NF-κB localization in multiple myeloma plasma cells and mesenchymal cells

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A B S T R A C T

Several reports demonstrated that the activation of Nuclear Factor-kappa B NF-κB is essential for the pathogenesis of multiple myeloma (MM). We analyzed the nuclear localization of NF-κB in MM-cells derived from 60 different patients with MM at presentation and in relapse, as well as in three myeloma cell lines. Nuclear localization (the active form) of NF-κB was detected in only one MM-sample from a refractory patient and in two samples from relapsed patients, while all the other samples, including the MM-cell lines, almost exclusively express the cytoplasmic (inactive) form of NF-κB. In mesenchymal cells from MM-patients NF-κB was clearly present in the nucleus. In addition, the proteasome inhibitor Bortezomib, which is described to antagonize NF-κB activity, had a consistent antitumor activity against both chemoresistant and chemo-sensitive MM-cells, regardless the NF-κB localization, thus suggesting the existence of other molecular targets of proteasome inhibitors in MM.

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1. Introduction

Multiple myeloma (MM) is a clonal B-cell malignancy characterized by accumulation of terminally differentiated B-lymphocytes, malignant plasma cells (PCs), within the BM. MM PCs localize into the BM where cell adhesion-mediated autocrine or paracrine activation of different cytokines, such as interleukin 6 (IL-6) and insulin-like growth factor 1 (IGF-1), results in PCs accumulation [1]. A major role in the pathogenesis of MM is attributed to NF-κB [2].

The NF-κB family of transcription factors is composed by an array of homo- and heterodimers (containing p50, p52, c-Rel, p65/RelA, and RelB). These proteins are held in the cytoplasm of most normal cells as an inactive latent form by specific proteins, the inhibitors of NF-κB (IκBs). Several stimuli activate NF-κB through kinase-dependent phosphorylation of IκB and its subsequent degradation by the 26S proteasome [3,4]. This process leaves NF-κB free to translocate into the nucleus, where it activates the transcription of numerous genes, such as cyclin D1 [5], Bcl-xL, and IAPs [6], and activates numerous transcriptional processes. The active form of the transcription factor plays an important role in inflammation, immune and stress responses, oncogenesis, cell migration, and angiogenesis.

Two major pathways lead to the activation of NF-κB: a classical and an alternative pathway. The classical pathway is defined by activation of p50–p65 complexes upon degradation of associated IκB. The alternative pathway is characterized by processing of an inactive p100–RelB dimer to active p52–RelB through proteosomal degradation of inhibitory C-terminal IκB-like sequences of NF-κB2 p100 [7,8].

In both pathways, NF-κB activation can be effectively blocked by proteasome inhibitors, which interrupt NF-κB translocation from the cytoplasm to the nucleus by inhibiting degradation of IκB proteins (Fig. 1). This mechanism has been demonstrated in different malignancies, where NF-κB is aberrantly activated and contributes...
The classical pathway
Proinflammatory cytokines, viruses, antigen receptors

The alternative pathway
Cytokines LTβR, BAF, CD40L

IKK2

IKK1

Inflammation
Lympoid organogenesis

NF-κB activation pathways.
The so-called classical/canonical NF-κB pathway is triggered by many inflammatory stimuli to induce IKK2-containing IKK complexes that specifically phosphorylate the three canonical κB proteins, thereby marking them for ubiquitination and proteasome-mediated proteolysis. Cytoplasmic RelA (A) as well as cRel-containing dimers are thereby released to translocate to the nucleus and activate gene mains involved in inflammatory processes. The alternative/non-canonical pathway is mediated by IKK1, is strictly dependent on IKKs homodimers and is activated by lymphotoxin β receptor (LTβR), B cell-activating factor belonging to the TNF family (BAFF), and CD40 ligand (CD40L). This pathway induces the release of RelB (B)-containing dimers to the nucleus where NF-κB plays a central role in the expression of genes involved in development and maintenance of secondary lymphoid organs. Crosstalk between canonical and non-canonical signalling pathways is of current research interest.

to the drug resistance, and where proteasome inhibitors block κB proteasomal degradation, thus inhibiting NF-κB nuclear translocation [9,10].

