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Apoptosis and TRAF-1 cleavage in Epstein-Barr virus-positive nasopharyngeal carcinoma cells treated with doxorubicin combined with a farnesyl-transferase inhibitor

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Abstract

Epstein-Barr virus (EBV)-associated nasopharyngeal carcinomas (NPC) are much more sensitive to chemotherapy than other head and neck carcinomas. Spectacular regressions are frequently observed after induction chemotherapy. However, these favorable responses are difficult to predict and often of short duration. So far there have been only few experiments to investigate the mechanisms which underline the cytotoxic effects of anti-neoplastic drugs against NPC cells. In addition, these studies were performed almost entirely on EBV-negative cell lines therefore not truly representative of NPC cells. For the first time, we have used two EBV-positive NPC tumor lines derived from a North African (C15) and a Chinese (C666-1) patient as *in vitro* targets for a panel of anti-neoplastic agents. Doxorubicin, taxol and in a lesser extent *cis*-platinum efficiently inhibited NPC cell proliferation at clinically relevant concentrations, but all three agents failed to induce apoptosis. However, massive apoptosis of C15 cells was achieved when doxorubicin (1 μM) was combined with a farnesyl-transferase inhibitor, BIM 2001 (5 μM). Moreover, this apoptotic process was associated with a caspase-dependent early cleavage of the TNF-receptor associated factor 1 (TRAF-1) molecule, a signaling adaptor which is specifically expressed in latently EBV-infected cells. TRAF-1 cleavage might become a useful indicator of chemo-induced apoptosis in EBV-associated NPCs.

Keywords: Nasopharyngeal carcinoma; TRAF-1; Doxorubicin; Farnesyl-transferase inhibitors; Chemotherapy; Apoptosis

1. Introduction

Undifferentiated NPC have several remarkable features which make them unique among human epithelial malignancies [1]. They are rare in most countries but they occur with a high incidence in some selected areas, especially Southeast Asia and North Africa. They are consistently associated with the EBV regardless of patient geographic origin. On tissue sections, NPC appears to be heavily infiltrated by non-malignant lymphocytes mostly of the T-lineage. The full length genome of EBV is contained in all malignant epithelial cells and consistently encodes several viral products which are likely to contribute to the malignant phenotype. However, there is no production of viral particles in tumor cells; in other words, NPC cells are mainly in a state of ''latent'' EBV-infection. Some of

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Abbreviations: EBERs, EBV-encoded RNAs; EBNA1, Epstein-Barr nuclear antigen 1; EBV, Epstein-Barr virus; Ftase, farnesyl-transferase; GGTase, geranylgeranyl-transferase; FTI, farnesyl-transferase inhibitors; LMP1, latent membrane protein 1; NPC, nasopharyngeal carcinoma; TRAF, TNF-receptor associated factor.

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the latent viral products are the EBNA1 protein and two species of small untranslated RNAs, called EBER 1 and 2. Another EBV-protein called LMP1 is produced in about 50% NPC. Other viral RNA messengers and their corresponding proteins are under investigation [2–4]. NPC oncogenesis is also promoted by cellular gene alterations. Inactivation of the p16/INK4 gene either by homozygous deletion and/or hypermethylation is frequently observed in NPC [5,6]. Chromosome 3p deletions also occurs at a high frequency, suggesting that this region contains tumor-suppressor genes which are critical for NPC-oncogenesis [7]. One of the recently identified tumor suppressors, RASSF1A on 3p21.3, was found to be promoter hypermethylated in about 70% of the primary tumors but not in the normal nasopharyngeal epithelia. Inactivation of both p16 on chromosome 9p21 and RASSF1A on chromosome 3p21.3 was detected in 75–85% of the microdissected NPC primary tumors [8]. In contrast with data recorded in most other human epithelial malignancies, p53 is rarely mutated in NPC [9]. A functional inactivation of p53 has been suspected by some authors but the mechanism of this potential inactivation remains to be elucidated [10,11].

Malignant NPC cells have a short doubling time and a high metastatic potential. Nevertheless, they are prone to enter apoptosis *in situ*. In the majority of cases, the apoptotic index is high on tissue sections of NPCs [12]. In addition, we have shown that NPC cells strongly express CD95 and are exquisitely sensitive to CD95-mediated apoptosis *in vitro* [13]. This vulnerability to apoptosis may in part explain why NPC have a higher sensitivity to radiotherapy and chemotherapy than most other head and neck carcinomas. Radiotherapy is the basic therapeutic arm for treatment of the primary tumors, but in a growing number of cases, it is combined with induction, concomitant or adjuvant chemotherapy [14]. Short term results of induction chemotherapy are often remarkable. The rate of complete remissions is routinely of 10–25% following two to three courses of induction chemotherapy [15,16]. In some studies, the rate of complete lymph node regressions can reach 50% [17]. However, these good responses are unpredictable and often of short duration. In addition, in only a few studies, were good short term responses to induction chemotherapy associated with some improvement in long term survival [15]. So far, despite its major role in the treatment of NPC, the effects of chemotherapy on EBV-positive NPC cells have been poorly investigated *in vitro*. One major reason for this delay is the extreme difficulty to grow NPC cells *in vitro*. For several decades, the only source of experimental NPC material were tumor lines propagated into nude mice as xenografts, although only a small fraction of clinical NPC specimens could be successfully grafted [18]. More recently, there were reports of NPC cell lines propagated *in vitro* but many of these lines do not contain EBV and one can question whether they are truly representative of *in vivo* NPC cells [19,20]. Significant progress was made by derivation from a Chinese NPC xenograft called xeno-666, of a subclone called C666-1 which can be permanently grown *in vitro* and retains the EBV genome [21]. We have also made slow but constant progress in improving short term *in vitro* cultures of cells derived from other NPC xenografts, especially the C15 North African NPC xenograft [13].

