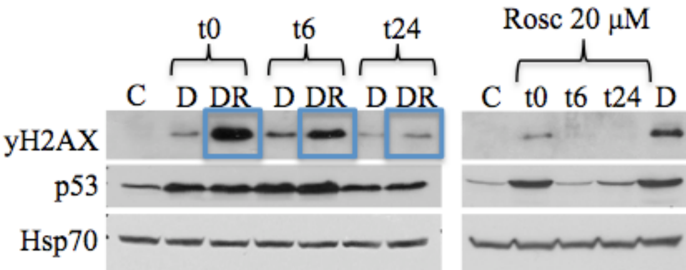


Fig. 19 Western blot analysis of DNA DSBs by phosphorylated γH2AX (serine 139) immediately (t0) or 6 (t6) and 24 (t24) hours following a 6 hour treatment with either 750 nM Doxorubicin (D) or 20 μM Roscovitine alone or in combination (DR)

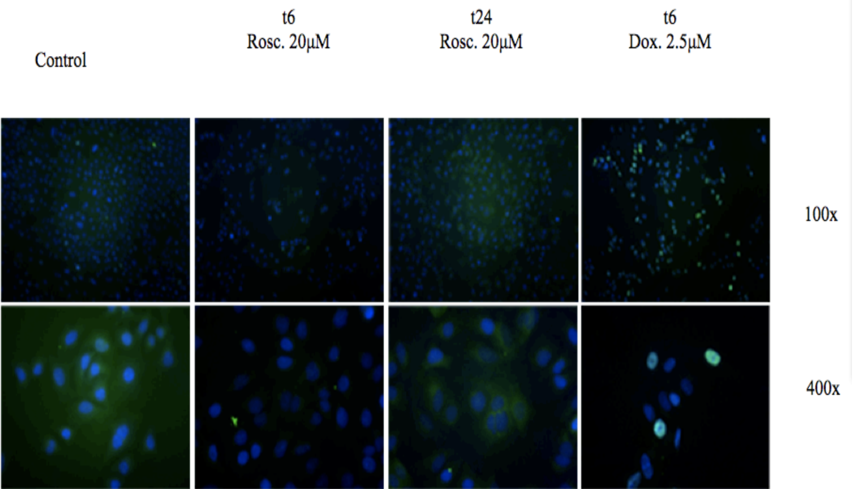


Fig. 20 Immunofluorescence analysis by fluorescent microscopy of phosphorylated γH2AX (serine 139) at the abovementioned time points following 6 hours of treatment with 20 μM Roscovitine or 2.5 μM Doxorubicin (as a positive control for DSBs). Images shown are γH2AX (FITC) and DAPI merges under 100x (upper panels) and 400x (lower panels) magnifications.

In addition to γH2AX, we observed overall DNA damage on a single-cell level utilizing the alkaline comet assay. The comet assay revealed no significant differences in DNA damage between cells treated with only Doxorubicin and those treated with both Doxorubicin and Roscovitine six hours-post drug removal. However, 24 hours after drug removal, while Doxorubicin-only treated cells had completely repaired the damage, cells treated with both Doxorubicin and Roscovitine contained a greater amount of DNA damage ($p \leq 0.0001$) (Figure 21). These data further support the hypothesis that Roscovitine can augment Doxorubicin-induced DNA damage by hindering DSB repair over time.

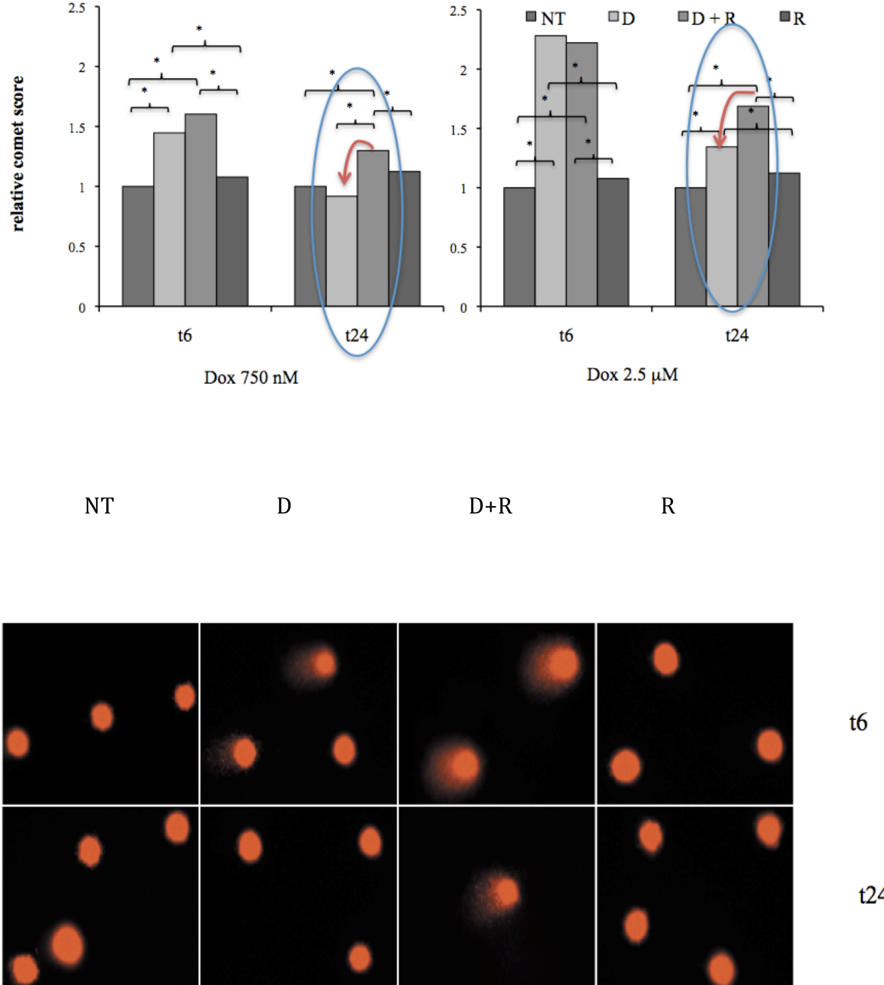


Fig. 21 Alkaline comet assay images (400x magnification) and d) respective quantification, 6 (t6) and 24 (t24) hours following a 6 hour incubation with abovementioned treatments (Control, NT; Doxorubicin, D; Doxorubicin + Roscovitine, D+R; Roscovitine, R) to measure overall DNA damage.

Combined treatment leads to global changes in DNA repair pathways

To assess the global effects of combination treatment, we performed genome-wide microarray analysis on cDNA from A549 cells treated for 24 hours with either 1 μM Doxorubicin alone or in combination with 20 μM Roscovitine. Here we focus our analysis primarily on genes involved in the DNA repair pathways: mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and NHEJ. We grouped the genes related to these pathways that changed in a statistically significant manner ($p\text{-value} \leq 0.05$) after combination treatment respect to Doxorubicin treatment in Table 1 and Figure 22. The most significant changes were observed in the NHEJ and HR pathways. In particular in HR we observed a decrease in BRCA1 (fold change: -0.46), BRCA2 (-0.34) and RAD50 (-0.75). Furthermore, there were significant variations in key proteins involved in NHEJ. In particular, we observed a significant decrease in the expression levels of Ku80 (XRCC5 -0.61), DNA-activated protein kinase (PRKDC -0.61), and NHEJ1 (-0.80) (Table 1 and Figure 6). These data support the reduced NHEJ activity observed with the in vitro NHEJ plasmid re-ligation assay. Moreover, they demonstrate a more global affect on DNA repair pathways as a result of combination treatment with Roscovitine.

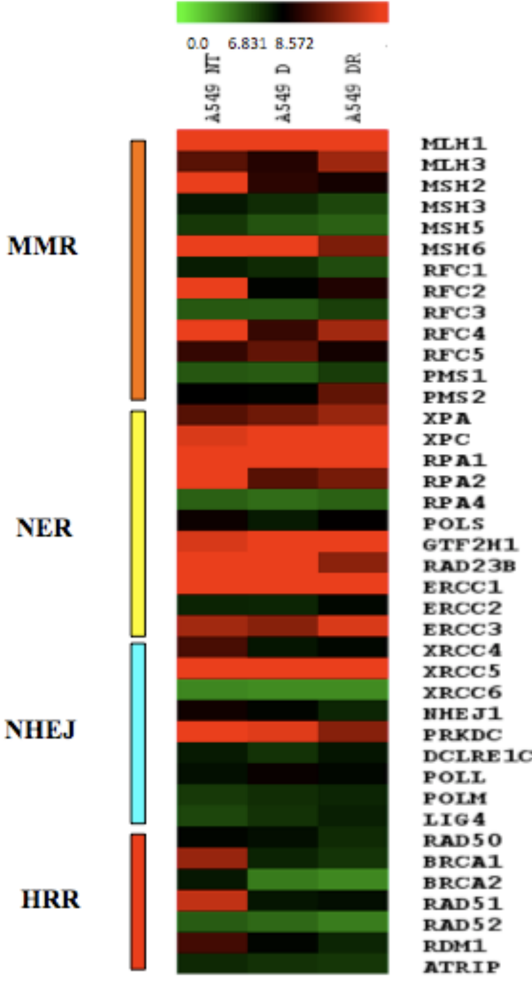


Fig. 22 Corrected microarray signal values of genes involved in DNA repair clustered by specific DNA repair pathway of A549 cells treated for 24 hours with 1 μM Doxorubicin alone or in combination with 20 μM Roscovitine in comparison to control cells

ID AFFYMETRIX	Gene symbol	A549 D1	A549 D2	A549 DR1	A549 DR2	M	P.Value
		Signal					
223598_at	*RAD23B*	8.83	8.91	7.68	7.88	-1.09	0.000223
202996_at	*POLD4*	10.01	10.14	8.89	9.29	-0.98	0.001349
209084_s_at	*RFC1*	5.67	5.77	4.87	4.76	-0.90	0.000436
219418_at	*NHEJ1*	6.76	6.55	5.75	5.96	-0.80	0.001689
211450_s_at	*MSH6*	8.46	8.47	7.61	7.76	-0.78	0.001138
209349_at	*RAD50*	6.40	6.48	5.63	5.75	-0.75	0.001394
203720_s_at	*ERCC1*	9.57	9.65	8.78	8.98	-0.73	0.002189
205887_x_at	*MSH3*	5.71	5.56	5.03	4.85	-0.69	0.003738
219715_s_at	*TDP1*	7.94	7.81	7.26	7.12	-0.68	0.002669
210543_s_at	*PRKDC*	8.36	8.36	7.78	7.72	-0.61	0.00473
208643_s_at	*XRCC5(Ku80)*	9.94	10.06	9.31	9.46	-0.61	0.00434
213734_at	*RFC5*	7.64	7.37	6.91	7.03	-0.53	0.014248
212525_s_at	*H2AFX*	6.05	6.17	5.51	5.69	-0.51	0.011937
211851_x_at	*BRCA1*	5.84	5.93	5.39	5.46	-0.46	0.022329
204752_x_at	*PARP2*	7.89	7.95	7.50	7.65	-0.34	0.049
205672_at	*XPA*	7.63	7.54	7.89	7.87	0.29	0.03678
221143_at	*RPA4*	3.79	4.06	4.25	4.26	0.33	0.01878
1053_at	*RFC2*	6.83	6.61	7.05	7.07	0.34	0.049
227766_at	*LIG4*	5.56	5.40	6.11	5.88	0.52	0.025825
202176_at	*ERCC3*	7.84	7.70	8.31	8.30	0.54	0.006878
209903_s_at	*ATR*	8.11	7.93	8.64	8.53	0.57	0.009919
202451_at	*GTF2H1*	8.60	8.55	9.29	9.07	0.61	0.01218
232134_at	*POLS*	6.32	6.00	6.98	6.75	0.71	0.008367
231119_at	*RFC3*	4.31	4.56	4.95	5.35	0.72	0.008497
204023_at	*RFC4*	7.26	7.17	8.04	7.84	0.72	0.00282
222233_s_at	*DCLRE1C*	5.50	5.44	6.41	6.10	0.78	0.00239
213468_at	*ERCC2*	5.82	5.85	6.58	6.64	0.78	0.000828
209805_at	*PMS2*	6.67	6.74	7.56	7.43	0.79	0.000908
209805_at	*PMS2*	6.67	6.74	7.56	7.43	0.79	0.000908
1554743_x_at	*PMS1*	4.32	4.51	5.29	5.16	0.81	0.002444
204838_s_at	*MLH3*	7.13	7.05	7.97	7.86	0.83	0.001711

Tab. 1 Statistically significant genes involved in DDR after combination treatment. Genes involved in DNA repair mechanisms, those shown in blue decreased and those in red increased in expression level (p value ≥ 0.05) after combination treatment with 1 μM Doxorubicin and 20 μM Roscovitine as compared to 1 μM Doxorubicin only, in A549 cells after 24 hours of treatment.

Discussion

Under genotoxic conditions the CDK2/cyclin A1 complex increases its functional kinase activity and the ability to phosphorylate Ku70. In addition, here we demonstrated upon treatment with different DNA damaging agents (doxorubicin or γ-irradiation) a marked dose dependent increase in the RNA and protein levels of cyclin A1, which is independent of the cell cycle phase redistribution. Conversely cyclin A2 (whose expression is tightly related to the S and G2-M phases of the cell cycle) is down-regulated under genotoxic stress conditions as a result of the check-point activation and consequent decrease of the S phase fraction. This switch in the respective levels of the A-family cyclins may be functionally relevant to redirect CDK2 activity toward DNA DSB repair, especially given the findings that the ectopic over-expression of cyclin A1 increased the in-vitro NHEJ activity and that cyclin A1 depletion, as demonstrated by others [muller tidow], results in an impaired DNA DSB repair ability.

DNA DSBs are considered the most lethal form of DNA damage and CDK inhibition has been shown to potentially affect the two major DSB repair pathways (HR and NHEJ). Various mechanisms have been proposed to explain this effect such as the deregulation of the DNA damage-induced checkpoint signalling cascade[13] or the down-regulation of specific genes involved [35, 36]. Roscovitine is an oral 2,6,9 trisubstituted purine analog currently under phase II investigation, which competes with ATP for the catalytic binding site on CDK2 (but also CDKs 1, 7 and 9 with a much lower affinity) with a demonstrated antitumor activity in many human cancer models and a nice toxicity profile.

One of the most prominent effects of the drug is the inhibition of CDK2/cyclin E complexes, which causes a decrease in Rb phosphorylation and a consequent inactivation of E2F family members, thus leading to cyclin transcriptional downregulation and ultimately to

cell cycle arrest. This strong transcriptional depression of most of the cell cycle related cyclins further enforces the drug's inhibitory effect on CDK/cyclin complexes. Furthermore, Roscovitine has been shown to down-regulate several other genes involved in a wide spectrum of cellular functions[31, 32], probably as a result of partial CDK7/cyclin H and CDK9/cyclin T inhibition[33]. In addition, whole genome ChIP-on-chip analysis recently mapped E2F transcription factor family members to the promoters of many more genes than were traditionally associated to the cell cycle[34], suggesting an alternative mechanism to explain these transcriptional effects.

We investigated the effect that Roscovitine may have on cyclin A1 transcription as one of the possible mechanisms through which CDK2 inhibition may curb DNA DSB repair activity. The promoter of the cyclin A1 gene, CCNA1 is not E2F-dependent and, consistently, increasing doses of Roscovitine did not repress cyclin A1 basal transcription levels in contrast to cyclins A2, D and E. However, we demonstrated that Roscovitine at doses preferentially inhibiting CDK2 but not CDK7 and 9 completely abolished cyclin A1 DNA damage-induced up-regulation, thus suggesting that residual CDK2 activity is required for cyclin A1 up-regulation. In addition Roscovitine co-administered with doxorubicin was able to largely modify the patterns of cell cycle phase distribution in comparison to doxorubicin only treatment. This resulted in an augmented S phase and consequently in an increased expression of cyclin A2. The combined treatment thus resulted in the complete inversion of the doxorubicin-induced switch between cyclin A1 and cyclin A2. Moreover, Roscovitine post-transcriptionally down-regulated cyclin A1.

Such transcriptional and post-transcriptional repression was observed in different NSCLC, prostate and breast cancer cell lines and we propose that this potentiates and synergizes the Roscovitine-mediated CDK2 inhibition thus resulting in a significant decrease of cellular NHEJ ability. In fact, we observed that combination treatment led to an increase in DNA DSBs and overall DNA damage over-time, further substantiating, not only the importance of CDK-inhibitors in combination therapy but also the role of CDKs in DNA repair mechanisms. While these findings

were supported by genome-wide mircroarray analysis, we also observed a significant effect on key genes involved in other DNA repair pathways.

Conclusions

Given the role of CDK2 in multiple DDR pathways, the down-regulation of cyclin A1, may further explain the effective inhibition of a broader range of DNA repair mechanisms by Roscovitine. Furthermore, through its inhibition of CDKs and thus E2F transcriptional activity, Roscovitine appears to play a role in the inhibition of DNA repair on a more global scale. Moreover, since NHEJ is considered the major pathway for the repair of γIR-induced DNA DSBs in human cells[38], we believe our data support further investigation on the therapeutic advantages of combination therapy with Roscovitine and Radiotherapy.

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Appendix 1

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RESEARCH Open Access

R-Roscovitine (Seliciclib) prevents DNA damage-induced cyclin A1 upregulation and hinders non-homologous end-joining (NHEJ) DNA repair

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Abstract

Background: CDK-inhibitors can diminish transcriptional levels of cell cycle-related cyclins through the inhibition of E2F family members and CDK7 and 9. Cyclin A1, an E2F-independent cyclin, is strongly upregulated under genotoxic conditions and functionally was shown to increase NHEJ activity. Cyclin A1 outcompetes with cyclin A2 for CDK2 binding, possibly redirecting its activity towards DNA repair. To see if we could therapeutically block this switch, we analyzed the effects of the CDK-inhibitor *R*-Roscovitine on the expression levels of cyclin A1 under genotoxic stress and observed subsequent DNA damage and repair mechanisms.

Results: We found that *R*-Roscovitine alone was unable to alter cyclin A1 transcriptional levels, however it was able to reduce protein expression through a proteosome-dependent mechanism. When combined with DNA damaging agents, *R*-Roscovitine was able to prevent the DNA damage-induced upregulation of cyclin A1 on a transcriptional and post-transcriptional level. This, moreover resulted in a significant decrease in non-homologous end-joining (NHEJ) paired with an increase in DNA DSBs and overall DNA damage over time. Furthermore, microarray analysis demonstrated that *R*-Roscovitine affected DNA repair mechanisms in a more global fashion.

Conclusions: Our data reveal a new mechanism of action for *R*-Roscovitine on DNA repair through the inhibition of the molecular switch between cyclin A family members under genotoxic conditions resulting in reduced NHEJ capability.

Background

The cell cycle is comprised of a series of highly coordinated events culminating in cell growth and division. Cyclin-dependent kinases (CDK) and their cyclin counterparts strictly regulate and drive cell cycle progression and different CDK/cyclin complexes are responsible for the timely occurrence of each phase transition in order to maintain genetic integrity throughout generations. Cancer cells have been frequently found to have a deregulated CDK activity allowing them to escape the normal cell cycle and proliferate uncontrollably. For these reasons CDKs have been considered attractive targets for cancer therapy and several CDK-inhibitors have been developed and are under intense investigation[1].

R-Roscovitine (Seliciclib, CYC202; herein referred to as Roscovitine), one of the most promising members of the CDK-inhibitor family, is an orally available adenosine analogue prominently targeting CDK2 (also affecting CDKs 1, 7 and 9 at a much lower rate)[2] with a low off-target effect on other members of the human kinome[3], and a nice toxicity profile[4]. In preclinical studies Roscovitine has shown significant *in vitro* and *in vivo* antitumor activity on a wide panel of human cancers and is currently in phase II clinical trials[5]. Since preclinical experimentation, it has become evident that, CDK-inhibitors, such as Roscovitine, may actually curb the activity of DNA repair machinery[6,7], hence

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becoming an attractive candidate for therapeutic association with either radiation therapy[8,9] or genotoxic agent-based chemotherapy[10]. However, the mechanism of this inhibition is still elusive.

One of the proposed means for CDK-inhibitors to affect DNA repair is through checkpoint deregulation [11-13], but increasing evidence supports a complex network of direct interactions between individual CDKs and proteins that play a key role in DNA damage repair (DDR). It is known that different DNA repair pathways are preferentially activated at specific stages of the cell cycle possibly suggesting a functional crosstalk between CDK/cyclin complexes and DNA repair mechanisms [14]. In particular, CDK2 has been shown to interact with p53[15], BRCA1[16], BRCA2[17], Ku70[18] and both, CDK1 and CDK2, can modulate BRCA1-BARD1 activity[13,19]. Moreover, CDK2 knock-down cells have an attenuated capacity to repair DNA damage suggesting a pivotal role for CDK2[7] in DDR. Given the ability of CDKs to compensate for each other *in vivo*, overall CDK activity has been proposed to be influential in DDR regulation[20] however CDK2 function seems to have a specific role in some survival pathways[21].