Moreover, it has been widely demonstrated that proteasome inhibitor Bortezomib induces apoptosis and reverses drug resistance of MM cells by affecting cytokine circuits, cell adhesion and angiogenesis in BM microenvironment. However, there are several experimental evidences that Bortezomib may also acts with mech-

angiogenesis in BM microenvironment. However, there are several

tance of MM cells by affecting cytokine circuits, cell adhesion and

inhibitor Bortezomib induces apoptosis and reverses drug resis-

tolerance from patients with MM at diagnosis or at relapse who gave informed consent

and were managed at the Division of Hematology, Ospedale Ferrarotto, University of Catania. One sample of MM PCs was obtained from the paracentesis of one MM patient resistant to chemotherapy with neoplastic ascites. All samples were selected for PCs infiltration from 50 to 99%. BM biopsies were fixed with 10% buffered formalin or fixed and decalified in Lowy’s solution. Four micron sections were dewaxed and antigen retrieval was carried out by treating sections for 30 min in pH 6 citrate buffer at 98 °C. BM aspirates were collected in tubes containing EDTA and cells were isolated by Ficoll Hypaque (Cedarlane labs, Ontario, Canada) density gra-

ditrified centrifugation. The CD138+ cells were separated as described by Hata et al. [11].

Cells were maintained in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 2 mM-l-glutamine and 1000 µl penicillin–streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Cells were kept in a 5% CO 2 atmosphere at a density of 5 × 10 6 cells/ml. BM mononuclear cells separated by Ficoll-Hypaque density sedimentation were also used to establish long-term BM mesenchymal cells (MCs) cultures as described previously [12]. Briefly, BM cells were cultured (1 × 10 6 cells/ml) for 24 h to obtain a confluent adherent cell mono-

layer of BM MCs. After BM MCs formed a confluent adherent layer, remaining non-adherent cells were washed with PBS.

2.2. Cell lines

The human MM cell lines KG-1, RPMI-8226 and KMS18 both plasmocytomas of B-cell origin, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in RPMI 1640 medium (Gibco, Invitro-

gen) supplemented with 2 mM-l-glutamine and 1000 µl penicillin–streptomycin and 10% FBS (Invitrogen) and were kept in a 5% CO 2 atmosphere at a density of 5 × 10 6 cells/ml.

2.3. Reagents

Drugs were purchased from Sigma–Aldrich (St. Louis, MO) and resuspended in DMSO (Etospose) or in water (Cytarabine, Daunorubicine and Bortezomib). Borte-

zomb (also known as PS-341) was purchased from Millennium Pharmaceuticals (Cambridge, MA).

2.4. Cell viability assay

Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufac-

turer’s instructions. Cells were seeded into 3 wells of 96-well plates at 5 × 10 4 cells/well and treated with different combinations of the drugs tested. After 24 and 48 h of incubation the culture medium was removed and the cells were washed with PBS (ph 7.4); 100 µl of fresh culture medium without drugs and 20 µl of assay solution were added to each well and the cells were incubated for 3 h. The plates were read on a Microplate Reader (Synergy HT, BIO-TEK). Survival was expressed as the percentage of viable cells in the treated sample versus the untreated control cells.

2.5. Flow cytometry

10 6 BM aspirate cells were washed with cold PBS containing 1% BSA and incubated for 1 h at 4 °C with control or specific primary antibodies: goat anti-

human CD138 (1:50, R&D Systems, Minneapolis, MN). After washing, the cells were incubated for 40 min at 4 °C with phycoerythrin-conjugated anti–goat secondary antibodies (1:100, Jackson Laboratories, West Grove, PA). Labeled cells were washed twice with PBS/BSA and the fluorescence intensity was evaluated by a FACScan (Beckman Coulter, Fullerton, CA).

