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Bcl-2–Enhanced Efficacy of Microtubule-Targeting Chemotherapy through Bim Overexpression: Implications for Cancer Treatment[1]

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Abstract

Bcl-2 is commonly overexpressed in tumors, where it is often associated with unfavorable outcome. However, it has also been linked to a favorable sensitivity to microtubule-targeting agents (MTAs). We show that Bcl-2–overexpressing lung and breast cancer cells were more sensitive to both paclitaxel and vinorelbine. Bcl-2 overexpression also significantly potentiated *in vivo* efficacy of paclitaxel, in terms of tumor volume decrease and survival benefits, in models of nude mice bearing lung cancer xenografts. To further investigate this favorable effect of Bcl-2, a genomic approach was taken. It revealed that Bcl-2 overexpression induced up-regulation of the proapoptotic protein Bim in lung cancer cells and that, conversely, Bcl-2 silencing decreased Bim expression level. A gene regulation study implicated the transcription factor Forkhead box-containing protein, class O3a in Bim up-regulation. Lastly, we show that Bim was responsible for MTA-triggered lung cancer cell death through a dynamin-related protein 1–mediated mitochondrial fragmentation. The Bcl-2–governed Bim induction evidence offers for the first time an explanation for the favorable higher sensitivity to treatment shown by Bcl-2–overexpressing cells. We suggest that Bim could be a powerful predictive factor for tumor response to MTA chemotherapy. Our data also give new insight into some failures in the efficacy of therapies targeted against Bcl-2.

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Introduction

Microtubule-targeting agents (MTAs) are known to inhibit cancer expansion through both antitumor and antiangiogenic properties. This therapeutic class is used to treat a broad range of solid tumors, including neuroblastoma and lung and breast cancers. It is well known that MTAs commonly disturb dynamics of the microtubule plus ends [1,2] and induce cell death through the mitochondrial apoptotic pathway [3,4]. Whether a cell survives or dies through apoptosis is determined by the relative levels of Bcl-2 family proteins. The antiapoptotic members, such as Bcl-2, secure mitochondrial integrity, whereas the proapoptotic members, such as Bim, facilitate the release of apoptogenic factors from the intermembrane space of mitochondria to cytosol [5]. In MTA-treated cells, the mitochondrial network undergoes very marked morphologic changes, from long tubular to short punctiform structures [3]. This fragmentation may contribute to mitochondrial injury and release of apoptogenic factors in the cytosol [6,7]. The dynamin-related protein 1 (Drp1) is crucial for fission [8,9], but growing evidence suggests that members of the Bcl-2 family may also be involved in the regulation of the mitochondrial network organization [10,11].

Overexpression of Bcl-2 is commonly found in various types of cancer and is generally regarded as a biomarker of resistance to both radiotherapy and chemotherapy [12–14]. Accordingly, targeted therapies directed to Bcl-2, such as the Bcl-2 antisense oligodeoxynucleotide oblimersen, are now available for phase I to III clinical

Abbreviations: MTAs, microtubule-targeting agents; Drp1, dynamin-related protein 1; FoxO, Forkhead box-containing protein, class O

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trials [15]. However, these therapies have produced inconsistent results. For example, addition of oblimersen to carboplatin and etoposide did not improve clinical outcome measured in patients with small cell lung cancers [16]. Recent studies in breast and prostate cancers also concluded that the combination of oblimersen with docetaxel did not display any efficacy [17,18].

These discrepant results must be reanalyzed in the light of the controversial role of Bcl-2 in its resistance to anticancer drugs. Bcl-2 overexpression paradoxically enhanced *in vitro* sensitivity to docetaxel in non–small cell lung cancer [19] and to vinca alkaloids in breast cancer cells [20]. Consistent with this finding, low Bcl-2 expression levels were responsible for ovarian cancer cell resistance to paclitaxel and vinflunine, while reintroducing Bcl-2 restored cell sensitivity to treatment [21,22]. All these data support an equivocal role for Bcl-2 in the mechanism of action of MTAs. Conversely, there is no doubt about the involvement of Bim in MTA-induced apoptosis [23–25]. In healthy cells, Bim can bind to LC8 and is thereby sequestered to the microtubule-associated dynein motor complex [26]. In a previous study, we showed that MTA treatment caused Bim translocation to mitochondria to trigger neuroblastoma cell death. Studies that aim at deciphering the molecular mechanisms underlying Bim activation during MTA-induced apoptosis have revealed an important role for the transcriptional regulation of *bim* gene expression [27]. Bim transcription is mainly controlled by transcription factors of the Forkhead box-containing protein, class O (FoxO) family, which are inhibited by phosphorylation through phosphatidylinositol 3 kinase (PI3K), AKT, and serum and glucocorticoid-inducible kinases (SGKs) [28,29].