Cyclins, similarly to CDKs, have been correlated to DDR. Cyclin E levels are upregulated under genotoxic stress conditions[22] and a post-translational cleavage generates an 18-amino acid peptide, which has been shown to interact with Ku70[18] promoting the release of the pro-apoptotic factor Bax from the inactivating complex Bax/Ku70. Moreover, an increasing amount of data suggests an important role in DDR for the A-type cyclins, and in particular for cyclin A1. Differing from cyclin A2, ubiquitously expressed during the S and G2/M phases of the cell cycle, cyclin A1 is a testis-specific cyclin, which interacts with CDK2 and is involved in germ cell meiosis and spermatogenesis[23]. Cyclin A1 may have a role in carcinogenesis, as it has been found to be over-expressed in acute myeloid leukemia and various other tumour types[23-25], however, its role in cancer is still particularly obscure. In somatic non-testicular tissues, cyclin A1 is not expressed or is expressed at very low basal levels. After genotoxic insult, cyclin A1 mRNA is upregulated *in vitro*[26] and *in vivo*[27]. At a molecular level, human CDK2/cyclin A1 complexes interact with members of the Ku family and phosphorylate Ku70[27,28], a pivotal player in the non-homologous end-joining (NHEJ) double strand break (DSB) repair pathway. Furthermore, under genotoxic conditions the kinase activity of CDK2/cyclin A1 complex increases, while the relative kinase activity of CDK2/cyclin A2 decreases and the CDK2/cyclin A1 complex out-competes with CDK2/cyclin A2 for Ku70 binding [28]. Moreover, it has recently been found that CDK2 phosphorylation status and structure changes upon the cyclin A family member with which it is bound [29] suggesting a non-redundant function between CDK2/cyclin A1 and CDK2/cyclin A2 complexes. Finally cyclin A1 knockout mice and *Xenopus* embryos exhibited a clear defect in DNA repair[27,30] and are more prone to undergo apoptosis[31].

Taken together these data support that during genotoxic stress differential transcriptional levels and activity of cyclin A family members may redirect CDK2 toward DNA repair resulting in a modulation of NHEJ. Since one of the most relevant effects of CDK inhibitors is the downregulation of cell cycle related cyclins, we investigated if the inhibition of DNA repair mechanisms by Roscovitine may also occur through the modulation of the expression levels of cyclin A family members. Physiological CDK-inhibition, in fact, results in cyclin downregulation through the inhibition of E2F-family transcription factors, which drive and regulate cell cycle-related cyclin transcription. Given that the promoter of the cyclin A1 gene, *CCNA1*, is different from the other cell cycle-related cyclins, not being under the regulation of E2Fs[32], here we analyzed the effects of Roscovitine on cyclin A1 expression and modulation of DNA repair mechanisms. We demonstrated that under DNA damaging conditions cyclin A1 is strongly upregulated and localizes to the nucleus. Although Roscovitine alone was not sufficient to reduce the basal levels of cyclin A1, in contrast to cell cycle related cyclins, Roscovitine treatment could abolish the DNA damage-induced cyclin A1 upregulation, reducing NHEJ and significantly hindering DNA repair over time.

Results

DNA damage induces a switch in the respective levels of A-family cyclins

We first compared mRNA levels of both members of the cyclin A family after treatment with increasing doses of Doxorubicin (from 250 nM up to 5 μM), a well-known inducer of DNA DSBs. We found that cyclin A1 upregulation is dose dependent with a plateau that is reached around 2.5 μM (IC90). On the contrary, Doxorubicin treatment caused a downregulation of cyclin A2 mRNA levels with a nadir that is reached at the dose of 750 nM (IC50) followed by a relative increase close to basal levels (that are not reached) at a dose of 2.5 μM (IC90) and further followed by a constant decline at higher doses (Figure 1A).

These finding were congruent with protein levels of both cyclins A1 and A2 (Figure 1B). The cyclin A1 antibody we utilized detected two bands, which both augmented upon treatment. The upper band we hypothesized to be a phosphorylated or hyper-phosphorylated form of cyclin A1, which was barely detectable when phosphatase inhibitors were excluded from

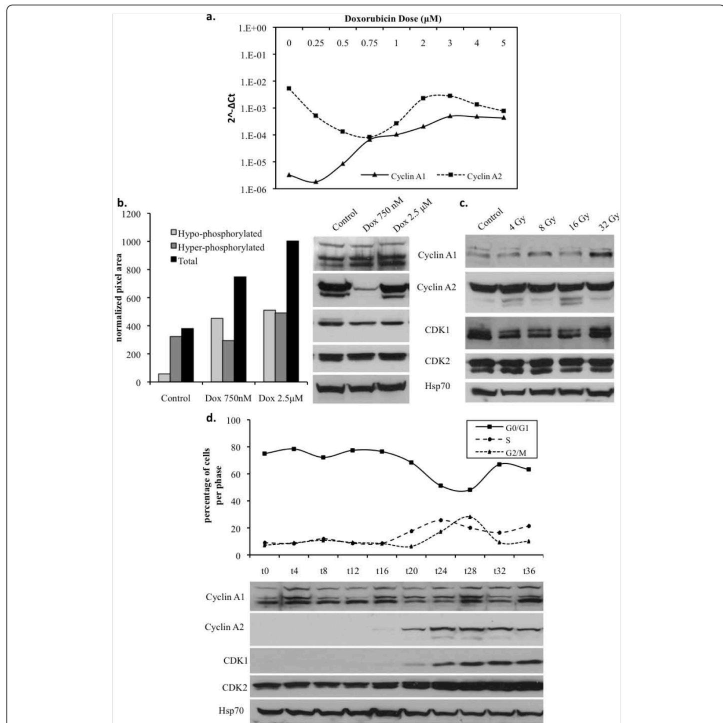

Figure 1 DNA DSBs induce an upregulation of cyclin A1 but not cyclin A2 in A549 cells in a cell cycle-independent manner A) Relative expression levels respect to GAPDH ($2^{\wedge-\Delta Ct}$) of cyclin A1 (*CCNA1*) vs. cyclin A2 (*CCNA2*) mRNA after 24 hours of treatment with increasing doses of Doxorubicin (250 nM to 5 μM). B) Western blot analysis of cyclin A1, cyclin A2, CDK1 and CDK2 expression levels with Hsp70 as a loading control after 24 hours of treatment with Doxorubicin (Dox 750 nM and 2.5 μM). Quantification of cyclin A1 expression levels as normalized pixel area respect to Hsp70. C)Western blot analysis of protein expression 1 hour after administration of increasing doses of γ-irradiation (4 Gy to 32 Gy). D) Flow cytometry cell cycle analysis with corresponding western blot showing cyclin A1, cyclin A2, CDK1 and CDK2 expression levels over the course of the synchronous cell cycle induced by serum starvation.

the lysis buffer. The lower band a hypo-phosphorylated or non-phosphorylated form, which was detectable when cell lysis was performed with or without phosphatase inhibitors (Additional File 1). Relative quantification of bands showed that Doxorubicin, while inducing a slight increase in the hyper-phosphorylated form of cyclin A1, induced a marked dose-dependent increase in the hypo-phosphorylated form. These finding were also noted in A549 cells 1 hour after gamma-irradiation (Figure 1C) suggesting that cyclin A1 upregulation is

not specific to doxorubicin treatment and that the timing of its upregulation is compatible with DNA repair events.

To ensure that the increase in cyclin A1 expression observed was not a result of cell cycle redistribution, we analyzed the expression of cyclin A family members during the synchronous cell cycle in the A549 NSCLC cell line. We observed that unlike cyclin A2, which, as expected, was expressed during the S and G2/M phases, cyclin A1 remained fairly constant throughout the cell cycle (Figure 1D). Cell cycle analysis by flow cytometry was also performed on asynchronous A549 cells treated for 24 hours with Doxorubicin (750 nM and 2.5 μM) in comparison to untreated controls, and as expected Doxorubicin treatment resulted in an activation of DNA damage cell cycle checkpoints at G1-S and G2-M phase transitions (Additional File 2). Cells treated with 750 nM Doxorubicin exhibited a decrease in the percentage of cells in S phase, which is duly noted by the observed decrease in cyclin A2 expression levels. However, treatment with 2.5 μM Doxorubicin resulted in a relative increase in the percentage of cells in S phase, which mirrors the increase in cyclin A2 expression at higher doses of Doxorubicin as seen by western blot. These data confirm that cyclin A1 is strongly induced under DNA damaging conditions and also supports a DNA damage-induced molecular switch between cyclin A2 and cyclin A1 during genotoxic stress.

Cyclin A1 localizes to the nucleus during genotoxic conditions and its overexpression increases in vitro NHEJ activity

To determine if cyclin A1 upregulation under DNA damaging conditions was specific to a sub-population or was found in all cells we performed flow cytometry analysis of Doxorubicin treated A549 cells. Cyclin A1 upregulation was observed in all cells, further confirming that this was independent of the cell cycle (data not shown). We also analyzed Doxorubicin treated A549 cells by immunofluorescence staining and microscopy noting not only a dose-dependent increase in fluorescent signal but also a nuclear localization of cyclin A1 protein at higher doses of Doxorubicin (2.5 μM) treatment (Figure 2A). The nuclear localization and the dose-dependent increase in cyclin A1 expression could speak further towards a specific role for cyclin A1 in DNA repair mechanisms.

To address the role of cyclin A1 in DNA DSB repair mechanisms, we used an *in vitro* plasmid re-ligation assay based on the ability of the whole cellular extract to re-join a linearized plasmid. Wortmannin, a known inhibitor of DNA dependent protein kinase (DNA PK), was used as a control to demonstrate the dependency of re-ligation upon NHEJ. Quantification of plasmid re-ligation was performed by real-time PCR utilizing primers, which bound both upstream and downstream of the enzymatic cut site, amplifying only upon re-ligation of plasmid DNA, and values were normalized on the quantity of plasmid in each reaction by primers which bound an intact region of plasmid DNA. We analyzed the NHEJ capability of HEK293FT cells (utilized for their optimal transfection efficiency), transiently transfected to overexpress cyclin A1 or enhanced yellow fluorescent protein (YFP, negative control). In cells overexpressing cyclin A1 there was a significant increase (approximately 6-fold) in NHEJ activity respect to YFP controls (Figure 2B).

Roscovitine, at doses primarily inhibiting CDK2, but not CDK7 or 9 prevents DNA damage-induced cyclin A1 transcriptional upregulation and increases protein degradation

Roscovitine, being a CDK2 inhibitor, can depress E2F-dependent transcription by blocking the phosphorylation of Rb-family proteins. Cyclin A1 expression is not E2F-dependent[30], therefore we investigated the effects of Roscovitine on cyclin A1 basal expression and eventually on the DNA damage-induced upregulation. First we analyzed the mRNA expression levels of cyclins A1, A2, B, D, and E after 24 hours of incubation with increasing doses (up to 60 μM) of Roscovitine. We found that all cyclin mRNA expression levels were greatly reduced respect to untreated controls (Figure 3A), except for cyclin A1, whose basal levels were substantially lower than the other cyclins and were not downregulated but remained fairly constant upon Roscovitine treatment consistent with its E2F-independent transcriptional regulation (Figure 3A). Therefore, we treated A549 cells for 24 hours with increasing doses of Doxorubicin (as previously stated) alone or in combination with a fixed dose of 20 μM Roscovitine. We chose to use the dose of 20 μM as it is not only a dose commonly utilized in the literature but also as it was experimentally proven to preferentially inhibit CDK2 resulting in a hypo-phosphorylation of p130/Rb2, while it is the highest dose with a limited effect on CDK7 and CDK9, as shown by the phosphorylation of the C-terminal domain (CTD) of RNA Polymerase II on serine 5 and 2 respectively (Figure 3B). Roscovitine was able to completely abolish the Doxorubicin-induced cyclin A1 mRNA and protein upregulation (Figure 3C&3D) suggesting that a residual CDK2 activity is required for cyclin A1 upregulation. Furthermore, co-administration of Doxorubicin and Roscovitine resulted in a change in cyclins A2, B, D and E mRNA expression levels, respect to Doxorubicin treatment alone (Additional File 3). In particular, cyclin A2 mRNA levels demonstrated an attenuated variation during combination treatments, which is consistent with

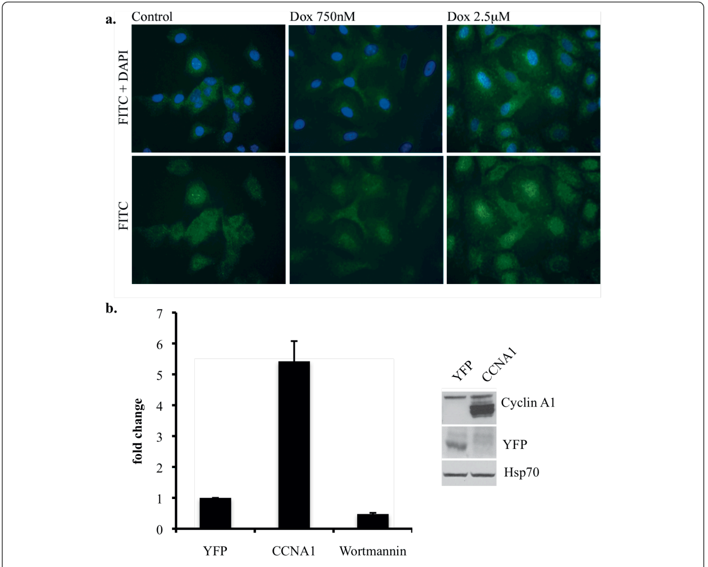

Figure 2 Nuclearization of cyclin A1 under DNA DSB conditions and its role in NHEJ. A) Immuno-fluorescence analysis by fluorescent microscopy of cyclin A1 localization in A549 cells after treatment with Doxorubicin (750 nM and 2.5 μM). Lower panels show FITC-stained cyclin A1 expression (green) and upper panels show FITC and DAPI (blue) merge at 400× magnification. B) Fold change, respect to YFP, of *in vitro* NHEJ plasmid re-ligation activity as quantified by real time PCR in HEK293FT cells transiently transfected with *YFP* (control) or cyclin A1 (*CCNA1*) and respective western blot and ponceau S staining verifying overexpression respect to Hsp70.

the cell cycle distribution as observed by flow cytometry (Additional File 2). At the protein level, the combination of Roscovitine with Doxorubicin resulted in an inversion of the Doxorubicin-induced molecular switch between cyclin A1 and cyclin A2 (Figure 3D).

Unlike cyclin A1 mRNA levels, treatment with Roscovitine alone also resulted in a decrease in cyclin A1 protein expression over time (Figure 3D&3E), suggesting that, aside from transcriptional regulation, Roscovitine may also regulate cyclin A1 on a post-transcriptional level. To confirm this hypothesis we treated A549 cells with Doxorubicin and Roscovitine respectively as well as 10 μM of the proteosome inhibitor MG-132. Inclusion of MG-132 significantly prevented the downregulation of cyclin A1 protein levels after treatment with 20 μM Roscovitine (Figure 3E). The transcriptional and post-transcriptional regulation of cyclin A1 by Roscovitine was confirmed in a panel of NSCLC (A549 and H23), breast (MCF-7 and MDA-MB-231) and prostate cancer (LNCAP and DU145) cell lines (data not shown).

Combined treatment with Roscovitine and Doxorubicin results in a downregulation of NHEJ capability

Cyclin A1 knockout MEFs have shown a reduced NHEJ capability[27]. To determine if Roscovitine may have a comparable effect on NHEJ mechanisms, we incubated untreated A549 cell lysates with 20 μM

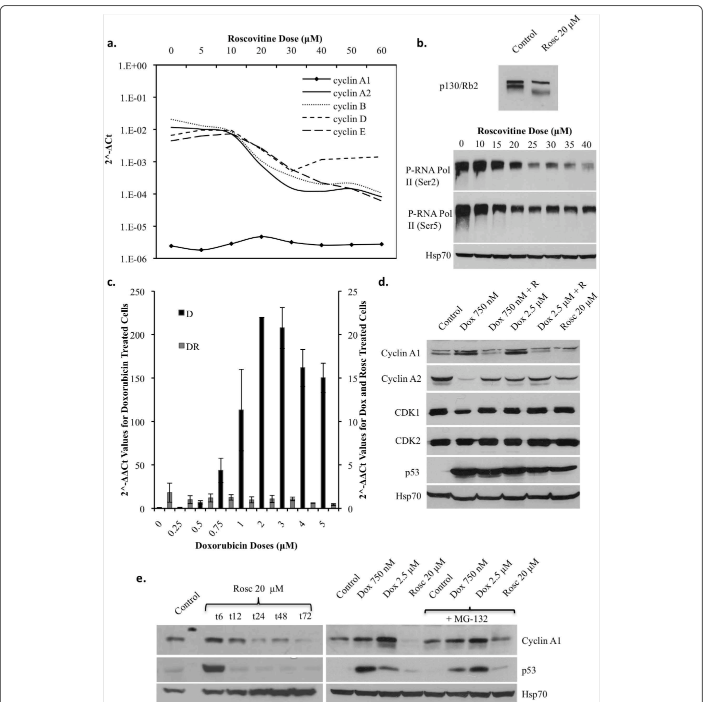

Figure 3 Roscovitine inhibits DNA DSB-induced upregulation of cyclin A1 mRNA at doses primarily affecting CDK2 and post-translationally downregulates cyclin A1 protein levels over time in A549 cells. A) Expression levels respect to GAPDH ($2^{-\Delta Ct}$), in mRNA of cyclin A1, cyclin A2, cyclin B, cyclin D and cyclin E after 24 hours of treatment with increasing doses of Roscovitine (5-60 μM). B) (Upper blot) Western blot analysis of inhibitory activity of Roscovitine (Rosc) against CKD2 phosphorylation of p130/Rb2 as shown by a shift in p130/Rb2 band height from hyper-phosphorylated in control cells to hypo-phosphorylated in Roscovitine treated cells, upper band is non-specific. (Lower blot) Western blot analysis of Roscovitine inhibition of CDK7 and CDK9 phosphorylation of the C-terminal domain (CTD) of RNA polymerase II, on serine 5 and serine 2 respectively, in cells treated for 24 hours with increasing doses of Roscovitine (10-40 μM). C) Fold change, respect to control ($2^{-\Delta\Delta Ct}$), of cyclin A1 mRNA expression levels in cells treated with either increasing doses of Doxorubicin alone (250 nM to 5 μM) or increasing doses of Doxorubicin in combination with 20 μM Roscovitine for 24 hours. Note that black bars represent Doxorubicin only treated cells and correspond to the vertical axis on the left-hand side of the graph, while grey bars represent Doxorubicin and Roscovitine treated cells and correspond to the vertical axis on the right-hand side of the graph. D) Western blot analysis of cyclin A1, cyclin A2, CDK1 and CDK2 protein expression in cells treated for 24 hours with either Doxorubicin (750 nM or 2.5 μM) alone, 20 μM Roscovitine alone, or in combination (Dox 750 nM/2.5 μM + R). p53 protein expression was included as a control for drug treatments. E) Post-translational inhibition of cyclin A1 protein levels over time. (Left-side blot) cyclin A1 and p53 protein expression in cells treated for increasing amounts of time (6-72 hours) with 20 μM Roscovitine. (Right-side blot) cyclin A1 and p53 expression in cells treated for 24 hours with either Doxorubicin (750 nM and 2.5 μM) or 20 μM Roscovitine alone or in combination with 10 μM of the proteosome inhibitor MG-132.

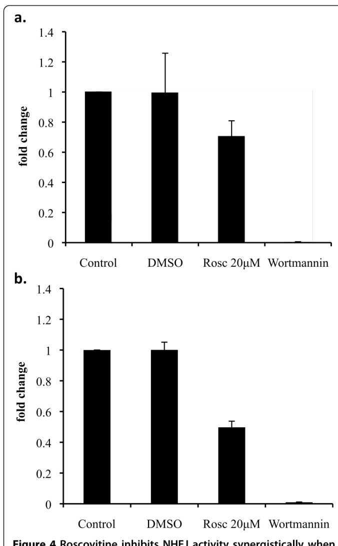

Figure 4 Roscovitine inhibits NHEJ activity synergistically when combined with Doxorubicin treatment in A549 cells. A) Analysis by real time PCR of NHEJ plasmid re-ligation activity of untreated A549 cell lysate with the addition of 20 μM Roscovitine, DMSO or Wortmannin. B) Analysis by real time PCR of NHEJ plasmid re-ligation activity in A549 cells treated for 12 hours with 20 μM Roscovitine. Wortmannin was added to untreated cell lysate as a negative control for NHEJ activity *in vitro*.

Roscovitine, DMSO, or Wortmannin for 15 minutes prior to incubation with linearized plasmid. While Wortmannin was able to almost completely inhibit NHEJ activity, DMSO had no effect and Roscovitine resulted in an approximate 25% diminution in plasmid re-ligation, which can be accounted for by a direct inhibition of CDK activity and eventual off-target effects of the drug (Figure 4A). However, when lysates from A549 cells treated for 12 hours with 20 μM Roscovitine were assayed for NHEJ capability, they demonstrated an approximate 45% reduction in plasmid re-ligation (Figure 4B) as a result of an additional biological mechanism to the pharmacological inhibition of CDK2.

Roscovitine enhances Doxorubicin-induced DSBs and delays DNA damage repair over time

To determine if the inhibition of NHEJ activity led to an overall increase in DNA DSBs we analyzed the quantity of phosphorylated γH2AX by western blot (Figure 5A). After six hours of incubation with respective drug treatments, we removed the drug-containing medium and analyzed A549 cells for γH2AX phosphorylation immediately following the six hour treatment(t0), then six(t6) and 24(t24) hours after drug removal with respect to control cells. Doxorubicin treatment induced an activation of γH2AX, which was significantly augmented following combined treatment with Roscovitine over time (Figure 5A), even though Roscovitine alone did not significantly activate γH2AX as shown by western blot and immunofluorescence staining (Figure 5A&5B).

In addition to γH2AX, we observed overall DNA damage on a single-cell level utilizing the alkaline comet assay. The comet assay revealed no significant differences in DNA damage between cells treated with only Doxorubicin and those treated with both Doxorubicin and Roscovitine six hours-post drug removal. However, 24 hours after drug removal, while Doxorubicin-only treated cells had completely repaired the damage, cells treated with both Doxorubicin and Roscovitine contained a greater amount of DNA damage ($p \le 0.0001$) (Figure 5C&5D). These data further support the hypothesis that Roscovitine can augment Doxorubicin-induced DNA damage by hindering DSB repair over time.