2.6. Immunostaining procedure

Immunohistochemical staining was performed on 2-μm thick paraffin-em-

bedded BM biopsies. After deparaaffination-hydration, sections were permeabi-

lized with PBS containing 0.4% Triton X-100 for 30 min and blocked with PBS containing 5% BSA for 30 min. The samples were incubated, over night at 4 °C, with rabbit anti-human NF-κB/p65 primary antibody (1:50, Santa Cruz). The day after, slides were first incubated for 1 h with biotinylated anti-goat secondary antibody, and then with streptavidin-HRP (Dako Corp., Carpintera, CA). Staining was detected using diaminobenzenedine (DAB) as chromogen. Sections were counterstained with hematoxylin.

Immunofluorescence staining was performed on cells suspension seeded on glass slides. Cells were washed in PBS buffer and fixed with 4% paraformaldehyde for 10 min. After rinsing, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and nonspecific staining was blocked in 4% BSA in PBS for 30 min. Cells were then incubated with rabbit anti-human NF-κB/p65 (1:50, Santa Cruz, CA) for 1 h at room temperature. Subsequently, cells were washed and incubated with Cy3-conjugated mouse anti-rabbit immunoglobulins (1:500 Jackson Laboratories). Nuclei were stained with DAPI (Sigma, St. Louis, MO).
2.7. Western Blotting

Western Blot analysis was carried out on proteins isolated after cellular fractionation. Cytoplasmic proteins were obtained by lysing cells in hypotonic buffer (10 mmol/l Trizma Base, 10 mmol/l KCl, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 2× Protease Inhibitor Cocktail, 0.2% NP40). After 2 min incubation in ice, cytosolic proteins were recovered by centrifugation at 2000 rpm for 10 min at 4 °C. Nuclear proteins were extracted resuspending the residual pellet in nuclear lysis buffer (10 mmol/l Trizma base, 10 mmol/l KCl, 100 mmol/l NaCl, 7 mmol/l β-mercaptoethanol, 2× protease inhibitor cocktail), and subsequently harvesting the proteins by centrifugation at 14,000 rpm for 15 min at 4°C. 30 µg cytoplasmic and nuclear proteins were loaded onto 10% SDS-polyacrylamide gels. Gels were electroblotted onto nitrocellulose membranes (Hybond C-extra, Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked for 1 h in 5% nonfat dry milk in TBS-T (2.5 mM Tris–HCl, 15 mM NaCl, 0.05% Tween 20) and then incubated for 1 h with the primary antibody rabbit anti-human NF-κB/p65 (1:50, Santa Cruz), monoclonal anti-Tubulin and polyclonal anti-Histone 2B antibodies (Santa Cruz). Filters were then rinsed and incubated for 1 h with the corresponding secondary antibodies conjugated with peroxidase (Amersham Biosciences). After washing, proteins were detected by the enhanced chemiluminescent method (ECL plus; Amersham Biosciences).

2.8. Statistical analysis

A two-tailed paired t-test was used to analyze the statistical significance of the results. Values of \( p < 0.05 \) was considered statistically significant. One asterisk indicates \( p < 0.05 \), two asterisks \( p > 0.05 \). Data are presented as mean values ± SD of the mean.

3. Results

3.1. Samples

A total of 48 samples from patients with MM at diagnosis and 12 samples from resistant/refractory MM were examined. Patients’
characteristics such cytogenetic features, PCs percentage and prior therapies [13] are detailed in Table 1.

3.2. NF-kB resides in the cytoplasm of PCs from newly diagnosed MM patients

A total of 37 samples from newly-diagnosed (34) or relapsed/resistant (3) MM patients were obtained from BM biopsies. In order to determine the sub-cellular localization of NF-kB, immunohistochemical analysis was performed on formalin fixed-paraffin embedded sections of BM biopsies having a PCs count ranging from 50 to 99%. The analysis revealed almost exclusively a cytoplasmic localization of NF-kB with a very small percentage (less than 2%) of PCs showing a weak nuclear staining (Fig. 2A and Supplementary figure). Accordingly, immunofluorescence experiments performed on 22 BM aspirates from patients at diagnosis (14) or relapsed/resistant (9) (Fig. 2B) and on 3 MM cell lines (XG-1, RPMI8226, KMS18) showed mainly a cytoplasmic localization for NF-kB, indicating the presence of the inactive form of NF-kB (Fig. 2C).

