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Pasteurella multocida toxin is a potent activator of anti-apoptotic signalling pathways

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Summary

Toxigenic *Pasteurella multocida* strains produce a 146 kDa protein toxin (PMT) that due to its high mitogenic activity is thought to possess carcinogenic properties. PMT affects several signal transduction pathways related to cancer by constitutively stimulating heterotrimeric G proteins. Downstream of $G\alpha_q$, $G\alpha_{13}$ and $G\alpha_i$, the toxin activates the small GTPase RhoA, MAP kinases and signal transducer and activator of transcription (STAT) proteins. PMT also stimulates $G\beta\gamma$ signalling and activates phosphoinositide 3-kinase (PI3K)-related pathways, which play a crucial role in proliferation and apoptosis. We show that treatment of HEK293 cells with PMT inhibits staurosporine-mediated apoptosis through PI3K-dependent phosphorylation of Akt and constitutive expression of Pim-1 kinase. Simultaneous activation of these survival kinases allows the activation of pro-survival pathways, such as GSK3β, Mcl-1, Bcl-xL and Bcl-2, as well as the downregulation of apoptotic signals by Bax or Puma. Only the combined inhibition of Akt and Pim reverses the PMT-induced protection from staurosporine-induced apoptosis. In addition, we show that apoptosis induced by tumour chemotherapeutic agents is blocked by PMT in human cancer cell lines. Our data indicate that PMT is a highly potent anti-apoptotic agent, which supports the view of a carcinogenic potential of the toxin.

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Introduction

The 146 kDa protein toxin (PMT) from *Pasteurella multocida* is one major virulence factor of the pathogen (Wilson and Ho, 2006). The toxin has been shown to cause various diseases of wild-life and domestic animals, including the atrophic rhinitis of pigs, which is characterized by major destruction of the nose turbinate (Dominick and Rimler, 1986; Harper *et al.*, 2006), but the role of PMT in human *P. multocida*-induced diseases, such as bite-wound infections, abscesses, septic arthritis or osteomyelitis (Lax and Grigoriadis, 2001; Harper *et al.*, 2006), is not well established. PMT is one of the most potent mitogens (e.g. for cell culture fibroblasts, epithelial cells or osteoclasts) (Rozengurt *et al.*, 1990; Martineau-Doize *et al.*, 1993; Mullan and Lax, 1996). The toxin stimulates several signalling pathways involved in cell proliferation, such as RhoA-, MAP kinases-, phosphoinositide 3-kinase (PI3K)- and JAK-STAT-dependent pathways (Harper *et al.*, 2006). These toxin effects are caused by constitutive activation of the heterotrimeric G proteins $G\alpha_q$, $G\alpha_{13}$ (Orth *et al.*, 2004) and $G\alpha_i$ (Orth *et al.*, 2008). The molecular mechanism of G protein activation involves toxin-catalysed deamidation of a glutamine residue essential for GTP hydrolysis (Orth *et al.*, 2009).

One target of STAT proteins is the *Pim-1* (provirus integration site for Moloney murine leukaemia virus) proto-oncogene that encodes two isoforms of a serine/threonine protein kinase with molecular weights of 33 and 44 kDa (Saris *et al.*, 1991). The Pim family (Pim-1-3) (van der Lugt *et al.*, 1995) is involved in multiple cellular functions such as proliferation, differentiation, cell survival, apoptosis and tumorigenesis (Wang *et al.*, 2001; Bachmann and Moroy, 2005). Pim kinase expression is elevated in several human cancers, including multiple myeloma, lymphoma, prostate cancer, leukaemia and pancreatic cancer (Yoshida *et al.*, 1999; Dhanasekaran *et al.*, 2001; Claudio *et al.*, 2002; Li *et al.*, 2006). After growth factor or cytokine receptor activation, Akt is phosphorylated and thereby enabled to transit from the membrane to the nucleus to affect transcription of target genes (Pekarsky *et al.*, 2000). Previous results indicate that Pim and Akt family members are critical components of overlapping signalling pathways involved in cell growth and survival (Amaravadi and Thompson, 2005). Indeed, both kinases can cooperate to promote increases in cell size, cell survival and tumorigenesis in transgenic animals

(Hammerman *et al*., 2005). Both serine/threonine kinases participate in the regulation of Bcl-2 (B-cell leukaemia/lymphoma-2) family members. The Bcl-2 family regulates apoptosis through control of mitochondrial membrane permeability and release of cytochrome *c*. Besides the pro-survival members Bcl-xL, Bcl-2 and Mcl-1, also pro-apoptotic members such as Bad, Bax and Puma belong to the Bcl-2 family. Abnormal levels of Bcl-2 proteins can be found in human cancers, where Bcl-2 protects from programmed cell death, a mechanism to control excess proliferation and tumour development.

Interestingly, also viruses and bacterial pathogens are able to induce an anti-apoptotic proliferative state that protects the infected cells from apoptosis induced by the immune system (Lax and Thomas, 2002; Nougayrede *et al*., 2005). In the case of *Helicobacter pylori* this has been associated with the development of cancer. Other bacteria, which contain proteins with the ability to act as mitogens (e.g. *Bacteroides fragilis* toxin or cytotoxic necrotizing factors from *Escherichia coli*), are also discussed as potential carcinogens (Lax, 2005).

Here, we investigated how the bacterial toxin PMT is able to rescue cells from induced programmed cell death. Our studies show that *P. multocida* toxin has the ability to suppress apoptosis induced by kinase inhibition after staurosporine treatment or DNA damage, presumably through activation of the PI3K, Akt and Pim pathway. Only simultaneous inactivation of both kinases restored staurosporine-induced apoptosis. Our data show that PMT mediates expression of anti-apoptotic and suppression of pro-apoptotic members of the Bcl-2 family. Therefore, we present *P. multocida* toxin as a potent inhibitor of apoptosis and we hypothesize that this is an immunomodulatory mechanism to enable infected cells to escape programmed cell death.

Results

PMT suppresses staurosporine-induced apoptosis

To investigate the potential influence of PMT on the process of programmed cell death, we induced apoptosis through treatment with the kinase inhibitor staurosporine that triggers both caspase-dependent and -independent apoptotic pathways.

HEK293 cells were pre-incubated with PMT^{wt} or inactive PMT^{C1165S} and, thereafter, the induction of apoptosis by staurosporine was analysed. As expected, treatment of HEK293 cells with staurosporine for 2 h strongly increased Annexin-V binding as a marker for apoptotic membrane blebbing (Fig. 1A, curve b). However, this effect was completely inhibited by pre-treatment of the cells with PMT^{wt} (curve c) and the cells were indistinguishable from untreated cells (curve a). On the other hand, an inactive mutant of PMT (PMT^{C1165S}), in which the active-site cysteine-1165 is changed to serine, was ineffective and did not prevent apoptosis (curve d).

