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ORIGINAL PAPER

TAT-RasGAP$_{317–326}$-mediated tumor cell death sensitization can occur independently of Bax and Bak

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Abstract The increase of cancer specificity and efficacy of anti-tumoral agents are prime strategies to overcome the deleterious side effects associated with anti-cancer treatments. We described earlier a cell-permeable protease-resistant peptide derived from the p120 RasGAP protein, called TAT-RasGAP$_{317–326}$, as being an efficient tumor-specific sensitizer to apoptosis induced by genotoxins in vitro and in vivo. Bcl-2 family members regulate the intrinsic apoptotic response and as such could be targeted by TAT-RasGAP$_{317–326}$. Our results indicate that the RasGAP-derived peptide increases cisplatin-induced Bax activation. We found no evidence, using in particular knock-out cells, of an involvement of other Bcl-2 family proteins in the tumor-specific sensitization activity of TAT-RasGAP$_{317–326}$. The absence of Bax and Bak in mouse embryonic fibroblasts rendered them resistant to cisplatin-induced apoptosis and consequently to the sensitizing action of the RasGAP-derived peptide. Surprisingly, in the HCT116 colon carcinoma cell line, the absence of Bax and Bak did not prevent cisplatin-induced apoptosis and the ability of TAT-RasGAP$_{317–326}$ to augment this response. Our study also revealed that p53, while required for an efficient genotoxin-induced apoptotic response, is dispensable for the ability of the RasGAP-derived peptide to improve the capacity of genotoxins to decrease long-term survival of cancer cells. Hence, even though genotoxin-induced Bax activity can be increased by TAT-RasGAP$_{317–326}$, the sensitizing activity of the RasGAP-derived peptide can operate in the absence of a functional mitochondrial intrinsic death pathway.

Keywords RasGAP · Tumor sensitization · Bcl-2 family members · Bax

Introduction

The modulation of tumor cell sensitivity to genotoxic agents is one major issue in anticancer research [1]. The development of small molecules able to selectively increase the susceptibility of cancer cells to genotoxin-induced cell death would ameliorate the efficacy of chemotherapy and offer substantial benefits to individuals with cancer. A few compounds have been demonstrated to act as tumor sensitizers. These compounds are generally devoid, on their own, of any cytostatic or cytolytic effect on cells but they increase the ability of known anti-cancer agents to kill tumor cells. ADD70 corresponds to a fragment of the apoptosis-inducing factor (AIF) protein that binds the Hsp70 heat shock protein [2]. When expressed in colon carcinoma and lymphoma, ADD70 sensitizes them to cisplatin [3]. However, ADD70 does not have the ability to enter cells and cannot therefore be used as is as a tumor sensitizer in vivo. SmacN7 is a short seven amino acid long peptide derived from Smac/Diablo, a protein released by the mitochondria in response to apoptotic stimuli that inhibits inhibitor of apoptosis proteins (IAPs) and hence favors the apoptotic process [4]. While apparently not inducing

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apoptosis of cancer cells by itself, a cell-permeable version of SmacN7 potentiates the action of genotoxins [5–7].

Our laboratory has developed an anti-tumor peptide that selectively sensitizes several cancer cell lines, but not non-cancer cell lines, to genotoxin-induced apoptosis [8]. The anti-tumor activity of this peptide is carried by a ten-amino acid long sequence derived from the SH3 domain of p120 RasGAP. This sequence corresponds to residues 317–326 of the RasGAP protein [8]. The $RasGAP_{317–326}$ peptide was made cell permeable by hooking it to a short sequence of the HIV TAT protein (residues 48–57) that allows polypeptides to penetrate cells [9], resulting in the so-called TAT-$RasGAP_{317–326}$ peptide. This compound by itself, does not affect cell viability [8]. The RasGAP-derived peptide is also capable of increasing the anti-cancer activity of cisplatin and doxorubicin against human tumor xenografts [10] and to augment the killing efficiency of photodynamic therapy in mesothelioma cells [11]. Recently, TAT-$RasGAP_{317–326}$ was shown to inhibit migration and invasion in vitro, indicating that it has the potential to function as an anti-metastatic compound [12].

Genotoxins are DNA-damaging substances that exert their anti-tumor activity by causing apoptosis in cancer cells mainly through the mitochondrial pathway [13–15]. The genotoxin-induced DNA damage response is orchestrated by a series of kinases (e.g. the ataxia-telangiectasia mutated, ATM) that sense the extent of the damage and activate downstream effectors [16, 17]. One of the key DNA damage response effector is p53, a transcription factor that regulates the expression of genes involved in cell cycle arrest (e.g. p21) and apoptosis (e.g. Puma and Bax) [18]. Puma and Bax both belong to the Bcl-2 family that tightly controls, directly or indirectly, the release of pro-apoptotic molecules from the mitochondria [19]. Members of this family share up to four BH (Bcl-2 Homology) domains and are conventionally subdivided into three groups on the basis of their pro- or anti-apoptotic activity and the BH domains they contain. Anti-apoptotic players (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1) and pro-apoptotic players (Bax, Bak and Bok) possess four BH domains, whereas the pro-apoptotic BH3-only proteins (Bid, Bim, Bad, Bmf, Bik, Noxa, Puma and Hrk) display only the BH3 domain [20]. When the balance between pro- and anti-apoptotic members of this family tips in favor of the former, mitochondrial membrane integrity is lost, cytochrome *c* is released in a Bax/Bak-dependent manner and this leads to caspase activation and apoptosis [21]. There are currently two main models describing how these proteins determine cell fate. These models are not necessarily mutually exclusive [22–24]. According to the 'indirect model', pro-apoptotic Bax and Bak are neutralized by anti-apoptotic proteins (e.g. Bcl-2) under normal conditions. When cells are subjected to apoptotic insults, BH3-only proteins displace Bax and Bak from their guardians allowing them to oligomerize in the outer mitochondrial membrane, leading to cytochrome *c* release and cell death [25]. Conversely, the 'direct model' supports a direct Bax/Bak activating role for a subgroup of the BH3-only members (tBid, Bim and Puma) called the 'activators'. In basal conditions, these are sequestered by the anti-apoptotic proteins but in response to apoptotic stimuli they are displaced by the BH3-only 'sensitizer' members (e.g. Bad and Noxa). The freed 'activators' can then promote the oligomerization of Bax and Bak [26, 27].

We showed earlier that TAT-$RasGAP_{317–326}$ does not modulate MAPK signaling pathways (p38, ERK and JNK), NF-κB transcriptional activity or Akt protein levels and phosphorylation status [8, 28]. Here we show that the activity of Bax can be modulated by TAT-$RasGAP_{317–326}$ in the context of tumor cells sensitization to genotoxin-induced apoptosis. Our data indicate however that Bcl-2 family members, including Bax and Bak, are dispensable for the capacity of the peptide to increase forms of death distinct from the classical mitochondrial apoptotic pathway.

Materials and methods

Chemicals

Cisplatin (Sigma, ref. no. P4394) was dissolved in water at a 1 mg/ml concentration and stored at −80 °C. TNFα (Pierce, ref. no. RTNFA10) was dissolved in water at a concentration of 10 μg/ml, aliquoted and stored at −80 °C. Cycloheximide (CHX) (Sigma, ref. no. C7698) was dissolved in methanol at 20 mg/mL and stored at −20 °C. Paraformaldehyde (PFA) and HCl were from Acros (ref. no. 30525-89-4 and 7647-01-0, respectively). Hoechst 33342 (Molecular Probe, Invitrogen, ref. no. H21492) was prepared as a 10 mg/ml water solution, stored at 4 °C and used at a final concentration of 10 μg/ml. The pan-caspase inhibitor MX1013 was a kind gift from Maxim Pharmaceuticals (San Diego, CA). Tris base [2-Amino-2-(hydroxymethyl)-1,3-propanediol], sodium dodecyl sulfate (SDS), mannitol, sucrose and trichloroacetic acid were from Sigma (ref. no. T1503, L4390, M4125, S0389 and T9159 respectively). HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] and polyoxyethylene (20) sorbitan monolaurate (Tween 20) were from AppliChem (ref. no. A3724 and 1873580, respectively). NaCl, EGTA [ethylenebis(oxyethylenenitrilo)tetraacetic acid] and bromophenol blue were from Acros (ref. no. 7647-14-5, 67-42-5, and 115-39-9, respectively). CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} was from Fluka and AppliChem (ref. no. 26680 and A1099.0050 respectively). EDTA (ethylenediaminetetraacetic acid), dithiothreitol (DTT), and glycerol were from Fluka (ref. no. 03620,

43817, and 49780, respectively). G-Sepharose beads were from GE Healthcare (ref. no. 17-0618-01). Dynabeads Protein G was from Life Technologies (ref. no. 10004D). Nitrocellulose membranes was from Biorad (ref. no. 162-0115). $MgCl_2$ was from Eurobio (ref. no. 018023). EDTA-free Protease Inhibitor Cocktail Tablets were from Roche Applied Science (ref. no. 1873580). SM-164 was synthesized as described [29]. DSS (disuccinimidyl suberate) was from Thermo Scientific Pierce (ref. no. 21555).