On the contrary, on CD138+ cells obtained from the paracentesis of a MM resistant patient with neoplastic ascites and from two BM aspirates from resistant/relapsed MM patients (PCs percentage was about 98%), immunofluorescence analysis revealed a nuclear localization of NF-kB/p65 (Fig. 2D).

Sub-cellular localization of NF-kB was also studied by Western Blotting analysis in BM PCs of 3 newly diagnosed MM patients. Cytoplasmic and nuclear lysates were isolated from PCs. The different fractions were then immunoblottedted with antibodies against NF-kB/p65, showing a cytoplasmic localization of NF-kB/p65 in the PCs. Western Blots for tubulin and histone 2B confirmed the purity of the cytoplasmic and nuclear extracts (Fig. 2E). The results are representative of 3 independent experiments.

Fig. 2. (Continued)
3.3. NF-κB localization is nuclear in BM mesenchymal cells (MCs) from newly diagnosed MM patients

The bone-marrow microenvironment plays a key role in myeloma cells growth and survival and the interactions of myeloma cells with the microenvironment are widely believed to be critical in the pathophysiology of MM. Several studies have investigated mesenchymal cells from multiple myeloma patients with particular attention to cell surface antigens, cytokines, and growth factors expression, concluding that MM mesenchymal cells are phenotypically and functionally distinguishable from normal donor mesenchymal cells.

On the basis of these studies and the studies that describe a role of new drugs such as Bortezomib counteracting the inter-
action between MM plasma cells and mesenchymal cells [14],
we decide to investigate the NF-κB status of MM mesenchymal
cells.

Sub-cellular localization of NF-κB was therefore studied in
BM MCs from 3 newly diagnosed MM patients. The study
was performed, either by immunocytochemistry and Western
Blotting experiments, on MCs. Immunocytochemistry analysis
carried out on BM MCs, isolated from cell culture by their abil-
ity to adhere to the plate within 48–72 h, showed NF-κB/p65
in the active status by nuclear localization (Fig. 3A). Similar
results were obtained by Western Blotting analysis. Cytoplas-
mic and nuclear lysates were isolated from MCs. The different
fractions were then immunoblotted with antibodies against NF-
κB/p65, showing a nuclear localization of NF-κB/p65 in the MCs
(Fig. 3B) compared to PCs where the signal was clearly visible
in the cytoplasmic fraction (Fig. 2E). Western Blots for tubu-
lin and histone 2B confirmed the purity of the cytoplasmic and
nuclear extracts. The results are representative of 3 independent experiments.

3.4. MM PCs exhibit a great dose-dependent sensitivity to
Bortezomib

We next studied the effect of proteasome inhibitor Bortezomib
on MM PCs. For this purpose, MM PCs were incubated in vitro in the
presence of 0.01 μM Bortezomib for 48 h (Fig. 4A) or in the presence
of decreasing concentrations (10–0.01 μM) of Bortezomib for 24 h
(Fig. 4B). These biologically active concentrations are fully compa-
rable with those achieved in vivo in the clinical setting. Cell viability
was measured by MTS assay. After 24 h of treatment, Bortezomib induced, even at lowest doses (0.01 μM), reduction of
50% of cell viability. At this concentration, Bortezomib in combina-
tion with Melphalan induced 85% of cytotoxicity, while Melphalan
alone reached only 15% of reduction of viability (Fig. 4C) thus con-
firming a recent study on synergistic effect of alkylating substances
with Bortezomib [16].

4. Discussion

In the present study, we have demonstrated, in contrast to other
reports, that at least the p65 (RelA) member of the NF-κB family,
that belongs to canonical pathway, resides mainly in the cytoplasm
(the inactive form) of PCs from all newly diagnosed MM patients,
the majority of relapsed patients and some MM cell lines. We have
found a nuclear localization of NF-κB/p65 (active form) only in
three samples from refractory patients and in BM mesenchymal
cells from MM patients. However, we do not exclude that other
members of the NF-κB family belonging to noncanonical pathway
might be found in the active form (nuclear localization) in the same
MM patients.
Fig. 4. Comparison of Bortezomib with standard chemotherapeutics on MM PCs.