This study had three aims: (1) to use C15 and C666-1 cells as prototype targets to assess the short term (72 hr) cytotoxic effects of pharmacological agents currently used in NPC chemotherapy; (2) to investigate whether cytotoxicity was due to apoptotic processes and (3) whether chemo-induced apoptosis of NPC cells could be enhanced by combination with novel molecularly targeted drugs. Among a panel of drugs, we found that doxorubicin, taxol and in a lesser extent *cis*-platinum had short term cytotoxicity on NPC cells *in vitro* at clinically relevant concentrations. Doxorubicin and taxol on their own did not induce apoptosis. However, the cytotoxic effect of doxorubicin was dramatically enhanced when it was combined with a novel inhibitor of human FTase, BIM 2001. In C15 cells, this combination induced massive apoptosis. It was associated with early cleavage of TRAF-1, a signaling protein whose expression is strongly induced by latent EBV-infection.

2. Materials and methods

2.1. Drugs

BIM 2001 was designed and synthesized at Biomeasure. Expansia kindly provided us FTI-277 and GGTI-286. Taxol, *cis*-platinum, doxorubicin and 5-fluorouracyl were purchased from Sigma.

2.2. NPC tumor lines

C15 is an undifferentiated NPC tumor line propagated by subcutaneous passage into nude mice [18]. It was established from the biopsy of a primary nasopharyngeal tumor in a 13-year-old girl born in Morocco. The biopsy was collected prior to any therapeutic procedure, according to the institutional guidelines concerning the use of clinical material. This patient had a voluminous primary tumor associated with lymph node and bone metastases. She was treated by induction chemotherapy with a combination of vincristine, cyclophosphamide, doxorubicin and methyl-prednisolone, prior to nasopharyngeal and cervical radiotherapy. A 70% tumor response was achieved in both the primary tumor and cervical lymph node metastases after 2 months of chemotherapy. Complete clinical remission was obtained after radiotherapy. It lasted only 5 months, due to recurrence of bone metastases. Presently, after more than 100 passages on nude mice, the C15 cells have consistent expression of EBV latent genes, especially the genes encoding the EBERs, the EBNA1 and LMP1 proteins

[22]. Their chromosomal modal number is 47 [23]. They retain a wild type p53 protein [9]. C666-1 is an EBV-positive NPC cell line propagated *in vitro*, related to an NPC xenograft, called xeno-666, derived from an undifferentiated NPC tumor biopsy from a Southern Chinese patient [24]. A primary *in vitro* culture was derived from xeno-666 at passage 18 and named C666. Subsequently, C666 was adapted to low density growth and several subclones were isolated. One of them, named C666-1, was extensively characterized and later used in this study [21]. This subcloned C666-1 cell line has consistent expression of EBV latent genes, especially the genes encoding the EBERs and the EBNA1 protein. However, the LMP1 protein is not detectable in C666-1 [21]. The cells are 100% positive for cytokeratin and characterized with a chromosomal modal number of 45 [21]. C666-1 cells express a mutated p53 protein [25,26]. Presently at their passage 80, they retain all the above-mentioned EBV latent genes/proteins and form tumors when inoculated into athymic nude mice.

2.3. In situ hybridization of EBERs

Pieces of xenografted C15 and C666-1 tumors were fixed (in a mix of acetic acid, formaldehyde and ethanol), paraffin-embedded and cut in 4 μm sections. EBERs detection was performed by *in situ* hybridization with a mixture of peptide nucleic acid (PNA) probes reacting with both EBER 1 and 2 and labeled with fluorescein (Dako EBER-PNA probe, Dako). The hybridization process was done as recommended by the manufacturer, using RNAse-free water. Hybridized probes were detected with anti-fluorescein antibodies conjugated to alkaline phosphatase (PNA ISH detection kit, Dako).

2.4. Preparation of NPC cells for in vitro experiments

Prior to *in vitro* experiments, C15 xenografted tumors were minced and treated with type II collagenase for cell dispersion, as previously reported [13]. Residual cell aggregates were removed by filtration on a nylon cell strainer with 100 μm pores. C666-1 cells were permanently propagated *in vitro* in plastic flasks coated with collagene I (Biocoat, Becton-Dickinson). *In vitro* culture medium was Hepes-buffered RPMI with 5% fetal calf serum for both C15 and C666-1 cells. C666-1 cells which were derived from a single malignant clone were free of contaminating murine fibroblasts. In contrast, C15 cell suspensions were often contaminated by murine fibroblasts. Therefore, for some experiments, C15 cell suspensions were grown on plastic coated with poly(2-hydroxyethylmethacrylate), an anti-adhesive polymer which inhibits cell attachment [27] (PolyHEMA, Sigma, Saint-Quentin Fallavier). Using this coating procedure, fibroblast proliferation was completely inhibited whereas C15 cells grew as non-anchored spheroids or aggregates of 150 μm average diameter at 72 hr.

2.5. Ki 67 immunostaining

C15 cell aggregates were deposited on glass slides by cytospinning, fixed in acetone at 4° for 10 min and stained with a mouse monoclonal antibody against the Ki 67 antigen (Dako). This antibody is specific of the human antigen and does not cross-react with its murine counterpart. Immunoreactivity was detected with peroxidase-conjugated antibodies (Power vision kit, ImmunoVision Technologies). Slides were counterstained with hematoxylin.