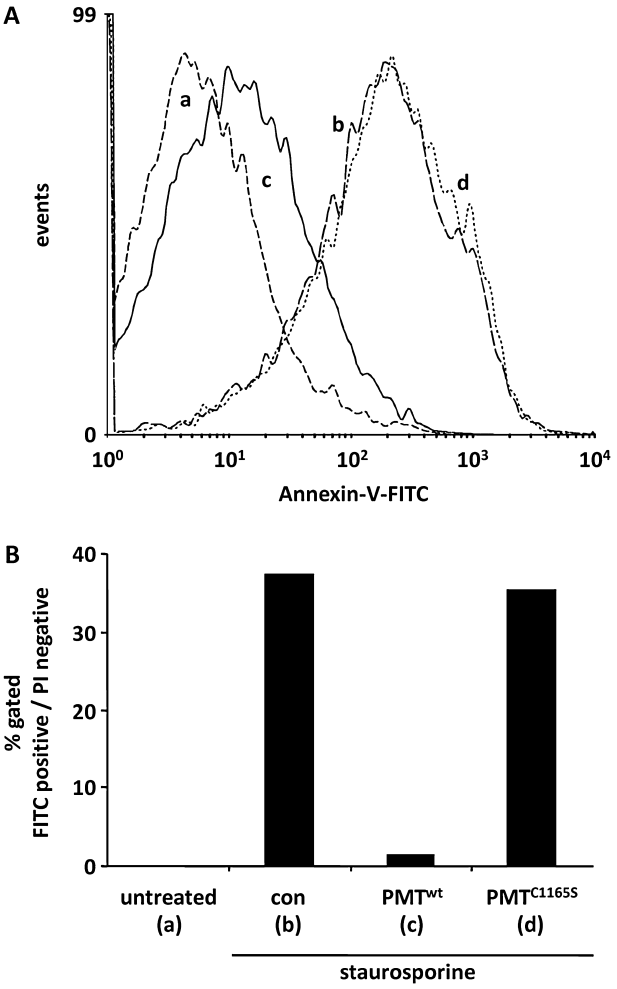


Fig. 1. PMT exhibits strong anti-apoptotic effects.
A. Apoptotic HEK293 cells identified in FACS analysis after staining with Annexin-V-FITC and propidium iodide (PI). HEK293 cells were incubated with staurosporine (1 μM) for 2 h to induce apoptosis, or left untreated. To investigate the ability of PMT to protect cells from apoptosis, cells were pre-incubated for 2 h with PMT^{wt} (6.5 nM) or the inactive mutant PMT^{C1165S} (6.5 nM) before staurosporine was added (a: untreated; b: staurosporine; c: staurosporine + PMT^{wt}; d: staurosporine + PMT^{C1165S}).
B. Quantification of results. Cells, which are Annexin-V-FITC positive and propidium iodide (PI) negative, are calculated and shown as per cent of gated cells.

PMT activates Akt

The strong mitogenic effect of PMT and the toxin-induced activation of the STAT and β-catenin/Notch1 pathway designate the toxin as a potential carcinogenic agent (Aminova and Wilson, 2007; Orth *et al*., 2007). Because PI3K and Akt play a critical role in carcinogenesis by

inducing anti-apoptotic effects (Dudek *et al*., 1997; Kauffmann-Zeh *et al*., 1997; Testa and Bellacosa, 2001; Hennessy *et al*., 2005), we studied the effect of PMT on the PI3K/Akt pathway in HEK293 cells. As shown in Fig. 2A, treatment of HEK293 cells with PMT induced a strong phosphorylation of Akt. After ~30 min of toxin treatment, activation of Akt was detected. After 2 h a maximal activation was observed, which remained stable for at least 6 h.

Akt phosphorylation (activation) induced by PMT was blocked by LY-294002, which is a general inhibitor of PI3Ks, indicating that the effect of PMT occurred via the PI3K pathway (Fig. 2B). Inhibition was also observed after treatment with AS-252424, a specific inhibitor of PI3Kα and γ.

PMT induces Pim-1 and Pim-2 accumulation

Previous data from our groups showed that PMT constitutively activates the JAK-STAT pathway (Orth *et al*., 2007). One target gene of the signal transducer and activator of transcription proteins is the *Pim* gene that is well known to protect from apoptosis (Wang *et al*., 2001; Bachmann and Moroy, 2005). Interestingly, Pim kinases and Akt show an overlap in their downstream targets. Pim-1 and Pim-2 are known to be activated through the JAK-STAT pathway, while there are currently no data available on Pim-3 signal transduction cascades. Therefore, we analysed Pim-1 and Pim-2 expression levels after PMT treatment after 6 and 18 h by Western blot analysis. Figure 2C shows that cell treatment with PMT induced protein expression of Pim-1 and Pim-2. In contrast to Akt, which is regulated through post-translational modification, Pim kinases are primarily regulated by expression and rapid degradation (Saris *et al*., 1991). PMT-induced Pim-1 and Pim-2 expression, however, was stable and the proteins accumulated over time.

PMT suppresses staurosporine-induced apoptosis via Pim-1 and Akt

Because PMT induced an upregulation and activation of Pim-1 and Akt, respectively, we next checked whether the ability of PMT to suppress apoptosis is dependent on Pim-1, Pim-2 and/or Akt. Therefore, HEK293 cells were transfected with Pim-1 and/or Pim-2 siRNA and lysed 30 min after stimulation with IL6. The Western blot in Fig. 3A shows that the knock-down of Pim-1 and Pim-2 was efficient and specific for each gene. Cells with a knock-down of Pim-1 and/or Pim-2 were then stimulated with PMT and treated with staurosporine to induce apoptosis. Propidium iodide (PI) and Annexin-V-FITC staining (Fig. 3B) showed that the knock-down of Pim-1 only partially prevented the anti-apoptotic effect of PMT against staurosporine-induced apoptosis (apoptotic cells: 59% in staurosporine-treated cells, 4.2% in staurosporine- and PMT-treated cells and 41% in Pim-1 knock-down cells treated with staurosporine and PMT). In addition, the experiment did not support a significant role for Pim-2 in PMT-induced cell rescuing. As the Pim-1 knock-down did not completely reverse the protective effect of PMT, we hypothesized that another survival kinase might be involved in this effect. To study whether Akt was involved, we next treated cells with either the PI3K inhibitor wortmannin, a specific Pim-1 inhibitor (Pim-1 IBII), or both. Then, cells were activated by PMT and subsequently treated with staurosporine. The FACS data of Fig. 3C confirmed that inhibition of Pim-1 alone is not sufficient to block staurosporine-induced apoptosis after PMT treatment. However, simultaneous inhibition of both serine/threonine kinases completely abrogated the PMT-mediated protection of HEK293 cells from apoptosis (56.1% apoptotic cells with Pim-1 and PI3-kinase inhibition compared with 57.8% apoptotic cells under control staurosporine treatment). These results show that both, Pim-1 and Akt, play a pivotal role in PMT-dependent protection from apoptosis and that these two pathways are sufficient to allow cell survival.

PMT modulates expression of Bcl-2 family members

Bcl-2 family members regulate apoptosis by controlling mitochondrial membrane integrity and the subsequent release of cytochrome *c*, a main inducer of apoptosis. Members of the Bcl-2 family can be divided into pro-apoptotic and anti-apoptotic members. Anti-apoptotic members are Bcl-xL, Bcl-2 or Mcl-1. They are indirectly regulated by Akt. Another directly Akt-activated pro-survival factor is the glycogen synthase kinase-3β (GSK3β). We explored the effect of PMT treatment on these pro-survival regulators (Fig. 4A). To this end, we treated HEK293 cells with 10 nM PMT and determined phosphorylation of GSK3β and Bcl-2 after 3 h or analysed expression of Mcl-1, Bcl-xL and Bcl-2 after 18 h. The data show a strong induction of phosphorylation of GSK3β and Bcl-2 by PMT. Additionally, PMT induced expression of *Mcl-1*, *Bcl-xL* and *Bcl-2* on the mRNA level (Fig. 4B).