Combined treatment leads to global changes in DNA repair pathways

To assess the global effects of combination treatment, we performed genome-wide microarray analysis on cRNA from A549 cells treated for 24 hours with either 1 μM Doxorubicin alone or in combination with 20 μM Roscovitine. Here we focus our analysis primarily on genes involved in the DNA repair pathways: mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and NHEJ. We grouped the genes related to these pathways that changed in a statistically significant manner (p-value ≤ 0.05) after combination treatment respect to Doxorubicin treatment in Table 1 and Figure 6. The most significant changes were observed in the NHEJ and HR pathways. In particular in HR we observed a decrease in *BRCA1* (fold change: -0.46) and *RAD50* (-0.75). Furthermore, there were significant variations in key genes involved in NHEJ. In particular, we observed a significant decrease in the expression levels of Ku80 (*XRCC5* -0.61), DNA-activated protein kinase (*PRKDC* -0.61), and NHEJ1 (-0.80) (Table 1 and Figure 6). These data support the reduced NHEJ activity observed with the *in vitro* NHEJ plasmid re-ligation assay. Moreover, they demonstrate a

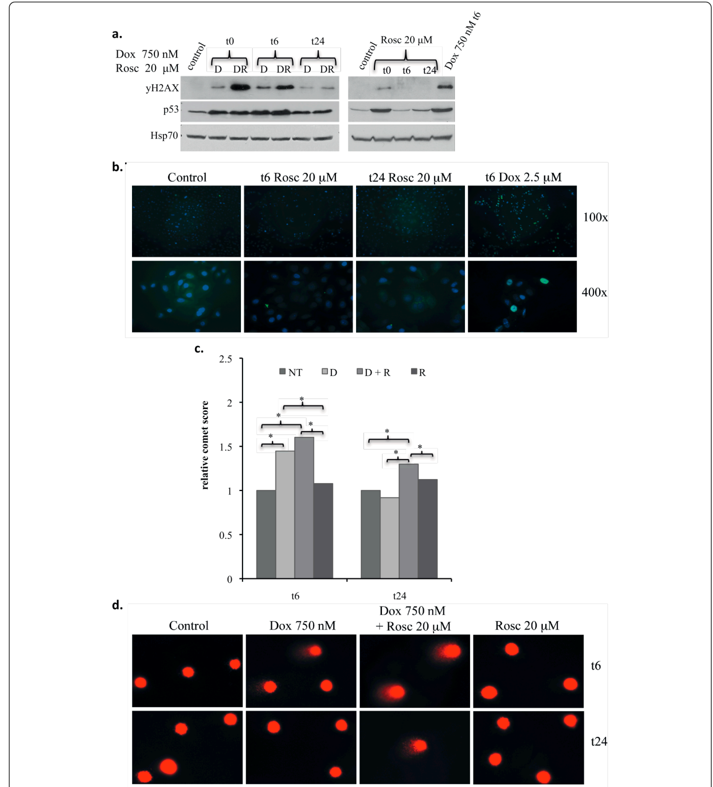

Figure 5 Roscovitine when combined with Doxorubicin increases DNA DSBs and overall DNA damage over time in A549 cells. A) Western blot analysis of DNA DSBs by phosphorylated γH2AX (serine 139) immediately (t0) or 6 (t6) and 24 (t24) hours following a 6 hour treatment with either 750 nM Doxorubicin (D) or 20 μM Roscovitine alone or in combination (DR). B) Immunofluorescence analysis by fluorescent microscopy of phosphorylated γH2AX (serine 139) at the abovementioned time points following 6 hours of treatment with 20 μM Roscovitine or 2.5 μM Doxorubicin (as a positive control for DSBs). Images shown are γH2AX (FITC) and DAPI merges under 100× (upper panels) and 400× (lower panels) magnifications. C) Alkaline comet assay quantification and D) respective images (400x magnification), 6 (t6) and 24 (t24) hours following a 6 hour incubation with abovementioned treatments (Control, NT; Doxorubicin, D; Doxorubicin + Roscovitine, D+R; Roscovitine, R) to measure overall DNA damage.

Table 1 Statistically significant genes involved in DDR after combination treatment

ID AFFYMETRIX	Gene symbol	A549 D1	A549 D2	A549 DR1	A549 DR2	M	P.Value
		Signal					
223598_at	*RAD23B*	8.83	8.91	7.68	7.88	-1.09	0.000223
202996_at	*POLD4*	10.01	10.14	8.89	9.29	-0.98	0.001349
209084_s_at	*RFC1*	5.67	5.77	4.87	4.76	-0.90	0.000436
219418_at	*NHEJ1*	6.76	6.55	5.75	5.96	-0.80	0.001689
211450_s_at	*MSH6*	8.46	8.47	7.61	7.76	-0.78	0.001138
209349_at	*RAD50*	6.40	6.48	5.63	5.75	-0.75	0.001394
203720_s_at	*ERCC1*	9.57	9.65	8.78	8.98	-0.73	0.002189
205887_x_at	*MSH3*	5.71	5.56	5.03	4.85	-0.69	0.003738
219715_s_at	*TDP1*	7.94	7.81	7.26	7.12	-0.68	0.002669
210543_s_at	*PRKDC*	8.36	8.36	7.78	7.72	-0.61	0.00473
208643_s_at	*XRCC5 (Ku80)*	9.94	10.06	9.31	9.46	-0.61	0.00434
213734_at	*RFC5*	7.64	7.37	6.91	7.03	-0.53	0.014248
212525_s_at	*H2AFX*	6.05	6.17	5.51	5.69	-0.51	0.011937
211851_x_at	*BRCA1*	5.84	5.93	5.39	5.46	-0.46	0.022329
204752_x_at	*PARP2*	7.89	7.95	7.50	7.65	-0.34	0.049
205672_at	*XPA*	7.63	7.54	7.89	7.87	0.29	0.03678
221143_at	*RPA4*	3.79	4.06	4.25	4.26	0.33	0.01878
1053_at	*RFC2*	6.83	6.61	7.05	7.07	0.34	0.049
227766_at	*LIG4*	5.56	5.40	6.11	5.88	0.52	0.025825
202176_at	*ERCC3*	7.84	7.70	8.31	8.30	0.54	0.006878
209903_s_at	*ATR*	8.11	7.93	8.64	8.53	0.57	0.009919
202451_at	*GTF2H1*	8.60	8.55	9.29	9.07	0.61	0.01218
232134_at	*POLS*	6.32	6.00	6.98	6.75	0.71	0.008367
231119_at	*RFC3*	4.31	4.56	4.95	5.35	0.72	0.008497
204023_at	*RFC4*	7.26	7.17	8.04	7.84	0.72	0.00282
222233_s_at	*DCLRE1C*	5.50	5.44	6.41	6.10	0.78	0.00239
213468_at	*ERCC2*	5.82	5.85	6.58	6.64	0.78	0.000828
209805_at	*PMS2*	6.67	6.74	7.56	7.43	0.79	0.000908
209805_at	*PMS2*	6.67	6.74	7.56	7.43	0.79	0.000908
1554743_x_at	*PMS1*	4.32	4.51	5.29	5.16	0.81	0.002444
204838_s_at	*MLH3*	7.13	7.05	7.97	7.86	0.83	0.001711

Genes involved in DNA repair mechanisms, those shown either decreased or increased in expression level (p value ≤ 0.05) after combination treatment with 1 μM Doxorubicin and 20 μM Roscovitine as compared to 1 μM Doxorubicin only, in A549 cells after 24 hours of treatment.

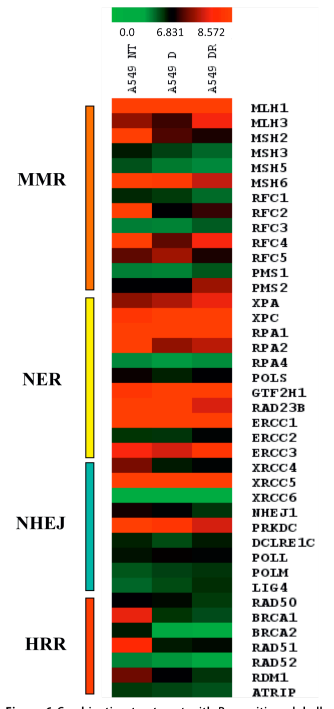

Figure 6 Combination treatment with Roscovitine globally affects DNA repair pathways. Corrected microarray signal values of genes involved in DNA repair clustered by specific DNA repair pathway of A549 cells treated for 24 hours with 1 μM Doxorubicin alone or in combination with 20 μM Roscovitine in comparison to control cells.

more global affect on DNA repair pathways as a result of combination treatment with Roscovitine.

Discussion

Under genotoxic conditions the CDK2/cyclin A1 complex increases its functional kinase activity and the ability to phosphorylate Ku70. In addition, here we demonstrated upon treatment with different DNA damaging agents (doxorubicin or γ-irradiation) a marked dose dependent increase in the RNA and protein levels of cyclin A1, which is independent of cell cycle phase redistribution. Conversely cyclin A2 (whose expression is tightly related to the S and G2-M phases of the cell cycle) is downregulated under genotoxic stress conditions as a result of the check-point activation and

consequent decrease of the S phase fraction. This switch in the respective levels of the A-family cyclins may be functionally relevant to redirect CDK2 activity toward DNA repair, especially given the findings that the ectopic overexpression of cyclin A1 increased *in-vitro* NHEJ activity and that cyclin A1 depletion, as demonstrated by others[27], results in an impaired DNA DSB repair ability.

DNA DSBs are considered the most lethal form of DNA damage and CDK inhibition has been shown to potentially affect the two major DSB repair pathways (HR and NHEJ)[7]. Various mechanisms have been proposed to explain this effect such as the deregulation of the DNA damage-induced checkpoint signalling cascade [13] or the downregulation of specific genes involved [33,34]. Roscovitine is an oral 2,6,9 trisubstituted purine analog currently under phase II investigation, which competes with ATP for the catalytic binding site on CDK2 (but also CDKs 1, 7 and 9 with a much lower affinity) with a demonstrated antitumor activity in many human cancer models and a nice toxicity profile.

One of the most prominent effects of the drug is the inhibition of CDK2/cyclin E complexes, which causes a decrease in Rb phosphorylation and a consequent inactivation of E2F family members, thus leading to cyclin transcriptional downregulation and ultimately to cell cycle arrest. This strong transcriptional depression of most of the cell cycle related cyclins further enforces the drug's inhibitory effect on CDK/cyclin complexes. Furthermore, Roscovitine has been shown to downregulate several other genes involved in a wide spectrum of cellular functions[35,36], probably as a result of partial CDK7/cyclin H and CDK9/cyclin T inhibition[37]. In addition, whole genome ChIP-on-chip analysis recently mapped E2F transcription factor family members to the promoters of many more genes than were traditionally associated with the cell cycle[38], suggesting an alternative mechanism to explain these transcriptional effects.

We investigated the effects that Roscovitine may have on cyclin A1 transcription as one of the possible mechanisms through which CDK2 inhibition may curb DNA DSB repair activity. The promoter of the cyclin A1 gene, *CCNA1* is not E2F-dependent and, consistently, increasing doses of Roscovitine did not repress cyclin A1 basal transcription levels in contrast to cyclins A2, B, D and E. However, we demonstrated that Roscovitine at doses preferentially inhibiting CDK2 but not CDK7 and 9 completely abolished cyclin A1 DNA damage-induced upregulation, thus suggesting that residual CDK2 activity is required for cyclin A1 upregulation. In addition Roscovitine co-administered with doxorubicin was able to largely modify the patterns of cell cycle phase distribution in comparison to doxorubicin only treatment. This resulted in an augmented S phase and consequently in an increased expression of cyclin A2. The combined treatment thus resulted in the complete inversion of the doxorubicin-induced switch between cyclin A1 and cyclin A2.

Roscovitine, alone or under DNA damaging conditions, was able to diminish cyclin A1 protein levels as well. Such transcriptional and post-transcriptional repression was observed in different NSCLC, prostate and breast cancer cell lines and we propose that this potentiates and synergizes the Roscovitine-mediated CDK2 inhibition thus resulting in a significant decrease of cellular NHEJ ability. In fact, we observed that combination treatment led to an increase in DNA DSBs and overall DNA damage over-time, further substantiating, not only the importance of CDK-inhibitors in combination therapy but also the role of CDKs in DNA repair mechanisms. While these findings were supported by genome-wide mircroarray analysis, we also observed a significant effect on key genes involved in other DNA repair pathways.

Conclusions

Roscovitine has shown to be able to significantly modify the DDR response. Even considering the many genes that are potentially involved, the putative role of CDK2 in multiple DDR pathways along with the downregulation of cyclin A1, may further explain the effective inhibition of a broad range of DNA repair mechanisms by Roscovitine. In particular since NHEJ is considered the major pathway for the repair of γIR-induced DNA DSBs in human cells[39], we believe our data support further investigation on the therapeutic advantages of combination therapy with Roscovitine and Radiotherapy.

Methods

Cell Culture and Serum Starvation

The following solid cancer human cell lines were purchased from and authenticated by American Type Culture Collection (ATCC; Manassas, VA) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air, within the appropriate medium according to supplier recommendations supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA) and 100U of Penicillin and 100 μg/ml of Streptomycin (Sigma-Aldrich; St. Louis, MO): NSCLC cell lines A549 and H23, breast cancer cell lines MCF-7 and MDA-MB-231, prostate cancer cell lines LNCAP and DU145, and the adenovirus transformed human embryonic kidney epithelial cells HEK293FT. Cells were regularly sub-cultured according to ATCC recommendations with a 0.25% trypsin-EDTA solution (Sigma). To obtain synchronous populations of cells, confluent plates of A549 cells were incubated in media supplemented with 0.1% (v/v) heat-inactivated fetal bovine serum for

96 hours. Cells were then sub-cultured into serum-containing medium and time points were taken every four hours.

Drugs, irradiations and treatments

Doxorubicin was obtained from BioMol International (Plymouth Meeting, PA). Lyopholized drug was re-suspended into a 1:1 mixture of dimethyl sulfoxide (DMSO; Fisher Scientific; Pittsburgh, PA) and MilliQ filtered H_2O (Millipore; Bellerica, MA) to a concentration of 4.31 mM, aliquoted for use and stored at -20°C. Roscovitine was obtained from Signa Gen Laboratories (Gaithersburg, MD). Lyophilized drug was re-suspended into DMSO to a concentration of 14.1 mM, aliquoted and stored at -20°C until use. Fresh dilutions from the stock solutions were prepared for each treatment. Taxol was obtained from USB Corporation (Cleveland, OH). Lyophilized drug was re-suspended into DMSO to a concentration of 5.86 mM, aliquoted and stored at -20° C until use. MG-132 (Z-Leu-Leu-Leu-al) was obtained from Sigma. Lyophilized drug was re-suspended into DMSO to a concentration of 10 mg/ml, aliquoted and stored at -20°C until use. Irradiations were performed in an AECL Gamma Cell 40, Cs-137 irradiator at a dose rate of 1 Gy/minute for respective doses. In treatments throughout this article the control samples refer to cells treated with an equal concentration (v/v) of DMSO as in the highest drug concentration used per experiment.

Western Blot Analysis and SDS-PAGE

Equal amounts (50-100 μg) of whole cell lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Whatman Inc., Piscataway, NJ) by wet electrophoretic transfer. Non-specific binding sites were blocked for 1 hour at room temperature with 3% non fat dry milk (NFM) in tris-buffered saline containing 0.01% Tween-20 (TBS-T) and probed with the following primary antibodies in 3% NFM in TBS-T overnight at 4° C; rabbit anti-cyclin A1 (sc-15383; Santa Cruz Biotechnology Inc.; Santa Cruz, CA), mouse anti-cyclin A2 (CY-A1; Sigma), mouse anti-cdc2 (A17; Abcam, Cambridge, MA), rabbit anti-CDK2 (sc-163; Santa Cruz), rabbit anti-p53 (sc-6243; Santa Cruz), mouse anti-Hsp70 (sc-24; Santa Cruz), mouse anti-p130/Rb2 full length (610262; BD Biosciences, San Jose, CA), rabbit anti-serine 952 phosphorylated p130/Rb2 (sc-16298; Santa Cruz), rabbit anti-serine-2 phosphorylated RNA polymerase II (A300-654A; Bethyl Laboratories Inc., Montgomery, TX), rabbit anti-serine-5 phosphorylated RNA polymerase II (A300-655A; Bethyl), mouse anti-α-tubulin (sc-58666; Santa Cruz), and mouse anti-ser139 phosphorylated histone γH2AX (Millipore cat. #05636; lot# DAM1567248). Membranes were washed for 15 minutes in TBS-T and then incubated for 1 hour with either goat anti-mouse (31432; Pierce; Rockford, IL) or mouse anti-rabbit (31464; Pierce) horseradish peroxidase conjugated IgG at a dilution of 1:10,000 in 3% NFM in TBS-T. This was followed by 15 minutes of wash in TBS-T and enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. All western blot images included in article are representative of at least three consecutive independent experiments.

Immunostaining

Following respective drug treatments, cells grown directly on sterilized glass coverslips were fixed and permeabilized for 10 minutes in 70% cold methanol (MeOH), immunostained (for cyclin A1 and γH2AX) and analyzed as previously described[40].

Flow cytometry

Cells (1×10^6) were collected, after respective drug treatments, washed, resuspended in 1 ml of PBS and fixed and permeabilized for at least 10 minutes in 70% cold ethanol. After fixation cells were pelleted, washed 3 times with PBS, re-suspended into a primary antibody solution (10 μg/ml antibody diluted in PBS) and incubated on ice for 15 minutes. Cells were then pelleted, washed 3 times with PBS, re-suspended into FITC-conjugated secondary antibody solution (10 μg/ml) and incubated for 15 minutes on ice protected from the light. Cells were washed 3 times in PBS and re-suspended in propidium iodide staining solution, 10 μg/ml propidium iodide (from stock of 0.5 mg/ml in 0.38 mM sodium citrate pH 7.0) and 25 μg/ml DNase-free RNase A (from stock of 10 mg/ml RNase A in 10 mM Tris pH 7.5 and 15 mM NaCl) diluted in PBS. Cells were incubated at 37°C for a minimum of 30 minutes protected from light and analyzed immediately by flow cytometry utilizing an Epics XL-MCL BeckmanCoulter (The Wistar Institute, Philadelphia, PA). Graphs represent average fluorescence intensity or average percentage of cells found in cell cycle phase over three consecutive independent experiments.

Reverse Transcriptase-PCR and Real time (RT-PCR)

Total RNA from cell lines was extracted using the High Pure RNA Isolation Kit (Roche) following the manufacturer's instruction. cDNA was synthesized from 1 μg of total RNA by using random hexamers as primers and moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) according the manufacturer's protocol in a final volume of 20 μl. As a control for genomic contamination a reverse transcription (RT) reaction was carried out without the addition of the reverse transcriptase (RT-). After cDNA synthesis, samples were diluted 1:10 and 4 μl was used in each real

time polymerase chain reaction (real time PCR). cDNA was amplified using species specific intragenic primers for *CCNA1*[23], *CCNA2*, *CCNB1*, *CCND3*, *CCNE1*, *TP53* and *GAPDH* genes (Additional File 4). Real time PCR was carried out utilizing SybrGreen Master Mix (Roche, Basel, Switzerland) following the manufacturer's instructions in a final reaction volume of 10 μl. Reactions were performed on a LightCycler 480 II (Roche Diagnostics, Indianapolis, IN) with an initial denaturation of 5 minutes at 95°C; 45 cycles of 10 seconds at 95°C, 20 seconds at 60°C, and 10 seconds at 72°C where fluorescence was acquired. Each sample was run in triplicate and data was analyzed using the comparative Ct method with GAPDH as the endogenous control and control cells as the reference sample in each experiment. Final data points represent the average fold change respect to control ($2^{\wedge-\Delta\Delta Ct}$) or expression levels respect to GAPDH ($2^{\wedge-\Delta Ct}$) of at least three consecutive independent experiments.

Alkaline Comet Assay

After appropriate drug treatments, cells were harvested and analyzed utilizing the alkaline comet assay as previously described[41,42]. Briefly, cells were mixed in a suspension of low melting point agarose and spread on agarose-coated slides. Once the agarose solidified, slides were incubated in lysis buffer followed by electrophoresis to allow migration of DNA and detection of DNA damage. Cells were then stained with 1 μg/mL ethidium bromide and analyzed using the fluorescence microscope Olympus BX40 (Melville, NY) with a Spot-RT digital camera and software (Webster, NY). At least 200 cells were evaluated per experimental point. Visual scoring of comet images using fluorescence microscopy was performed according to Norbury[43]. Briefly, each nucleus is assigned a score from 0-4 depending on the relative intensity of DNA fluorescence in the tail (0 = no damage, 4 = >80% of DNA found in the tail) and the final score is calculated as the average DNA damage found in all cells counted from three consecutive independent experiments. Statistical analysis was carried out using a standard student's t test.

Transient transfections

The human cyclin A1 IMAGE clone 5172478 (GenBank: BC036346.1) was purchased from ATCC (MGC-34627) transformed into DH5α heat-shock competent *E. coli* cells and grown on LB agar plates or in broth with 100 μg/ml Ampicillin (Fisher) at 37°C. Plasmid DNA was extracted using the Genopure Plasmid Midi Kit (Roche) following manufacturer's instructions then verified by restriction enzyme digestion and gel electrophoresis. HEK293FT cells were transiently transfected using a 6:2 ratio of Fugene HD (Roche) and plasmid DNA (2 μg) following manufacturer's protocol. Enhanced yellow fluorescent protein (pEYFP) plasmid DNA was utilized as a control for transfection efficiency at the same concentration. Cells were analyzed after 36 hours of transfection by western blot and fluorescence microscopy to confirm expression of transfected protein and then utilized in experiments as described.