The aim of this study was to decipher the role of Bcl-2 in human cancer cell sensitivity or resistance to MTAs. The study design, using bcl-2 gene transfer in tumor cell lines of different origins (lung, breast, and neuroblastoma), enables us to efficiently induce Bcl-2 overexpression, as observed in many patients [30–32]. This study may help us determine the best treatment for tumors that intrinsically overexpress Bcl-2 and so improve the outcome of these patients. First, we found that Bcl-2 overexpression selectively increased efficacy of paclitaxel and vinorelbine in lung and breast carcinoma cells. This finding was validated in nude mice bearing non–small cell lung cancer xenografts, where Bcl-2 overexpression potentiated *in vivo* paclitaxel efficacy. A genomic approach then showed that Bcl-2 overexpression was responsible for a significant increase in *bim* expression levels. We also found that Bim up-regulation was mediated by FoxO3a activation. Lastly, we show that Bim involvement in cell sensitivity to treatment relies on its ability to trigger the mitochondrial network fragmentation. We thus offer, for the first time, an explanation for the higher sensitivity to MTAs displayed by Bcl-2–overexpressing cancer cells. Taken together, our data strongly suggest that Bim could serve as a biomarker of choice in predicting treatment efficacy.

Materials and Methods

Cell Lines and Reagents

Human non–small cell lung carcinoma (A549), neuroblastoma (SHEP), and breast cancer (MCF-7) cells were purchased from ATCC (Molsheim, France). These three lines were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with an empty plasmid pUse (Upstate Biotechnology, Lake Placid, NY) for control cells (A549 pUse, SHEP pUse, or MCF-7 pUse) or with a plasmid containing human Bcl-2 cDNA (pUse Bcl-2 vector) kindly given by Ferlini [21]. Various stable transfectants were obtained after geneticin selection (A549 Bcl-2, SHEP Bcl-2, and MCF-7 Bcl-2). All these lines were routinely cultured in RPMI supplemented with 10% FBS, 2% L-glutamine, and 1% penicillin and streptomycin, at 37°C with 5% CO_2. A stock solution of paclitaxel (Alexis, Lausen, Switzerland) was prepared in DMSO. Vinorelbine (Sigma, Steinheim, Germany) and doxorubicin (Dakota, Paris, France) were prepared in aqueous solutions.

Transfection Studies

siRNA Bim (signalSilence Bim siRNA I; Cell Signaling Technology, Beverly, MA) and siRNA Bcl-2 (HP validated siRNA 1027400, target sequence: AAC CGG GAG ATA GTG ATG AAG; Qiagen, Hilden, Germany) were transfected into A549 Bcl-2 cells. SiRNA Bcl-2 was designed and validated to minimize the risk of off-target effects. siRNA Bim specifically inhibits Bim expression using RNA interference, a method whereby gene expression can be selectively silenced through the delivery of double-stranded RNA molecules into the cell. Nontargeting siRNA [Signalsilence control siRNA (Cell Signaling Technology) concerning Bim studies and AllStars Negative control siRNA (Qiagen) for Bcl-2 studies] was used as controls for non–sequence-specific effects of the transfected siRNA.

A549 wild-type cells (A549 w) were transfected with $pcDNA_6$ plasmid ($pcDNA_6$) or containing Bim_{EL} cDNA ($pcDNA_6$-Bim), kindly given by A. Vazquez [33]. These transfections were achieved using Lipofectamine 2000 (Invitrogen) according to the protocol supplied by the manufacturer.

Cytotoxicity Assay

Cells were seeded in 96-well plates to be treated for 48 or 72 hours with different agents: paclitaxel, vinorelbine, and doxorubicin. Doses of MTAs used to treat cells *in vitro* were in the range of plasma concentrations measured in the patient [34]. For A549 Bcl-2 cells, we tested the effect of mdivi-1, a small-molecule mitochondrial division inhibitor, at 1 μM with paclitaxel. Growth inhibition was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. This colorimetric assay allows the monitoring of cell survival *in vitro*. Each experiment included vehicle-treated cells (controls) that proliferate following an exponential model. For each dose of anticancer drug tested, results were expressed as percentage of survival cells, according to the following equation: $OD_{\text{treated cells}} \times 100/OD_{\text{control cells}}$.

Animal Studies

All the experimental procedures and animal care complied with French official guidelines. Female BALB/c nude mice 6 to 8 weeks old were obtained from Charles River Laboratories (L'Arbresle, France). The animals were housed in a temperature- and humidity-controlled room with a 12-hour on-off light cycle and given free access to food and water.

A total of 5×10^6 A549 pUse or A549 Bcl-2 cells were inoculated subcutaneously into the right dorsal flanks of the nude mice. Experimental treatments were started when tumors reached about 50 mm^3 in volume (day 0). Mice were then randomly divided into four groups: group 1 (A549 pUse–bearing mice, $n = 8$) and group 2 (A549 Bcl-2–bearing mice, $n = 7$) received vehicle solution (control groups); group 3 (A549 pUse–bearing mice, $n = 8$) and group 4 (A549 Bcl-2–bearing mice, $n = 7$) were injected with paclitaxel.

We selected an administration schedule of consecutive daily intravenous (i.v.) injections for 5 days for paclitaxel, based on effective similar schedules previously published [35,36]. Paclitaxel was given to tumor-bearing mice at a dose of 24 mg/kg/day, slightly less than