In order to investigate whether PMT might also be able to downregulate pro-apoptotic signals mediated by the Bcl-2 family members Puma and Bax, immunoprecipitations of these proteins were performed. Cells were treated with staurosporine to induce apoptosis, and stimulated with PMT simultaneously, lysed and precipitated with the corresponding antibodies. Figure 4C shows that PMT was able not only to prevent but also to downregulate staurosporine-induced Bax and Puma expression, as cellular effects of PMT show a time lag of about 90 min compared with staurosporine-mediated effects.

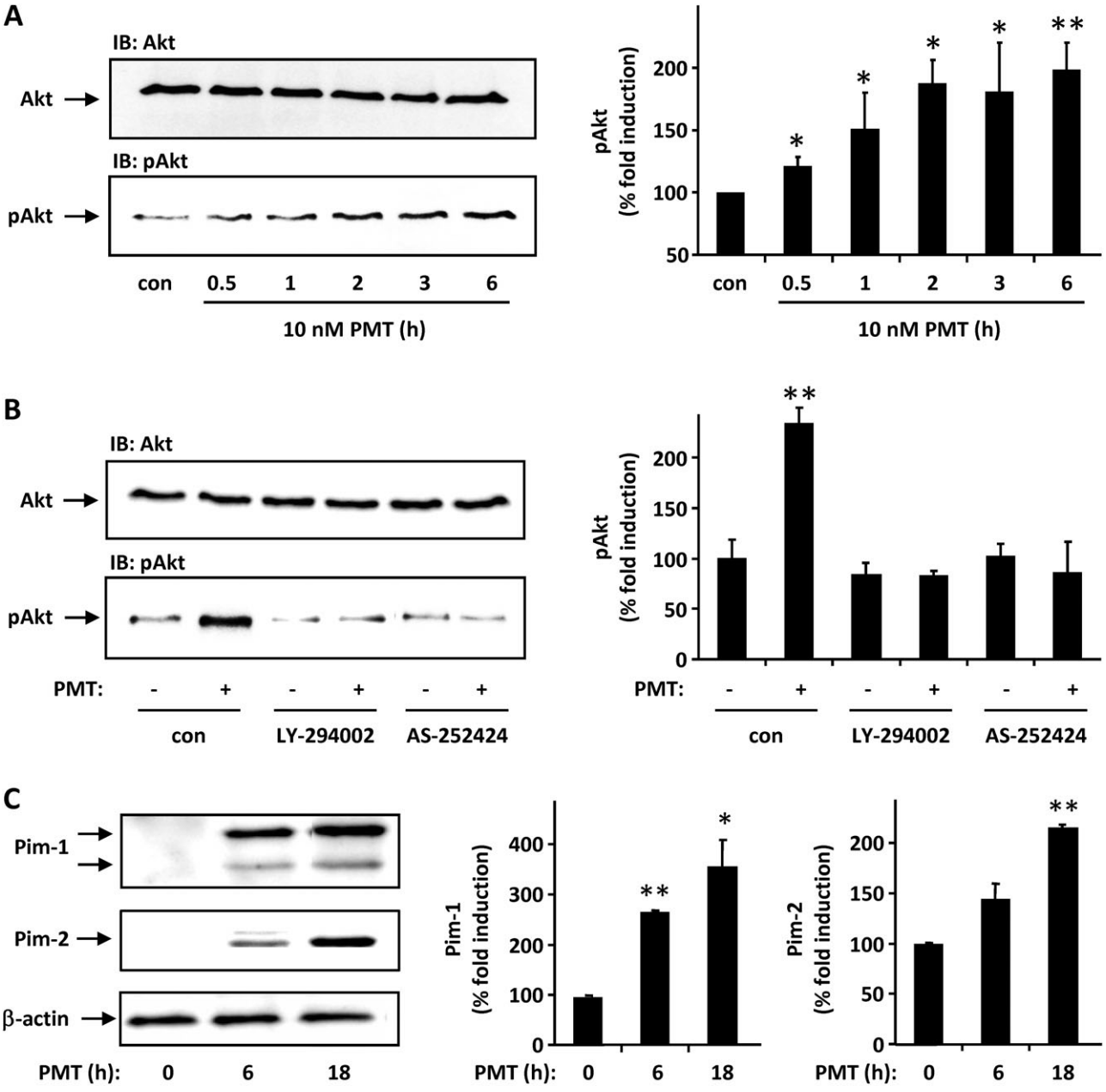


Fig. 2. PMT leads to phosphorylation of Akt and expression of Pim-1.
A. HEK293 cells were serum-starved for 24 h and stimulated with 10 nM PMT for the indicated period of time. Cells were lysed and extracted with RIPA buffer as described under *Experimental procedures*. Total Akt and Ser473 phosphorylation of Akt was determined by subsequent immunoblotting with a general anti-Akt antibody or phosphospecific anti-Akt antibody. Quantification of immunoblots was performed using Multi Gauge software (Fujifilm). The intensity of signals were calculated against untreated samples of controls and presented as per cent of control (mean ± SEM; n = 3). Statistical significance was assessed by using ANOVA, with $*P < 0.05$ and $**P < 0.01$.
B. HEK293 cells were serum-starved for 24 h and pre-treated without (con), with AS-252424 (5 μM) or LY-294001 (20 μM) for 30 min. Then, cells were stimulated with PMT (10 nM). After 3 h, cells were lysed and phosphorylation of Akt was determined as described under (A). Quantification of immunoblots was performed using Multi Gauge software (Fujifilm). The intensity of signals was calculated against untreated samples of controls and presented as per cent of control (mean ± SEM; $n = 3$). Statistical significance was assessed by using ANOVA, with $*P < 0.05$ and $**P < 0.01$.
C. HEK293 cells were stimulated with 6.5 nM PMT for the indicated time points and lysed with RIPA buffer. Expression levels of Pim-1 and Pim-2 were determined with specific antibodies. Equal loading was confirmed by immunoblotting against β-actin. Quantification of immunoblots was performed using the Image Analysis System Bioprofil (Fröbel, Germany) Bio ID software version 12.06. The intensity of signals was calculated against untreated samples of controls and is presented as per cent of control. Statistical significance was assessed by using a paired Student's *t*-test, with $*P < 0.01$ and $**P < 0.005$.