Buffers

The composition of phosphate buffered saline (PBS) is 116 mM NaCl, 10.4 mM Na_2HPO_4, 3.2 mM KH_2PO_4 (pH 7.4). The composition of the isotonic mitochondrial buffer (MB) is 10 mM HEPES (pH 7.4), 210 mM mannitol, 70 mM sucrose, 1 mM EDTA supplemented with one tablet of protease inhibitor cocktail per 50 ml. CHAPS lysis buffer is made of 5 mM $MgCl_2$, 137 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % CHAPS, 20 mM Tris-base, supplemented with one tablet of protease inhibitor cocktail per 50 ml and adjusted to pH 7.4 with HCl. MonoQ-c buffer is made of 70 mM β-glycerophosphate, 0.5 % Triton X-100, 2 mM $MgCl_2$, 1 mM EGTA, 100 μM Na_3VO_4, 1 mM DTT, 20 μg/ml aprotinin supplemented with one tablet of protease inhibitor cocktail per 50 ml. RIPA buffer contains 50 mM Tris base pH 8.0, 150 mM NaCl, 0.5 % deoxycholate, 1 % NP-40, 0.1% SDS supplemented with one tablet of protease inhibitor cocktail per 50 ml.

Peptides

TAT and TAT-RasGAP$_{317–326}$ are retro-inverso peptides (i.e. synthesized with D-amino acids in the opposite direction compared to the natural sequence). The TAT moiety corresponds to amino acids 48–57 of the HIV TAT protein (RRRQRRKKRG) and the RasGAP$_{317–326}$ moiety corresponds to amino acids 317–326 of the human RasGAP protein (DTRLNTVWMW). These two moieties are separated by two glycine linker residues in the TAT-RasGAP$_{317–326}$ peptide. The peptides were synthesized at the department of biochemistry, University of Lausanne, Switzerland, using FMOC technology, purified by HPLC and tested by mass spectrometry.

Cell lines

U2OS, HCT116 were cultured in DMEM (Invitrogen, ref. no. 61965) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Invitrogen, ref. no. 10270-106) in 5 % CO_2 at 37 °C. HeLa cells were cultured in RPMI (Invitrogen, ref. no. 61870) supplemented with 10 % heat-inactivated FBS in 5 % CO_2 at 37 °C. Culturing of the cells prior to performing the experiments described in this article was performed in six-well plates by seeding 100′000 cells (Figs. 1, 5b), 150,000 (Fig. 2, U2OS and HeLa cells), 200,000 (Fig. 2, HCT116 cells), or 250,000 cells (Figs. 5a, 6) in the wells 24 h before being treated as indicated in the figures. HeLa cells were infected with LeGO-iT2 empty and LeGO-iT2-Bcl-X_L lentiviruses. Knock-out (KO) mouse embryonic fibroblasts (MEFs) for Bcl-X_L, Bcl-2, Mcl-1, Bim, Bmf and Bid were transformed by infecting them with a SV40 large T-encoding lentivirus [30, 31]. KO MEFs for Bax, Bak, Bax/Bak and Bad were provided by the late Dr. Stanley Korsmeyer. Culturing of the KO MEFs prior to performing the experiments described in this article was performed during 24 h in six-well plates by seeding 150,000 cells (Fig. 4). For starvation, cells were washed three times with PBS and incubated for 24 h with medium lacking FBS.

TRAIL-induced apoptosis

Cells were incubated for 24 h with recombinant Flag–TRAIL and 2 μg/ml anti-FLAG M2 antibody diluted in culture medium.

UV illumination

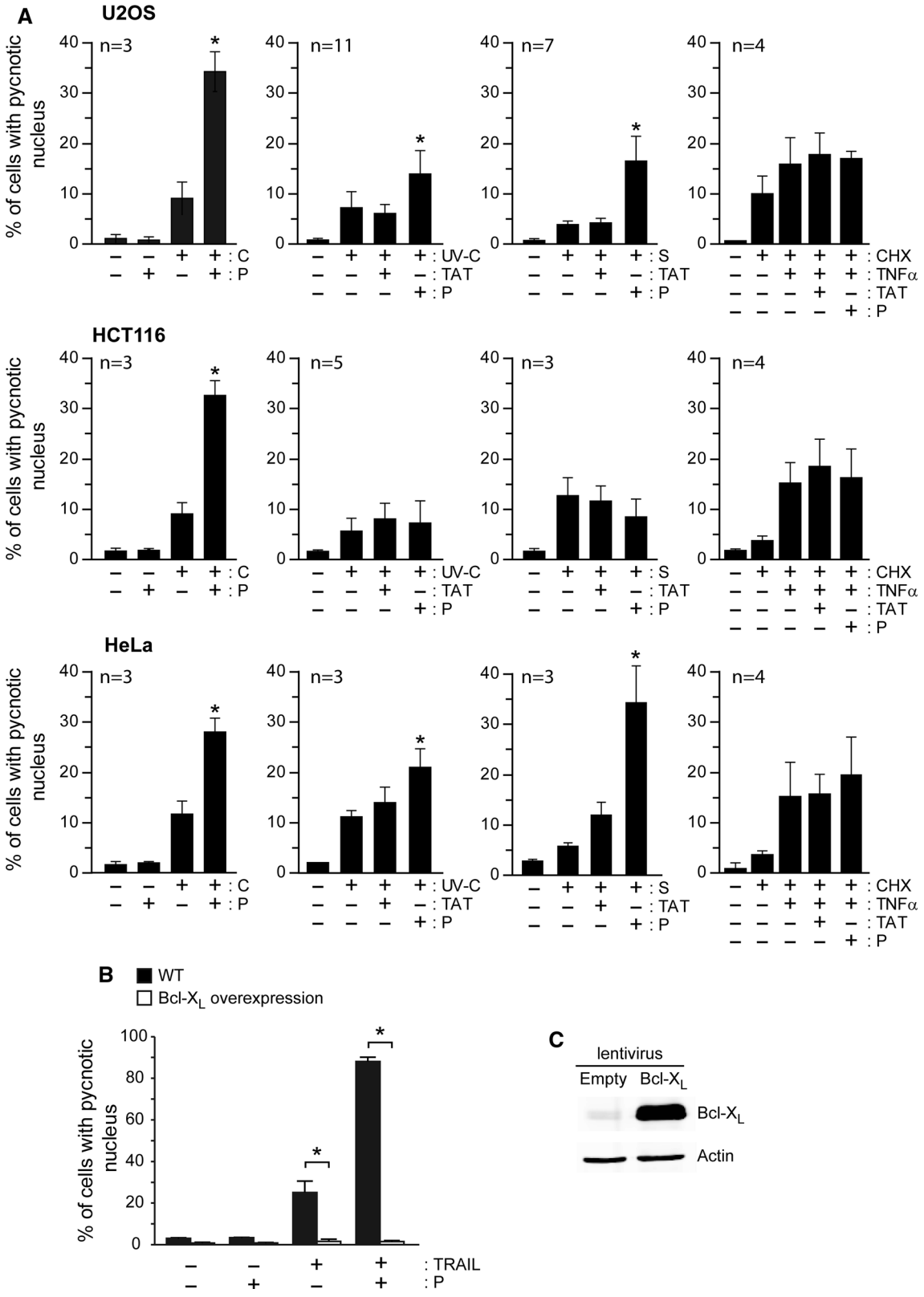
Cells in culture dishes with the lid removed were UV-C-illuminated using the UV lamp of a cell culture hood (Fortuna from Scanlaf 1200) delivering an intensity of 0.25 J/m^2 per second. The cells were then incubated for the indicated periods of time in 5 % CO_2 at 37 °C.

Apoptosis quantitation

Cells were fixed in 2 % PFA and nuclei labeled with Hoechst 33342. Apoptotic cells (i.e. cells displaying a pycnotic nucleus) were counted under a Nikon Eclipse TS100 microscope. When apoptosis was assessed on a population of transfected cells, only green cells (i.e. cells having incorporated the transfected plasmids) were counted.