In vitro NHEJ assay

The *in vitro* NHEJ assay was performed on respectively treated cell lysates as previously described[44] utilizing 120 μg of protein extract and 60 μg of purified BamHI (Roche) digested pCI-neo plasmid DNA (Promega). A reaction including the incubation of 20 μM Wortmannin with whole cellular lysate for 15 minutes on ice before the addition of digested plasmid DNA was included as a negative control for NHEJ activity in each experiment. After incubation samples were diluted 1:10, phenol chloroform 25:24:1 (Fisher) extracted, and ethanol precipitated overnight at 4°C. DNA was resuspended into 20 μl of Tris-EDTA buffer and 1 μl was utilized in each real time PCR reaction. To detect plasmid re-ligation one set of primers amplified an intact region of the plasmid to act as the endogenous control, while a second set of primers bound both up-stream and down-stream of the enzymatic cut site. Samples were run in triplicate with each primer pair following the real-time PCR protocol described above. Final results represent the average fold change ($2^{\wedge-\Delta\Delta Ct}$) in re-ligation respect to control, over three consecutive independent experiments.

Microarray Analysis

Total RNA was isolated by Trizol (Invitrogen). Fifteen μg of total RNA was converted to cDNA by using Superscripts reverse transcriptase (Invitrogen), and T7-oligo-d(T)24 (Geneset) as a primer. Second-strand synthesis was performed using T4 DNA polymerase and E.Coli DNA ligase and them blunt ended by T4 polynucleotide kinase. cDNA was purified by phenol-chloroform extraction using phase lock gels (Brinkmann). Then cDNAs were *in vitro* transcribed for 16 hours at 37°C by using the IVT Labelling Kit (Affymetrix) to produce biotinylated cRNA. Labelled cRNA was isolated by using the RNeasy Mini Kit column (QIAGEN). Purified cRNA was fragmented to 200-300 mer using a fragmentation buffer. The quality of total RNA, cDNA synthesis, cRNA amplification and cRNA fragmentation was monitored by capillary electrophoresis (Bioanalizer 2100, Agilent Technologies). Fifteen micrograms of fragmented cRNA was hybridised for 16 hours at 45°C with constant rotation, using a human oligonucleotide array U133 Plus 2.0 (Genechip, Affymetrix, Santa Clara, CA). After hybridisation, chips were processed by using the Affymetrix GeneChip Fluidic Station 450 (protocol

EukGE-WS2v5_450). Staining was made with streptavidin-conjugated phycoerythrin (SAPE)(Molecular Probes), followed by amplification with a biotinylated anti-streptavidin antibody (Vector Laboratories), and by a second round of SAPE. Chips were scanned using a GeneChip Scanner 3000 G7 (Affymetrix) enabled for High-Resolution Scanning. Images were extracted with the GeneChip Operating Software (Affymetrix GCOS v1.4). Quality control of microarray chips was performed using the AffyQCReport software[45]. A comparable quality between microarrays was demanded for all microarrays within each experiment.

Microarray Statistical Analysis

The background subtraction and normalization of probe set intensities was performed using the method of Robust Multiarray Analysis (RMA) described by Irizarry et al.[46]. To identify differentially expressed genes, gene expression intensity was compared using a moderated t test and a Bayes smoothing approach developed for a low number of replicates[47]. To correct for the effect of multiple testing, the false discovery rate, was estimated from p-values derived from the moderated t test statistics[48]. The analysis was performed using the affylmGUI Graphical User Interface for the limma microarray package[49].

Abbreviations Used

CDK: cyclin-dependent kinase; DDR: DNA damage response; NHEJ: non-homologous end-joining; DSB: double strand break; HR: homologous recombination; NER: nucleotide excision repair; MMR: mismatch repair.

Additional material

Additional file 1: Western blot analysis of cyclin A1 protein expression with and without the inclusion of phosphatase inhibitors in lysis. Phosphatase inhibitor activity was confirmed by probing for phosphorylated p130/Rb2 in comparison to full-length p130/Rb2. After 24 hours of Doxorubicin treatment (750 nM and 2.5 μM), cyclin A1 protein levels clearly augment in cells lysed with the inclusion of phosphatase inhibitors, whereas the increase is not as notable in cells lysed without the inclusion of phosphatase inhibitors.

Additional file 2: Flow cytometry analysis of cell cycle breakdown after treatment. Flow cytometry analysis of cell cycle breakdown in A549 cells treated for 24 hours with respective treatments of Doxorubicin (750 nM or 2.5 μM) or 20 μM Roscovitine alone or in combination and graph representing average cell cycle distributions from three consecutive independent experiments.

Additional file 3: Drug induced changed in cyclin mRNA expression levels. Expression levels respect to GAPDH ($2^{\wedge-\Delta Ct}$), in mRNA of cyclin A1, cyclin A2, cyclin B, cyclin D and cyclin E after 24 hours of treatment with either increasing doses of Doxorubicin (250 nM to 5 μM) alone or in combination with 20 μM Roscovitine.

Additional file 4: Table of gene specific primer sequences utilized in this manuscript.

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Authors' contributions

MF and CES designed experiments, performed the research, analyzed the data and wrote the paper. DF performed microarray experiments and analysis. FR performed experiments and analyzed the data. LB, AR and AG designed experiments and wrote the paper. All authors critically reviewed and edited the paper.

Competing interests

The authors declare that they have no competing interests.

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Palliative splenic irradiation in primary and post PV/ET myelofibrosis: outcomes and toxicity of three radiation schedules

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Abstract

Splenectomy and Splenic Irradiation (SI) are the sole treatment modalities to control drug resistant splenomegaly in patients with Myelofibrosis (MF). SI has been used in poor surgical candidates but optimal total dose and fractionation are unclear. We retrospectively reviewed 14 MF patients with symptomatic splenomegaly. Patients received a median of 10 fractions in two weeks. Fraction size ranged from 0.2-1.4 Gy, and total dose varied from 2-10.8 Gy per RT course. Overall results indicate that 81.8% of radiation courses achieved a significant spleen reduction. Splenic pain relief and gastrointestinal symptoms reduction were obtained in 94% and 91% of courses respectively. Severe cytopenias occurred in 13% of radiation courses. Furthermore patients were divided in three groups according the radiation dose they received: 6 patients in the low dose group (LDG) received a normalized dose of 1.67 Gy; 4 patients in the intermediate dose group (IDG) received a normalized dose 4.37 Gy; the remaining 4 patients in the high dose group (HDG) received a normalized dose of 9.2 Gy. Subgroup analysis showed that if no differences in terms of treatment's efficacy were seen among dose groups, hematological toxicity rates distributed differently. Severe cytopenias occurred in 50% of courses in the HDG and in the 14.3% and in 0% of the IDG and LDG respectively. Spleen reduction and pain relief lasted for a median of 5.5 months in all groups. Due to the efficacy and tolerability of the low dose irradiation 4 patients from the LDG and IDG were retreated and received on the whole 12 RT courses. Multiple retreatments did not show decremental trends in terms of rates of response to radiation nor in terms of duration of clinical response. Moreover, retreatment courses did not cause an increased rate of adverse effects and none of the retreated patients experienced severe hematological toxicities. The average time of clinical benefit in retreated patients was extremely longer (21 months, range 44-10) than in comparison to patients who were not retreated (5,75 months, range 3-6).

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Key words: myelofibrosis, splenomegaly, palliation, radiotherapy, low dose irradiation.

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Introduction

Primary myelofibrosis[1] (PM) is a Philadelphia negative chronic myeloid disorder (CMD) currently classified with polycythemia vera (PV) and essential thrombocytemia (ET) as a chronic myeloproliferative diseases[2] (MPDs). PM is a rare disease mainly affecting older people[3] with a median survival of 3.5-5 years.[4] The pathogenetic mechanism is not clearly understood but probably relates to a clonal stem-cell disorder that leads to ineffective erythropoiesis, dysplastic megakaryocyte hyperplasia and an increased ratio of immature to total granulocytes.[5] These findings are characteristically accompanied by reactive bone marrow (BM) fibrosis that develops and is mediated by megakaryocyte-derived fibrogenic cytokines.[6]

Collagen fibrosis, presumably along with many other factors, interferes with normal hematopoietic processes, ultimately leading to erythroid hypoplasia.[7,8] Due to BM fibrosis, in MF patients as well as those with post ET/PV MF, extramedullary hematopoietic starts in the spleen or in multiple organs as an attempt to override BM failure, often leading to the development of splenomegaly or hepatosplenomegaly. Moreover splenomegaly exacerbates cytopenias through the sequestration and destruction of hematopoietic elements.[9]

Progressive high-grade splenomegaly occurs in the majority of MF patients. Unfortunately the standard current pharmacologic therapeutic options, due to their short periods of response, fail to control organomegaly and organomegaly-associated symptoms (abdominal pain and early satiety, weight loss, portal hypertension and profound fatigue), which account for much of the patient's discomfort. Also if a new generation of "target drugs" are currently under intense investigation with some encouraging results splenomegaly control still remains a crucial step for patients' quality of life improvement.

To date, splenectomy or splenic irradiation (SI) are the sole treatment modalities to control drug resistant splenomegaly in MF patients. When technically achievable splenectomy is currently the preferred treatment modality for MF based upon good, long-lasting outcome in term of organomegaly-related symptoms palliation.[9,10] Unfortunately, it is consistently associated with a significant rate of mortality as well as intra- and peri-operative complications.[9,10] SI, instead, has been generally preferred in patients not undergoing surgery due to a poor general status or decline and allows for a good but transitory splenomegaly palliation. In fact, the major shortcoming of radiation is that its palliative effect on splenomegaly generally does not last longer than 6 months.

There is a general agreement that emerges from the literature to use RT at dose levels lower than in other hematological malignancies, however, few studies have taken a retrospective look at SI.[11] The indication for SI is still controversial[12] and there is not a precise univocal definition of the optimal total dose and fractionation, mainly due to the limited number of patients included in existing studies and the wide range of radiation schedules adopted. Moreover, it is unclear if re-irradiation of MF patients is a safe strategy to extend the overall time of clinical benefit that a single SI course allows. Here we aim to assess outcomes and complication rates of splenic irradi-

ation in three cohorts of patients treated with different "low dose" irradiation schedules.

Patients and Methods

After approval from our institutional research review committee, we retrospectively reviewed data concerning 15 patients (10 male, 5 female, median age at diagnosis 61 years, median age at first irradiation 67 years), 11 with a histologically proven diagnosis of PM and 4 with a post ET- MF complaining of a high grade symptomatic splenomegaly that were consecutively referred to our institution from 1997-2007 (Table 1). All patients had a drug resistant, splenomegaly and lacked any further treatment options. Before being admitted to radiation, patients had previously been judged unfit for surgery due to their general status or had refused splenectomy. Fourteen out of fifteen underwent splenic irradiation and one was excluded due to preexisting advanced heart failure (patient 14). In the 14 irradiated patients the first course of radiation occurred at a median of 58 months from the diagnosis of MF. All fourteen treated patients had a severe splenomegaly with splenic pain, abdominal discomfort, and weight loss; 11 patients (84%) had in concurrence constitutional symptoms such as night sweats, low-grade fever and an initial state of cachexya. All except 3 required red blood cell transfusions (≥2 units per month).

Patients were scored (at the time of their first irradiation) on the basis of Dupriez's prognostic parameters[13] (Hb levels <10 g/dL and WBC <4 or >30×10³/μL) in three categories: high, intermediate and low risk. Four patients belonged to the high-risk, 4 to the low-risk and 6 to the intermediate-risk groups respectively. All patients had already undergone a cytoreductive pharmacological treatment: 8 received hydroxyurea as a single modality treatment, 1 received hydroxyurea plus Ara-C, 2 patients received hydroxyurea plus 6-mercaptopurine, 1 received hydroxyurea and melphalan, 1 received busulphan and 3 patients were given Thalidomide in association with conventional cytoreductive treatments. Radiation treatment was delivered by a Siemens 15 MV Linac with multi leaf collimator; all patients had a CT scan simulation (slice thickness 10 mm) in the supine position. The treatment planning system (Plato system v 2.6.3.) was used and no patient's immobilization devices were adopted during the simulation and treatment.

Two portal arrangements were alternatively used to encompass the entire spleen volume: antero-posterior (AP-PA), opposed parallel or opposed tangential in the attempt to reduce the dose to the left kidney. If the left kidney was displaced posteriorly, a tangential arrangement was provided; if the kidney was displaced medially an antero-posterior approach was arranged. In the plan evaluation process between target coverage and kidney sparing we assigned priority to left kidney sparing in order to reduce the total dose to the organ in case of multiple courses of splenic irradiation.

Since our institutional standards of radiation for MF have changed during the past ten years, patients received different total doses and dose per fraction. To compare the various RT treatments we used the Normalized Tumor Dose[14] (NTD10), defined as the total dose delivered in 2 Gy fractions that corresponds to a particular biologically effective dose level and calculated according to the formula:

$$NTD_{10} = nd\left(\frac{1+\frac{d}{\alpha/\beta}}{1+\frac{2}{\alpha/\beta}}\right)$$

where n is the number of RT fractions and d the fraction size in Gy. The α/β value of the Linear Quadratic Model[15] was empirically fixed to 10 as for early responding tissues. By standardizing the delivered dose of all 22 administered treatments into a 2 Gy isoeffective treatment, we were able to make a correct radiobiological comparison among different RT schedules. On the basis of the NTD values, patients were divided into three different groups but it should be underlined that RT schedules were not chosen on the basis of patients' clinical parameters but rather were dependent on the progressive modification of our institutional treatment philosophy.

The initial patients, who had received a total dose of 10 Gy with a dose per fraction in the order of 1 Gy, were designated as our high dose group (HDG). Patients who had received our current standard of treatment (0.2 Gy fraction up to a total dose of 2 Gy in 10 fractions) were designated as our low dose group (LDG). Whereas the intermediate dose group (IDG)

Table 1. Patients' characteristics at the time of first irradiation.

Patient no.	Age at irradiation and sex	Interval diagnosis irradiation (intent to treat)	Dupriez score	Previous treatments	Symptoms at time of radiation	RBC transfusion U/month	Pre irradiation WBC ×10³/mL	Pre irradiation PLT ×10³/mL
1*	53 F	14 y.	HR	HU	S , P	2U	39.9	232
2*	65 F	16 y.	IR	HU	S , P, CS	4U	6.48	381
3	62 F	3 y.	LR	HU; MPH	S , P	2U	9.55	21
4*	75 F	8 y.	LR	HU	S , P, CS	2U	8.36	44
5	67 M	4 y.	IR	HU	S , P, CS	2U	5.7	210
6	67 M	8 y.	IR	HU; Ara-C	S , P	NT	38.1	190
7	77 M	8 y.	LR	HU; 6-MP	S , P, CS	2U	10.3	423
8	87 M	1 y.	HR	B; Th	S , P, CS	2U	2.63	119
9	46 M	4 y.	IR	HU	S , P, CS	NT	29.6	307
10	67 M	1 y.	IR	HU	S , P, CS	2U	4.9	121
11	70 M	2 y.	HR	HU	S , P, CS	2U	89	143
12	58 M	7 y.	LR	HU; Th	S , P, night sweats	NT	10.89	329
13*	65 M	2 y.	IR	HU; 6-MP; Th	S , P, CS	2U	8	673
14	76 M	4 y.	HR	HU	S , P, CS	4U	1.04	67
15	55 F	4 y.	HR	HU; Th	S , P, CS	2U	42.51	143

*S: Splenomegaly; P: splenic pain; CS: constitutional symptom; HU: Hydroxyurea; Th: Thalidomide; 6-MP: 6-Mercaptopurine; ARA-C: Arabinosylcytosine; MPH: Melphalan; B: Busulphan; HR: High risk; IR: Intermediate risk; LR: Low risk; NT:Not transfused.*Post ET - MF.*

reflected the transition or better our "dose finding effort" toward lower doses with the aim to reduce treatment related toxicities (Supplemental Table 1). The IDG encompasses patients who had received a wide range of treatment with radiobiological characteristics, which, in some cases, may partially overlap with the LDG. However we decided to aggregate our patients in this way in order to obtain homogeneity in the low and in the high dose groups.

Patients in which three or more of the following criteria were present were considered responsive to treatment: subjective absence of MF-related gastrointestinal symptoms (bulky effect), absence of splenic pain, consistent reduction of the spleen volume (not less than 50% of the initial size) assessed by clinical examination (according the formula: spleen volume = $4/3\pi$ 1° diameter · 2° diameter · 3° diameter) and, finally, reduction of the RBC transfusion units required per month.

To evaluate toxicity and response to treatment, patients had undergone clinical examination and blood cell count twice a week during the period of irradiation and for the following 2 weeks. If no toxicity occurred, blood tests were scheduled weekly for the following month. The evaluation of the spleen reduction was carried out 20 days after patients had completed radiation.

Treatment related toxicity was limited to myelosuppression and was measured on the basis of RTOG acute toxicity scoring criteria. An RT course after which had developed a post radiation grade 4 (WBC count $\leq 1\times10^3/\mu L$ and/or PLTs count $\leq$20×103/μL) acute cytopenia was considered too toxic. RBC count was excluded from toxicity scoring because almost all patients were already heavily transfused long before receiving RT. Due to the small size of the study cohort and the inhomogeneities in patients' characteristic due to the accrual criteria we did not perform any statistical data analysis, as it would not be statistically representative or pertinent.

Results

Total delivered dose per RT course ranged from 2 to 10.8 Gy, the dose per fraction varied from 0.2-1.4 Gy. RT courses were generally administered over a two week period (median number of fraction per RT course was 10), patients received RT five days per week continuously; four patients had multiple courses of RT, and one patient received 4 courses. In the first group (low dose group, LDG), 6 patients received a median NTD of 1.67 Gy (0.6 standard deviation). In the second (intermediate dose group, IDG), 4 patients had a median NTD of 4.37 Gy (1.89 standard deviation). The third group (high dose group, HDG) contained 4 patients who received a normalized median dose of 9.2 Gy (0.46 standard deviation).

According to the above-defined criteria, 12 patients were considered responsive. Overall

response rates after all 22 RT treatments indicate that 81.8 % of courses achieved a significant spleen size reduction; however, better results were achieved on splenic pain relief (94.45% of RT courses) and reduction of gastrointestinal symptoms (91% of courses). No significant difference in terms of spleen size reduction and splenic pain relief emerged after subgroup analysis. Patients in the LDG had spleen size reduction and splenic pain relief in 91% and 100% of courses respectively, while in the IDG and in the HDG, 76.5% and 75% of courses obtained a spleen size reduction. Pain relief was achieved in 86% and 100% respectively (Table 2).

After completing radiation all responsive patients had an improvement in their body weight while SI was less effective in reducing patients' transfusion requirements. In only 35.3% (6/17) of courses there was a slight improvement of anemic state, but this was transient and shorter than spleen size reduction and pain relief.

Within the entire study population, grade 4 RTOG life-threatening cytopenias occurred in 21.5 % of patients (3/14) or 13.6% of RT courses. In all cases it developed in the first week after completing radiation and required hospitalization. Interestingly, RT complications distributed differently among groups. In the LDG, no grade 4 RTOG adverse effects occurred. Patients in the IDG experienced 14.3% of RT courses followed by severe cytopenias (1/ 7 courses), while in the HDG, 50% of RT treatments were too toxic (Supplemental Figure 1). Both non-responding patients (patients 9 and 8) experienced severe acute complications. One (patient 8) appeared to rescue from cytopenia but 3 months later developed a leukemic transformation that lead to death. The second patient (patient 9), complained of a massive splenomegaly, did not respond to SI and underwent splenectomy 12 months after RT. One month after splenectomy the patient died as a result of sepsis.

The median time of symptom relief after a single RT course was 5.5 months and no differences were found among dosage groups. According to the patients' general conditions, the cumulative RT dose delivered and the rate of spleen shrinkage in response to previous irradiation, retreatment after splenic relapse was considered in 4 patients. The four retreated patients received on the whole 12 RT courses and one patient received 4 courses without any acute toxicity. Two of the retreated patients belonged to the LDG and the remaining two to the IDG. However, it is important to note that of the patients retreated from the IDG, one received treatments of 0.3 up to 3 Gy

Table 2. Splenic irradiation results (by NTD group).

	Number of patients		Median	Number	Median Dose	NTD10		Response			
	PM	Post ET MF	dose delivered	of courses	per fraction	m	sd	% of courses with reduction in spleen size	% of courses with pain relief	Median duration of response (In months)	Hematol toxicity Gradxe 4 RTOG
LDR	3	3	200 cGy (2-4 Gy range)	11	20 cGy	1.67 Gy	0,60	91%	100%	6 months	0% (range 3-12)
HDR	4	0	1000 cGy range (980-1080cGy)	4	110 cGy	9.20 Gy	0.46	75%	100%	4 months (range 6-0)	50% (2/4)
IDR	3	1	500 cGy Range	7	50 cGy	4.37 Gy	1.89	76,5%	86%	5 months (range 6-0)	14,3% (1/7)
							(300-800 cGy)				

Low dose group (LDG); high dose group (HDG); intermediate dose group (IDG); median (m) standard deviation (sd).

in 10 fractions total, which could be considered radiobiologically partially overlapping with the treatment dosages of the LDG.

In comparison to the first irradiation, multiple retreatments did not show decremental trends in terms of rates of response to radiation nor in terms of duration of clinical response. Even in the case of one patient, who received 4 RT courses, there was no change in the duration of symptoms' palliation. Moreover, after retreatment courses we did not observe an increased rate of adverse effects and none of the retreated patients experienced severe hematological toxicities. The average time of clinical benefit (Supplemental Figure 2) in retreated patients was extremely longer (21 months, range 44-10) than in comparison to patients who were not retreated (5,75 months, range 3-6).