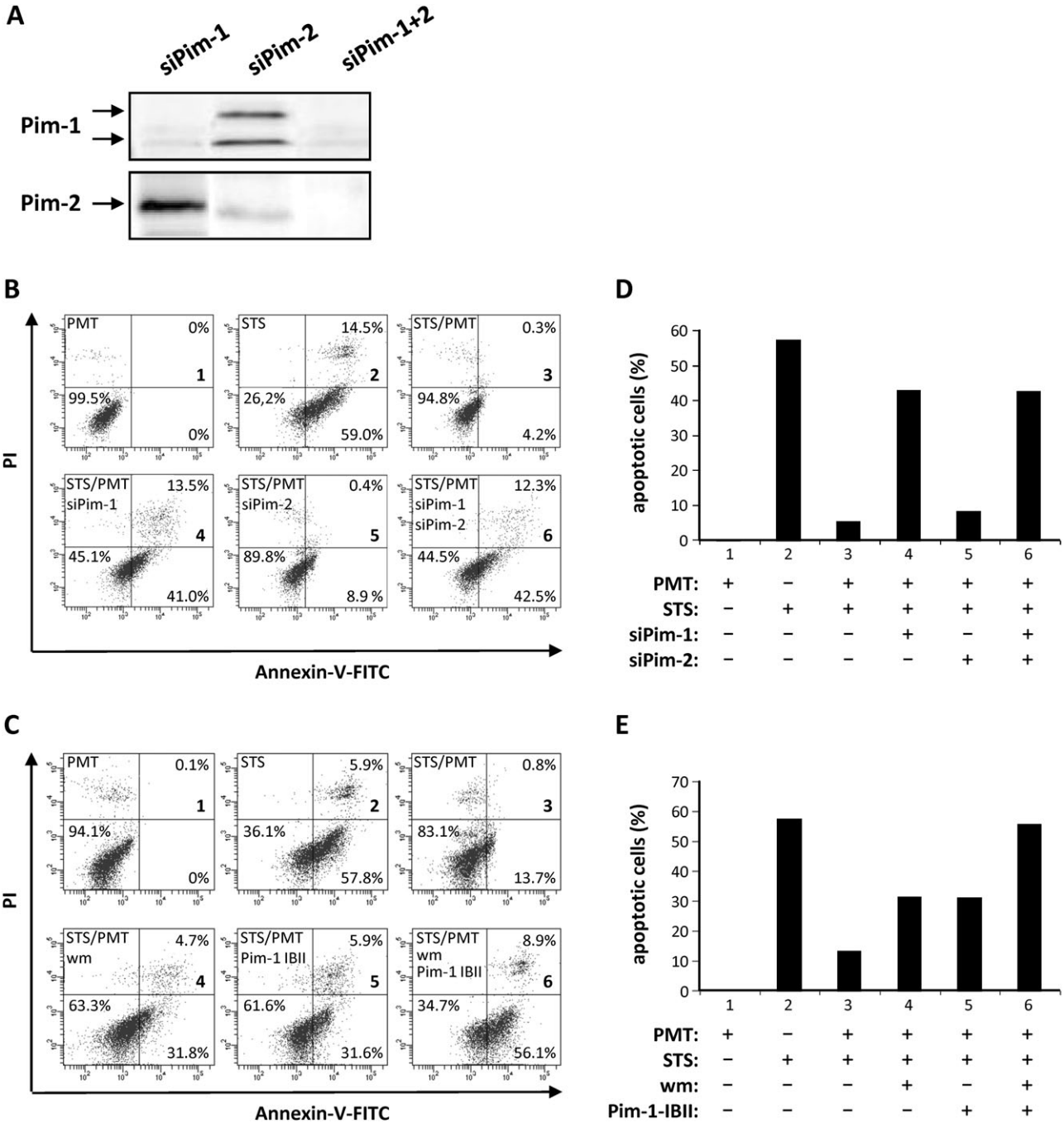


Fig. 3. Anti-apoptotic effect of PMT depends on Pim- and Akt-signalling pathways.
A. HEK293 cells were transfected with Pim-1 and/or Pim-2 siRNA, stimulated with 100 ng ml^{-1} IL6 for 30 min and lysed with RIPA buffer. Expression levels of Pim-1 and Pim-2 were determined by subsequent immunoblotting with specific antibodies.
B. Pim-1/Pim-2 knocked-down HEK293 cells (siPim-1, siPim-2) were stimulated with 6.5 nM PMT for 2 h and treated with 1 μM staurosporine (STS) for additional 2 h, or left untreated. Cells were double-stained with Annexin-V-FITC and propidium iodide (PI). The upper right gates contain the necrotic cells (PI/FITC double positive), the lower right gates the apoptotic cells (PI negative, FITC positive).
C. HEK293 cells were treated with 100 nM PI3K inhibitor wortmannin (wm) and/or 100 nM Pim-1 inhibitorII (Pim-1 IBII) 30 min prior to PMT stimulation and double-stained with Annexin-V-FITC and propidium iodide (PI). Upper right gate: necrotic cells, lower right gate: apoptotic cells.
D and E. Quantification of apoptotic cells of results presented in (B) and (C).

To corroborate the data obtained on the protein level, RNA from PMT-stimulated and staurosporine-treated cells were prepared and quantitative PCR was performed to monitor changes in gene expression. Figure 4D confirms that PMT alters the gene transcription of *Bax* and *Puma* as anti-apoptotic Bcl-2 family members.

PMT prevents DNA damage-induced apoptosis

To confirm that the anti-apoptotic effect of PMT is not restricted to staurosporine-induced apoptosis, we studied the action of PMT on cell death induced by anti-tumour chemotherapeutics. In contrast to staurosporine, which induces apoptosis by inhibiting protein kinases, doxorubicin and busulfan damage DNA to cause apoptotic cell death. These two chemotherapeutics act in different ways on DNA. Doxorubicin intercalates between DNA strands and also inhibits topoisomerase II, whereas busulfan alkylates DNA leading to cross-links. As shown in Fig. 5A, doxorubicin is a strong inducer of apoptosis in lung carcinoma cells as measured by caspase-3 and caspase-7 cleavage. This chemotherapeutic-induced apoptosis was concentration-dependently inhibited by PMT. Pretreatment of lung carcinoma cells for 6 h with 100 pM toxin reduced doxorubicin-induced cleavage of caspases and treatment with 1 nM PMT nearly abolished caspase cleavage. In addition, PMT reduced the busulfan-induced activation of caspases in melanoma cells (Fig. 5B).