Statistics

The statistical analyses were done with Microsoft Excel (XP edition) using the unpaired Student's *t* test. Significance is indicated by an asterisk when $P < 0.05/n$, where P is the probability derived from the *t* test analysis and n is the number of comparisons done (Bonferroni correction). For Figs. 4, 6, and 8, the statistical test used was one-way ANOVA followed by Bonferroni's post hoc comparison tests.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Bcl-X$_L$ overexpression
: TRAIL
C
lentivirus
Empty
Bcl-X$_L$
Actin

◀**Fig. 1** Ability of TAT-RasGAP$_{317–326}$ to sensitize tumor cells to various cell death stimuli. **a** U2OS, HeLa and HCT116 were subjected to UV-C illumination or treated during 24 h with various apoptotic stimuli as indicated in the figure. Cells were then fixed and apoptosis was counted by scoring pycnotic nuclei. *C* 30 μM cisplatin; *P* 20 μM TAT-RasGAP$_{317–326}$; *TAT* 20 μM TAT; *UV-C* 15 J/m^2 (U2OS), 25 J/m^2 (HCT116 and HeLa); *S* starvation; *CHX* 10 μg/ml CHX; TNFα: 1 ng/ml (U2OS and HeLa), 0.5 ng/ml (HCT116). The results correspond to the mean ± 95 % CI of independent experiments. The *asterisks* indicate a statistically significant difference between cells treated or not with TAT-RasGAP$_{317–326}$ in the presence of the apoptogenic stimuli. **b** HeLa cells overexpressing Bcl-X$_L$ or not were treated during 24 h with 20 μM TAT-RasGAP$_{317–326}$ (*P*), 200 ng/ml TRAIL (TRAIL). Cells were then fixed and apoptosis was counted by scoring pycnotic nuclei. Results correspond to the mean ± 95 % CI of three independent experiments. The *asterisk* denotes a statistical significant difference. **c** HeLa cells were infected with empty virus or virus encoding Bcl-X$_L$. Bcl-X$_L$ expression levels were assessed by Western blotting

Transfection

Cells were transfected using Lipofectamine 2000 (GibcoBRL, ref. no. 18324-012) according to the manufacturer's instructions. Briefly, the indicated plasmids, together with 0.5 μg of GFP-encoding plasmid, were diluted in 250 μl DMEM without FBS. The amount of DNA used in the transfection was kept constant to 4 μg by adding the appropriate quantities of the pcDNA3 empty vector. In parallel, 8 μl of lipofectamine were added to 250 μl of DMEM without FBS and kept at room temperature for 5 min. Finally, the DNA-containing medium was added drop by drop to the lipofectamine-containing medium and the resulting 500 μl mix was added to cells. Five hours later, cells were washed once with fresh medium (DMEM supplemented with FBS) and cultured for an additional 20 h period in the presence of the treatments indicated in the figures.

Coimmunoprecipitation

7.5 × 10^5 U2OS and HeLa cells and 10^6 HCT116 cells were seeded in 10 cm plates and the next day treated as shown in the figures. Sixteen hours later, the cells were lysed in 300 μl of 1 % CHAPS lysis buffer and 700 μg of the lysate proteins were immunoprecipitated overnight with 1.5 μg of the 6A7 anti-Bax antibody. Immunoprecipitates were captured on 30 μl of protein G-Sepharose at 4 °C for 2 h. Immunocomplexes were then washed three times in CHAPS lysis buffer and eluted in 30 μl of 2× sample buffer (25 mM Tris-HCl pH 7.5, 10 % glycerol, 6 % SDS, 0.02 % of bromophenol blue and 100 mM DTT). The immunoprecipitates and 50 μg of total cell lysates were separated by SDS-PAGE and then transferred onto nitrocellulose membrane. The membranes were blocked with TBS (20 mM Tris base, 130 mM NaCl, pH 7.6) containing 0.1 % Tween 20 and 5 % non-fat milk (TBS–TM) and incubated overnight at 4 °C with a 1:1000 dilution of the N-20 anti-Bax antibody. Blots were then washed with TBS containing 0.1 % Tween 20 (TBS-T), incubated 1 h at room temperature with the appropriate fluorophore-conjugated secondary antibody (1:5,000 dilution) and subsequently visualized with the Odyssey infrared imaging system (LICOR Biosciences, Bad Homburg, Germany). Bak immunoprecipitation was performed as described above with the following modifications: 2,000 μg of the lysate proteins were immunoprecipitated overnight with 1.5 μg of the Ab-2 anti-Bak antibody; immunoprecipitates were captured with 15 μl of Dynabeads Protein G; and the G23 anti-Bak antibody was used as the primary antibody for immublotting.

Crosslinking of mitochondrial proteins

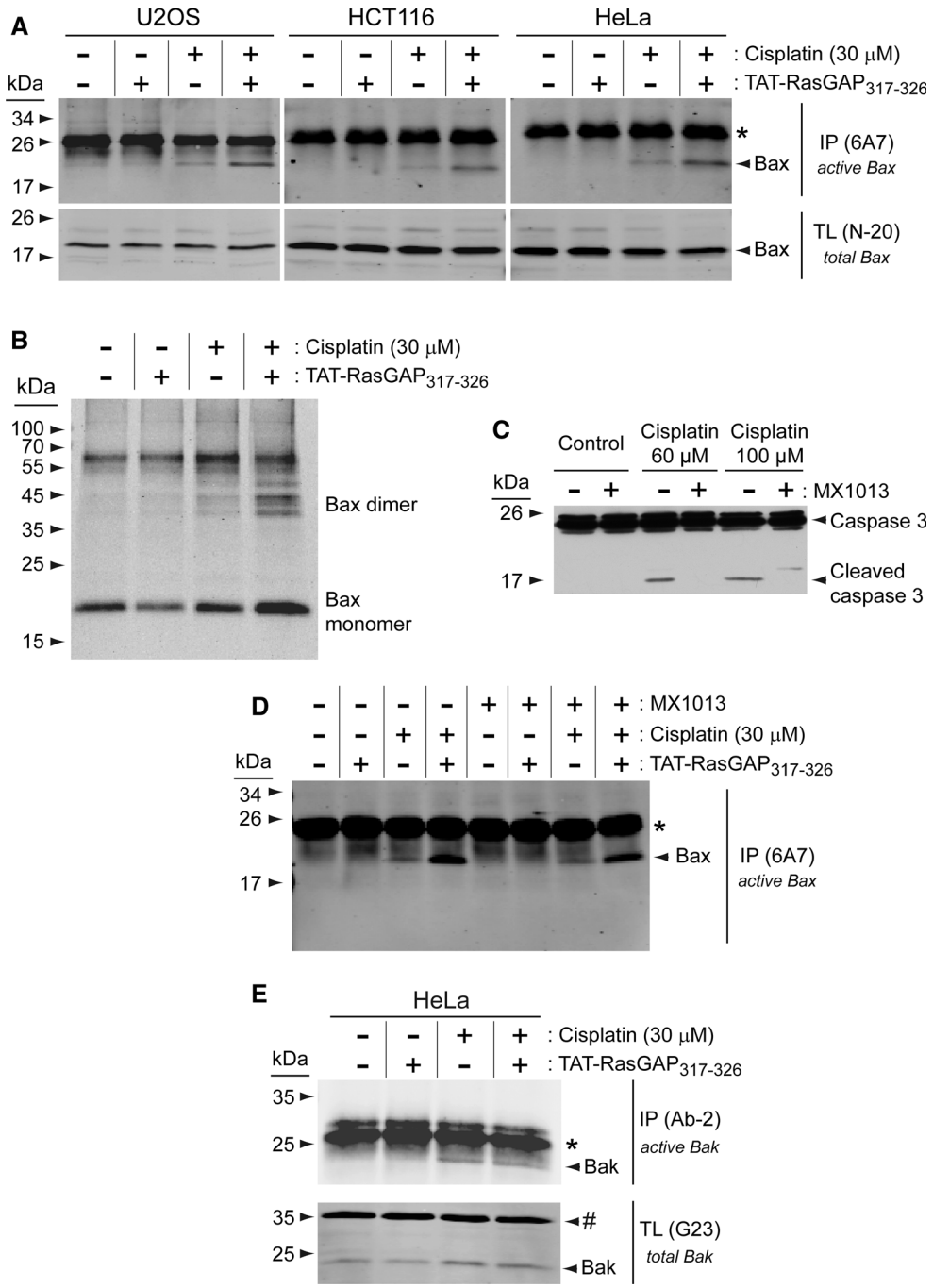
HeLa cells were harvested in PBS and centrifuged 10 min at 1,000×*g*. Cells were then resuspended in isotonic MB, broken by five passages through a 25G1 0.5- by 2.5-mm needle fitted on a 2 ml syringe and centrifuged at 1,500×*g* for 5 min. This procedure was repeated twice and supernatants from each step were pooled and centrifuged 5 min at 1,500×*g*. Supernatant was collected, centrifuged 5 min at 2,000×*g* and further centrifuged 10 min at 9,000×*g*. Pellet was resuspended in MB (100 μl), centrifuged 10 min at 7,000×*g* and the pellet, representing the mitochondrial fraction, was finally resuspended in a volume of 85–100 μl of MB. Mitochondria (40 μg) were incubated for 30 min at room temperature with 5 mM DSS. The reaction was stopped by addition of 1 M Tris base pH 8 quenching buffer (final Tris concentration of 20 mM). Mitochondria were centrifuged, resuspended in 30 μl RIPA lysis buffer and incubated on ice for 15 min. After centrifugation at 16,000×*g*, the supernatant was collected, mixed with 7.5 μl 5X loading buffer and analyzed by Western blot using the N-20 anti-Bax antibody.

Western blotting

Cells were lysed in 100 μl of monoQ-c buffer. Primary and secondary antibodies were used at a 1:1000 and a 1:5000 dilution, respectively. Visualization of the bands was performed using the Odyssey infrared imaging device and software (Licor, Homburg, Germany). Protein levels were quantitated using ImageJ software.