Discussion

Splenomegaly rapidly occurs in all MF patients and is one of the causes of major discomfort. Curative treatments are to-date still limited in MF. Allogenic bone marrow transplantation (allo-BMT) has shown promising results in younger patients but its role in elderly patients is controversial. In particular, several studies suggest that in individuals older than 45 the treatment's risk-related mortality outweigh the benefits.[16] On the contrary, other studies, more recently, explored the use of allo-BMT also in patients older than 60 with some interesting results.[36,37] Actually, BMT in the elderly is still a matter of debate since the number of patients accrued in clinical trials is limited and the follow-up time short. It derives that since MF remains a disease of the elderly, standard and palliative treatments to manage cytopenias and massive organomegaly still retain a relevant role in a consistent proportion of patients.

Splenomegaly can be effectively controlled by conventional cytotoxic chemotherapy[39] until patients become drug resistant. More recently atiangiogenic drugs and target drugs are expected to offer a new chance of treatment for all patients. In particular a new class of molecules designed to inhibit Jak have been tested in different phase II trials with positive results.[38] Jak inhibitors have shown a significant activity on splenomegaly but there is no reason to think that, along with their use, also resistant patients will be selected.

After massive splenomegaly is established, splenectomy is considered the principle palliative measure because it offers a lengthy relief of symptoms. Unfortunately splenectomy is weighted by significant morbidity and mortality rates. The two largest single institution series from Barosi[10] and Tefferi[9] reported a mortality rate of 8.4% and 9% respectively, with the latter increasing to 26% when the three-month post splenectomy period was considered, and a morbidity rate of 39.3% and 31% respectively. After splenectomy, up to 25% of patients may experience accelerated hepatomegaly and extreme thrombocytosis.[17] Moreover splenectomy has been also correlated to a significantly higher incidence of blast transformation.

A large Italian study demonstrated a crude transformation rate in splenectomized patients of 26.4% in comparison to 11.9% in non-splenectomized patients with the cumulative actuarial transformation rate of 55% in splenectomized *vs.* 27% in non splenectomized patients at 12 years after diagnosis. The overall relative risk of blast transformation was therefore 2.61 times higher among splenectomized patients.[18] In conclusion, despite the impact on symptoms, no overall survival benefit has been demonstrated after splenectomy[9,19] on the contrary, this procedure is associated with a substantial risk of operative mortality, early and late morbidity and is contraindicated in patients with thrombocytosis. Furthermore splenectomy has been shown to be a predictor of treatment failure in case of allo-BMT16. Alternative treatments to manage splenomegaly, with lower morbidity and mortality rates, would offer a significant improvement in the clinical management of MF patients.

Radiotherapy has been used in selected situations to control extramedullary hematopoiesis, as in spinal localizations,[20] in pulmonary hypertension[21] or in symptomatic hepatomegaly[22] with promising results. However, its role in splenic palliation remains controversial because of the lack of robust data (Table 3). It has been shown that splenic irradiation can be very effective in reducing spleen size and splenic pain with response rates comparable to splenectomy.[23] The major shortcoming of radiotherapy is the reliance on its transient effect that normally does not exceed 6 months.

As an alternative to splenectomy, SI has been considered in poor surgical candidates or in patients who declined surgery. In these patients, that generally are in worse condition compared to those that undergo surgery, palliative splenic irradiation have shown mortality rates that are comparable to splenectomy.[24] On the other hand an high rate of severe life-threatening cytopenias has been reported in patients that underwent splenic irradiation, ranging from 32% (16/50 courses) of the Mayo Clinic series[24] where lower doses of RT were used (median dose per course 2.775 Gy) to 35% (6/17 courses) of a French series25 where a more aggressive treatment was delivered (median dose per course 9.8 Gy).

Although a general trend in favor of low doses is emerging in the literature, the wide variability of total radiation doses, the different number of fractions as well as the different schedule of irradiation reported makes it difficult to define a standard of treatment.[23]

In order to be able to make dose-effect comparisons the major drawback of some of the published series is that the total dose and the fractionation scheme seem not to be decided up-front the treatment but modified during the irradiation on the basis of the single patient response with a consequent high variability in the total dose, fractionation and overall treatment time. Some authors[25] used the common 5 daily fractions per week schedule but increased the fraction size during the radiation course (from 0.4-0.5 Gy/fraction in the first week of treatment, up to 0.8-1 Gy/fraction during the following weeks) until the palliative effect or toxicity is reached. Other authors[35] give radiation 2-3 times per week with an altered time factor. Both such approaches can be empirically effective but generate data that are difficult to compare with the common radiobiology algorithms that are based on larger daily fraction sizes (around 2 Gy) and with a time of inter-course sub-lethal DNA damage repair of 24 hours between fractions. Given that it is hard to make radiobiological comparisons among some published series, it is clear that, still now, the most critical issue regarding a rational use of RT is the definition of an optimal total dose and fractionation.

The leading idea of our approach to SI has been to adopt a relative long fractionation schedule of 10 fractions in two weeks independent of the total dose delivered with the intent to generate comparable results also in case of treatments differing in total dose and dose per fraction. This approach should also minimize the incidence rate of post-attinic severe cytopenias and favor a rapid recovery of early blood precursors from RT. In fact, since a strong dose-sparing effect of fractionation on bone marrow precursors[26,27] has been proven, we believed that it would be meaningful to also apply this concept to extramedullary hematopoiesis sites. Therefore, we decided to utilize a long RT schedule (median 10 fractions) even when it could appear unjustified to do so due to the minimal total dose delivered.

Regarding the total dose, at the beginning of our experience, we adopted an aggressive RT regimen (1 Gy per fraction up to a total dose of 10 Gy) but we observed a high incidence of severe side effects. This raised the concern that the same stem clonal disorder that underlies MF could make hematopoietic precursors more sensitive to radiation. In order to reduce the incidence of acute cytopenias we progressively reduced total RT doses until we established our actual standard of care (0.2 Gy per fraction up to 2 Gy total dose).

Our findings show that extremely low dose

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Table 3. Synoptic table of published data on palliative SI in myelofibrosis.

Author	Number of patients MF	Post PV/ET	Median dose delivered	# of RT courses	Median dose per fraction	Estimated NTD10 Median	Dev standard	% of courses with reduction in spleen size	Response % of courses with pain relief	Median duration of response (In months)
Elliot[24]	18	5	277.5cGy range (30-1365 Gy)	50	50 cGy	3,162 Gy	2,784	94%	96%	6 range (1-41)
Greenberger[32]	13	1	650 cGy range (40-1728 cGy)	21	57.14 cGy	5,807 Gy	3,204	95%	100%	NV range (1-73)
Parmentier[33]	5	4	690 cGy range (180-2900 cGy)	12	25cGy range (12,5-75)	5,845 Gy	6,451	92%	NA	NA
Wagner[35]	6	0	NA From 200-450 cGy in 25-50 cGy fraction 3 times per week	NA	NA	NA	NA	80%	63%	NA
Bouabdallah[25]	15	0	980cGy (60-3050 cGy)	17	Daily fr. 40-100 cGy median duration 22 days	NA	NA	81%	90%	Spleen size reduction 6 months range (1-24 months) Splenic pain 7 months Range (1-19 months)
Mc Farland[34]	4	2	range 300-600 cGy	13	Irradiation twice wk: 1stw50cGy 2ndw75cGy 3rdw100cGy	NA	NA	92%	NA	MF: 1-16 months Post PV/MF: 2-12 months
Present study LDG	3	3	200 cGy range (200-400 cGy)	11	20 cGy	1.67 Gy	0.603	91%	100%	6 months (range 3-12)
HDG	4	0	1000 cGy range (980- 1080 cGy)	4	110 cGy	9.205 Gy	0.465	75%	100%	4 months (range 6-0)
IDG	3	1	500 cGy range (300-800 cGy)	7	50 cGy	4.375 Gy	1.892	76,5%	86%	5 months (range 6-0)

NA: not assessable.

treatments are isoeffective as compared to higher dose regimens in effectively reducing splenomegaly. Unfortunately we cannot explain the functionality of low dose treatment regimens in being so effective as compared to high dose treatments; however, these findings are in concordance with the hypothesis of low dose hypersensitivity.[28] The suggestive issue of radiobiology has been intensely investigated *in vitro*[29] and postulates a hypersensitivity state of cells when irradiated at low doses (<0.4-0.5 Gy). Recently, there have been several indirect confirmations of this theory in clinical studies, linking low dose hypersensitivity to tumor regression[30] as well as to the occurrence of adverse effects,[31] at dose levels under the threshold generally accepted for toxicity or tumor control.

Since in our series, as well as in others reported,[24,25,32,33] there is an inherent discrepancy due to variability in total dose delivered, fraction number, and fraction size, to be able to correctly compare different treatments we used the NTD formula, a radiobiologic tool commonly used in the clinic to evaluate the biologic effectiveness of modified RT fractionations. The overall NTD10 of all 22 RT courses in our series is 2.59 Gy, a value comparable with the median NTD10 estimated from the Mayo series (3.16 Gy). Interestingly our patients seem to have a lower overall incidence rate of grade 4 RTOG (13.6% of courses vs. 32%). This discrepancy is somehow difficult to be explained since there are just slight differences in the normalized radiation dose that patients of the two groups received. Even a slighter difference in terms of patient characteristics can be found between the Mayo Clinic series and ours (median age at the time of the first irradiation 65 *vs.* 67 years; time intercurring between diagnosis and irradiation 44 vs. 58 months respectively). A possible explanation could be that in the definition of toxicity criteria, differing from the Mayo report, we did not consider hemoglobin levels since the majority of our patients were transfused from long time before receiving radiotherapy. Another possible explanation could rely on the medical treatment that patients received before undergoing radiation: in fact it is interesting to note that the only two patients in both series that received melphalan as medical treatment before radiation later experienced severe post-attinic cytopenias.

To compare outcomes after different radiation doses we stratified our patients into three groups according to the NTD_{10} value they

received. We found that, if no differences in terms of spleen shrinkage or pain relief emerged among patients who underwent different RT regimens, daily fractions of 0.2 Gy up to 2 Gy is significantly the safer fractionation scheme since it is not associated to grade 4 hematological toxicities. In our patients, independently from the dose, radiotherapy was very effective in reducing massive splenomegaly, but did not resolve completely the spleen enlargement (Supplemental Table). It is possible to argue that, since we found a safe RT schedule, it would be meaningful to prolong the radiation treatment until a complete splenomegaly remission. On the contrary we decided to maintain a conservative approach and to stop the treatment once the planned final dose was achieved. Two main considerations led to our decision: first of all the fact that the palliative effect of Radiotherapy seems to last no longer than 6 months independently from the dose delivered. We were concerned that reducing the spleen size until normalization could result in a small increase of the time free from symptoms at the cost of a probably higher incidence of severe cytopenias. Secondly, since the aim of our treatment was strictly palliative, we considered it meaningful, once symptom relief was achieved, to stop the treatment with the intent to minimize the patient's absorbed dose per course of RT in order to potentially be able to repeat the treatment in the future.

In fact, because of the low incidence of mild adverse effects in the LDG (and in the lower dose burden of the IDG) we were able to repeat the irradiation several times thus prolonging the clinical benefit much more than expected.

Four patients safely underwent 12 RT courses with no occurrence of grade 4 RTOG hematological toxicity. All the retreated patients belong to the low or intermediate risk group. In these patients the intensity and the persistence of splenic response to irradiation did not change under multiple retreatment courses. However, retreatment increased the average time of symptoms relief four fold longer than in un-retreated patients (21 months *vs.* 5.75). It could be argued that a possible bias in our work is that all the post ET-MF patients were allocate in the LDG or in the inferior burden of other IDG but this fact could not modify the consistency of the presented data; especially because just one patient with post ET-MF have been retreated so far. We propose that, the results regarding the average time of clinical benefit in retreated patients can be considered substantially valid for primary MF patients. Furthermore, it deserves to be mentioned that, in comparison to MF patients, a shorter interval free from symptoms has been reported after SI[24] in post-ET MF patients.

With all the limitations inherent to the small number of patients examined, we found that in our series Dupriez's score (calculated at the time of patient's referral to the radiotherapy department) is not predictive of response to palliative radiotherapy or occurrence of toxicity. We conclude that our actual standard of 2 Gy delivered in 10 fractions over two weeks has a NTD of 1.67 Gy, a value two- to three-fold lower than other published series. This schedule of treatment has shown to be extremely well tolerated and to date in our experience is not associated with severe hematological toxicities. Such optimal treatment compliance encouraged repeating irradiation in responsive patient and this favored a drastic increase of the average time of clinical benefit.

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Review Article

Histone Deacetylase Inhibitors in the Treatment of Hematological Malignancies and Solid Tumors

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The human genome is epigenetically organized through a series of modifications to the histone proteins that interact with the DNA. In cancer, many of the proteins that regulate these modifications can be altered in both function and expression. One example of this is the family of histone deacetylases (HDACs), which as their name implies remove acetyl groups from the histone proteins, allowing for more condensed nucleosomal structure. HDACs have increased expression in cancer and are also believed to promote carcinogenesis through the acetylation and interaction with key transcriptional regulators. Given this, small molecule histone deacetylases inhibitors have been identified and developed, which not only inhibit HDACs, but can also lead to growth arrest, differentiation, and/or apoptosis in tumors both *in vitro* and *in vivo*. Here, we will discuss some of the recent developments in clinical trials utilizing HDACs inhibitors for the treatment of both hematological malignancies as well as solid tumors.

1. Introduction

DNA is woven together with proteins into an intricate organization of both extended euchromatin and condensed heterochromatin. The posttranslational modifications of the histone proteins involved in this structure regulate the epigenetic organization of the genome. This genomic organization is often altered on an epigenetic level, including the phosphorylation, acetylation, methylation, ubiquitination, sumoylation, and ADP-ribosylation of the eight histones within the nucleosome (H2A, H2B, H3, and H4).

In 1964, Mirsky and Allfrey published the first reports of histone acetylation and methylation being involved in RNA synthesis in a reversible fashion and being highly associated with open chromatin [1, 2]. Today, it is known that histone acetyltransferases transfer the acetyl group from acetyl-CoA forming ε-N-acetyl lysine on conserved lysines of the N-terminal tails of histones H3 and H4 (and to a lesser extent H2A and H2B), resulting in an open nucleosomal structure. This can be reversed by histone deacetylases (HDACs) of which, in mammals, there are currently 18 identified and have been divided into four classes based on cellular localization and function [3]. Class I includes HDACs 1, 2, 3, and 8 which are all nuclear and ubiquitously expressed. Class II, being able to shuttle back and forth between the nucleus and the cytoplasm and believed to be tissue restricted, includes HDACs 4, 5, 6, 7, 9, and 10; within this class, HDACs 6 and 10 (class IIb) have two catalytic sites, are expressed only in the cytoplasm, and are involved in a variety of biological processes. Class III contains the structurally diverse NAD^+-dependent sirtuin family, which does not act primarily on histones [4]. Finally, the ubiquitously expressed HDAC11 represents Class IV, which has previously been characterized as being part of both Class I and Class II (Figure 1). Nonhistone targets of HDACs include p53, E2F, GATA-1, YY1, RelA, Mad-Max, c-Myc, NF-κB, HIF-1α, Ku70, α-tubulin, STAT3, Hsp90, TFIIE, TFIIF, and hormone receptors explaining the diverse biological

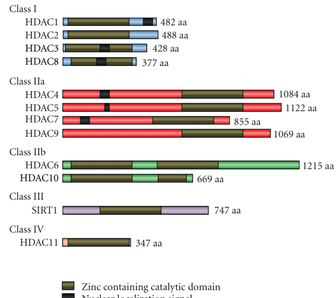

FIGURE 1: The histone deacetylase, family. Schematic representations of class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, and 10), class III (SIRT1), and class IV (HDAC11). Structure and Length of HDACs are shown. The total number of amino acid residues (aa) is depicted on the right, next to each HDAC. The enzymatic domains and the nucleus localization sequences are highlighted in brown and black, respectively.

effects that HDACs can impart to the cell ([5–17] for review, see [18, 19]).

Knockout mice for HDACs 1 and 2 display embryonic or perinatal lethality and class II HDACs knockouts, while viable and fertile (except for HDAC7) have significant developmental abnormalities [20–22]. HDACs expression, and activity can be altered in many cancers and in both lymphoma and leukemia HDACs is associated with the function of oncogenic-translocation products, such as PML-RARα in acute promyelocytic leukemia [23–25]. Furthermore, with the discovery of specific pan-HDACs inhibitors, it has been shown that blocking HDACs function can cause cell-cycle arrest and differentiation through the increased expression of p21$^{WAF1/CIP1}$ [26, 27], affect tumor survival by blocking angiogenesis through the increased acetylation of HIF-1α [9], affect protein degradation through the acetylation of Hsp90 [13], and increase the expression of pro-apoptotic factors [28–31], making HDACs inhibitors a good candidate for single-agent cancer therapy and even combination therapy with conventional chemotherapeutics and radiation. Here, we will discuss the latest clinical advances in HDACs inhibitors.

2. HDACs Inhibitor Classifications

Riggs and colleagues identified the HDACs inhibitor prototype sodium butyrate to be an effective inhibitor of deacetylase activity [32, 33]. This was found to be noncompetitive, reversible and specific for HDACs activity [34–36]. Sodium butyrate was also found to induce differentiation, RNA synthesis and strongly inhibit cell growth in the G1 phase of the cell cycle [37]. These findings paved the road for development of more specific and effective HDACs inhibitors to use in the clinic. HDACs inhibitors can be divided into four major structural classes: (1) small molecular weight carboxylates; (2) hydroxamic acids; (3) benzamides; and (4) cyclic peptides [19, 38, 39]. Pan-HDACs inhibitors include vorinostat, panobinostat, belinostat and isotype/class-specific HDACs inhibitors include romidepsin, mecetinostat (MGCD0103) and entinostat [39]. Vorinostat (Zolinza) and Romidepsin (Istodax) are the only HDACs inhibitors currently approved by the U.S. Food and Drug Adminitration (FDA) for the treatment of refractory cutaneous T-cell lymphoma (CTCL) [40, 41].

All HDACs inhibitors available or in development target the zinc molecule found in the active site of Class I, II, and IV HDACs and are characterized by their ability to inhibit the proliferation of transformed cells in culture and tumor growth in animal models by inducing cell-cycle arrest, differentiation, and/or apoptosis (Figure 2). It has been shown that HDACs inhibitors can selectively induce the expression of less than 10% of genes, some of which are involved in the inhibition of tumor growth (e.g., p21^{WAF1}, p27^{Kip} and p16^{ink4a}) [19, 26, 38]. Furthermore, evidence shows that more genes may be repressed after HDACs inhibitors treatment than activated, this could be due to a chromatin conformation in a hyperacetylated state that represses transcription, the release of transcriptional repressors from HDACs protein complexes, the activation or inactivation of nonhistone transcriptional repressors and many other plausible explanations. Unfortunately, the mechanism of action is not completely elucidated, and there are also no substantiated HDAC or HAT measurements that can predict tumor response to HDACs inhibitors treatment.

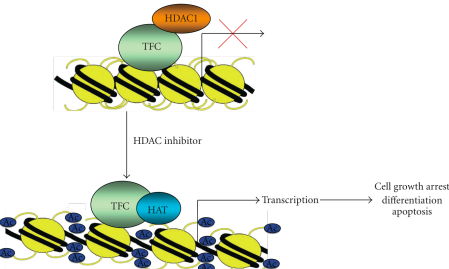

Figure 2: Mechanism of action of histone deacetylases inhibitors. It has been proposed that there are specific sites in the promoter region of a subset of genes that recruit the transcription factor complex (TFC) with histone deacetylases (HDACs). With inhibition of HDACs by HDACs inhibitors, histones are acetylated, and the DNA that is tightly wrapped around a deacetylated histone core relaxes. The accumulation of acetylated histones in nucleosomes leads to increased transcription of this subset of genes, which, in turn leads to downstream effects that result in cell-growth arrest, differentiation, and/or apoptosis.

Otherwise, HDACs inhibitors induce broad hyperacetylation in both tumor and normal tissues, which can be used as a biomarker for drug activity. However, steps will need to be taken to further characterize the molecular mechanisms behind HDACs inhibitors function as well as predictive markers of response to further implement them functionally in the clinic.

3. HDACs Inhibitors in Clinical Trials

From the initial discovery of sodium butyrate, there has been tremendous interest and investigation in HDACs inhibitors, today there are at least 15 HDACs inhibitors that are currently under clinical investigation for both hematological malignancies and solid tumors, both for single-agent and combination therapy [42]. Initial molecules included valproic acid, phenyl-butyrate, SAHA (vorinostat), trapoxin A, oxamflatin, depudepsin, depsipeptide (romidepsin, Istodax) and trichostatin A [38, 43], which have paved the way to the second-generation HDACs inhibitors such as the hydroxamic acids: belinostat (PDX101), LAQ824, and panobinostat (LBH589), and the benzamides: entinostat (MS-27-275), CI994, and MGCD0103 (mocetinostat) [44]. Here, we will discuss some of the recent clinical trials regarding several of the most promising HDACs inhibitors (Table 1).

4. Vorinostat

In 2006, two phase II trials led vorinostat (Zolinza) to be approved by the U.S. FDA for the treatment of refractory cutaneous T-cell lymphoma CTCL [40]. A multicenter phase IIB trial enrolled a total of 74 patients for progressive, persistent, or recurrent CTCL who had received at least two prior therapies. Patients were treated daily with 400 mg of orally administered vorinostat and showed an overall response rate of 29.7%, a 6.1 month median duration of response, and a 9.8 month median time to progression [45]. Similar findings were published in a phase II study with a similar patient population [46]. When considering all patients from these trials together, 26% of patients experienced thrombocytopenia, 14% anemia, and only 5% of patients experienced grade 3 to 5 adverse events, including thrombocytopenia, pulmonary embolism, fatigue, and nausea. The most common adverse events were diarrhea, fatigue, and nausea. From the larger multicenter trial, 6 patients continued treatment with vorinostat for 2 years or longer with continued clinical effect (one complete remission (CR), four partial remission (PR), and one stable disease (SD)) [47].