2.6. In vitro prenyl-transferase assays

The effect of BIM 2001 and other drugs on FTase activity was assayed *in vitro* with FTase from human brain cytosol (ABS Reagents) as target enzyme and recombinant human H-Ras protein containing the wild type CAAX box (Biomol) as a specific protein substrate. The incubation mixture (25 μL) for [^{3}H]farnesylation contained 50 mM Tris–HCl (pH 7.5), 5 mM dithiothreitol, 20 μM $ZnCl_2$, 40 mM $MgCl_2$, 0.6 μM [^{3}H]farnesyl pyrophosphate (22.3 Ci/mmol) (NEN), 4 μM recombinant H-Ras and 10 μg FTase. After 60 min at 37°, the reaction was stopped by adding 150 μL of absolute ethanol. The mixture was then filtered on Unifilter GF/B microplate (Packard) and washed six times with ethanol prior to scintillation counting. After adding 50 μL of Microscint 0, plates were counted with a Packard Top Count scintillation counter. FTI-277 was used as a positive control for the FTase inhibition assay. Drug effects on GGTase I activity were assayed by a similar method with GGTase I from human brain (ABS reagent) as target enzyme and human recombinant H-Ras containing a mutated CAAX box (CVLL) (Biomol) as a specific substrate. The incubation mixture contained 4 μM recombinant H-Ras, 0.6 μM [^{3}H]geranylgeranyl-pyrophosphate (19.3 Ci/mmol) (NEN) and 100 μg of GGTase I. GGTI-286 was used as a positive control for the GGTase-I inhibition assay. Results were expressed as the concentrations of drugs required to inhibit 50% of prenyl-incorporation into the recombinant H-Ras proteins (IC_{50}).

2.7. Assessment of drug effect on NPC cell viability

Cell viability assays were performed in 96 well plastic microplates, either uncoated (C666-1) or coated with PolyHEMA (C15). For each cell line, the number of cells distributed per well was optimized in order to achieve the highest metabolic activity while keeping exponential growth till the end of the test. C15 and C666-1 cells were seeded at 10^5 and 35×10^3 per well, respectively, in 150 μL culture medium. Following an overnight pre-incubation culture, serial dilutions of chemotherapy drugs were added in quadruplicate (final concentration 50 nM–50 μM in 250 μL per well). At the completion of a 72 hr incubation with pharmacological agents, cell viability was evaluated using the WST-1 assay (Roche Molecular). The

WST-1 assay is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases. In contrast with other tests, it does not require washing the cells in microplate wells and is compatible with spheroid culture. Cells were incubated with 10 μL/well of the WST-1 reagent added to the culture medium, for 3–6 hr, at 37°. The plates were subsequently read on an ELISA reader (Dynatech MR7000) using a 450 nm filter. The mean and standard deviation were determined for quadruplicate samples. For each compound, values falling in the linear part of a sigmoid curve were included in a linear regression analysis and were used to estimate the 50% inhibitory concentration (IC_{50}).

2.8. Assessment of nuclear fragmentation and measurement of caspase activity in drug-treated NPC cells

For apoptosis induction, C15 and C666-1 cells were seeded in 24 well plates, in the absence of PolyHEMA at 10^6 and 3×10^5 cells per well, respectively, pre-incubated overnight and treated with drugs for 48 hr. Nuclei were visualized in live cells incubated with Hoescht 33342, 1 mg/mL in PBS, for 5 min at 37°. Caspase activity was assessed at single cell level by flow cytometry using the CaspaTag kit according to manufacturer instructions (Quantum-Appligene). This procedure involved a cell-permeable, general caspase inhibitor (VAD-fluoromethyl ketone) labelled with carboxyfluorescein. This fluorescent inhibitor irreversibly binds to active caspases and therefore is selectively retained in apoptotic cells. In order to discriminate human malignant cells from contaminating murine fibroblasts, C15 cell suspensions were submitted to additional staining with an anti-human HLA A, B, C directly conjugated to allo-Phyco-Cyanine (Becton Dickinson). In a first step, C15 cells were incubated for 1 hr at 37°, in their culture plates, with fluorescent VAD-FMK, in 300 μL of culture medium. Cells were then washed, trypsinized and further incubated with the anti-HLA antibody. Finally, both caspase and HLA fluorescence were analyzed using a FACS calibur flow cytometer (Becton Dickinson).

2.9. Assessment of PARP and TRAF-1 cleavage

Whole cell extracts were prepared from drug-treated and control C15 and C666-1 cells in RIPA-SDS buffer [13]. A total of 30–50 μg of total protein extract were submitted to electrophoresis on SDS–polyacrylamide gels (7.5% polyacrylamide gels for PARP detection; 12% continuous or 8–16% gradient gels for TRAF-1). Separated proteins were transferred to Immobilon membranes (Millipore) which were probed with anti-PARP (Oncogene) or anti-TRAF-1 (Santa Cruz) antibodies, revealed with horseradish peroxidase-conjugated antibodies (Amersham). Detection was performed with the ECL chemiluminescence system (Amersham). Two types of antibodies from Santa Cruz were used for study of TRAF-1-cleavage: the H-3 monoclonal and the H-132 polyclonal; a positive control was provided by C15 cells treated for 24 hr with the CD95-agonist antibody, 7C11 (Immmunotech). In order to confirm caspase involvement in TRAF-1 cleavage, cells were treated with the Z-VAD-FMK inhibitor (50 μM) starting 1 hr prior to the exposition to apoptosis-inducing drugs (Calbiochem).

3. Results

3.1. Detection of EBERs in NPC tumor lines

In order to check that both C15 and C666-1 retained latent EBV-infection, EBERs expression was detected by *in situ* hybridization in both tumor lines (Fig. 1A and B) (C666-1 xenografted tumors were reformed by cell injection into nude mice at *in vitro* passage 40). As expected, EBERs staining was essentially nuclear. Most but not all malignant cells were positively stained, an observation which is consistent with previous reports about fresh NPC biopsies [28].