Antibodies

The anti-Puma rabbit polyclonal IgG antibody, the anti-Bid rabbit polyclonal antibody, the anti-Bcl-X$_L$ rabbit polyclonal IgG antibody, and the anti-caspase-3 rabbit polyclonal IgG antibody were from Cell Signaling (ref. no. 4976, 2002, 2764 and 9665, respectively). The 6A7 anti-active Bax mouse



monoclonal IgG1, the N-20 anti-total Bax rabbit polyclonal IgG, and the G23 anti-total Bak rabbit polyclonal IgG antibodies were from Santa Cruz (ref. no. sc-23959, sc-493 and sc-832, respectively). The anti-Bcl-2 mouse monoclonal IgG1 antibody was from Upstate Biotechnology (ref. no. 05-341), the Y37 anti-Mcl-1 rabbit monoclonal IgG antibody was from Abcam (ref. no. ab32087), the 3C5 anti-Bim rat monoclonal IgG2a antibody and the 1E1-1-10 anti-cIAP1 rat monoclonal IgG2a antibody were from Enzo Life Sciences (ref. no. ALX-804-527-C100 and ALX-803-335-C100, respectively). The 48/hILP/XIAP anti-XIAP mouse monoclonal IgG1 antibody was from BD Biosciences (ref. no. 610762). The Ab-2 anti-active Bak mouse monoclonal IgG2b antibody was from Merck (ref. no. AM04). The mouse monoclonal anti-FLAG M2 antibody was from Sigma (ref. no. F1804). Secondary antibodies were goat anti-mouse IRDye800-conjugated antibody (Rockland, ref. no. 610-132-121), goat anti-rabbit Alexa Fluor 680-conjugated antibody (Molecular Probes, ref. no. A21109) and donkey anti-rabbit HRP-conjugated antibody (Jackson ImmunoResearch, ref. no. 711-035-152).

◀ **Fig. 2** TAT-RasGAP$_{317–326}$ favors, in a caspase-independent manner, the ability of cisplatin to activate Bax. **a** U2OS, HeLa and HCT116 were treated for 16 h with 20 μM TAT-RasGAP$_{317–326}$ and/or with 30 μM of cisplatin as indicated in the figure. The cells were then lysed and Bax was immunoprecipitated with an antibody (*6A7*) that specifically recognizes the active conformation of Bax. Western blots were revealed with the N-20 antibody recognizing all forms of Bax. *IP* immunoprecipitate; *TL* total lysate; *asterisk* immunoglobulin light chains. **b** HeLa cells were treated during 16 h with 20 μM TAT-RasGAP$_{317–326}$ and 30 μM cisplatin alone or in combination. Mitochondria were isolated and cross-linked with DSS. Mitochondrial proteins were extracted and Bax was analyzed by Western blotting. **c** HCT116 were either left untreated or treated with the indicated concentrations of cisplatin for 20 h, in the presence or in the absence of the MX1013 pan-caspase inhibitor. The cells were then lysed and the cleavage of caspase-3 was assessed by Western blotting. **d** U2OS cells were processed as in panel A but in the presence or in the absence of 10 μM MX1013. **e** HeLa cells were treated for 16 h with 20 μM TAT-RasGAP$_{317–326}$ and/or with 30 μM of cisplatin as indicated in the figure. The cells were then lysed and Bak was immunoprecipitated with an antibody (*Ab-2*) that specifically recognizes the active conformation of Bak. Western blots were revealed with the G23 antibody recognizing all forms of Bak. *IP* immunoprecipitate; *TL* total lysate; *asterisk* immunoglobulin light chain; *hash* non-specific band

Plasmids

The pcDNA3 expression vector is from Invitrogen. pEGFP-C1 (Clontech) encodes the green fluorescent protein. Puma.cmv (Origene) encodes human Puma. hTP53.dn3 (#703) encodes human p53. It was generated by subcloning the 1,193 bp EcoRI/BamHI fragment from hTP53.lti (#384; gift from Richard Iggo) into pcDNA3 opened with the same two enzymes. TRIP-PGK-IRESNEO-WHV (#350) is a lentiviral vector bearing the neomycine resistance. SV40LargeTantigen.pBABE-puro (#731) encodes the SV40 large T antigen (Addgene; plasmid 13970). SV40LargeTantigen.lti-neo (#738) similarly encodes the large T antigen but in a lentiviral expression vector. It was constructed by subcloning the BamHI 2187 base pairs fragment of SV40LargeTantigen.pBABE-puro into TRIP-PGK-IRESNEO-WHV (#350) opened with the same enzyme. LeGO-iT2-Bcl-xL (#821) was constructed by subcloning the 771 bp EcoRI fragment from hBcl-XL.dn3 (#274) into LeGO-iT2 (#809; Addgene: plasmid 27343). The extension .lti indicates that the backbone is a lentiviral vector. The extension .dn3 indicates that the backbone plasmid is pcDNA3.

Results

Sensitizing activity of TAT-RasGAP$_{317–326}$ to various apoptotic stimuli

We demonstrated earlier that TAT-RasGAP$_{317–326}$ sensitizes several cancer cell lines to apoptosis induced by genotoxins such as cisplatin and doxorubicin [8]. These compounds are described to induce apoptosis mostly via the intrinsic apoptosis pathway [13, 14]. To gain further insight about the manner by which TAT-RasGAP$_{317–326}$ exerts its tumor sensitization effect, HeLa, U2OS, and HCT116 cancer cells were treated with additional pro-apoptotic compounds. Figure 1a shows that TAT-RasGAP$_{317–326}$ favored cisplatin-induced apoptosis in the three different cancer cell lines, confirming earlier results [8, 28]. TAT-RasGAP$_{317–326}$ sensitized some, but not all, tumor cell lines to UV- or growth factor deprivation-induced apoptosis, despite the fact that these treatments can activate the intrinsic apoptotic pathway [32, 33]. None of the tumor cell lines was sensitized by the peptide to TNFα, an extrinsic pathway stimulator [34, 35] in presence of CHX, a protein synthesis inhibitor that prevents the expression of anti-apoptotic proteins that would otherwise counteract the pro-apoptotic abilities of TNFα [36, 37]. These results suggest that TAT-RasGAP$_{317–326}$ exerts a pro-apoptotic role in conditions where the intrinsic cell death pathway is engaged. However since HeLa and HCT116 are type II cells [38, 39] (see also Fig. 1b), i.e. they need the mitochondrial pathway to fully activate caspase-3 in response to extrinsic death stimuli [40], it is unclear why TAT-RasGAP$_{317–326}$ does not have any sensitizing effect on these cell lines when incubated with TNFα. One possibility is that CHX prevents the expression of a protein that is targeted by TAT-RasGAP$_{317–326}$ to mediate its sensitization effect. To test this hypothesis, we used another extrinsic pathway stimulator, TNF-related apoptosis-inducing ligand (TRAIL) [41]. Unlike TNFα + CHX, the apoptotic response induced by TRAIL was increased by TAT-RasGAP$_{317–326}$ in HeLa cells (Fig. 1b). These cells were confirmed to be type II cells by the complete protection against TRAIL-induced apoptosis conferred by Bcl-X$_L$ over-expression (Fig. 1c).

TAT-RasGAP$_{317–326}$ increases cisplatin-induced Bax activation

Tumor cells sensitized by TAT-RasGAP$_{317–326}$ to genotoxin-induced apoptosis show increased caspase-3 activation and more pronounced mitochondrial membrane depolarization [28]. This suggests that the RasGAP-derived peptide acts upstream of cytochrome *c* release from mitochondria. Consequently, Bax, which is required for mitochondrial outer membrane permeabilization, cytochrome *c* release, and caspase activation [21], should be more activated by genotoxins in the presence of TAT-RasGAP$_{317–326}$. To study the status of Bax activation, we performed immunoprecipitation experiments with an antibody (Bax 6A7) that only binds the active conformation of Bax. Figure 2a shows that while TAT-RasGAP$_{317–326}$ alone does not affect Bax activation, it increases cisplatin-induced Bax stimulation. Consistent with these observations, the peptide did not induce

Bax oligomerization by itself but amplified cisplatin-induced Bax oligomerization (Fig. 2b). Figure 2a (lower blots) shows that neither cisplatin nor TAT-RasGAP$_{317–326}$, alone or in combination, alter total Bax cellular levels. However, the amount of Bax associated with the mitochondria, either as monomers or dimers, is increased in cells treated with cisplatin and TAT-RasGAP$_{317–326}$ compared to cells treated with cisplatin alone (Fig. 2b). It appears thus that the RasGAP-derived peptide can facilitate cisplatin-mediated Bax recruitment to the mitochondria.