A phase II clinical trial tested the use of vorinostat in other hematological malignancies, including relapsed diffuse large B-cell lymphoma (DLBCL), where out of 18 patients, one resulted in a CR and one in SD with grade 1 and 2 toxicities, but was concluded to have an overall minimal effect in treating DLBCL [48]. A second trial tested vorinostat in patients with lymphoma showing promising results. Out of 17 patients with relapsed indolent non-Hodgkin's lymphoma four patients achieved CR, two had PRs and four patients remained with SD [49].

A dose-escalation phase I trial was also performed for oral vorinostat as a single-agent therapy in acute myeloid leukemia (AML). Out of 41 total patients enrolled, 31 with AML, three with myelodysplastic syndrome (MDS), four with chronic lymphocytic leukemia (CLL), two with acute lymphoblastic leukemia, and one with chronic myeloid

Table 1: Table of HDACs inhibitors discussed in this paper, organized by class (refer to text for references).

HDACs inhibitor class	HDACs inhibitors	Other common identifiers	Clinical trial phase	Structure
Small molecular weight carboxylates	Valprioc acid	Depakene, Depakote, Depakote ER, Depakote Sprinkle	FDA-approved for epileps, seizures, mania, bipolar disorders, migranes Phase I/II in hematological malignancies and solid tumors	
Hydroxamic Acids	Vorinostat	Suberoylanilide hydroxamic acid, (SAHA), Zolinza	FDA-approved for CTCL Phase I/II in hematological malignancies and solid tumors	
	Panobinostat	LBH589	Phase I/II in hematological malignancies and solid tumors	
	Belinostat	PXD101	Phase I/II in hematological malignancies and solid tumors	
Benzamides	Entinostat	MS-27-275, MS-275, SNDX-275	Phase I/II in hematological malignancies and solid tumors	
	MGD0103	Mocetinostat	Phase I/II in hematological malignancies and solid tumors	
Cyclic tetrapeptides	Romidepsin	Depsipeptide, Istodax, FK228, FR901228	FDA-approved for CTCL Phase I/II in hematological malignancies and solid tumors	

leukemia (CML). The maximum tolerated dose (MTD) was 200 mg when given twice daily and 250 mg when given three times daily, each given for 14 days in a 21-day cycle. The dose limiting toxicities (DLT) were again nausea, vomiting, and diarrhea. Seven of the patients with AML showed hematologic responses, including two CRs and two CRs with incomplete recovery [50].

Vorinostat has also been tested for use in treating several solid tumors, including platinum-resistant epithelial ovarian cancer, primary peritoneal carcinoma, and nonsmall cell lung carcinoma (NSCLC). After encouraging results from a phase I dose-escalation trial of vorinostat combined with carboplatin and paclitaxel in advanced solid malignancies, resulting in 11 out of 25 patients (10 of 19 with NSCLC and 1 of 4 with head and neck cancer) achieving a PR [51], a phase II National Cancer Institute-sponsored study has been carried out and results recently published [52]. This phase II randomized, double-blinded, placebo-controlled trial enrolled 94 patients with previously untreated stage IIIB or IV NSCLC to receive Carboplatin and Paclitaxel with either Vorinostat (400 mg daily on days 1 through 14 of each treatment cycle) or placebo. In the Vorinostat arm, a favorable trend toward improvement in median PFS (6 months versus 4,1 months in the placebo arm) and OS (13 months in the Vorinostat arm versus 9,7 months in the placebo arm) was clearly shown although at the price of an increased toxicity. Grade 4 thrombocytopenia was more frequent in the Vorinostat arm (18% versus 3% in the placebo arm) as well as grade 2-3 nausea, diarrhea, and fatigue. Moreover, 26% of patients in the Vorinostat arm discontinued therapy after the first cycle in comparison to 16% of the ones enrolled in the placebo arm. Comparably, the proportions of patients who completed all 6 cycles scheduled were 41% and 29%, respectively, for the placebo and Vorinostat arm.

Several trials also tested the efficacy of Vorinostat as single agent in different solid tumor sites (head and neck, breast, colorectal, and prostate cancer) and all reported a considerably high rate of adverse effects limiting the possibility of a reliable efficacy assessment. The most common adverse event reported in those trials were: fatigue (from 62% to 81%), nausea (from 58% to 74%), anorexia (from 58% to 81%), vomiting (from 33% to 56%), and thrombocytopenia (from 17% to 50%) [53–55].

Vorinostat is potentially also an attractive candidate for association with radiation since HDACs inhibition decreases cellular ability to repair DNA double-strand breaks both by Homologous Repair (HR) and Non-Homologous End Joining (NHEJ) [56, 57], thus resulting in a potent *in vivo* radiosensitizing effect [58]. A Phase I trial recently tested Vorinostat in combination with pelvic palliative radiotherapy (3 Gy per fraction up to 30 Gy) for gastrointestinal tumors. Vorinostat was administered orally once daily 3 hours before each radiotherapy fraction at doses ranging from 100 mg to 400 mg. The most common, any grade, adverse effects reported were fatigue, nausea, anorexia, and vomiting, respectively, in 94%, 65%, 59%, and 47% of patients [59].

5. Romidepsin

Romidepsin (Istodax, Gloucester Pharmaceuticals) is a natural compound isolated from *Chromobacterium violaceum*. It is a bicyclic tetrapeptide and is sometimes referred to as depsipeptide after the class of molecules to which it belongs. It was first tested for antibacterial activity, but it was found to have strong cytotoxic activity against different tumor cell lines, and later on mice. Romidepsin is mainly targeting class I HDACs, and it has also been recently approved by the FDA for treatment of CTCL. Two phase II multicentric single arm trials collected cumulatively 167 patients with refractory CTCL (mostly in advanced stages) treated with Romidepsin at a starting dose of 14 mg/m^2 infused over 4 hours on days 1, 8, and 15 every 28 days. The endpoint for both studies was the overall response rate (ORR). Median time to first response was 2 months in both studies and ORR was 34% and 35%, respectively. The median duration of response was 15 and 13.7 months, respectively. Adverse effects observed in both studies were similar to the toxicities observed in phase I trials. Common (any grade) adverse effects included nausea (56% and 86%, resp.), fatigue (53% and 77%), vomiting (34% and 52%), and anorexia (23% and 54%). Furthermore, consistently with the toxicity pattern shown by Romidepsin in Phase I studies [60], ECG changes were also noted in a large proportion of patients of the study (up to 50%) consisting of T-wave flattening, ST tract depression, and QT interval prolongation. Cardiotoxicity, which has not been frequently found after Vorinostat treatments, seems to be a more specific side effect of Romidepsin and has been explained as being dependent upon the interaction of the drug with the HERG K^+ channels [3]. Romidepsin has also been initially tested clinical conditions other than CTCL. In some Phase I/II trials, single-agent Depsipeptide has shown a limited clinical benefit in treating refractory neoplasms, including AML/MDS, CLL, lung cancer, hormone refractory prostate cancer, and renal cell cancer [61–66].

6. Mecetinostat (MGCD0103)

Mecetinostat (MGCD0103) is a class I isotype-selective orally available benzamide HDACs inhibitor. Early clinical trials have demonstrated activity in hematological malignancies, including myeloid leukemia and lymphoma and was well tolerated with DLTs of fatigue, nausea, vomiting, and diarrhea. A phase I trial resulted in a bone marrow CR in three of 29 patients with AML at a MTD of 60 mg/m^2 administered three times weekly [67]. A phase II study in adults with relapsed or refractory DLBCL (33 patients) or follicular lymphoma (FL—17 patients) also demonstrated significant anticancer activity. Most of the 17 patients with DLBCL that were reassessed by CT after treatment showed a decrease in tumor volume, as well as one CR and 3 PRs. Out of ten patients with FL, one achieved PR. Grade 3 toxicities or greater included fatigue, neutropenia, thrombocytopenia, and anemia [68]. A phase II trial was also conducted in patients with relapsed or refractory Hodgkin's lymphoma. A treatment schedule of 110 mg or 85 mg three times per week in a 4-week cycle were given to 23 and 10 patients, respectively. From the 21 patients

evaluated from the 110 mg cohort, there was an ORR of 38% (2 had CRs, and 6 had PRs). The patients who had CRs remained with progression free survival for >270 and >420 days, respectively. From the 10 patients in the 85 mg cohort, all 5 that were evaluated demonstrated tumor reductions of ≥30%, with one PR and 2 SDs [69].

Aside from the beneficial effects demonstrated in hematological malignancies, MGCD0103 also demonstrated clinical benefits in solid tumor treatment. A phase I trial in patients with advanced solid tumors given MGCD0103 three times per week for 2 of every 3 weeks showed tolerable DLTs of fatigue, nausea, vomiting, anorexia, and dehydration. After four or more cycles, SD was observed in five of 32 patients. A phase II dose of 45 mg/m^2/day was recommended [70]. Phase I/II studies in solid tumors were also conducted in combination with gemcitabine. Phase I included patients with refractory solid tumors. Phase II was limited to gemcitabine naive patients with locally advanced or metastatic pancreatic cancer. During a 28-day cycle patients received MGCD0103 three times per week in a dose ascending 3 + 3 design targeting a DLT of <33%. Gemcitabine was administered three times per cycle weekly at 1000 mg/m^2. Out of the 14 patients evaluated, there were 2 PRs in patients with pancreatic carcinoma, one PR in a patient with nasopharyngeal cancer, and one PR in a patient with cutaneous T-cell lymphoma. The phase II trial is ongoing at a dose of 90 mg for patients with pancreatic cancer [71].

7. Panobinostat (LBH589)

Panobinostat is a hydroxamate that has shown potential in early phase I and II clinical trials. In an initial trial, 15 patients with AML, ALL, or MDS were treated with 4.8 to 14 mg/m^2 panobinostat administered intravenously as a 30-minute infusion. Transient blast cell reductions occurred in 8 of 11 patients with peripheral blasts. Four patients exhibited a DLT of grade 3 QTcF prolongation at 14 mg/m^2, which were asymptomatic and cleared after treatment ended. Common toxicities included nausea, diarrhea, vomiting, hypokalemia, loss of appetite, and thrombocytopenia [72]. CTCL patients (stage IB-IVA), including Mycosis Fungoides (MF) and Sezary Syndrome (SS), who have failed two or more previous therapies were enrolled in a phase II clinical trail. Panobinostat was administered at 20 mg orally on days 1, 3, and 5 weekly until disease progression or intolerance to two groups of patients, one who had received prior treatment with oral bexorotene and a second without. The best overall responses were 3 PRs and 4 SDs. ECG monitoring of QTcF prolongation was performed, without any >500 ms [73].

8. Belinostat (PXD101)

Belinostat has shown promising anticancer activity in both hematologic malignancies as well as solid tumors. In a trial enrolling 16 patients with advanced hematological neoplasms, belinostat was administered intravenously at one of three dose levels: 600, 900, and 1000 mg/m^2/d. While no CRs or PRs were noted, intravenous administration was well tolerated, and five patients (including two with DLBCL) achieved SDs after 2–9 treatment cycles. There were no grade 3 or 4 hematological toxicities (except one case of grade 3 lymphopenia), and the most common adverse effects were nausea, vomiting, fatigue and flushing. There were two grade 4 renal failures in patients with multiple myeloma (MM). The recommended dose for phase II studies was 1000 mg/m^2/d, intravenously administered on days 1–5 of a 21-day cycle for patients with hematological neoplasia [74].

For solid tumors, Belinostat was tested in a phase I study of patients with advanced refractory cancers. The 46 patients received six dose levels, ranging from 150 to 1200 mg/m^2/d over a 5-day cycle. DLTs were fatigue, diarrhea, atrial fibrillation, and grade 2 nausea/vomiting, which led to inability to complete the full cycle. 39% of patients resulted in SD. Of the 24 patients treated at the MTD, which was determined to be 1000 mg/m^2/d, 50% achieved SD [75]. Patients with platinum resistant epithelial ovarian cancer (EOC) are resistant to conventional chemotherapy. Belinostat was administered intravenously at 1000 mg/m^2/d on days 1–5 of a 21-day cycle to metastatic or recurrent platinum resistant EOC and low malignant potential (LMP) ovarian tumors. Of the 18 patients with LMP, 1 had PR, 10 had SDs. Median PFS in LMP was 13.4 months. Patients with EOC 9 had SD with a median PFS of 2.3 months [76].

9. Entinostat (MS-27-275)

Clinical trials of Entinostat, a benzamide derivative, initiated in 2005 with a Phase I study enrolling patients with advanced solid tumors or lymphoma. Entinostat was administered to a total of 22 patients once a week for 4 weeks during a 6-week cycle. The MTD was determined to be 6 mg/m^2, and the common DLTs were hypophophatemia, hyponatremia, and hypoalbuminemia, which were all reversible [77]. After the analysis of three different dose schedules, 4 mg/m^2 weekly or 2 to 6 mg/m^2 every other week, for three weeks in a 28-day cycle; the biologically relevant plasma concentrations and antitumor activity were determined [78].

In solid tumors, a phase I combination therapy trial was performed on ten patients with an advanced NSCLC. Patients were treated with 5-azacitidine (AZA), a DNA methyltransferase inhibitor, subcutaneously on days 1–6 and 8–10 along with a fixed dose (7 mg/m^2) on day 3 and 10 of a 28-day cycle of entinostat. The dose of AZA was varied by cohort using a standard 3 + 3 dose assessment. No DLTs were observed in the 30 mg/m^2 dose cohort. However, in the 40 mg/m^2 cohort, after one week, a patient was replaced due to rapidly progressing disease, and another patient experienced a grade 3 neutropenia and thrombocytopenia. The common toxicities included injection site reactions, nausea/vomiting, constipation, fatigue, and cytopenias. One patient had a PR, which continued longer than 8 months. Two patients had SDs and the remaining patients had PODs [79].

10. Valproic Acid

Valproic acid (VPA) has been increasingly studied in clinical trials for a variety of cancer types as a single agent or in

combination with other therapies. In solid tumors, VPA was analyzed for activity in 12 patients with cervical cancer. Three four-patient dose cohorts were formed, for 20 mg/kg, 30 mg/kg, and 40 mg/kg administered orally for five days over a six-day protocol. Tumor-deacetylase activity decreased in eight patients in a statistically significant manner. A grade 2 depression in level of consciousness was registered in 9 patients [80]. Another phase I study in 26 patients revealed neurocognitive impairment, with grade 3 or 4 neurological side effects in 8 of the 26 patients. When administered intravenously the MTD was determined to be 60 mg/kg/d [81]. A phase II study for the treatment of advanced solid tumors with hydralazine and VPA revealed clinical benefit in 80% (12) of patients with cervix, breast, lung, testis, and ovarian carcinomas. Four patients had PRs and eight SDs, and the most common toxicity was hematological [82].

VPA has been more frequently studied in the use of combination therapies, specifically with all transretinoic acid (ATRA). From a study of 75 patients with AML/MDS, 66 were initially treated with VPA monotherapy followed by ATRA in nonresponsive or relapsed patients. VPA was administered for a median treatment duration of 4 months and ATRA, 2 months. 24% of patients showed hematological improvement with a median response duration of 4 months. Four out of 10 relapsed patients, when administered ATRA had a second response and both treatments were well tolerated [83]. VPA was also combined with both AZA as well as ATRA in patients with AML or high-risk MDS. A total of 53 patients were treated with AZA at the fixed dose of 75 mg/m^2 daily for 7 days, ATRA at 45 mg/m^2 orally daily for 5 days starting on day 3, and VPA, which was dose escalated and administered orally daily for 7 days concomitantly. The ORR was found to be 42%, the median remission duration was 26 weeks, the MTD for VPA was 50 mg/kg daily for 7 days and the DLT was reversible neurotoxicity [84]. In another study of patients with AML/MDS, increasing doses of VPA administered orally and concomitantly with a fixed dose of decitabine (15 mg/m^2 by intravenous daily infusion) for 10 days revealed a safe daily dose of 50 mg/kg. 22% (12) of patients had an objective response, this included 10 CRs and 2 CRs with incomplete platelet recovery [85].

11. Associations of HDACs Inhibitors with Other Target Drugs

Despite the very high number of gene products potentially deregulated in solid tumors, high throughput screening analyses suggest that mutations often occur in genes that collaborate in a relatively limited pool of common cell signaling pathways [86]. This hypothesis may have a great relevance in the clinic. In fact, having at hand several classes of effective "pathway-oriented" target drugs, and admitting that a tumor may be driven by a limited number of deregulated pathways, it possible that the concomitant use of a combination of drugs directed against different pathways functionally related may result in an improved antineoplastic effect or in the overcoming of drug resistance.

Recent studies on multiple myeloma (MM) models suggest that HDACs inhibitors may synergize with proteasome inhibitors. Although the molecular mechanism underlying this effect is not completely understood several means have been proposed [87] and encouraging data has come from the early clinical experimentation, including a phase I trial [88] of randomized patients with relapsed/refractory MM to receive Vorinostat (200 mg twice daily or 400 mg once daily for 14 days) in combination with bortezomib (0.7 or 0.9 mg/m^{2}on days 4, 8, 11, and 15 or 0.9 or 1.1 or 1.3 mg/m^2 on days 1, 4, 8, and 11). Among 34 evaluable patients, the best response to vorinostat plus bortezomib was a partial response (PR) in 9 (26%) patients, minimal response (MR) in 7 (21%) patients, and stable disease (SD) in 18 (53%) patients. Mean duration of SD was 89 days, range 9–369 days. Of the 13 evaluable patients who had previously been treated with bortezomib, 5 achieved a PR, 1 had an MR, and 7 had SD. Eleven of the 34 patients enrolled (32.4%) discontinued treatment due to adverse effects (AEs). Most common AEs were fatigue, nausea, diarrhea, and hematological toxicities. A phase II open label study from the same group is currently ongoing. Another Phase I trial accrued 23 heavily pretreated (median of 7 previous regimens) patients with relapsed/refractory MM to receiving escalating doses of Bortezomib (1 or 1.3 mg/m^2 on days 1, 4, 8, and 11 and Vorinosat 100 mg twice daily, 200 mg twice daily, and 400 mg once daily, or 500 mg once daily for 8 days each 21-day cycle). Overall response rate was 42%, two patients receiving 500 mg vorinostat had prolonged QT interval and fatigue as dose-limiting toxicities. The most common grade >3 toxicities were myelosuppression ($n = 13$), fatigue ($n = 11$), and diarrhea ($n = 5$). In the same setting of patients with relapsed/refractory MM, the combination of Romidepsin and Bortezomib and Dexamethasone has also shown promising results. In a Phase I/II trial, of 18 evaluable patients, this schedule resulted in a overall response rate of 67%. The most common drug related grade 3 toxicities included fatigue (2 pts.), neutropenia (1 pts.), sepsis (2 pts.), and peripheral neuropathy (1 pts). Preclinical data seems to confirm a synergic effect of Panobinostat and Bortezomib, and a Phase I trial is currently ongoing (NCT00532389). These encouraging results are paving the way to a relevant number of trials testing the association of different HDAC and Proteasome inhibitors, and results are expected in a relatively short time.

12. HDACs Inhibitor-Related Toxicity

The relationship between the toxicity of HDACs inhibitors and their pharmacodynamic/pharmacokinetic properties is still largely unknown. This makes it difficult to optimize HDACs inhibitors treatment. Studies in preclinical models have shown that HDACs inhibitors are a class of agents that has been generally well tolerated and proved a very good toxicity profile in comparison with other chemotherapeutic drugs used in cancer therapy. The main adverse effect is fatigue, which is generally mild and tolerable in most patients, but in 30% of patients, it can be severe enough to cause drug discontinuation. Gastrointestinal toxicities are also common side effects and include anorexia, nausea, vomiting, and diarrhea. Overall, they are mild and controllable

with symptomatic treatment. Biochemical disorders such as hypokalemia, hyponatremia, hypocalcemia, hyperglycemia, hypophosphatemia, and hypoalbuminemia are common with various HDACs inhibitors, while neurocortical disturbances including somnolence, confusion, and tremor are observed mainly with phenylbutyrate and valproic acid. All these side effects are generally reversible upon cessation of administration of the drug.

Another side effect of histone deacetylase inhibitors is transient thrombocytopenia that is relatively common with most HDACs inhibitors [89], it is generally mild, although has been dose limiting in some studies.

A significant adverse reaction regards the cardiotoxicity. Early studies in preclinical animal models have shown that various HDACs inhibitors such as Romidepsin are able to cause myocardial inflammation and cardiac enzyme elevation. These studies represent a controversial issue since high doses of HDACs inhibitors were used [90, 91] compared to the doses that were confirmed appropriate for use in Phase I trials. Specifically, the effect of Romidepsin on cardiac function was assessed in 42 patients with T-cell lymphoma. They received a total of 736 doses of Romidepsin and an intensive cardiac monitoring was evaluated [92]. Grade I (T-wave flattening) and grade II (ST segment depression) ECG changes occurred in more than half of the ECGs obtained post treatment; however, these changes were reversible and of short duration, with no elevation in cardiac enzymes and no significant changes in left ventricular ejection fraction.