3.2. Assessment of C15 and C666-1 proliferation

As previously reported, C666-1 cells consistently proliferated *in vitro*, growing as cell monolayers in various types of plastic vessels [21]. In order to prevent increasing contamination by murine fibroblasts, tumor-derived C15 cells were seeded in microplates coated with PolyHEMA matrix and grown as floating aggregates. These aggregated cells remained proliferating as demonstrated by immunocytological detection of the human Ki 67 antigen in a significant fraction of them (Fig. 1C). In addition, repeated measurements of WST-1 tetrazolium salt reduction during 3 consecutive days of culture demonstrated a consistent, steady increase of viable cells (Fig. 1D). C15 cell doubling time was estimated at 1.5 days. In the same plates, without plastic coating, the doubling time of C666-1 cells was about 3.5 days (Fig. 1D).

3.3. Short term cytotoxicity of conventional drugs applied on NPC cells in vitro

cis-Platinum, bleomycin, 5FU, doxorubicin and taxol are among the drugs most frequently used in chemotherapy of NPC [14]. Their short term cytotoxic effect was assessed *in vitro* on the C15 and C666-1 cells, using a cell viability assay based on WST-1 reduction. Cultured cells were incubated in the presence of various concentrations of each therapeutic agent for 72 hr. As shown in Table 1, both C15 and C666-1 were highly sensitive to doxorubicin with IC_{50} below 500 nM. C666-1 cells were also highly sensitive to taxol, whereas C15 cells were resistant to this drug. On the other hand, C15 was mildly sensitive to the

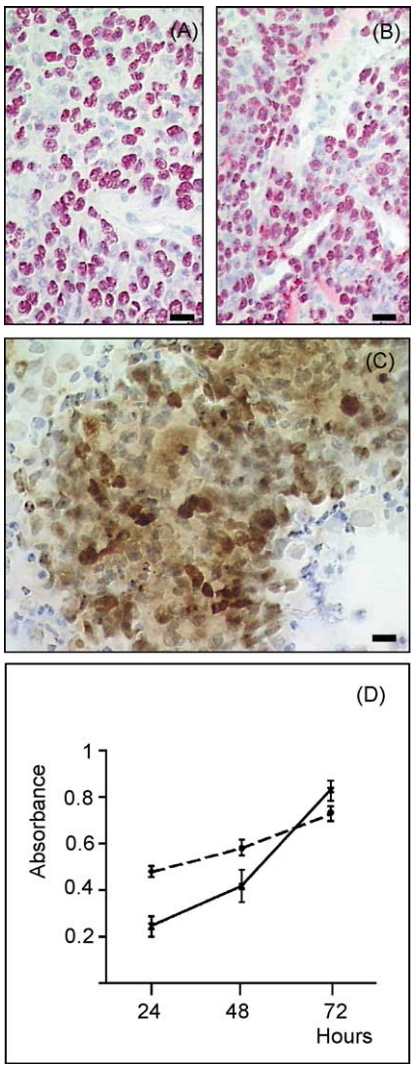

Fig. 1. Detection of the EBERs in NPC tumor lines and assessment of NPC cell proliferation *in vitro*. (A and B) *In situ* hybridization of the EBERs on tissue sections of xenografted tumors formed by C15 (A) and C666-1 (B) NPC cells (scale bar: 20 μm). (C) Ki 67 immunostaining of C15 cells grown *in vitro* for 72 hr on PolyHEMA matrix; cell aggregates (average size 150 μm) were cytospined and fixed in acetone (10 min/4°) prior to immunostaining (scale bar: 10 μm). (D) Proliferation of C15 (solid line) and C666-1 (dotted line) cells *in vitro* demonstrated by sequential WST-1 reduction assays. Cells were seeded in 96 well plates at 10^5 per well on plastic coated with PolyHEMA (C15) and 35×10^3 per well without plastic coating (C666-1). The WST-1 reaction was performed at 24, 48 and 72 hr to assess the evolution of cell viability reflected by absorbance at 450 nm. Data are the means (±SD) of quadruplicates. Similar results were obtained in three separate experiments.

cytotoxic effect of *cis*-platinum (1 μM IC_{50}) whereas C666-1 cells were five times more resistant. Finally, at clinically relevant concentrations, there were no significant effects of bleomycin and 5-FU in either cell line.

3.4. Characterization of a novel farnesyl-transferase inhibitor (BIM 2001)

Although doxorubicin was very active on both C15 and C666-1, it did not induce massive apoptosis in these cell lines, at least at clinically relevant concentrations (next figures and data not shown). Therefore, we considered the possibility of using molecularly targeted agents, especially FTIs in combination with doxorubicin, to increase its cytotoxic effect and attempt to induce apoptosis. We turned to a novel FTI which had been designed to be highly selective of farnesyl-transferase: BIM 2001. Its chemical structure is depicted in Fig. 2. It is a peptido-mimetic related to BIM-46068 and -4622829 [29,30]. The biological activity of this novel compound was first assayed for its effect on purified human prenyl-transferase activities and its inhibition of Ras-processing in intact cells. Table 2 shows that BIM 2001 is a potent inhibitor of human brain

Table 1
Growth-inhibition of NPC cells treated with conventional drugs *in vitro*

	C15 cells	C666-1 cells
Doxorubicin	150 nM	200 nM
cis-Platinum	1 μM	5 μM
Bleomycin	5 μM	5 μM
5-fluorouracyl	5 μM	5 μM
Taxol	25 μM	200 nM

Determination of the IC_{50}. Following an overnight pre-incubation culture, serial dilutions of drugs (final concentration 50 nM–50 μM) were added. At the completion of a 72 hr incubation with tested pharmacological agents, cell viability was evaluated using the WST-1 tetrazolium salt. After 3–6 hr, the plates were read on an ELISA reader using a 450 nm filter. The mean and standard deviation were determined. For each compound, values falling in the linear part of a sigmoid curve were included in a linear regression analysis and were used to estimate the 50% inhibitory concentration (IC_{50}). Data are the mean of quadruplicates. Similar results were obtained in three separate experiments.