There is evidence that caspase activation induces a positive feedback loop to increase Bax activation and cytochrome *c* release from the mitochondria [42]. To determine if TAT-RasGAP$_{317–326}$ modulates this feedback loop, the experiment shown in Fig. 2a was repeated in the presence of the MX1013 pan-caspase inhibitor [43, 44] that efficiently blocks executioner caspase activity (Fig. 2c). Figure 2d shows that caspase inhibition does not prevent TAT-RasGAP$_{317–326}$ from enhancing cisplatin-mediated Bax activation. The role of Bak, the other Bcl-2 family member involved in mitochondrial permeabilization was also investigated but we did not observe modulation of cisplatin-induced Bak activation by TAT- RasGAP$_{317–326}$ (Fig. 2e).

TAT-RasGAP$_{317–326}$ does not modulate Bcl-2 and IAP family members

Bax activation is controlled by pro- and anti-apoptotic members of the Bcl-2 family [19, 21]. Conceivably, TAT-RasGAP$_{317–326}$ could favor genotoxin-induced apoptosis by increasing the levels of pro-apoptotic Bcl-2 family members and/or decreasing the levels of the anti-apoptotic members. However, the peptide did neither modulate the expression of pro-apoptotic Bim, Puma and Bid proteins nor the anti-apoptotic Bcl-X$_L$, Bcl-2 and Mcl-1 proteins (Fig. 3). Another possibility that could explain how TAT-RasGAP$_{317–326}$ favors apoptosis is inhibition of IAPs. However, the peptide did not alter cellular levels of cIAP1, cIAP2 and XIAP (Fig. 3). Therefore, modulation of the expression of IAPs and Bcl-2 family members is not the mechanism used by TAT-RasGAP$_{317–326}$ to favor Bax activation and apoptosis. However, this does not exclude the possibility that the peptide affects the activity of these proteins.

Targeted disruption of individual Bcl-2 family members or IAPs does not prevent TAT-RasGAP$_{317–326}$-mediated tumor cell sensitization

Large T antigen-transformed MEFs lacking specific Bcl-2 family members were used to investigate the role of this protein family in the capacity of TAT-RasGAP$_{317–326}$ to sensitize tumor cells to genotoxin-induced apoptosis. MEFs are not cancer cells and hence are not susceptible to TAT-RasGAP$_{317–326}$-mediated genotoxin sensitization. However, transforming MEFs with the SV40 large T antigen renders them sensitive to the peptide [30]. Figure 4a shows that transformed MEFs lacking the pro-apoptotic BH3-only Bid, Bim, Bmf, or Bad proteins or the anti-apoptotic Bcl-X$_L$, Bcl-2, or Mcl-1 proteins were efficiently sensitized by TAT-RasGAP$_{317–326}$ to cisplatin-induced apoptosis. Furthermore, the pro-apoptotic family members Bax and Bak, crucial for outer mitochondrial membrane permeabilization are not individually required to mediate TAT-RasGAP$_{317–326}$ sensitization. As expected [45], transformed MEFs lacking both Bax and Bak were totally resistant to cisplatin-induced death.

We next examined the implication of cIAP1/2 and XIAP in TAT-RasGAP$_{317–326}$-mediated sensitization to cisplatin-induced apoptosis. We used the SM-164 Smac mimetic to induce cIAP1/2 degradation and XIAP inhibition [46]. In presence of the Smac mimetic, the sensitization to cisplatin induced by the peptide was not altered, despite strong reduction in cIAP1 and cIAP2 levels, indicating that cIAP1/2 and XIAP are not involved in TAT-RasGAP$_{317–326}$-mediated sensitization of cisplatin-induced apoptosis.

TAT-RasGAP$_{317–326}$ does not sensitize cells to Puma over-expression

We previously hypothesized that an intact p53/Puma axis was required for efficient genotoxin-induced apoptosis and for TAT-RasGAP$_{317–326}$ to favor genotoxin-induced apoptosis in cancer cells [28]. We reasoned that if Puma is necessary and sufficient for the sensitizing activity of the RasGAP-derived peptide, ectopic expression of Puma should render tumor cells sensitive to TAT-RasGAP$_{317–326}$ in the absence of cisplatin. Figure 5a shows that transfecting U2OS cells with amounts of Puma-encoding plasmids in the 0.5 to 3.5 μg range (open circles) led to similar levels of Puma expression than obtained by treating the cells with concentrations of cisplatin in the 2.5–30 μM range (closed squares). However, TAT-RasGAP$_{317–326}$ only sensitized cells incubated with cisplatin and not those transfected with the Puma-encoding plasmids (Fig. 5b).

Puma is a p53 transcription target that may, once synthesized, need p53 to exert its full pro-apoptotic actions [47]. Figure 6 shows that ectopic expression of p53 in U2OS cells induced apoptosis. However this was neither modulated by Puma nor by TAT-RasGAP$_{317–326}$. Taken together these data suggest that TAT-RasGAP$_{317–326}$ does not directly amplify the DNA damage-induced p53/Puma arm. Hence, even though cisplatin requires p53 and Puma to induce apoptosis [28], this genotoxin may modulate

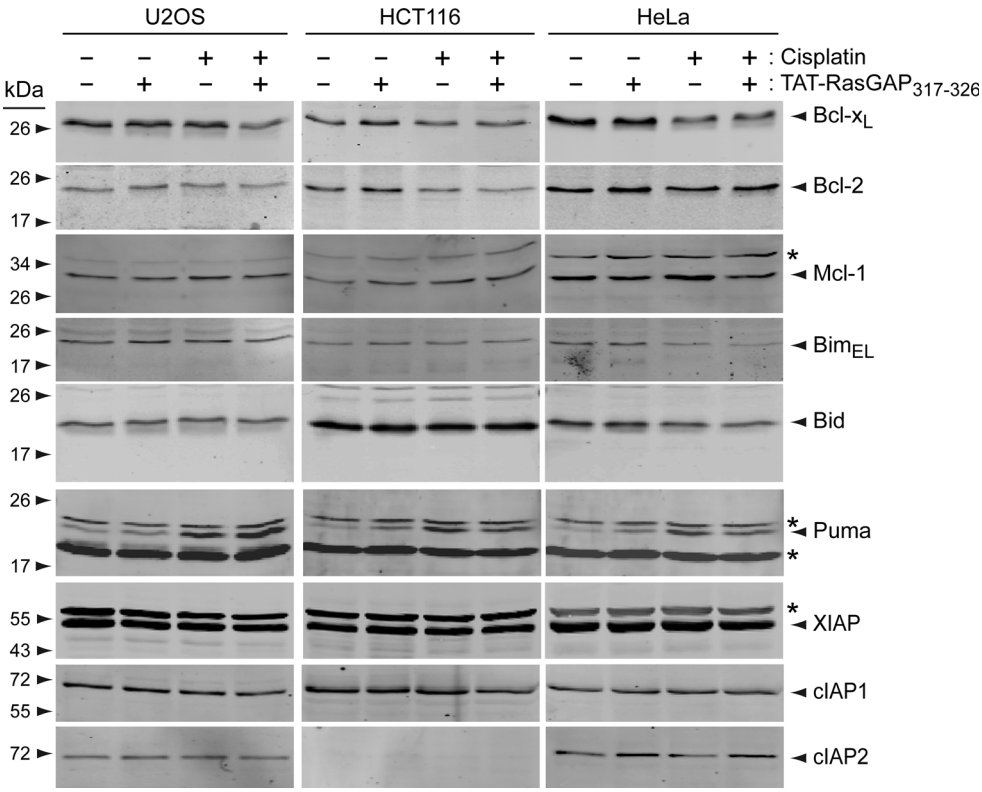


Fig. 3 TAT-RasGAP$_{317–326}$ does not modulate Bcl-2 family protein levels. U2OS, HCT116 and HeLa were treated as shown in the figure for 22 h (TAT-RasGAP$_{317–326}$, 20 μM; cisplatin, 30 μM). Cells were then lysed and total protein extracts were immunoblotted with antibodies specific for the indicated proteins (*asterisk* non-specific band). cIAP2 was not detected in HCT116 cells

other pro-apoptotic signals that are targeted by TAT-RasGAP$_{317–326}$ to induce cell death in cancer cells.

TAT-RasGAP$_{317–326}$ affects long term survival of cancer cells independently of p53

The above observation that the RasGAP-derived peptide does not amplify the p53/Puma axis led us to reconsider the implication of p53 in the TAT-RasGAP$_{317–326}$-mediated sensitization of cancer cells. Cells lacking an intact p53/Puma axis show a drastically reduced apoptotic rate when exposed to genotoxin treatment [28], but they may remain sensitive to the drugs on longer term exposure. Indeed, Fig. 7 shows that the survival of either p53-positive or p53-negative HCT116 cells, tested by colony formation assay, was reduced by cisplatin treatment to similar extent. Importantly, TAT-RasGAP$_{317–326}$ significantly augmented cisplatin-mediated cell survival impairment in both cases (Fig. 7). It was suggested, based on earlier observation that the HCT116 tumor cell line lacking either p53 or Puma did not undergo more cisplatin-induced apoptosis in the presence of TAT-RasGAP$_{317–326}$ [28], that TAT-RasGAP$_{317–326}$ required a functional p53-Puma axis to exert its sensitizing function. The data presented in Fig. 7 demonstrate that this initial assumption is not correct. Presumably, the absence of p53 or Puma by dramatically increasing the resistance of HCT116 cells to cisplatin-induced apoptosis [28] masks the sensitizing effect of the peptide in short-term experiments. Another difference to take into account is the method used to evaluate the sensitization to cisplatin. In previous report, sensitization was evaluated using apoptosis-specific assays (i.e. scoring cells with pycnotic nuclei). Here, HCT116 p53 KO cells were shown to be sensitized to cisplatin using colony formation assays. Apoptosis can affect the ability of cells to form colonies but other forms of death (e.g. mitotic catastrophe, necrosis) can too. Hence the results shown in Fig. 7 indicate that TAT-RasGAP$_{317–326}$ sensitizes tumors to several types of cell death.