In addition, cardiac dysrhythmias were observed in a small number of patients but most of these patients had pretreatment documented dysrhythmias. Similar ECG changes and QT-interval prolongation have been reported in other Phase I/II Romidepsin studies [60, 66, 93–96]. In other Romidepsin studies, there have been reports of sudden death; however, the relationship to the drug remains unclear. In particular, a Phase II study of 15 patients with metastatic neuroendocrine tumors, administered with standard doses of Romidepsin reported one sudden death in a 48-year-old patient [66]. However, this patient had a history of hypertension, and a biventricular hypertrophy was revealed by postmortem examination, both are known risk factors for sudden death. Cardiotoxicity may be a class effect of HDACs inhibitors, being more frequent with Romidepsin and other class-I inhibitors rather than Vorinostat and other pan-HDAC inhibitors but it is unlikely that these side reactions are limited just to those HDACs inhibitors. Additional parallel cardiotoxicity studies with other various HDACs inhibitors are necessary.

Possible room for improvement could be in the development of isoform-selective HDACs inhibitors (extensively reviewed in [97]). It is known from knockout studies that the deletion of some specific HDACs isoforms can cause precise phenotypic defects. In particular, mice lacking some of the HDACs isoforms (namely, HDAC2, HDAC3, HDAC5, and HDAC9) show severe cardiac malformations and dysfunctions [98, 99], suggesting that HDACs inhibitors, specific for other HDACs could possibly have a better cardiotoxicity profile still retaining the full pro-apoptotic action. Furthermore the introduction of reliable sensitivity biomarkers in the design of trials will allow a better stratification of patients thus minimizing the risk of exposure of the unresponsive subjects to HDACs treatment and toxicity. Recently, a genome-wide loss-of-function screening was undertaken to reveal genes that govern tumor cell sensitivity to HDAC inhibitors in a sarcoma cell model, and HR23B, a protein involved in shuttling ubiquitinated proteins to the proteasome was identified as a potential biomarker [100]. HR23B expression was further investigated in 21 skin biopsies from 20 patients with CTCL enrolled in a Vorinostat Phase II trial [46] and analyzed by immunohistochemistry. The proportion of patients with a strong HR23B staining who had a clinical response was 69%, thus suggesting a pretty high positive predictive value (PPV). Similar PPV for HR23B were obtained when looking at patients treated with other HDACs inhibitors [101].

13. Conclusions

HDAC, inhibitors represent a promising new group of anticancer agents, even though the mechanisms of HDAC inhibitor-induced tumor cell death require further elucidation. While vorinostat and romidepsin are the only US FDA-approved HDACs inhibitors currently utilized in cancer therapy, as we have shown here, there are many HDACs inhibitors that are presently under intense clinical investigation, both as single agents and combination therapies. These will hopefully be able to further improve the range of treatment options available for hematologic malignancies as well as for solid tumors.

As we come closer to understanding the molecular mechanisms inherently responsible for tumorigenesis, as well as the full range of HDACs inhibitor cellular actions, we will be able to target in a more appropriate way and be able to pair cancer therapies for clinical use. In order to establish rigorous patient selection criteria and optimal drug combinations to properly design further trials and maximize the clinical gain, the bridge between the biological function and the therapeutic benefit of these drugs needs to be further elucidated.

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Male breast cancer

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Abstract

Male breast cancer (MaleBC) is a rare disease, accounting for <1% of all male tumors. During the last few years, there has been an increase in the incidence of this disease, along with the increase in female breast cancer (FBC). Little is known about the etiology of MaleBC: hormonal, environmental and genetic factors have been reported to be involved in its pathogenesis. Major risk factors include clinical disorders carrying hormonal imbalances, radiation exposure and, in particular, a positive family history (FH) for BC, the latter suggestive of genetic susceptibility. Rare mutations in high-penetrance genes (*BRCA1* and *BRCA2*) confer a high risk of BC development; low-penetrance gene mutations (i.e. *CHEK-2*) are more common but involve a lower risk increase.

About 90% of all male breast tumors have proved to be invasive ductal carcinomas, expressing high levels of hormone receptors with evident therapeutic returns.

The most common clinical sign of BC onset in men is a painless palpable retroareolar lump, which should be evaluated by means of mammography, ultrasonography and core biopsy or fine needle aspiration (FNA).

To date, there are no published data from prospective randomized trials supporting a specific therapeutic approach in MaleBC. Tumor size together with the number of axillary nodes involved are the main prognostic factors and should guide the treatment choice. Locoregional approaches include surgery and radiotherapy (RT), depending upon the initial clinical presentation. When systemic treatment (adjuvant, neoadjuvant and metastatic) is delivered, the choice between hormonal and or chemotherapy (CT) should depend upon the clinical and biological features, according to the FBC management guidelines. However great caution is required because of high rates of age-related comorbidities.
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Keywords: Male breast cancer; Genetics; Therapy; Surgery; Radiotherapy; Chemotherapy; Hormonal treatment; Survival; Local recurrence

1. Introduction

Male breast cancer (MaleBC) is a rare disease, showing an increasing incidence trend rising along with that of female breast cancer (FBC). Even if male and female breast cancers seem to be similar, with regard to epidemiological aspects, they deeply differ because of the lower incidence and later onset of the former. Little is known about the etiology of MaleBC: hormonal, environmental and genetic factors are involved in the pathogenesis of breast cancer in men as well as in women. The major risk factor related to MaleBC is a positive family history for breast cancer, which indicates a relevant genetic component. In fact, MaleBC susceptibility can result from rare mutations in high-penetrance genes conferring a high risk, or from more common low-penetrance genes giving a lower risk increase.

From the clinical and biological point of view, male and female breast cancers differ mainly in the frequency of their histological types and in the expression of hormone receptors and of epidermal growth factor receptor 2 (HER2).

In the lack of randomized controlled trials, principles of management of MaleBC are mainly derived from randomized trials in female patients (pts). Since it is often late diagnosed, MaleBC remains a substantial cause of morbidity and mortality in men. This last consideration together with the increasing incidence made it urgent to comprehensively review the epidemiological, genetic, histopathological and clinical aspects of MaleBC, including the diagnosis, prognosis and treatment of the disease.

2. Epidemiology

In Western countries, MaleBC accounts for <1% of all cancers in men but its incidence varies greatly in different geographical areas and ethnic groups [1,2]. The worldwide variation of MaleBC resembles that of FBC, with higher rates in North America and Europe and lower rates in Asia. A substantial high proportion of MaleBC cases have been reported in Africa [3]. Although scarce, data from this continent have shown annual MaleBC incidence rates ranging from 5 to 15% [4–6]. These relatively high rates have been attributed to endemic infectious diseases, such as bilharziosis and hepatitis B/C that, by chronic liver infection, may cause liver damage leading to hyperoestrogenisms. By contrast, the annual incidence of MaleBC in Japan is significantly lower (5 per 1,000,000) than the average incidence, comparable to the lower than average incidence of FBC in this country [7]. Recent epidemiological studies indicate that MaleBC incidence is rising [8]. The incidence of MaleBC increases with age and the bimodal age distribution seen in women is absent in men, with a peak incidence in the sixth decade [3]. Overall, due to the absence of screening programs in men, MaleBCs are diagnosed at a more advanced age and with a more severe clinical presentation than in women, with greater tumor size and a more frequent lymphonodal involvement. The mean age at breast cancer diagnosis in males is 63.4 years [9]; in the SEER data, the median ages at diagnosis of breast cancer were 67 and 62 years in males and females, respectively [3]. The mortality rates for MaleBC have been shown to remain stable [1], however, survival rates differ significantly according to race/ethnicity [10] and are not significantly different from those observed in women [3]. In general, the prognosis for male and female patients with breast cancer is similar. Overall survival rates are lower for men, but this is due to an older age at diagnosis and more advanced stage at presentation [11]. Disease-specific survival rates are higher than overall survival rates due to the older average age and deaths from other comorbid diseases [12].

Table 1
Risk factors for male breast cancer.

High risk	Hormonal imbalance Testicular or liver damage Oestrogen intake	*BRCA2*
	Radiation exposure	Klinefelter's syndrome Breast cancer family history
Moderate/Low risk	Occupational exposure Heat	*BRCA1*
	Obesity	*CHEK2* Cowden syndrome
Suspected risk	Occupational exposure Exhaust emissions Magnetic fields	*AR*
	Alcohol intake	*CYP17*

3. Risk factors

Similar to breast cancer in women, MaleBC is likely to be caused by the concurrent effects of different risk factors, including clinical disorders relating to hormonal imbalances, certain occupational and environmental exposures, and genetic risk factors, for instance a positive family history (FH) of breast cancer (BC) and mutations in BC predisposing genes, such as *BRCA* genes, and possibly others. Environmental factors, particularly occupational carcinogen exposure, might well contribute to MaleBC risk by interacting with genetic factors. We reported a strong association between a specific occupation (truck driving) and breast cancer risk in male carriers of *BRCA1/2* mutations [13]. Risk factors for MaleBC are summarized in Table 1.

3.1. Hormonal risk factors

As is the case in female BCs, MaleBCs are highly sensitive to hormonal changes. In particular, hormonal imbalance between an excess of estrogen and a deficiency of testosterone increases the risk of the disease. This imbalance may occur endogenously due to testicular abnormalities, including, undescended testes, congenital inguinal hernia, orchitis, orchiectomy and testicular injury [14]. Liver diseases, such as cirrhosis, may also result in a hyperestrogenic state [15]. In general, liver damage and disease, caused by the effects of several drugs or their metabolites, may affect hepatic functions and lead to hyperestrogenism.

Obesity is one of the most common causes of hyperoestrogenization in men because of increased peripheral aromatization of androgens. Obesity, in fact, doubles the risk of breast cancer in men [16–18]. Recently it has been reported that first-born male children have a 1.71 times higher risk of MaleBC than their younger brothers, possibly because they have been exposed to higher levels of intrauterine estrogen [19].

Klinefelter's syndrome, characterized by 47XXY karyotype, testicular dysgenesis, gynecomastia, low testosterone concentrations and increased gonadotrophins, is strongly associated with MaleBC risk. Individuals with this syndrome have a 20–50 times higher risk over the general male population [20].

An upset in estrogen or androgen balance is a causal factor in gynecomastia, which is extremely common in pubescent boys, may occur in men over the age of 50 and is found in 6–38% of male pts affected by BC. However, the incidence of gynecomastia in MaleBC pts is no higher than in the general male population [6]; gynecomastia, therefore, does not in itself seem to represent a risk factor for MaleBC [17,21]. Conditions increasing exposure to estrogen or decreasing exposure to androgen, such as the exogenous administration of estrogen to trans-sexuals or the long-term use of antiandrogens and estrogens in the treatment of prostate cancer, have also been implicated as causative factors for MaleBC [22–24].

3.2. Occupation and environmental risk factors

As in women, ionizing radiations have been considered as possible causal cofactors in the etiology of MaleBC [25], with a modest positive trend with the increasing number of X-ray examinations performed on chest and adjacent body areas and with an induction period of at least 20–25 years, with a subsequent decrease of risk after the 30 or 40 years subsequent to the last exposure.

Occupational exposure to heat and electromagnetic radiation are postulated to be linked to MaleBC risk. A higher frequency of breast cancer is reported in men who have worked in hot environments, such as blast furnaces, steel works, rolling and finishing mills [26], possibly because long-lasting exposure to high ambient temperatures can lead to testicular failure. An increased MaleBC risk has been observed in men exposed to high electromagnetic fields [2] and a 1.31 relative risk in men with an exposure above the first quartile has been reported, although no clear trend of exposure and risk has emerged [27].

In a few studies, a certain degree of risk has been found to be associated also to polycyclic aromatic hydrocarbons (PAHs) [2], but the evidence is still too inadequate to draw any valid conclusions. Moreover, PAHs are usually found in environments contaminated by other pollutants with mutagenic effects, such as nitrogen oxides, nitrosamines and exhaust fumes, making it very difficult to disentangle the effect of any single pollutant.

3.3. Dietary risk factors

As for women, alcoholic beverages seem to represent a risk factor for the development of MaleBC, with an increase of 16% for each increase of 10 g/day of alcohol intake. Moreover, strong consumers of alcoholic beverages (more than 90 g/day) present a 6-fold increased OR to develop MaleBC

when compared to light consumers (<15 g/day) [28]. The available evidence for other components of diet is rather scarce. The consumption of animal fats and in particular red meat in relation to the risk of MaleBC has been investigated in several studies, but the results are still not clear. Inconsistent findings have also been provided by the evaluation of the effect of fruit and vegetable intake [28]. Overall, with the exception of alcohol consumption, dietary factors seem to play a marginal role in the etiology of MaleBC.

3.4. Family and personal history of cancer

Similar to FBC, a positive FH of BC is associated with increased risk of MaleBC. Data from population-based studies have shown that about 20% of all MaleBC pts have a history of BC in a first-degree female relative [17,18,29–31]. In general, a positive FH of either female or male breast cancer among first-degree relatives confers a 2–3-fold increase in MaleBC risk [17,32–34]. The risk increases with increasing numbers of first-degree relatives affected and with early onset in affected relatives. In addition to BC families, MaleBC cases have also been reported in families with the hereditary non-polyposis colorectal cancer (HNPCC) syndrome [35] and Cowden syndrome [36].

A personal history of a second primary tumor is reported in more than 11% of MaleBC pts [37]. Men diagnosed with a first primary breast cancer have a 16% increased risk of developing a second primary cancer in comparison with the general male population [37]. Data from the SEER program from the National Cancer Institute show that a history of MaleBC is associated with a 30-fold increased risk of breast cancer on the contralateral side [38], which is much higher than the 2–4-fold increase observed in women [39]. The risk of a second site-specific cancer is elevated also for gastrointestinal cancer, pancreas and prostate carcinomas, melanoma and non-melanoma skin tumors [37,40].

3.5. BRCA1 and BRCA2

MaleBC predisposition can result from germ-line mutations in the high-penetrance *BRCA2* (OMIM #6600185) and, with lower frequency, *BRCA1* (OMIM #113705) genes. The presence of MaleBC within high-risk BC families indicates a high likelihood of *BRCA2* mutations with a frequency ranging from 60 to 76%, whereas *BRCA1* mutations frequency ranges from 10 to 16% [41,42]. The frequency of *BRCA1* and *BRCA2* mutations are extremely different in ethnically diverse population- and clinic-based MaleBC series, ranging from 4 to 40% for *BRCA2* and up to 4% for *BRCA1* (Table 2), and resulting higher in the presence of founder effects [12,43]. *BRCA1* and *BRCA2* founder mutations have been identified in specific countries or ethnic groups, particularly in genetically isolated populations such as the Icelanders and Ashkenazi Jews. In Iceland, the *BRCA2* 999del5 founder mutation is involved in 40% of all MaleBC cases [44]. In Ashkenazi Jews the *BRCA1* 185delAG and the *BRCA2* 6174delT founder mutations found in women are also frequent in men. In fact, the combined prevalence of the *BRCA1* and *BRCA2* founder mutations among Askenazi Jewish men is slightly higher than for women, due to the higher frequency of *BRCA2* mutations [45]. However, even in heterogeneous countries, such as Italy, there is evidence of founder *BRCA1* and *BRCA2* mutations in regions that show a micro-homogeneity [46–50]. *BRCA2* mutations are currently considered as the major genetic risk factor for MaleBC, however, there is no evidence for a correlation between the location of the mutation within *BRCA2* gene and risk of MaleBC. The median age at BC diagnosis among *BRCA2* mutation carriers is earlier (median, 58.8 years) than that of negative cases (median, 67.9 years) [29]. Overall, *BRCA1* and *BRCA2* mutations are more prevalent in men with a positive first-degree FH compared with those without [29,51,52]. Since mutations are also identified in MaleBC cases without

Table 2
BRCA1 and *BRCA2* mutations prevalence from studies of male breast cancer patients.

Study	Center	*n* tested	*BRCA1* mutation *n* (%)	*BRCA2* mutation *n* (%)
Couch et al. Nat Genet 1996 [169]	Philadelphia, PA	50	ne	7 (14)
[a]Friedman et al. Am J Hum Genet 1997 [170]	Southern California	54	0	2 (4)
[a,§]Thorlacius et al. Am J Hum Genet 1997 [44]	Iceland	30	ne	12 (40)
Mavraki et al. Br J Cancer 1997 [171]	Leeds, UK	28	ne	2 (7.1)
Haraldsson et al. Cancer Res 1998 [172]	Sweden	34	ne	7 (21)
Csokay et al. Cancer Res 1999 [173]	Hungary	18	0	6 (33)
Tirkkonen et al. Genes Chrom Cancer 1999 [174]	Sweden	26	0	5 (19)
[§]Sverdlov et al. Genet Test 2000 [175]	Israel	31	1 (3)	1 (3)
Kwiatkowska et al. Hum Mut 2001 [176]	Poland	37	ne	4 (11)
[a]Basham et al. Breast Cancer Res 2002 [29]	Cambridge, UK	94	0	5 (5)
Frank et al. J Clin Oncol 2002 [42]	USA	76	8 (10)	14 (18)
Evans et al. Familial Cancer 2008 [51]	Manchester, UK	64	4 (6)	17 (27)
[§]Chodick et al. Eur J Med Genet 2008 [45]	Israel	261	8 (3)	21 (8)
[a]Ottini et al. Breast Cancer Res 2008 [86]	Italy	108	2 (2)	8 (7)

ne: not evaluated.
[a] Population-based study.
[§] Mutational analysis limited to founder mutations.

FH, from a clinical point of view, predictive genetic testing is not only beneficial in men from high-risk families but also among isolated MaleBC cases.

3.6. CHEK2

There is evidence supporting the implication of *CHEK2* (OMIM #604373), a cell cycle checkpoint kinase that along with *BRCA1* and *BRCA2* plays a role in DNA repair, in inherited MaleBC predisposition. In particular, it has been estimated that the *CHEK2* 1100delC mutation accounts for 9% of MaleBC cases and confers approximately a 10-fold increase of BC risk in men lacking *BRCA1* and *BRCA*2 mutations [53]. Although this mutation has been strongly associated with the increased MaleBC risk in high-risk BC families, this association is not so clear in MaleBC cases unselected for FH [54–57]. Furthermore, there is evidence that the contribution of the *CHEK2* 1100delC variant to MaleBC predisposition varies from one ethnic group and from one country to another [58].

3.7. AR

AR gene (OMIM # 313700), the gene encoding the androgen receptor, has been suggested to play a role in MaleBC predisposition. Germ-line mutations of *AR* and variation of the polyglutamine (CAG) repeat within *AR* exon 1 were found in MaleBC cases [59], However, these results were not supported by additional studies [60]. Overall, *AR* gene mutations do not seem to contribute significantly to the risk of MaleBC.

3.8. CYP17

The *CYP17* gene encodes for the cytochrome P450c17α enzyme that is involved in the synthesis of estrogens and androgens. A germ-line variant in the *CYP17* promoter region was found to be associated with an increased MaleBC risk [61]. Overall, a possible role for the *CYP17* promoter polymorphism in MaleBC risk may be suggested although studies are not conclusive because of the small sample size analyzed.

4. Lifetime risk for male breast cancer

Male carriers of *BRCA2* germ-line mutations have a higher risk of developing BC than men in the general population. Male *BRCA2* mutation carriers have been estimated to have a lifetime risk of 6.9% for developing BC, which is approximately 80–100 times higher than in the general population [62]. The association between *BRCA1* germ-line mutations and MaleBC risk has proved to be less clear. In a clinically based study of *BRCA1* mutation carriers, a lifetime risk of 5.8% for MaleBC has been estimated [63]. Recently, the risk of developing breast cancer for male *BRCA1* and *BRCA2* mutation carriers has been evaluated in the US population by means of an analysis of data from 1939 families collected within the National Cancer Institute's Cancer Genetics Network [64]. Data from this large study show that at all ages, the cumulative risks of MaleBC are higher in both *BRCA1* and *BRCA2* mutation carriers than in non-carriers (Table 3). The relative risk of developing BC is highest for men in their thirties and forties and decreases with increasing age. In particular, in *BRCA2* mutation carriers the relative risk at age 30 is 22.3 times that at age 70. Both the relative and cumulative risks are higher for *BRCA2* mutation carriers than for *BRCA1* mutation carriers. In particular, the estimated cumulative risk of MaleBC at age 70 is 1.2% for *BRCA1* mutation carriers and 6.8% for *BRCA2* mutation carriers (Table 3). Overall, these observations demonstrate that *BRCA1* mutations are associated with an increased risk of MaleBC, but such risks are substantially lower than those in *BRCA2* mutation carriers.

Table 3
Age-specific cumulative risk of developing breast cancer for general male population and male *BRCA1* and *BRCA2* mutation carriers (%)[a].

Age, year	General population	*BRCA1* carrier	*BRCA2* carrier
30	1.2×10^{-4}	1.7×10^{-2}	0.18
40	1.9×10^{-3}	0.12	1.2
50	8.5×10^{-3}	0.3	2.7
60	2.7×10^{-2}	0.62	4.7
70	6.7×10^{-2}	1.2	6.8
80	0.12	1.8	8.3

[a] Modified by Tai et al. [64].

Male carriers of *BRCA1* and *BRCA2* mutations are at increased risk of developing several cancer types, including prostate and pancreatic cancer. The prostate is the most consistently reported site for cancer susceptibility in male *BRCA1* and *BRCA2* mutation carriers, although the association between prostate cancer risk and *BRCA2* mutation is more consistent. A relative risk (RR) of 1–3 and of 2–5 has been estimated for *BRCA1* and *BRCA2* mutation carriers, respectively, and the RR risk has proved to be greater for men under 65 years of age [65,66]. Intriguingly, mutations in the ovarian cancer cluster region (OCCR), the central part of the *BRCA2* gene associated with a higher risk of ovarian cancer compared with breast cancer, are associated with a lower risk of prostate cancer than mutations outside the OCCR (19.2% vs. 33.6% before the age of 80) [62]. Pancreatic cancer is an established feature of the *BRCA2* phenotype. A significant increased risk of pancreatic cancer is reported also in relatives of *BRCA1* mutation carriers [63,67]. Overall, a RR of 2–3 and of 2–8 has been estimated for *BRCA1* and *BRCA2* mutation carriers, respectively [63,65,67]. Male carriers of *BRCA1* and *BRCA2* mutations are also at risk of developing colon and gastric carcinomas, melanoma and non-melanoma skin cancer. However data to determine the magnitude of excess cancer risk at these sites are limited [66].