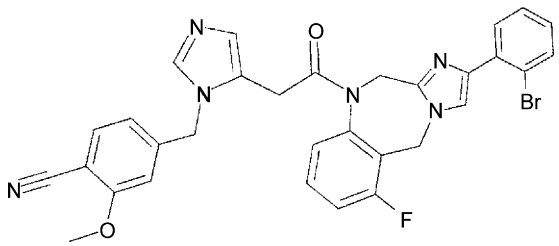

Fig. 2. Chemical structure of the FTase inhibitor BIM 2001.

Table 2
Inhibitory effects of BIM 2001 on purified human prenyl-transferase activities

	Enzyme assays IC_{50} (nM)	
	FTase	GGTase I
BIM-46068	91 (83–99)	268000 (15200–384000)
FTI-277	30 (26–34)	314 (305–323)
GGTI-286	365 (329–401)	114 (106–123)
BIM 2001	15 (13–16)	Inactive up to 100 μM

Values of IC_{50} represent the concentration required to inhibit 50% of the enzyme-catalyzed incorporation of labeled prenyl substrate into the recombinant human H-Ras with the wild type CAAX box (farnesyl-transferase target) or the modified CAAX box (CVLL; geranyl-geranyl transferase target). Assay results are reported as the mean of two experiments with the lowest and highest IC_{50} values observed in individual experiments in parentheses.

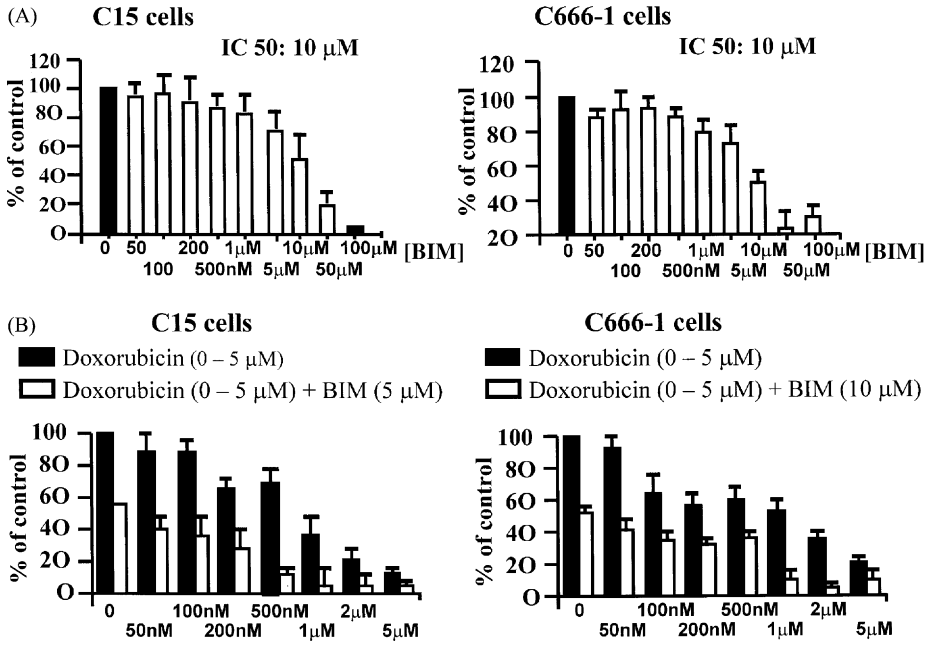

Fig. 3. Effect on NPC cell viability of BIM 2001, alone or in combination with doxorubicin. C15 and C666-1 cells were grown in 96 well plates, in the presence of various concentrations of pharmacological agents, prior to assessment of cell viability using the WST-1 assay. The observed results are reported as means (±SD) of quadruplicates and are representative of three similar experiments. (A) Treatment of NPC cells with various concentrations of BIM 2001 alone. (B) Treatment of NPC cells with various concentrations of doxorubicin with or without BIM 2001, 5 μM (C15) or 10 μM (C666-1).

FTase *in vitro*. The IC_{50} value is in the nanomolar range and compares favorably with the compounds of reference, FTI-277 and BIM-4606829 [29]. It is noteworthy that in contrast to other tested FTIs, no activity on GGTase-I was detected up to 100 μM of BIM 2001 thus showing its high selectivity for FTase.

3.5. Enhancement of doxorubicin cytotoxic activity on NPC cells when used in combination with BIM 2001

As shown in Fig. 3A, BIM 2001 had only limited toxicity on both C15 and C666-1 cells when it was used alone (IC_{50} = 10 μM). However, the cytotoxic effect of doxorubicin against C15 cells was dramatically enhanced when it was combined with BIM 2001 (Fig. 3B). A more than additive effect was obvious for doxorubicin concentrations of 500 nM or 1 μM with BIM 2001 at 5 μM. In contrast, for C666-1 cells, a higher concentration of the FTI drug was required to enhance the effect of doxorubicin. A more than additive effect was observed only with BIM 2001 at 10 μM for doxorubicin concentrations of 1 and 2 μM. In both C15 and C666-1 cells, there was no enhancement of *cis*-platinum and bleomycin cytotoxicity when they were combined with BIM 2001 in the same range of concentration (data not shown).