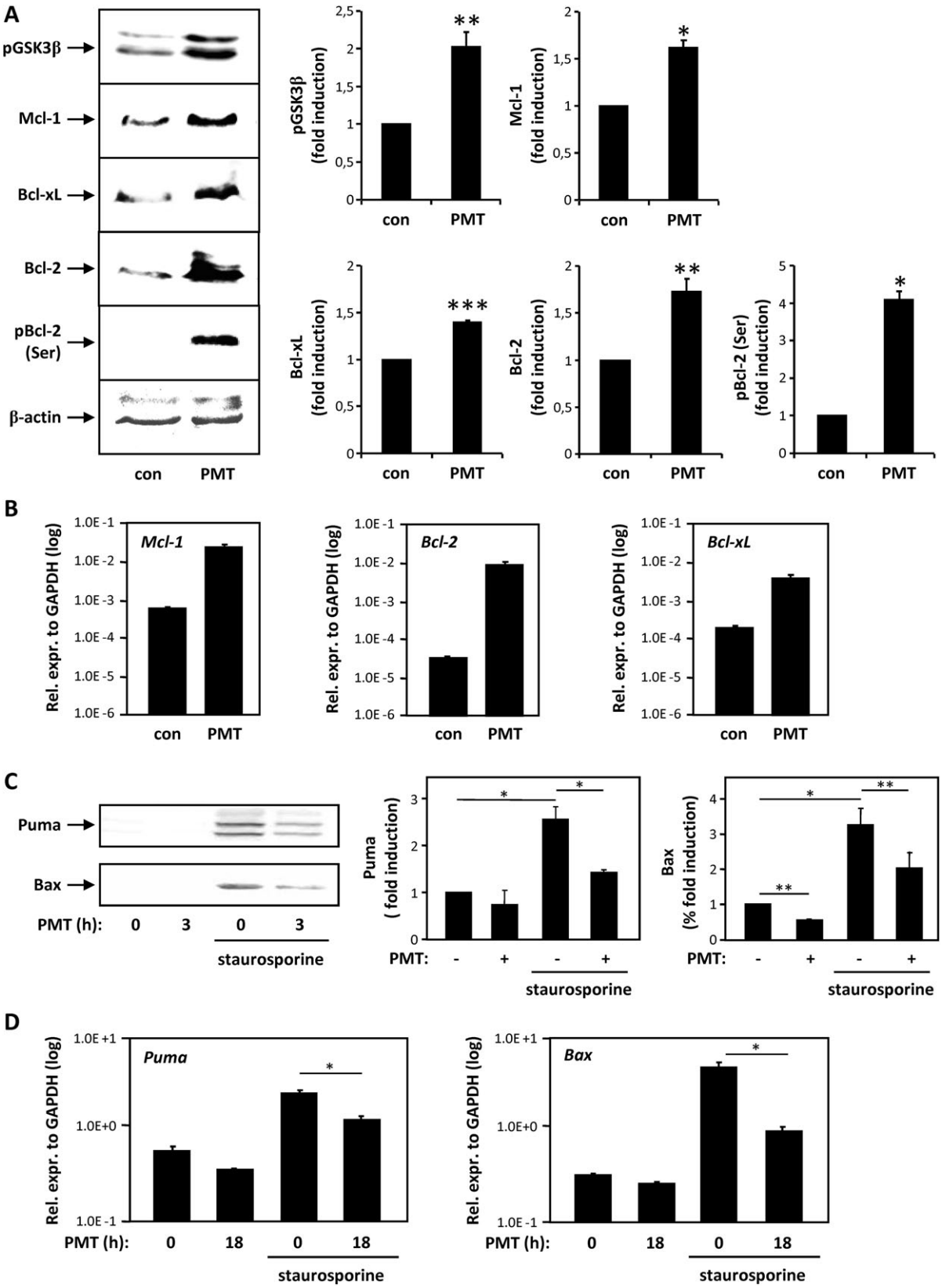
Discussion

Our findings show that PMT induces a strong anti-apoptotic effect in mammalian cells. The toxin prevented apoptosis mediated by staurosporine and chemotherapeutics. Apoptosis was determined by Annexin-V staining as well as by caspase-3 and/or -7 activation. PMT reduced both parameters efficiently. Staurosporine rapidly induces apoptosis in all mammalian cells. It is a strong protein kinase inhibitor, which is well known for its potent apoptotic effect. Only few agents are capable to protect cells from staurosporine-induced cell death (D'Alimonte *et al*., 2007; Javouhey *et al*., 2008). Therefore, we were surprised to find that PMT blocked this strong effect of staurosporine.

Previously, it has been reported that PMT-induced activation of G proteins concomitantly stimulates Gβγ signalling, including activation of PI3Kγ (Preuss *et al*., 2009). Because PI3Ks regulate survival signals, e.g. via Akt, we were prompted to study the effects of PMT on the activation of this kinase. Akt plays an important role in cellular proliferation, apoptosis and survival (Nicholson and Anderson, 2002; Jetzt *et al*., 2003; Katavic *et al*., 2003; New *et al*., 2007). Our data show that the toxin increased the phosphorylation of Akt and thereby activated the kinase. The involvement of PI3Ks in PMT-induced phosphorylation of Akt is shown by the inhibiting effect of the unspecific PI3K inhibitor wortmannin or LY-294002 and by the specific PI3Kγ and α inhibitor AS-254242. PI3Ks of the class I (α, β, γ and δ) are known to integrate signalling of heterotrimeric G proteins (e.g. via Gβγ) and growth factor receptors (e.g. via Ras) (Stein and Waterfield, 2000). Both pathways are strongly activated by PMT. In addition to the direct stimulation of G proteins, the toxin causes transactivation of growth factor receptors in a cell type specific manner (Seo *et al*., 2000; Sabri *et al*., 2002). Therefore, an activation of PI3Kγ through Gβγ might not be the only reason for a stimulation of Akt. Our studies did not intend to distinguish between these possible pathways. However, blockade of PI3K signalling by the inhibitors only partially restored the apoptotic effects induced by staurosporine, indicating the involvement of additional signal pathways.

Recently, we have shown that PMT causes a strong and prolonged activation of the JAK2/STAT3/5 pathway. STAT signalling was reported to regulate expression of Pim kinases, which possess anti-apoptotic activity. Pim is a proto-oncogene and Pim-1 and Pim-2 are upregulated in a number of human cancers (Hammerman *et al*., 2005). Interestingly, Akt and Pim share most of their substrates and hence activate overlapping but independent downstream pathways (White, 2003). Hammerman *et al*. (2005) hypothesized that Pim-2 and Akt might have synergistic oncogenic effects. In line with this view is their finding that mice, which are double transgenic for Pim-2 and Akt, exhibited 100% penetrance of tumour development, while the single transgenic mice developed no or only a few tumours.

Therefore, we studied a possible role of Pim kinases for the anti-apoptotic effects of PMT. Knock-down of Pim-1 and Pim-2 revealed that only Pim-1 is involved in the anti-apoptotic effect of PMT. Nevertheless, Pim-1 is just partially responsible for PMT-induced rescue. Using a specific Pim-1-kinase inhibitor in addition to PI3K inhibitors, we observed a complete restoration of staurosporine-induced apoptosis, suggesting that both signalling pathways are essentially involved in the anti-apoptotic effects of PMT. Furthermore, we show that PMT caused an upregulation of survival and also a downregulation of apoptotic pathways. The toxin induced the expression of anti-apoptotic Bcl-2 family members such as Bcl-xL, Mcl-1 or Bcl-2. Additionally, we could show a phosphorylation of Bcl-2, which is known to enhance its anti-apoptotic functions. GSK3β, a direct effector of Akt, was also found to be phosphorylated after PMT treatment. GSK3β is inactivated by phosphorylation leading in turn to a proliferative effect via Cyclin D1. The anti-apoptotic effect of PMT is strengthened by downregulation of the pro-apoptotic Bcl-2 family members Puma and Bax. All these data indicate that PMT


A
pGSK3β
Mcl-1
Bcl-xL
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pBcl-2 (Ser)
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pGSK3β (fold induction)
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Fig. 4. PMT stimulates pro-survival pathways with concomitant inhibition of pro-apoptotic pathways.
A. Activation of PMT on pro-survival pathways. HEK293 cells were treated with or without 10 nM PMT. Phosphorylation of pGSK3β and pBcl-2 was determined after 3 h of PMT treatment and expression of Mcl-1, Bcl-xL and Bcl-2 was determined after 18 h of toxin treatment. Cells were lysed in SDS sample buffer as described in *Experimental procedures*. Membrane was stained with Ponceau S to visualize actin and confirm equal loading. Quantification of immunoblots was performed by using Multi Gauge software (Fujifilm). The intensity of signals was calculated against untreated samples of controls and are presented as per cent of control (mean ± SEM; $n = 3$). Statistical significance was assessed using paired Student's *t*-test, with $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.
B. HEK293 cells were stimulated with PMT (6.5 nM) for 2 h. RNA was extracted, cDNA was prepared and used for quantitative PCR analysis with specific primers for *Mcl-1*, *Bcl-2* and *Bcl-xL*. All results were analysed as described in *Experimental procedures* and are presented as relative expression to the reference gene (*GAPDH*).
C. HEK293 cells were stimulated with 6.5 nM PMT for 3 h or left unstimulated. Then, cells were treated with 1 μM staurosporine and lysed with RIPA buffer. Immunoprecipitations were performed with anti-Puma or anti-Bax antibody followed by immunoblotting with the according antibodies. Quantification of immunoblots was performed using the Image Analysis System Bioprofil (Fröbel, Germany) Bio ID software version 12.06. Signal intensity was calculated against untreated control samples and is presented as per cent of control (mean ± STD; $n = 2$). Statistical significance was assessed by using a paired Student's *t*-test, with $^{*}P < 0.05$ and $^{**}P < 0.01$.
D. HEK293 cells were stimulated with PMT (6.5 nM) for 3 h and treated with 1 μM staurosporine. RNA was extracted, cDNA was prepared and used for quantitative PCR analysis with specific primers for *Puma* and *Bax*. All results were analysed as described in *Experimental procedures* and are presented as relative expression to the reference gene (*GAPDH*). Statistical significance was assessed by using a paired Student's *t*-test, with $^{*}P < 0.05$.