TAT-RasGAP$_{317–326}$ sensitizes HCT116 cells irrespectively of their Bax and Bak status

Recently, HCT116 cell lines lacking Bax and/or Bak were generated [48]. We aimed to determine if Bax and Bak were required for cisplatin-induced death and TAT-RasGAP$_{317–326}$-mediated sensitization in the HCT116 background as they were in the MEF background (see Fig. 4). Bax and Bak status in HCT116 wild-type, single KOs and double KO was first confirmed by Western blotting (Fig. 8a). In contrast to MEFs, the absence of Bax and Bak in HCT116 cells reduced, but did not abrogate the ability of cisplatin to induce apoptosis (Fig. 8b) and, importantly, did

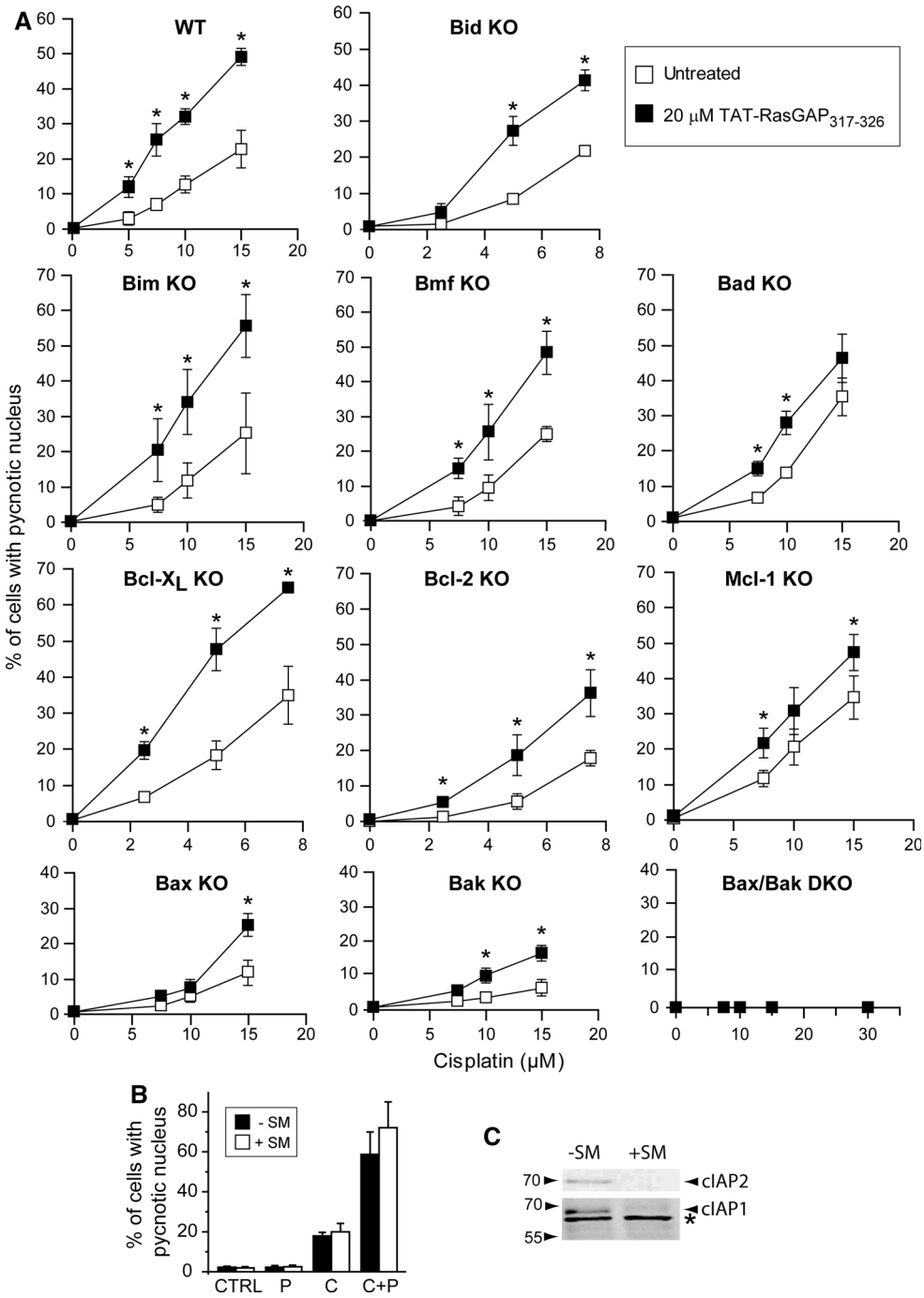


Fig. 4 Role of individual Bcl-2 family proteins in TAT-RasGAP$_{317–326}$-mediated sensitization to cisplatin-induced apoptosis. **a** SV40-transformed MEFs lacking the indicated Bcl-2 family proteins were treated as indicated in the figure for 22 h. Cells were then fixed and apoptosis was counted by scoring pycnotic nuclei. The results correspond to the mean ±95 % CI of at least three independent experiments. The asterisks indicate a statistically significant difference (as assessed by unpaired *t* test between cells treated or not with TAT-RasGAP$_{317–326}$ in the presence cisplatin). **b** HeLa cells were treated for 16 h with 20 μM TAT-RasGAP$_{317–326}$ (*P*) and/or with 30 μM of cisplatin (*C*) in the presence or in the absence of 100 nM Smac mimetic (SM) as indicated in the figure. Cells were then fixed and apoptosis was determined by scoring pycnotic nuclei. The results correspond to the mean ±95 % CI of three independent experiments. **c** Cells were lysed and cIAP1 and cIAP2 expression controlled by Western blotting

not prevent TAT-RasGAP$_{317–326}$ from sensitizing these cells to cisplatin-induced apoptosis. Using a colony formation assay, it was found that cisplatin reduced more or less to similar extents the survival of HCT116 cells expressing or not Bax and Bak (Fig. 8c). The absence of Bax and Bak did not alter the capacity of TAT-RasGAP$_{317–326}$ to favor cisplatin-mediated survival decrease. These results indicate that apoptosis and cell death induced by genotoxins such as cisplatin can occur in the absence of a functional mitochondrial apoptotic pathway (i.e. in the absence of Bax and Bak in HCT116 cells) and that these modes of death can be augmented by TAT-RasGAP$_{317–326}$.

Discussion

This study provides advances in the understanding on the mode of action of TAT-RasGAP$_{317–326}$. It shows that this peptide enhances cisplatin-induced Bax activation. The RasGAP-derived peptide can therefore positively modulate

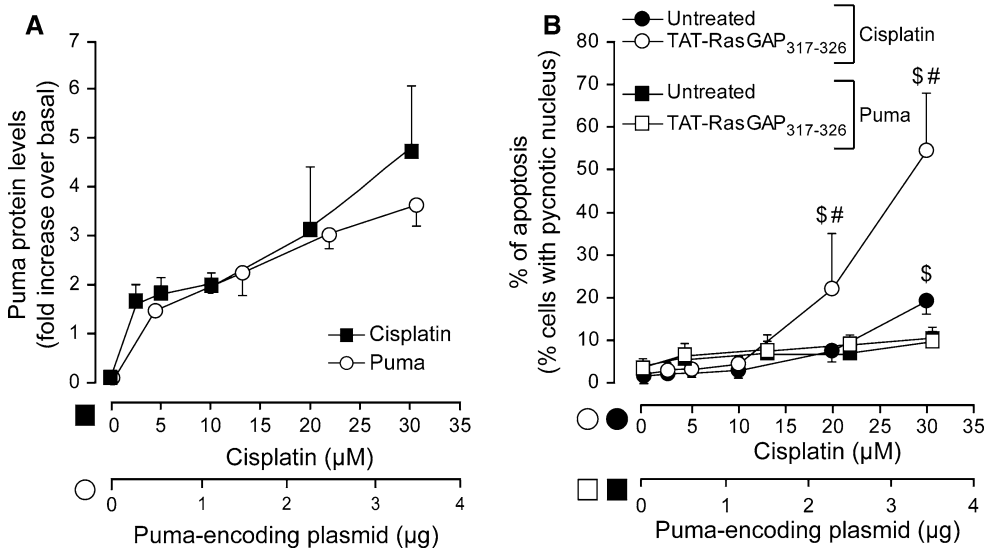


Fig. 5 Puma expression per se does not render tumor cells sensitive to TAT-RasGAP$_{317–326}$. **a** U2OS were treated with increasing concentrations of cisplatin or transfected with the indicated amounts of a Puma-encoding plasmid. Puma protein levels were then determined by quantitative Western blotting. **b** U2OS cells were treated as in *panel* **a** and then incubated or not with TAT-RasGAP$_{317–326}$ (20 μM) for 22 h. Apoptosis was then determined. Results correspond to the mean ± 95 % CI of three independent experiments. *hash* indicates a statistically significant difference between cells incubated or not with TAT-RasGAP$_{317–326}$ at given doses of cisplatin. *dollar* denotes significant differences to the "0 μM cisplatin" condition