3.6. Contribution of apoptosis to the cytotoxic effect of the doxorubicin/BIM 2001 combination

After 48 hr incubation with the doxorubicin/BIM 2001 combination, many C15 cells started to round up, retract their processes, and subsequently detach from culture dishes. Under nuclear staining with Hoechst 33342, typical changes related to nuclear apoptosis–nuclear condensation and fragmentation—were detected at 48 hr of incubation and became more obvious after 72 hr (Fig. 4A). Such morphological changes were much less apparent in C15 cells treated with either doxorubicin or BIM 2001 alone (data not shown). In contrast to C15 cells, significant changes in the morphology of the nucleus were not observed in C666-1 cells, even in the presence of both doxorubicin and BIM 2001. At best, some nuclei with partial chromatin condensation were recorded (data not shown). To provide more evidence that apoptosis was strongly enhanced by combination of BIM 2001 with doxorubicin, general caspase activity was monitored by flow cytometry, with an assay based on selective retention of a fluorescent membrane permeable inhibitor. As shown in Fig. 4B, caspase-related fluorescence was almost twice as high in cells treated with both drugs compared to cells treated with doxorubicin alone. In cells treated by BIM 2001 alone, caspase activity was identical to untreated controls. Apoptosis of C15 cells induced by the drug combination was further documented by Western blot analysis of the PARP-cleavage (Fig. 5). A strong PARP-cleavage was apparent after only a 24 hr period of combined treatment (Fig. 5). At 48 hr, the intact fragment of the PARP was almost undetectable. A moderate cleavage of the PARP was also detected in cells treated by doxorubicin or BIM 2001 alone, but a much lower fraction of the protein was affected in these experimental conditions. No PARP-cleavage were detected in C666-1 cells even

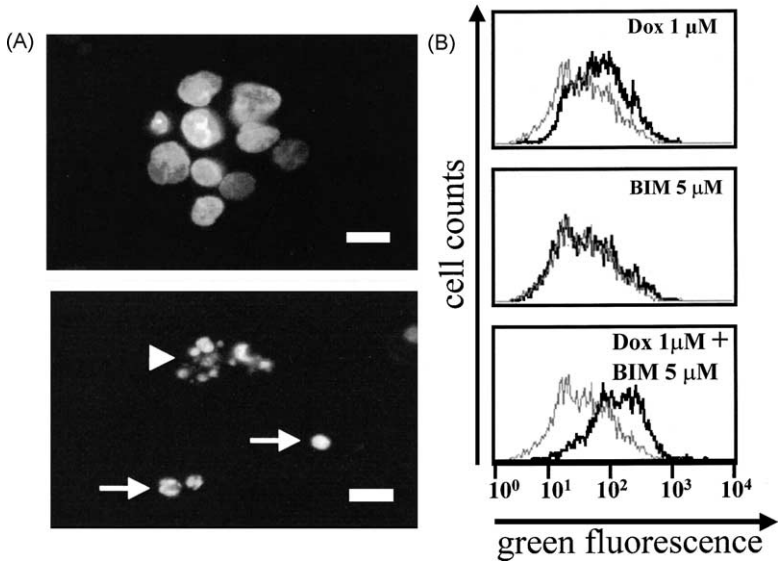

Fig. 4. Induction of nuclear fragmentation and caspase activation in C15 cells treated with doxorubicin combined to BIM 2001 (48 hr). (A) Nuclei visualized in live cells with Hoechst staining (scale bar: 10 µm). Upper panel: non-treated cells. Lower panel: cells treated for 48 hr with doxorubicin (1 µM) combined with BIM 2001 (5 mM); most nuclei shrank (arrows) and exhibited chromatin condensation and fragmentation (arrowhead). (B) Flow cytometry measurement of caspase-activation in C15 cells. Values of mean fluorescence channels were 56 (non-treated cells; gray line), 85 (cells treated with doxorubicin alone), 58 (BIM 2001 alone) and 147 (both drugs). This is one of three similar experiments.

when treated by doxorubicin combined with BIM 2001 (data not shown).

3.7. Cleavage of TRAF-1 in C15 cells treated with the BIM 2001/doxorubicin combination

Finally NPC cells treated with doxorubicin and/or BIM 2001 were investigated for TRAF-1-cleavage. TRAF-1 is a signaling adaptor which negatively regulates tumor necrosis factor signaling [31]. It has a restricted tissue distribution but its expression is ectopically induced by EBV-infection [32]. We have previously shown that TRAF-1 is strongly expressed by EBV-positive NPC cells [33]. On the other hand, Leo *et al.* (2001) have reported that TRAF-1 expressed from a transfected plasmid is cleaved at aspartate 163 in recipient cells undergoing apoptosis, especially death

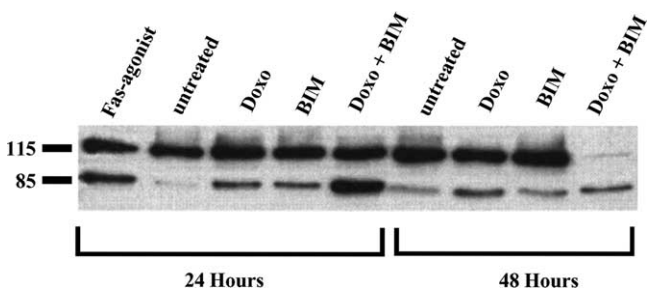

Fig. 5. Induction of PARP-cleavage in C15 cells treated with doxorubicin combined to BIM 2001. C15 cells were treated with doxorubicin (1 µM), BIM 2001 (5 µM) or a combination of both for the indicated periods. Corresponding cell lysates (50 µg protein per lane) were separated by 7.5% SDS–PAGE and analyzed by Western blot with an anti-PARP monoclonal antibody. The intact PARP was at 115 kDa whereas its cleaved product was at 85 kDa, as shown in the positive-control extract derived from C15 cells treated with the Fas agonist-antibody 7C11.