On the other side, we found a consistent dose- and time-dependent antitumor activity of Bortezomib against both myeloma cell lines and all the PCs from both relapsed and at diagnosis MM patients, independently of NF-κB/p65 localization, thus indicating that Bortezomib is able to induce cell death regardless to the NF-κB/p65 localization and suggesting the existence of other molecular targets of proteasome inhibitors in MM. In addition, we have confirmed the synergism of Bortezomib with Melphalan in the cytotoxicity of MM cells.

Constitutive nuclear localization (the active form) of NF-κB has been reported in different solid and haematological malignancies suggesting that targeting the NF-κB pathway may provide novel therapeutic strategies, particularly in haematological diseases [17,18]. We and others have demonstrated that constitutive nuclear localization of NF-κB has been associated with lymphoproliferative diseases such as B cell chronic lymphocytic leukaemia [19], a subgroup of B-cell non-Hodgkin’s lymphoma [20,21], acute myeloid leukaemia [22,23], and it is also likely that NF-κB activation contributes to malignant transformation of myelodysplastic syndromes [24].

Several experiments have clearly demonstrated that the activation of NF-κB contributes to the pathogenesis of MM by regulating the expression of many proteins that act as growth factors for MM plasma cells, of several antiapoptotic genes, of proteins that are involved in angiogenesis and in the interaction between myeloma cells and bone marrow mesenchymal cells. In addition, the clinical success of the proteasome inhibitor [25], Bortezomib, considered to act by inhibiting IkB proteasomal degradation [26], has further highlighted the relevance of NF-κB pathway in MM. However, the mechanisms that activate the NF-κB pathway in MM are not
yet clear. Recently, it has been shown that primary MM samples and MM cell lines may have mutations in genes encoding positive and negative regulators and effectors of NF-κB signaling, leading to chronic NF-κB target gene expression. However, mutations involved in the activation of NF-κB pathway were found in no more than 20% of MM samples and the level of NF-κB signature in MM and MGUS are comparable, on average, to those in normal PCs [27–29]. Ma et al. demonstrated that mutations in the IkB gene from two MM cell lines result in enhanced phosphorylation of IkB and, consequently, reduced NF-κB/IκB binding and nuclear active localization of NF-κB [30]. Activation of the NF-κB pathway in MM cells by mutation has been described only in a single MM patient with an activating mutation of NF-κB2 by Fracchiolla et al. [31]. Other authors have examined the potential structural alterations of the RelA gene in 50 MM patients but they found a single point mutation in just one patient, concluding that RelA gene alterations are involved only rarely in MM pathogenesis [32]. Indeed, nuclear localization of NF-κB has been shown in MM resistant cell lines and several studies correlated the expression of constitutively active NF-κB in MM primary or cell lines with chemoresistance of malignant MM cells [33,34]. In the majority of these studies experiments were performed with MM drug-resistant or cytokine-treated cell lines. Ni et al. have reported a nuclear localization of NF-κB in MM PCs but they have examined 13 biopsies of MM patients without describing the phase of disease and the nuclear localization shown in the manuscript is a rare staining in comparison with the percentage of malignant PCs infiltration indicated [35]. Bharti et al. have examined 4 MM cell lines and 22 MM samples in which a clear nuclear localization of p65 was found just in 4 cases by immunofluorescence and 3 by EMSA [36].

Our results diverge also from those of Markovina et al. [37] who found evidence of NF-κB activation in most plasma cell samples. In addition, such activation was insensitive to Bortezomib treatment in the majority of samples. The authors indicate the PIR (proteosome inhibitor-resistant) pathways as an alternative mechanism activating NF-κB in the samples studied. However, disease stage, treatment history and/or response, and other identifying patient characteristics are not available for the samples analyzed in this study. Reports from the literature on NF-κB localization in MM are summarized in Table 2.

Therefore, the outcome from these and other papers, was that NF-κB pathway is constitutively active in MM and that Bortezomib anti-MM activity is strictly due to inhibition of NF-κB by preventing proteasome degradation of IκBα.