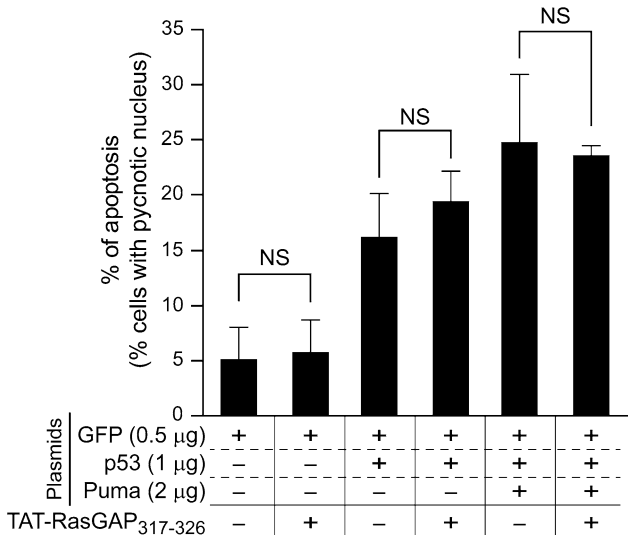


Fig. 6 Ectopic expression of p53 and Puma does not render cancer cells sensitive to TAT-RasGAP$_{317–326}$. U2OS were transfected with the indicated plasmids and treated or not with 20 μM of TAT-RasGAP$_{317–326}$ for 22 h. Apoptosis was then determined. Results correspond to the mean ± 95 % CI of four independent experiments. *NS* no statistically significant difference

the intrinsic apoptotic pathway activated by genotoxins. But the ability of TAT-RasGAP$_{317–326}$ to favor genotoxin-activated tumor cell death is not limited to the intrinsic apoptotic pathway because i) cisplatin-induced apoptosis and TAT-RasGAP$_{317–326}$-mediated sensitization can occur in cells lacking Bax and Bak and ii) tumor cells lacking p53 can be sensitized by the RasGAP-derived peptide to die by an apparently non-apoptotic form of cell death. Hence, TAT-RasGAP$_{317–326}$-mediated tumor cell sensitization to genotoxins can occur independently of Bax, Bak, and p53 through a death pathway that remains to be clarified.

Increased Bax stimulation in the context of TAT-RasGAP$_{317–326}$-mediated sensitization

Bax and Bak are Bcl-2 family members the activation and oligomerization of which induce permeabilization of the mitochondrial outer membrane, resulting in the release of cytochrome *c* [21]. This study shows that TAT-RasGAP$_{317–326}$ increases genotoxin-induced Bax activation. However, how this augmentation is accomplished remains unclear. First, the ability of TAT-RasGAP$_{317–326}$ to increase genotoxin-induced Bax activation did not require caspase activation (Fig. 2). Therefore, the peptide does not amplify a positive feedback loop on Bax activation induced by the stimulation of caspases following cytochrome *c* release [42]. Second, Bax activation does not result from a peptide-induced balance shift between the expression levels of pro- and anti-apoptotic Bcl-2 family members. Indeed, the protein levels of Bcl-2, Bcl-X_L, Mcl-1, Bim, Puma, and Bid were not modulated by TAT-RasGAP$_{317–326}$ (Fig. 3). Moreover, the expression of IAPs was not modified by the RasGAP-derived peptide. However, this does not exclude the possibility that the peptide affects the activity of these proteins. In addition, all the Bcl-2 family proteins we have tested (Bcl-2, Bcl-X_L, Mcl-1, Bim, Bid, Bmf and Bad) were shown to be individually dispensable for the sensitizing activity of the peptide.

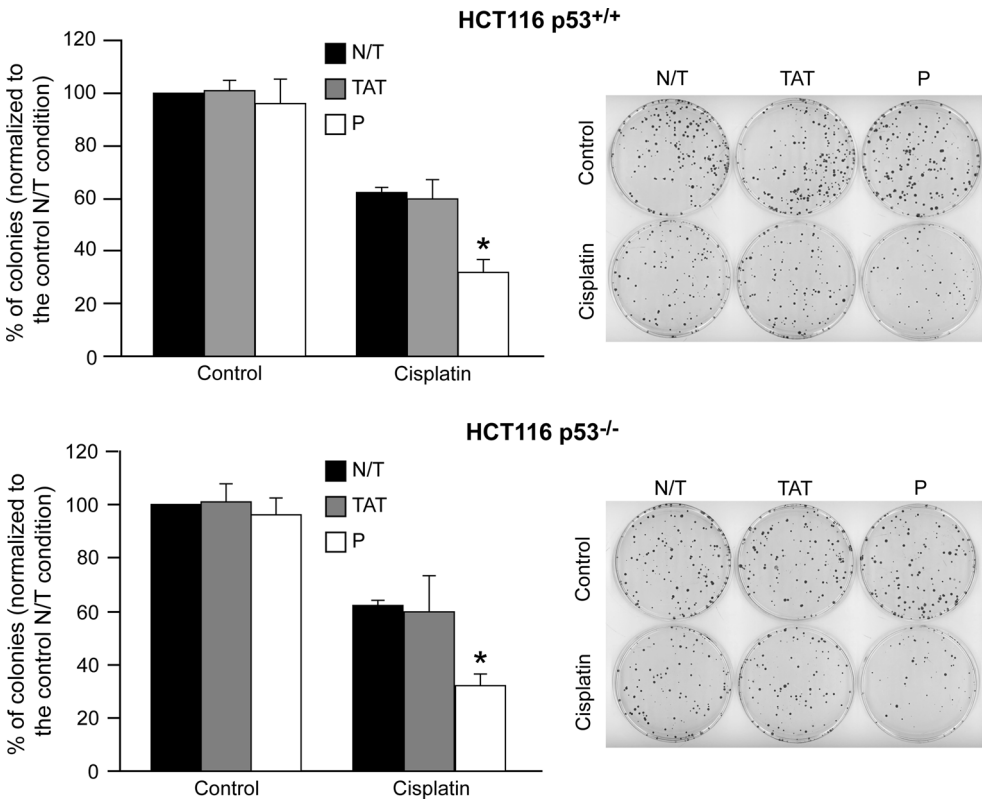


Fig. 7 TAT-RasGAP$_{317–326}$ sensitizes cancer cells to cisplatin-induced cell death irrespectively of their p53 status. Wild-type HCT116 and p53$^{-/-}$ HCT116 were treated with 1 μM cisplatin in the presence or in the absence of 20 μM TAT-RasGAP$_{317–326}$ (*P*) or 20 μM TAT for 3 days. Culture medium was then replaced with fresh medium and following an additional 10-day period the number of colonies was recorded. *N/T* untreated. The results correspond to the mean ± 95 % CI of at least three independent experiments. The *asterisks* indicate a statistically significant difference between cisplatin-treated cells incubated or not with TAT-RasGAP$_{317–326}$

This suggests that these proteins are not direct or indirect targets of TAT-RasGAP$_{317–326}$. However, the possibility that the RasGAP-derived peptide targets several Bcl-2 family members, which compensate each other when one is absent to mediate the sensitizing action of the peptide, cannot be excluded at the present time. Furthermore, HCT116 Bax KO cells were found to be sensitized to genotoxin-induced death. This indicates that TAT-RasGAP$_{317–326}$-mediated sensitization is not strictly restricted to increased Bax activation.

TAT-RasGAP$_{317–326}$-mediated sensitization independently of Bax and Bak (and p53)

In contrast to MEFs, HCT116 cells lacking Bax and Bak can still undergo genotoxin-induced apoptosis and this cell death response can be increased by TAT-RasGAP$_{317–326}$. Moreover, HCT116 cells lacking p53, despite being greatly compromised in their ability to apoptose in response to genotoxins, as evidenced by their resistance to pycnosis, are nevertheless more efficiently killed in long-term survival experiments (colony-formation assays) by genotoxins when incubated with the RasGAP-derived peptide. Thus, TAT-RasGAP$_{317–326}$ has the capacity to favor non-mitochondrial forms of cell death.