Overall, these observations indicate that the total cancer risk to male carriers of *BRCA1* and, particularly, *BRCA2* mutations, is high before the age of 65 and consists mainly in breast, prostate and pancreatic cancers.

5. Oncogenetic counseling for men at increased breast cancer risk

At present, oncogenetic counseling is available to women at increased risk of breast and ovarian cancer. These women usually have a first-degree FH of cancer and are offered screening for *BRCA1* and *BRCA2* mutations. *BRCA1/2* genes testing is an example of susceptibility testing, which is the assessment of the future risk determination in an asymptomatic individual. To date, attention has focused mainly on the women belonging to *BRCA1* and *BRCA2* families and little is known about the impact of genetic testing on men.

No universal guidelines have been established to determine the population of pts who should be tested for *BRCA* mutations. General adopted criteria consider families as eligible for *BRCA* mutations testing if they meet any of the following classifications: multiple pre-menopausal first or second-degree relatives with BC, bilateral BC, ovarian cancer and MaleBC. The criteria for testing of men should be similar to genetic testing criteria for women [66], and the following individuals should therefore be eligible for testing:

- men without cancer, if they have a FH of breast or ovarian cancer in first- or second-degree relatives with BC diagnosed before the age of 50;
- men with a diagnosis of breast cancer regardless of FH;
- men with a diagnosis of prostate cancer if they have a FH of breast or ovarian cancer in first- or second-degree relative with BC diagnosed before age 50;
- men of Ashkenazi Jewish descent, since the *BRCA* genes mutation prevalence is 2.5% in the general Ashkenazi Jewish population.

To date, fewer men than women have pursued *BRCA1* or *BRCA2* testing, most likely due to the misinformation about cancer risk in men. Generally, men have a clear understanding of genetic testing and often, rather than for their own cancer risk, their principal motivation for seeking it is concern for their families and children, specifically for their daughters [68]. In fact, male carriers of *BRCA1* and *BRCA2* mutations have an increased risk of developing breast, prostate and other cancers [66]. There are therefore important management implications for male *BRCA* carriers and there is a need to promote cancer screening recommendations, particularly with regard to breast and prostate cancer, to male carriers of *BRCA* mutations who are undergoing genetic counseling.

6. Histopathological features

About 90% of all male breast tumors prove to be invasive ductal carcinomas [11]. Since the male breast lacks terminal lobules, unless it is exposed to high doses of endogenous and/or exogenous estrogens, the lobular histotype accounts for only 1.5% of invasive cancers, whereas in women more than 10% of all breast carcinomas are lobular [11,12]. The lobular histotype has been reported in association with Klinefelter's syndrome [69]. In situ ductal and lobular in situ carcinomas account for almost 10% of all male breast carcinomas [11,70,71]. The vast majority of MaleBCs are low grade (68–78% G1–2) [72].

In large studies MaleBC has been found to express high levels of hormone receptors. The estrogen receptors are more likely to be positive in MaleBC than in FBC (80–90% vs. 75%) as are the progesterone receptors (73–81% vs. 65.9%), with evident therapeutic returns [73–77]. The proportion of hormone-receptor-expressing tumors increases with age, as occurs in post-menopausal women [11]. The expression of androgen receptors ranges from 39 to 95% according to the various reports in literature [1,78,79].

With regard to the over-expression of the proto-oncogene HER2/neu, it should be borne in mind that it is less likely to be present in MaleBC (about 5%) than in FBC (about 15%) [80,81]. Even though previous studies have reported equivalent over-expression rates for both sexes, it should be noted that they were performed prior to the standardization of the assessment method, thus leading to a possible overestimation of the findings [82,83]. Recently, an immunohistochemical HER2 expression has been found in about 15% of MaleBCs, confirmed by FISH in all cases presenting a 3+ Herceptest [84]. Furthermore, it has been observed that the HER2/neu status of the metastatic lesions may differ from that of the original primary tumor [85].

At present, little is known of the immunophenotypic characteristics of MaleBCs stratified according to *BRCA1* and *BRCA2* mutation status. *BRCA2*-related MaleBCs seem to show a significant association with HER2 over-expression and have higher histological grades [86]. These data suggest that specific phenotypic characteristics, indicative of aggressive behavior, could be associated with *BRCA2*-linked MaleBCs.

7. Clinical characteristics and diagnostic work-up

The most common clinical sign of breast cancer onset in men is a painless palpable retroareolar lump [87]. Other initial symptoms may include nipple involvement, with retraction and/or ulceration and/or bleeding, and axillary lymphoadenopathies [74,77,87–90]. The association between gynecomastia and MaleBC has been studied and a similar incidence has been found in MaleBC pts when compared to the general population [6,91].

The majority of pts (over 40%) presents with stage III/IV disease [1], often due to an early chest wall spread, not only as a consequence of low public awareness, but also with the scarcity of male breast parenchyma. It is interesting to note that the proportion of advanced stage disease reaches 50–60% when North African series are involved [92].

Clinically suspicious lesions referred for imaging should first be evaluated with mammography and with ultrasonography scans to select pts who will undergo to FNA or core biopsy (Fig. 1). Mammography can identify malignant breast

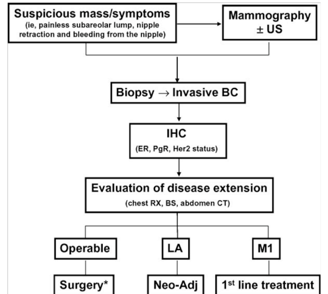

Fig. 1. Algorithm for the management of suspicious male breast mass. US = ultrasonography; BC = breast cancer; IHC = immunohistochemistry; ER = estrogen receptor; PgR = progesterone receptor; BS = bone scan; LA = locally advanced disease; Neo-Adj = neo-adjuvant treatment; M1 = metastatic disease; * = post-op treatment in Fig. 2.

tumors with a sensitivity of 92–100% and a specificity of 90% [93–95]. US of the axillary region could be helpful for staging as long as more than 50% of pts have positive axillary nodes at diagnosis [74].

8. Prognostic evaluation

Overall, men experience a worse prognosis than women [96], probably due to an advanced stage at diagnosis together with the higher age of male patients often leading to the coexistence of serious comorbidities. The overall 5- and 10-year survival rate of MaleBC patients are around 60 and 40%, respectively [11]. Nevertheless, when male or FBC pts are matched with respect to age and stage, no significant difference in terms of DFS or OS between the sexes is observed [97].

The number of histologically positive axillary nodes and the tumor diameter are significant prognostic factors [11]. The higher the number of lymph node metastases, the more unfavorable the prognosis will be. In fact, the survival rates at 5 years has been reported to be 90% for patients with node negative disease, 73% for those with 1–3 positive nodes and 55% for the group with 4 or more involved nodes [98]. It has to be mentioned that axillary nodes involvement has been reported in about the 50–60% of cases [99].

Another negative prognostic factor is the advanced age at the time of diagnosis, since the increased presence of comorbidities may limit the possibility of treatment [77,100]. Thus, the disease-specific survival (DSS) rates should be considered [74,98]. In a large French series, 5- and 10-year OS rates of 65 and 38%, respectively, were reported, whereas the DSS rates were 74 and 51%, respectively. In fact, only 113 (60.5%) out of the 187 deceased pts, died of breast cancer [74].

9. Locoregional treatments for male breast cancer

To date there are no published data from prospective randomized trials supporting a specific therapeutic approach in MaleBC. Most of the information regarding locoregional treatment derives from retrospective studies or those performed by individual institutions, with all the potential biases deriving from an analysis of data collected over a time span of several decades. This means, therefore, that almost all the treatment strategies that have been progressively adopted in MaleBC are based upon data resulting from female studies. A review of literature clearly shows that changes in treating MaleBC mirror the evolution of FBC care.

9.1. Surgery

Surgery is the cornerstone of treatment of MaleBC pts [75]. Until the 1970s, as for FBC, radical mastectomy was the treatment of choice for MaleBC; this approach was subsequently progressively substituted by less invasive surgical procedures, such as modified radical mastectomy, according to lesion extension [75,101,102].

Initial reports suggested that a less invasive approach might possibly have little effect on the patient's outcome [103–105]. More recently, in a retrospective study with 397 MaleBC cases, this topic has been reopened by Cutuli et al., who have reported that radical mastectomy is of no more value than modified radical mastectomy in terms of local relapse [74].

Since breast conservation has become the standard for the surgical management of FBC [106–110] new interest in minimally invasive surgical procedures has also arisen in the treatment in male pts.

Conservative breast surgery followed by radiotherapy, proposed in selected pts for the treatment of small tumors, has produced encouraging results, although there may be several technical difficulties when the procedure is used in males [111]; in fact, a larger tumor size and a higher rate of chest wall infiltration are found compared to female pts [112]. Moreover the usual central or retroareolar localization of the primary tumor in men, together with the paucity of the male breast parenchyma, makes a partial resection difficult to be planned. Nevertheless, in selected situations, for example when the breast tumor is associated with gynecomastia, even a lumpectomy could be a rational approach [111].

Radical mastectomy often leads to widespread skin removal, consequently causing problems in the management of the chest wall defect. Different options have been proposed such as the use of a transverse thoracoepigastric skin flap [113]. Other authors have suggested that a transverse rectum abdomini myocutaneous (TRAM) flap may be the best choice for male breast reconstruction, not only because it is

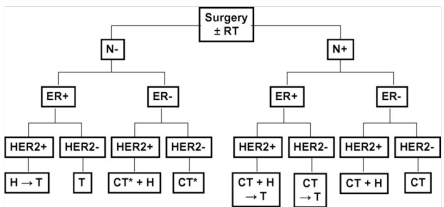

Fig. 2. Algorithm for the treatment of early male breast cancer. RT = radiotherapy; N = node involvement; ER = estrogen receptor; HER2 = epidermal growth factor receptor 2; T = tamoxifen; H = trastuzumab; CT = chemotherapy; * = consider CT according to risk level.

able to replace the missing skin and fat but also because it may be a source of hair-bearing skin similar to that of the male breast [114]. When the surgical wound is closed, the nipple can be reconstructed surgically or simply tattooed to restore the body image.

As for breast surgery, the surgical management of axillary lymph nodes has also undergone changes over the past years. Since axillary node involvement is one of the most relevant prognostic variables in MaleBC as in FBC [11], axillary lymph node dissection has been performed as part of the adjuvant treatment, but it is consistently associated with many late complications (i.e. lymphedema, paresthesias and reduced motility of the upper limb) [115].

Since several studies in FBC have shown that sentinel lymph node biopsy (SLNB) can reliably predict the status of axillary nodal involvement, so preventing useless larger dissections and ameliorating the quality of life [116], a minimally invasive approach has also became the standard treatment for men pts [117,118].

The first report regarding SLNB in a man with BC, was published by Hill et al. from the Memorial Sloan Kettering Cancer Center [119]. Larger single institution series, overall including <200 pts, have subsequently been collected by the leading American and European centers for breast cancer care, suggesting that SLNB in MaleBC pts is an extremely accurate tool providing a sentinel lymph node detection rate close to 100% [120–123]. The use of this technique could be indicated in pts with tumor size <2.5 cm and without clinical evidence of axillary node involvement [124].

9.2. Adjuvant radiotherapy

As MaleBC frequently presents at an advanced stage with early nodal involvement, locoregional relapse rates after surgery alone are quite high. In a comparative study published in the late-1990s by Scott-Conner, analyzing stage-specific differences in contemporary treatment strategies for highly comparable breast cancer pts of both sexes treated between 1985 and 1992, it was reported that radiotherapy after surgery was preferentially given to males [125].

Nevertheless, a subsequent large retrospective analysis of MaleBCs diagnosed between 1995 and 2005 have showed that, to date, male pts are more likely not to receive adjuvant radiotherapy compared to women [112].

Unfortunately, it is difficult to properly evaluate the real impact of adjuvant radiotherapy in MaleBC pts in terms of DFS and OS since most of the papers dealing with the question are statistically underpowered [96,126,127].

Notwithstanding this, several retrospective single institution studies have reported an excellent rate of local control after radiotherapy. Stranzl et al. have obtained a local control rate of 96.8% on a cohort of 31 pts who underwent postmastectomy adjuvant radiation with a 5-year DSS and DFS of 84% and 73%, respectively [128]. Similar results have been reported by Zabel et al. and Ober et al., the former with a local control rate of 96% after postoperative radiotherapy, the latter found that 5- and 10-year rates of local control were 90 and 85%, respectively, on a series of 41 pts [129,130].

Furthermore, these encouraging results concur with the two largest studies published so far. The first one by Cutuli et al. collected 690 pts coming from 20 French institutions over a time span of 30 years. In this series, the overall rate of locoregional relapse among the 496 evaluable pts was 9.5%, with a significant difference between irradiated and non-irradiated pts (7.3% vs. 13%, respectively) [131]. In the second one, on a historical cohort of 428 pts, Ribeiro et al. demonstrated a significant difference in 5-year DFS rates between pts receiving radical mastectomy alone or simple mastectomy plus radiotherapy (44.6% vs. 77.2%, respectively) [77]. Other studies have failed to show a significant impact of RT on local recurrence rates [89].

The drawbacks of all the cited studies should be borne in mind when planning the therapeutic strategy for pts treated outside controlled trials. All these retrospective data, in fact, collected over several decades, are not able to take into account the huge technical changes in RT planning and delivery. Moreover, RT can be used in association with various types of surgery on both the breast and the axilla and also with a wide range of systemic adjuvant treatments, hence the

same guidelines generally accepted for FBC can be followed [1,89,99,132–134].

Adjuvant radiotherapy should be mandatory after breast-conserving surgery and, on the chest wall, after mastectomy in cases of close or positive margins and tumors larger than 1 cm with areola, skin or pectoral muscle involvement. Moreover, histological parameters, such as lymph-vascular space invasion, high tumor proliferation rates, high grade, multifocality and nodal involvement should strongly recommend RT on primary site [124,127,135].

It has been proven that in male pts too, axillary nodal involvement is the most accurate predictor of locoregional failure [127,136] as well as of shorter DFS [75,101] and OS [89,137,138], which indicates that the fixed number of 3 involved axillary nodes requiring additional axillar irradiation in female pts might also be used for male pts [139]. Similarly, supraclavicolar area irradiation should be considered with 4 or more nodes involved.

10. Adjuvant chemotherapy

Whereas reliable data support the use of adjuvant CT in women [140], the few available data regarding men suggest that such strategy might be beneficial even in this subpopulation [141].

Great caution is required given the possibility of increased toxicities due to comorbidities and older age at diagnosis.

Several retrospective series have suggested that the use of adjuvant CT in male pts is associated with a reduced risk of relapse [142–144].

In 1987, Bagley et al. published the results of a small, prospective study involving 24 men with stage II breast carcinoma treated with adjuvant CMF and reported a 5-year survival rate of over 80% [145]. Yildirim and Berberoglu have found an increase of 5-year survival rate in 121 men treated with different regimens [144].

Since MaleBC is a rare disease, it is hardly possible to plan and carry out large randomized studies; nevertheless, given the confirmed results regarding FBC and the positive experiences in men, both men and women could share the same guidelines for adjuvant treatment [146]. So that, chemotherapy should be used in the absence or doubt about endocrine-responsiveness and the taxanes may be considered when lymph nodes are involved. Regarding the use of adjuvant trastuzumab, since no specific data exist, its use should be considered according to patients' and tumor characteristics, following FBC guidelines (Fig. 2).

11. Adjuvant hormonal therapy

As previously mentioned, MaleBC expresses hormone receptors in about 90% of cases, which makes adjuvant hormone treatment a basic part of the therapeutic management of the disease (Fig. 2). A great many retrospective studies have, in fact, evaluated the usefulness of tamoxifen, first in the metastatic setting [3], where it has proved to be extremely active, and subsequently in the adjuvant setting, where it has been associated with a reduction of the relapse and mortality rates [75,77,147,148]. Goss et al. in particular have reported a significant increase, both in DFS and OS, in a series of MaleBC pts treated with hormone therapy, even though often administered for <2 years [75]. Another study including 39 men with stage II/III BC has shown a 5-year survival rate of 61% in pts treated with adjuvant tamoxifen for 1 or 2 years, vs. 44% in the control cases [77]. Interestingly, in both these experiences the duration of the adjuvant therapy was shorter than the normal standard of 5 years; both these studies, therefore, might even have underestimated the real benefits deriving from adjuvant tamoxifen.

Moreover, in a recent British observational study, performed between 2002 and 2003 to evaluate the management of men with breast carcinoma, it has been noted that 126 pts out of the considered 161 (78%) had received adjuvant tamoxifen [149].

Tamoxifen has proved to lead to an increase in survival rates in women with hormone-responsive disease and to date is generally considered the standard adjuvant treatment for hormone-dependent MaleBC. The tolerance of the drug has not been sufficiently studied in men; its main side effects are deep venous thrombosis, reduction of libido, impotence, mood changes and hot flushes [150].

With regard to aromatase inhibitors, even fewer studies have been performed to evaluate their role in the adjuvant setting; in fact, preclinical data have led to doubts regarding their usefulness. When used in healthy male volunteers, anastrozole has not proved to bring about the complete estrogenic suppression it usually provides in women: only a 50% reduction of estradiol plasma levels associated with an increase in testosterone levels in the 58% of cases has been observed [151]. On the contrary, encouraging results have been obtained in two pts treated with letrozole for metastatic disease: an objective response has been obtained in both cases (one with complete response) [152,153].

To date, the use of aromatase inhibitors and/or GnRH analogues cannot be included in the adjuvant treatment strategy for men with breast cancer.

12. Neoadjuvant therapies

The main indications for the use of neoadjuvant treatments are the presence of an ulcerated neoplasia, its fixation to the surrounding tissues, a state of advanced lymph node involvement and the possibility of avoiding surgical treatment which would modify the body structure [134]. A further advantage is that it makes it possible to observe the drug efficacy *in vivo*: it is now known that those pts who achieve a histopathological complete response to neoadjuvant therapy generally have a more favorable prognosis. Since no specific data on this topic for MaleBC exist, FBC guidelines should be followed man-

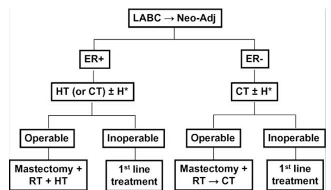

Fig. 3. Algorithm for the treatment of locally advanced inoperable male breast cancer. LABC = locally advanced breast cancer; Neo-Adj = neo-adjuvant treatment; ER = estrogen receptor; HT = hormonal therapy; CT = chemotherapy; H* = trastuzumab depending on HER2 status; RT = radiotherapy.

aging eventual peculiar situations. The choice of treatment depends essentially on the biological features of the tumor (Fig. 3).

13. Treatment of metastatic disease

In the past, the traditional management of metastatic MaleBC consisted in surgical interventions causing hormonal status modifications, such as orchiectomy, adrenalectomy or hypophysectomy, which did, in fact, lead to a positive response in 55–80% of the cases, depending on the performed procedure [1,154–158]. Obviously, these surgical approaches were effective only in the majority of pts with hormone-responsive breast carcinomas. Nowadays these methods have given way to various types of additive hormone treatment, the most important being tamoxifen, which leads to a response in about 50% of cases [159]. There have been reports of even complete response to LH–RH analogues, with or without antiandrogens [160–162]. Other possibilities to take into consideration include androgens, progestins, corticosteroids and high doses of estrogens, in order to obtain response rates ranging from 32 to 75%, according to the chosen drug [1]. The role of fulvestrant remains undetermined for MaleBC pts.

As already mentioned in the section regarding adjuvant therapy, the role of aromatase inhibitors in MaleBC has not yet been sufficiently evaluated and is therefore still not fully understood, although encouraging results have been obtained from single institution experiences [152,153,163].

In spite of the fact that the mean onset age in males is higher than in females, this alone cannot be considered as a valid criterion for excluding chemotherapeutic management; treatment choice should depend upon the clinical and biological features. At the present time, chemotherapy should be addressed to hormone-refractory disease, to young men and to cases of aggressive tumors, for example those with visceral metastases. It should be borne in mind that chemotherapy might also have a significant palliative effect [164]. Since very few reports can be retrieved from literature, there is no standard chemotherapeutic regimen, with response rates ranging from the 13% of 5-fluorouracile alone to the 67% of the combination of 5-fluorouracile, doxorubicin and cyclophosphamide [159].

With regard to male pts with HER2/neu over-expressing tumors, they should be treated with trastuzumab, on the basis of data coming from FBC both in the adjuvant and in the metastatic settings [165–168].

Practice points

- Major risk factors for the development of MaleBC include clinical disorders carrying hormonal imbalances, radiation exposure and a strong FH for BC.
- MaleBC can be linked to mutations in BRCA or in low-penetrance genes (i.e. CHEK-2).
- Men with BC should be referred for genetic counseling and potential genetic testing.
- Most MaleBCs are advanced stage ductal invasive carcinomas.
- MaleBC expresses hormone receptors in about 90% of cases and is less likely to over-express HER2/neu than FBC.
- Locoregional approaches include surgery and RT depending upon the initial clinical presentation.
- Systemic treatment must be administered according to the tumor biology:
  - Tamoxifen is the recommended therapeutic option for hormone sensitive MaleBCs, either as adjuvant or metastatic first-line treatment. Data on the efficacy of other hormonal therapies are not yet definitive, even though positive experiences have been reported.
  - CT should be prescribed in the absence or doubt about endocrine-responsiveness.
  - HER2/neu over-expressing tumors should be treated with trastuzumab.

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