affects both, survival pathways (e.g. stimulation of Akt and Pim) and apoptotic pathways (e.g. decrease in Puma and Bax). It is a commonly accepted model that viruses induce increased cell proliferation and survival signals, including Akt and Pim pathways, in order to preserve host cells for their replication process (Chen *et al*., 2008; Ji and Liu, 2008). Today, we can only speculate on the reasons why *P. multocida*, which is not an intracellular pathogen, has the ability to induce growth and prevent apoptosis of host cells. However, recently it was shown that Bcl-2 and Bcl-xL can directly bind and suppress the function of the Nod-like receptor (NLR) family member NALP-1 (Bruey *et al*., 2007; Faustin *et al*., 2009). NLR family proteins are involved in recognition of intracellular microbial components and constitute a critical component of the so-called inflammasome that ultimately leads to the processing of IL1β by caspase-1 and concomitant cell death. The PMT-induced change in regulation of cell survival and homoeostasis may therefore give the bacteria a growth advantage as it allows them to escape the host cell's immune system by blunting the innate immune response.

Akt and Bcl-2 family members also play an important role in maintaining the integrity of the skeletal system. Bcl-2 for example is needed for osteoclast differentiation and survival and Akt1-deficient mice suffer from osteopoenia (Kawamura *et al*., 2007; Nagase *et al*., 2009). Interestingly, this corresponds with the pathological phenotype of *P. multocida* infections in swine. In atrophic rhinitis, nasal turbinates are destroyed due to excessive osteoclast numbers and inhibition of ostoblast differentiation and cell function (Gwaltney *et al*., 1997; Lax *et al*., 2004), which is considered to be the characteristic effect of PMT. Our data give first evidence that in addition to the obvious effect on skeletal maintenance, the immune system might actually be the primary target for the toxin's actions.

Finally, we studied the apoptotic effects mediated by the anti-tumour agents doxorubicin and busulfan. Doxorubicin is a DNA-intercalating agent and also acts as a topoi-

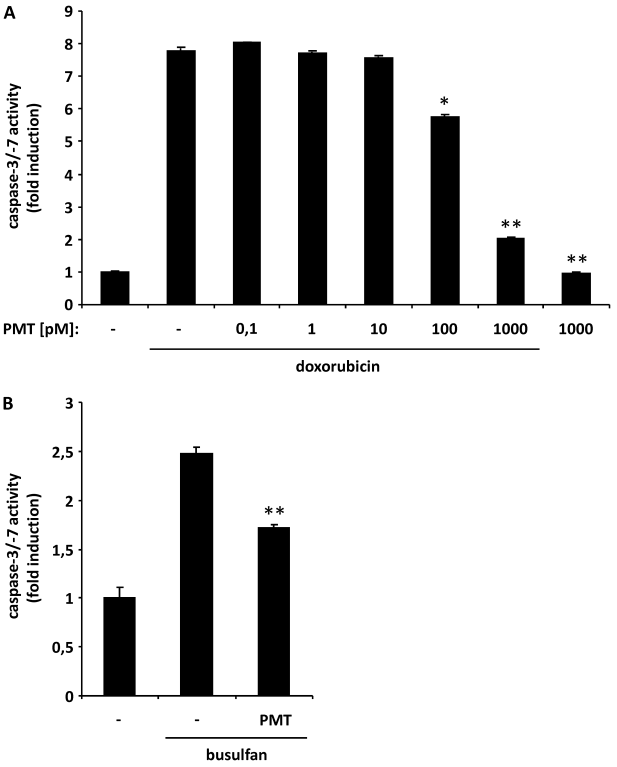

Fig. 5. PMT blocks chemotherapeutic-induced apoptosis in lung cancer cells and melanoma.
A. Lung carcinoma cells (A549/CCl-185) were treated with PMT at indicated concentrations for 6 h. Afterwards doxorubicin was added to a final concentration of 1 μM and incubated for 30 h. Finally, cleavage of caspase-3/-7 was measured by Caspase-3/7Glo assay. Data were calculated as fold induction of caspase cleavage over untreated cells (mean ± SEM; $n = 3$). Statistical significance was assessed by using ANOVA, with $^{*}P < 0.05$ and $^{**}P < 0.01$.
B. Melanoma cells (F01) were treated with PMT (1 nM) for 6 h. Afterwards busulfan was added to a final concentration of 100 μM for 48 h. Finally, cleavage of caspase-3/-7 was measured by Caspase-3/7Glo assay. Data were calculated as fold induction of caspase cleavage over untreated cells (mean ± SEM; $n = 3$). Statistical significance was assessed by using ANOVA, with $^{**}P < 0.01$.

somerase II inhibitor to induce apoptosis of target cells. Busulfan is a classical DNA-alkylating agent. The apoptotic effects of both compounds were inhibited by PMT, suggesting that the blockade of apoptosis by PMT was not staurosporine specific. Very recently, it was reported that in doxorubicin resistant cancer cells Jun kinase and STAT3 activity were elevated (Kim *et al*., 2009), suggesting an important role of these pathways in chemotherapeutic resistance. PMT is known to activate the JAK-STAT pathway via Ga_q leading to a long-term activation of STAT1, 3 and 5 (Orth *et al*., 2007). Therefore it is reasonable to presume a collaborative action of toxin-induced signalling (Akt, Pim, STAT) to protect cancer cells against doxorubicin-caused cell death.

The participation of heterotrimeric G proteins in regulation of apoptosis and cell survival is commonly accepted even though the mechanisms are not well understood (Yanamadala *et al*., 2009). In contrast to the short-lived stimulation of receptor-induced signalling caused by the binding of an agonist, PMT-mediated signal transduction cascades remain active for a longer time period, most likely due to the covalent modification of heterotrimeric G proteins of the G_i, G_q and $G_{12/13}$ families by PMT-induced deamidation (Orth *et al*., 2009). Interestingly, constitutive activation of heterotrimeric G proteins has also been described in a variety of tumours. The identical amino acid residue of Ga_{i2} (glutamine-205), which is observed to be mutated in pituitary tumours (Williamson *et al*., 1995), is deamidated by PMT. Moreover, in *Gnaq*, encoding for Ga_q, mutations of codon 209 were recently detected in melanoma of the uvea and in blue naevi (Van Raamsdonk *et al*., 2009). The same glutamine-209 residue of Ga_q is deamidated by PMT leading to constitutively active G_q (Williamson *et al*., 1995).