Recent findings in non-small cell lung cancer showed that Bax and Bak can be dispensable for cisplatin-induced apoptosis [49]. In this study, using cisplatin-sensitive and cisplatin-resistant cell lines lacking Bax and Bak, it was shown that cisplatin-induced apoptosis may rely on caspase-8 activation in an acid sphingomyelinase and Fas-associated death domain (FADD)-dependent manner. Incidentally, it is also noticed that Bax/Bak DKO MEFs were found to be totally resistant to cisplatin-induced apoptosis in line with the data presented in Fig. 4. One could hypothesize that apoptosis in Bax/Bak DKO HCT116 cells is mediated through caspase-8 activation and that TAT-RasGAP$_{317–326}$ increases this response. The implication of caspase-8 in TAT-RasGAP$_{317–326}$ cancer cell sensitization remains to be defined however.

The present report also clarifies the role played by p53 (and Puma) in TAT-RasGAP$_{317–326}$-mediated sensitization of tumor cells to genotoxin-induced apoptosis. Interpretations of earlier work were that TAT-RasGAP$_{317–326}$ required a functional p53/Puma axis to favor apoptosis of cisplatin-stimulated tumor cells [28]. We therefore hypothesized that reproducing expression of p53 and Puma in cancer cells, which occurs when cells are treated with cisplatin, would render them sensitive to TAT-RasGAP$_{317–326}$. However, this proved not to be the case (Figs. 5, 7). Moreover, tumor cells lacking p53, even though highly resistant to cisplatin-induced apoptosis in short-term experiments [28], were nevertheless less able to survive long-term cisplatin treatment when co-treated with TAT-RasGAP$_{317–326}$ (Fig. 7). This indicates that the RasGAP-derived peptide does not require p53 to exert its tumor sensitization activity. Hence, p53 and Puma are not

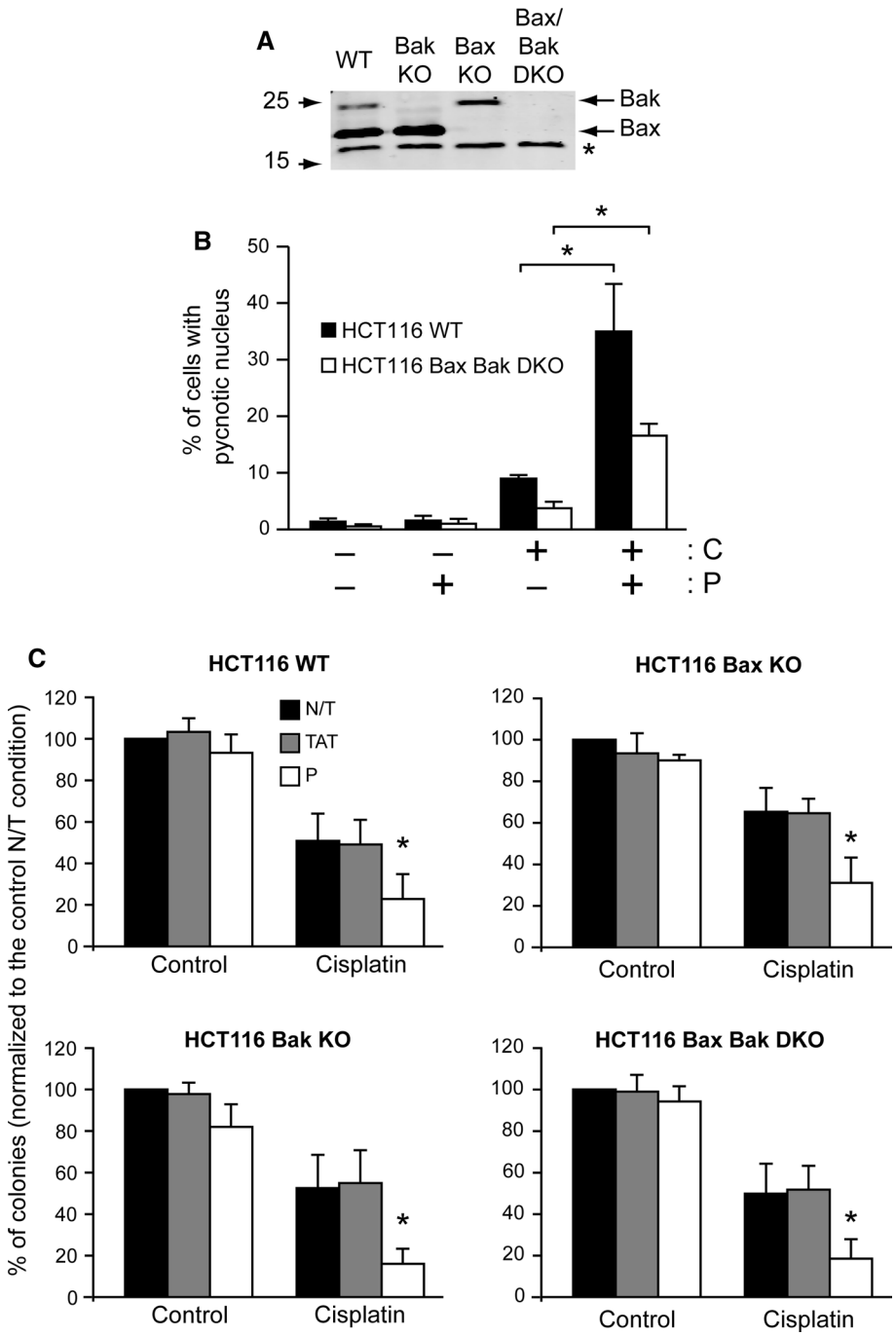


Fig. 8 TAT-RasGAP$_{317–326}$ sensitizes HCT116 cancer cells to cisplatin-induced cell death irrespectively of their Bax and Bak status. **a** Bax and Bak expression was assessed by Western blotting in wild-type (WT) HCT116, HCT116 Bak KO, HCT116 Bax KO and HCT116 Bax/Bak DKO cells (*asterisk* non-specific band). **b** WT HCT116 and HCT116 Bax/Bak DKO cells were treated during 24 h with 20 μM TAT-RasGAP$_{317–326}$ (*P*), 30 μM cisplatin (*C*). Cells were then fixed and apoptosis was assessed by scoring pycnotic nuclei. Results correspond to the mean ± 95 % CI of three independent experiments. The *asterisk* denotes a statistical significant difference. **c** The indicated HCT116 cell lines were treated with 2 μM cisplatin in the presence or in the absence of 20 μM TAT-RasGAP$_{317–326}$ (*P*) or 20 μM TAT for 3 days. Culture medium was then replaced with fresh medium and following an additional 10-day period the number of colonies was recorded. *N/T* untreated. The results correspond to the mean ± 95 % CI of four independent experiments. The asterisks indicate a statistically significant difference between cisplatin-treated cells incubated or not with TAT-RasGAP$_{317–326}$

targets of TAT-RasGAP$_{317–326}$. The apparent requirement of p53 and Puma for the genotoxin sensitizing function of the peptide in short-term apoptosis assays most likely results from the fact that genotoxins inefficiently induce apoptosis of tumor cells lacking a functional p53/Puma axis [50]. In other words, the absence of TAT-RasGAP$_{317–326}$-mediated sensitization observed in p53- or Puma-negative cells merely reflects the fact these cells cannot properly undergo genotoxin-induced apoptosis. Measuring cell death in short-term assays (e.g. within the first 24 h of treatment) might lead to an underestimation of the killing potential of genotoxins, as long-term cell survival is not taken into account. This may particularly be the case in cells bearing mutations in apoptosis-controlling proteins. Tumor cells lacking p53 can bypass the primary apoptotic decision, which takes place shortly after the administration of an apoptotic stimulus, and undergo several cell divisions before dying. This can occur either by p53-independent apoptosis or as a result of mitotic catastrophe or necrotic cell death [51]. The observation that

TAT-RasGAP$_{317–326}$, in combination with cisplatin, reduces the viability of p53-negative tumor cells demonstrates that this peptide can also positively reinforces p53-independent cell death mechanism(s) that occur in a more delayed manner compared to short-term apoptosis.

Earlier reports indicate that cisplatin [49] and TRAIL (reviewed in [52]) induce caspase-8 activation. Since the present work shows that TAT-RasGAP$_{317–326}$ sensitizes tumor cells to cisplatin and TRAIL-induced apoptosis, it raises the possibility that the peptide acts at the level of caspase-8 or of a caspase 8-containing complex. The activation of caspase-8 is modulated by several proteins, for instance positively by FADD and negatively by cellular FADD-like interleukin-1β–converting enzyme (FLICE)-like inhibitory protein (cFLIP) [53]. Hypothetically, TAT-RasGAP$_{317–326}$ could favor FADD activity to activate caspase-8 or the peptide could neutralize the cFLIP inhibitory effect. Further work is now needed to dissect the role played by caspase-8 and its binding partners in the tumor sensitizing activities of TAT-RasGAP$_{317–326}$.

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Conflict of interest CW is a coinventor of the TAT-RasGAP$_{317–326}$ compound as a genotoxin sensitizer (patent owned by the University of Lausanne) and may receive royalties from patent licensing if the compound is commercialized. Patent numbers and dates are WO 2005000887 (30.6.2003) and WO 2010097720 (30.1.2009). The other authors declare that they have no conflict of interest.

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