However, our results and a recently published paper of Hideshima strongly argue against this dogma.

Hideshima et al. have demonstrated that Bortezomib in MM cell lines and primary cells, while exerts an high cytotoxic effect, actually activates 2 upstream NF-κB-activating kinases (RIP2 and IKKβ), promotes down-regulation of NF-κB’s inhibitor (IκBα), and increases NF-κB DNA binding in vitro, thus improving NF-κB activity [38–40].

Activation of NF-κB is a dynamic phenomenon that can be induced by cytokines, chemokines, growth factors, and contact of cells belonging to the microenvironment and we do not doubt of its role in the pathogenesis of MM. However, in agreement with the new current dogma, we demonstrated that in a steady state, especially in patients at diagnosis, NF-κB resides mainly in the cytoplasm (the inactive form) but the PCs maintain the sensitivity to Bortezomib. Indeed we cannot exclude that a small amount of p65 (RelA) in the nucleus of MM cells it is not detected by the methods we have used in our experiments and this may contribute to the Bortezomib activity we observed. However, our results provide believable evidence that blocking of IκB degradation is not the only mechanism responsible for proteasome inhibitor-induced apoptosis and for the proven clinical activity of Bortezomib. In particular several studies demonstrated that NF-κB activation cannot be the unique mechanisms by which the proteasome inhibitor Bortezomib performs its anti-tumor activity. Indeed, Bortezomib as a proteasome inhibitor is able to inhibit the degradation of mult ubiquitinated target proteins, i.e., cell cycle regulatory proteins such as cyclins and cyclin-dependent kinase inhibitors, thus regulating cell cycle progression. In addition, several possible Bortezomib-induced proteasome-independent mechanisms have been described. Among these mechanisms, Landowsky et al. [41] have shown that Bortezomib treatment induces the expression of gene products associated with the endoplasmic reticulum secretory pathway with deregulation of intracellular Ca2+, leading to caspase activation and cell death. Pei et al. [42] and more recently Kikuchi et al. [43], indicate that Bortezomib target the HDAC in MM cells and can be used in association with HDAC inhibitors. Qin et al. [44] described the involvement of p53-independent BH3-only protein NOXA in mitochondrial-based apoptotic pathway induced by Bortezomib. Different studies [45] demonstrate that Bortezomib both directly induces apoptosis of human MM cells and abrogates paracrine growth of MM cells in the BM via altering cellular interactions and cytokine secretion in the BM milieu. The antiangiogenic effect of Bortezomib represents another mechanism of its anti-MM

### Table 2

<table>
<thead>
<tr>
<th>MM Cells</th>
<th>Methods</th>
<th>Results</th>
<th>References</th>
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<tr>
<td>Dexa-resistant cell lines (ARP-1, RPMI8226, AR1177)</td>
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<td>Nuclear</td>
<td>Feinman et al. [2]</td>
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<tr>
<td>MM primary cells U266, HS-Sultan, K620 RPMI8226</td>
<td>IHC, EMSA</td>
<td>Nuclear (2/4)</td>
<td>Ni et al. [35]</td>
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<td>RPMI8226</td>
<td>IF</td>
<td>Cytoplasmic</td>
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<tr>
<td>MM1S, RPMI8226</td>
<td>EMSA</td>
<td>Weakly nuclear</td>
<td>Hideshima et al. [33]</td>
</tr>
<tr>
<td>MM1S</td>
<td>ELISA</td>
<td>Nuclear</td>
<td>Mitsiades et al. [6]</td>
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<td>EMSA, IF</td>
<td>Weakly nuclear</td>
<td>Ma et al. [30]</td>
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<tr>
<td>ARH177</td>
<td></td>
<td>Nuclear</td>
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<td>2G/doxR</td>
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<td>Nuclear (4/22)</td>
<td>Bharti [36]</td>
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<td>Markovina et al. [37]</td>
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</table>

Cell lines are undersigned.
activity [46] that should be considered in its possible therapeutic employment.

Conflict of interest

The authors do not have any conflict of interest.

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Contributions. Conticello Concetta and Raffaella Giuffrida equally contributed to this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2010.06.023.

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