Notably, not only mutations of Gα-subunits but also uncontrolled signalling of GPCRs appear to be involved in carcinogenesis. For example, lysophosphatidic acid (LPA), which acts as a lipid growth factor, is implicated in tumorigenesis. LPA activates G protein-coupled receptors leading to a stimulation of heterotrimeric G proteins of different families. Very recently, it was demonstrated that overexpression of the LPA-producing enzyme autotaxin or overexpression of LPA receptors increases tumorigenesis (Liu *et al*., 2009). Noteworthy, the subset of G proteins activated by LPA and PMT are comparable. LPA and PMT stimulate the G_i, G_q and $G_{12/13}$ families with the difference that LPA acts via GPCRs and the toxin directly on the G proteins.

Taken together, we show that PMT is a strong activator of PI3Ks, resulting in Akt stimulation and anti-apoptotic effects. Together with the strong mitogenic effects of PMT (Rozengurt *et al*., 1990), the toxin-induced activation of STAT (Orth *et al*., 2007) and the increase in Pim protein expression and activity (shown in this report) assign PMT as a potential carcinogenic agent and a useful tool to protect cells from apoptotic stimuli.

Experimental procedures

Material

PI3Ks inhibitors AS-252424 and LY-294002 were from Axxora and Sigma, respectively. All other reagents were of analytical grade and purchased from commercial sources.

Annexin-V staining of apoptotic cells

HEK293 cells were incubated with 1 μM staurosporine for 2 h to induce apoptosis or left untreated. To investigate the ability of PMT to protect cells from apoptosis, cells were pre-incubated for 2 h with PMT or the inactive mutant PMT^{C1156S} (each 6.5 nM) before staurosporine (Sigma-Aldrich, Munich, Germany, #S4400) was added. Subsequently, cells were incubated for 15 min in 100 μl of incubation buffer containing 5 μl of propidium iodide (PI) and Annexin-V-FITC staining solution respectively (Annexin-V-FLUOS Staining Kit, Roche, #11858777001). Apoptotic non-necrotic cells were quantified by flow cytometry on a FACScanto cytometer (Beckton Dickinson, Heidelberg, Germany) and the results were analysed using FACS Diva v 4.1.2 and Weasel v 2.5 software. To test the dependence of PMT-induced cell survival effect on Pim-1 and Akt, HEK293 cells were treated with 100 nM PI3K inhibitor wortmannin (Calbiochem) or 100 nM Pim-1 inhibitorII (Calbiochem) 30 min prior to PMT stimulation.

Caspase-3 and -7 activation

Cells growing in DMEM medium containing 1% FBS were incubated with indicated substances for 30 h (doxorubicin) or 48 h (busulfan) to induce apoptosis, or left untreated. To investigate the ability of PMT to protect cells from apoptosis, cells were pre-incubated for 6 h with PMT before doxorubicin (1 μM) or busulfan (100 μM) was added. Caspase-3 and -7 activity was measured using the Caspase-3/7Glo assay (Promega) in accordance to the manufacturer's instructions.

Expression of recombinant proteins

Recombinant PMT^{wt} and the inactive mutant PMT^{C1165S} were expressed and purified as described before (Busch *et al*., 2001).

Immunoblot analysis

For determination of Akt activation and Pim-1 and Pim-2 expression, cells were extracted at 4°C with RIPA buffer [50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1% Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, and complete protease inhibitors, Roche Applied Science] and analysed by Western blotting. For determination of pGSK3β, Mcl-1, Bcl-xL, Bcl-2 and pBcl-2 cells were lysed in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT and 0.01% bromophenol blue) and sonicated. Antibodies against Akt (Cell Signaling Technology, #9272), Ser473-phosphorylated Akt (Cell

Signaling Technology, #9271), phosphorylated GSK3β (Cell Signaling Technology, #9336), Pim-1 (Cell Signaling Technology, #2907), Pim-2 (Cell Signaling Technology, #4723), β-actin (Cell Signaling Technology, #4967), Mcl-1, Bcl-xL, Bcl-2 and serine-phosphorylated Bcl-2 were purchased from Cell Signaling Technology (Pro-Survival Bcl-2 Family Antibody Sampler Kit #9941). To test for Bax and Puma expression, HEK293 were stimulated for 3 h with 6.5 nM PMT in the presence of 1 μM staurosporine. After washing the cells with cold phosphate-buffered saline, cells were resuspended in lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Deoxycholat, 1 mM NaF and 25 mM Tris), containing phosphatase inhibitors (PhosSTOP, Roche Diagnostics) and incubated for 45 min at 4°C. After centrifugation, lysates were either immunoprecipitated overnight with 5 μg of Bax or Puma antibody (Cell Signaling Technology, #2722 and #4976) bound to Protein A/G Sepharose (Sigma) or used as whole cell extracts. Samples were separated on 12% SDS-PAGE, transferred to nitrocellulose and incubated with anti-Bax or anti-Puma antibodies at a dilution of 1:1000, followed by incubation with an anti-rabbit HRP-coupled antibody (all Cell Signaling Technology Pro-Apoptosis Bcl-2 Family Antibody Sampler Kit #9942) at a dilution of 1:5000 and detection by enhanced chemiluminescence.

Reverse transcriptase PCR

To investigate the role of PMT on mRNA expression of Bcl-2 family members, HEK293 cells were stimulated with PMT (6.5 nM) for 3 h and treated with 1 μM staurosporine where indicated. RNA was extracted with the 'High pure RNA Isolation Kit' (Roche), following the manufacturer's protocol. Total RNA was quantified by NanoDrop. cDNA was prepared using 'Reversed Aid First Strand cDNA Synthesis Kit' (Fermentas Life Science). Aliquots of the cDNA were used for quantitative PCR analysis with the 'SYBR Green Rox mix' (Thermo Scientific) with following primers: *GAPDH*, sense, 5′-acggatttggct-3′, antisense, 5′-ttgacg gtgccatggaatttg-3′, human *Bax*, sense 5′-ttcccctcaaccccgt-3′, antisense 5′-catccgctccctccaacc-3′, human *Bcl-xL*, sense, 5′-gggagtccccccagaagag-3′, antisense, 5′-ataggagtccaggtggc cgt-3′, human *Puma* sense 5′-ggagggtcctgtacaatctc-3′, antisense 5′-gctacatgtgcagagaaag-3′, human *Mcl-1* sense 5′-ttccaaggcat gcttcggaaac-3′, antisense 5′-tctgctaatggttcgatgcag-3′, human *Bcl-2* sense 5′-cgccctgtcgatgactgagta-3′, antisense 5′-cccatgct ccgttatcctg-3′.The results were analysed using the Fast Real-Time PCR System (Applied Biosystems). The mean values of the results (mean ± SEM; $n = 2$) were normalized and are presented as relative expression compared with *GAPDH*.

Transfection of siRNA

Pim-1 siRNA (Santa Cruz Technology sc-39145) and Pim-2 siRNA (Santa Cruz Technology sc-39145) transfection was carried out according to the manufacturer's protocol with lipofectamine™ 2000 (Invitrogen 11668027).

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References

Amaravadi, R., and Thompson, C.B. (2005) The survival kinases Akt and Pim as potential pharmacological targets. *J Clin Invest* **115:** 2618–2624.

Aminova, L.R., and Wilson, B.A. (2007) Calcineurin-independent inhibition of 3T3-L1 adipogenesis by *Pasteurella multocida* toxin: suppression of Notch1, stabilization of beta-catenin and pre-adipocyte factor 1. *Cell Microbiol* **9:** 2485–2496.

Bachmann, M., and Moroy, T. (2005) The serine/threonine kinase Pim-1. *Int J Biochem Cell Biol* **37:** 726–730.