receptor-mediated apoptosis but also doxorubicin-induced apoptosis [34]. Therefore, the cleavage of the endogenous TRAF-1 was investigated in drug-treated NPC cells. C15 cells treated by a CD95-agonist (7C11) for 24 hr were used as a positive control; a dramatic decrease in the amount of intact TRAF-1 was observed in the corresponding protein extracts (Fig. 6B). Simultaneously, there was a marked increase of the cleaved fragments characterized by Leo *et al.* [34]: fragment I (24 kDa, reacting with the H-132 antibody) and II (30 kDa, reacting with H-3) (Fig. 6B). In cells treated with doxorubicin or BIM 2001 alone, we could see no modifications of the TRAF-1 molecule. In contrast, a substantial increase of cleaved fragments similar to fragments I and II was observed in the extracts of cells treated with the combination of doxorubicin and BIM 2001 (Fig. 6B). A more careful examination revealed some differences in the pattern of cleavage induced either by the CD95-agonist or the drug combination. First, the cleaved fragment II consistently had a slightly bigger size in drug-treated cells than with the 7C11 agonist; however, this difference in size was tenuous and visible only on a 8–16% gradient gel. Next, in cells treated with 1 µM doxorubicin combined to BIM 2001, the amount of intact TRAF-1 molecule was only marginally reduced (Fig. 6B). However, in later experiments, we could obtain an obvious decrease of the intact TRAF-1 molecule by using 2.5 µM doxorubicin combined to BIM 2001 (Fig. 6C). Again, no increase in TRAF-1 cleavage was observed when doxorubicin was used alone, even at this higher concentration. In order to confirm that drug-induced cleavage of TRAF-1 was mediated by caspases, C15 cells were treated with the general caspase-inhibitor Z-VAD-FMK in addition to the combination of doxorubicin and BIM 2001. As one can see in Fig. 6D, the

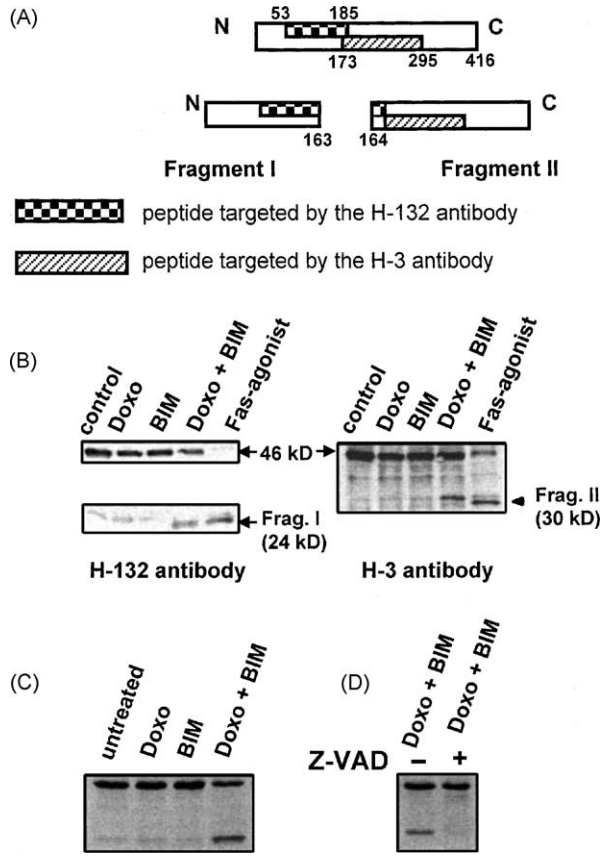

Fig. 6. Induction of TRAF-1-cleavage in C15 cells treated with doxorubicin combined to BIM 2001. (A) Diagram of Fas-mediated TRAF-1-cleavage as reported by Leo *et al.* (2001) [34]. Sequence location of target peptides used for production of the H-132 polyclonal and H-3 monoclonal antibody are indicated by a checked and an hatched box, respectively. The peptide targeted by H-3 was entirely contained in fragment II whereas the peptide targeted by H132 was mainly co-linear with fragment I. (B) C15 cells were treated for 24 hr with doxorubicin (1 μM), BIM 2001 (5 μM) or a combination of both. Control cells were treated with the Fas-agonist antibody 7C11. Cell lysates (50 μg protein per lane) were separated on a 8–16% SDS–PAGE linear gradient prior to Western blot analysis with anti-TRAF-1 antibodies. Both antibodies detected a main band at 46 kDa. Additional bands corresponding to fragments of smaller sizes were visible, mainly in samples treated with 7C11 or the combination of doxorubicin and BIM 2001. In the extracts of cells treated with 7C11, these smaller fragments had apparent molecular weights of 29 kDa (H-3) and 24 kDa (H-132) which were compatible with the sizes of TRAF-1 fragments reported by Leo *et al.* [34]. (C) C15 cells were treated for 24 hr with doxorubicin (2.5 μM), BIM 2001 (5 μM) or a combination of both. Cell lysates were separated on a 12% linear gel prior to Western blot analysis with the H3 monoclonal antibody. (D) C15 cells were treated with Z-VAD-FMK (50 μM) starting 1 hr prior to their exposition to doxorubicin (1 μM) and BIM 2001 (5 μM) for 24 hr. Cell lysates were separated on a 12% linear gel prior to Western blot analysis with the H3 monoclonal antibodies. Identical results were obtained in three similar experiments.

amount of TRAF-1 cleaved fragment II was dramatically reduced by this procedure.

4. Discussion

There are only few reports about the biological events which underline the cytotoxic effects of anti-neoplastic drugs in NPC cells. These reports are mainly focused on the effects of *cis*-platinum and taxol. *cis*-Platinum was shown to induce growth arrest in the CNE1 NPC cell line. CNE1 growth arrest was observed at relatively low concentrations (about 1 μM) and was accompanied by increased expression of senescence-associated β-galactosidase [35,36]. Taxol was shown to induce growth arrest and/or apoptosis in TWO1, TW039 and HNE1 NPC cell lines [37–39]. However, it is important to remember that these studies were performed on EBV-negative cell lines which either were derived from rare forms of EBV-negative differentiated NPCs (CNE1, TW039) or had lost the EBV-genome after a few passages *in vitro* (TWO1; HNE1) [19,20]. Therefore, these target cell lines could not be regarded as truly representative of undifferentiated EBV-positive NPCs. Testing anti-neoplastic drugs on genuine EBV-positive NPC cells *in vitro* was recently made possible by technical developments in the handling of the C666-1 and C15 NPC tumor lines. C666 was derived from the xeno-666 transplanted NPC and it was subcloned in order to stabilize its association with the EBV-genome. C666-1 is one of the resulting clones. In contrast, no permanent *in vitro* cell line was derived from the C15 transplanted NPC. However, we greatly improved the *in vitro* handling of the C15 cells by the use of a PolyHEMA matrix which prevented the proliferation of murine fibroblasts while allowing selective growth of malignant cells in the form of small floating aggregates.

Interestingly, in our cell viability assay, both C15 and C666-1 were remarkably sensitive to the cytotoxic effects of doxorubicin used at clinically relevant concentrations (below 500 nM). It is noteworthy that doxorubicin had been included in the combination of drugs which had achieved a marked—although transient—tumor regression in the patient who was the donor of the C15 tumor line (see Section 2). In the current therapeutic context, anthracyclins remain major arms for chemotherapy of NPCs, especially in the treatment of advanced forms [40]. Using the same cell viability assay, C15, and even more C666-1, were less sensitive to *cis*-platinum than to doxorubicin. While this study was in progress, another report also showed a poor sensitivity of C666-1 to *cis*-platinum using a short term cytotoxicity assay [25]. This might look surprising since *cis*-platinum is currently regarded as the most effective agent in the chemotherapy of NPCs [14]. One possible explanation might be that the effect of *cis*-platinum against NPC cells is not properly explored by short term assays. Clonogenic assays probably will be required to better explore the impact of low concentrations of *cis*-platinum on C15 and C666-1. Presently, this type of assay is precluded by the very low clonogenic performance of both cell lines.

In order to both increase cytoxicity and achieve chemo-induced apoptosis of NPC cells, we attempted treatments combining doxorubicin with FTIs. FTIs were considered because they are relatively recent molecularly targeted

drugs which are known to induce apoptosis of malignant cells in several experimental systems [41,42]. They are actively investigated in clinical trials and there are some indications that they can increase the benefit of chemotherapy or radiotherapy without parallel increase of undesirable side-effects [30,41–43]. We have used a novel FTI, BIM 2001, which is potentially active by oral administration. We confirmed that this compound was a selective inhibitor of the farnesyl-transferase enzyme. We then demonstrated that the cytotoxic effect of doxorubicin against C15 cells was dramatically enhanced when it was used in combination with BIM 2001. The cytotoxic effect against C15 cells was underlined by massive apoptosis 48 hr after starting cell exposure to doxorubicin and BIM 2001. In contrast, when used against C666-1 cells, the combination of doxorubicin and BIM 2001 was less toxic and failed to induce apoptosis; the decrease of cell viability was apparently underlined by growth arrest and non-characterized cell death (data not shown). The Epstein-Barr virus oncoprotein LMP1 has been reported to antagonize apoptosis in several experimental conditions [10]. However, we observed chemo-induced apoptosis in C15 cells which are LMP1-positive but not in C666-1 cells which are LMP1-negative [21]. This suggests that LMP1 does not play a critical role in the NPC cell response to the doxorubicin/BIM 2001 combination. In terms of therapeutic investigations, the strong cytotoxic effect of the doxorubicin/BIM 2001 combination against C15 cells and its ability to induce massive apoptosis provide a strong incentive to evaluate the interest of farnesyl-transferase inhibitors for NPC treatment in future clinical trials.

Although FTIs were initially designed to inhibit the farnesylation and membrane anchoring of Ras proteins, it is now clear that they interfere with farnesylation of other proteins especially Rho B, Rap 2, lamin A and B [41,42]. In some cellular models, cell treatment by FTIs results in the accumulation of geranylated Rho B which induces apoptosis of transformed cells [42]. Alternatively, in other models, FTIs have been shown to inhibit the PI-3-kinase pathway [41]. Classically, anthracyclins such as doxorubicin exert their cytotoxic effect by inducing production of reactive oxygen species (ROS). ROS can induce DNA lesions, resulting in p53-activation and up-regulation of p21/waf1, GADD 45, CD95 and Bax [44]. ROS also have some impact on membrane lipids and can induce production of lipid second messengers [45]. Presently it is difficult to know where and how the FTI- and doxorubicin-activated pathways can intersect in such a way that they induce apoptosis. Our observation about TRAF-1 cleavage may provide interesting clues for future investigations on this subject. Leo *et al.* could demonstrate than caspase 8 is a key-effector of TRAF-1 cleavage during CD95-mediated apoptosis [34]. However, we found that the cleavage of TRAF-1 induced by drugs had some distinct specific features when compared to the cleavage induced by CD95-agonists, especially a relatively high ratio of uncleaved to cleaved forms in drug-treated samples. This suggests that an increase in TRAF-1 production occurs in drug-treated NPC cells, in addition to the cleavage process. Interestingly, the same high ratio of cleaved to uncleaved molecules was reported by Leo *et al.* for the HT1080 cell line expressing exogenous TRAF-1 and treated with doxorubicin [34].

Finally, it is noteworthy that TRAF-1 is strongly expressed not only in NPCs, but also, according to recent reports, in other EBV-associated malignancies, especially post-transplant lymphomas and EBV-associated Hodgkin lymphomas [46,47]. Through additional studies, the cleavage of TRAF-1 might become a useful marker for prediction and monitoring of the anti-tumor effect of chemotherapy in EBV-associated malignancies.

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