Bruey, J.M., Bruey-Sedano, N., Luciano, F., Zhai, D., Balpai, R., Xu, C., *et al*. (2007) Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. *Cell* **129:** 45–56.

Busch, C., Orth, J., Djouder, N., and Aktories, K. (2001) Biological activity of a C-terminal fragment of *Pasteurella multocida* toxin. *Infect Immun* **69:** 3628–3634.

Chen, J.L., Limnander, A., and Rothman, P.B. (2008) Pim-1 and Pim-2 kinases are required for efficient pre-B-cell transformation by v-Abl oncogene. *Blood* **111:** 1677–1685.

Claudio, J.O., Masih-Khan, E., Tang, H., Goncalves, J., Voralia, M., Li, Z.H., *et al*. (2002) A molecular compendium of genes expressed in multiple myeloma. *Blood* **100:** 2175–2186.

D'Alimonte, I., Ballerini, P., Nargi, E., Buccella, S., Giuliani, P., Di Iorio, P., *et al*. (2007) Staurosporine-induced apoptosis in astrocytes is prevented by A1 adenosine receptor activation. *Neurosci Lett* **418:** 66–71.

Dhanasekaran, S.M., Barrette, T.R., Ghosh, D., Shah, R., Varambally, S., Kurachi, K., *et al*. (2001) Delineation of prognostic biomarkers in prostate cancer. *Nature* **412:** 822–826.

Dominick, M.A., and Rimler, R.B. (1986) Turbinate atrophy in gnotobiotic pigs intranasally inoculated with protein toxin isolated from type D *Pasteurella multocida*. *Am J Vet Res* **47:** 1532–1536.

Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., *et al*. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **275:** 661–665.

Faustin, B., Chen, Y., Zhai, D., Le Negrate, G., Lartigue, L., Satterthwait, A., and Reed, J.C. (2009) Mechanism of Bcl-2 and Bcl-X(L) inhibition of NLRP1 inflammasome: loop domain-dependent suppression of ATP binding and oligomerization. *Proc Natl Acad Sci USA* **106:** 3935–3940.

Gwaltney, S.M., Galvin, R.J., Register, K.B., Rimler, R.B., and Ackermann, M.R. (1997) Effects of *Pasteurella multocida* toxin on porcine bone marrow cell differentiation into osteoclasts and osteoblasts. *Vet Pathol* **34:** 421–430.

Hammerman, P.S., Fox, C.J., Birnbaum, M.J., and Thompson, C.B. (2005) Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival. *Blood* **105:** 4477–4483.

Harper, M., Boyce, J.D., and Adler, B. (2006) *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *FEMS Microbiol Lett* **265:** 1–10.

Hennessy, B.T., Smith, D.L., Ram, P.T., Lu, Y., and Mills, G.B. (2005) Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov* **4:** 988–1004.

Javouhey, E., Gibert, B., Arrigo, A.P., Diaz, J.J., and Diaz-Latoud, C. (2008) Protection against heat and staurosporine mediated apoptosis by the HSV-1 US11 protein. *Virology* **376:** 31–41.

Jetzt, A., Howe, J.A., Horn, M.T., Maxwell, E., Yin, Z., Johnson, D., and Kumar, C.C. (2003) Adenoviral-mediated expression of a kinase-dead mutant of Akt induces apoptosis selectively in tumor cells and suppresses tumor growth in mice. *Cancer Res* **63:** 6697–6706.

Ji, W.T., and Liu, H.J. (2008) PI3K-Akt signaling and viral infection. *Recent Patents Biotechnol* **2:** 218–226.

Katavic, V., Grcevic, D., Lee, S.K., Kalinowski, J., Jastrzebski, S., Dougall, W., *et al*. (2003) The surface antigen CD45R identifies a population of estrogen-regulated murine marrow cells that contain osteoclast precursors. *Bone* **32:** 581–590.

Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997) Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385:** 544–548.

Kawamura, N., Kugimiya, F., Oshima, Y., Ohba, S., Ikeda, T., Saito, T., *et al*. (2007) Akt1 in osteoblasts and osteoclasts controls bone remodeling. *PLoS ONE* **2:** e1058.

Kim, J.H., Lee, S.C., Ro, J., Kang, H.S., Kim, H.S., and Yoon, S. (2009) Jnk signaling pathway-mediated regulation of Stat3 activation is linked to the development of doxorubicin resistance in cancer cell lines. *Biochem Pharmacol* **79:** 373–380.

Lax, A.J. (2005) Opinion: bacterial toxins and cancer – a case to answer? *Nat Rev Microbiol* **3:** 343–349.

Lax, A.J., and Grigoriadis, A.E. (2001) *Pasteurella multocida* toxin: the mitogenic toxin that stimulates signalling cascades to regulate growth and differentiation. *Int J Med Microbiol* **291:** 261–268.

Lax, A.J., and Thomas, W. (2002) How bacteria could cause cancer: one step at a time. *Trends Microbiol* **10:** 293–299.

Lax, A.J., Pullinger, G.D., Baldwin, M.R., Harmey, D., Grigoriadis, A.E., and Lakey, J.H. (2004) The *Pasteurella multocida* toxin interacts with signalling pathways to perturb cell growth and differentiation. *Int J Med Microbiol* **293:** 505–512.

Li, Y.Y., Popivanova, B.K., Nagai, Y., Ishikura, H., Fujii, C., and Mukaida, N. (2006) Pim-3, a proto-oncogene with serine/threonine kinase activity, is aberrantly expressed in human pancreatic cancer and phosphorylates bad to block bad-mediated apoptosis in human pancreatic cancer cell lines. *Cancer Res* **66:** 6741–6747.

Liu, S., Umezu-Goto, M., Murph, M., Lu, Y., Liu, W., Zhang, F., *et al*. (2009) Expression of autotaxin and lysophosphatidic acid receptors increases mammary tumorigenesis, invasion, and metastases. *Cancer Cell* **15:** 539–550.

van der Lugt, N.M., Domen, J., Verhoeven, E., Linders, K., van der Gulden, H., Allen, J., and Berns, A. (1995) Proviral tagging in E mu-myc transgenic mice lacking the Pim-1 proto-oncogene leads to compensatory activation of Pim-2. *EMBO J* **14:** 2536–2544.

Martineau-Doize, B., Caya, I., Gagne, S., Jutras, I., and Dumas, G. (1993) Effects of *Pasteurella multocida* toxin on the osteoclast population of the rat. *J Comp Pathol* **108:** 81–91.

Mullan, P.B., and Lax, A.J. (1996) *Pasteurella multocida* toxin is a mitogen for bone cells in primary culture. *Infect Immun* **64:** 959–965.

Nagase, Y., Iwasawa, M., Akiyama, T., Kadono, Y., Nakamura, M., Oshima, Y., *et al*. (2009) The anti-apoptotic molecule Bcl-2 regulates the differentiation, activation and survival of both osteoblasts and osteoclasts. *J Biol Chem* **284:** 36659–36669.

New, D.C., Wu, K., Kwok, A.W., and Wong, Y.H. (2007) G protein-coupled receptor-induced Akt activity in cellular proliferation and apoptosis. *FEBS J* **274:** 6025–6036.

Nicholson, K.M., and Anderson, N.G. (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* **14:** 381–395.

Nougayrede, J.P., Taieb, F., De Rycke, J., and Oswald, E. (2005) Cyclomodulins: bacterial effectors that modulate the eukaryotic cell cycle. *Trends Microbiol* **13:** 103–110.

Orth, J.H., Lang, S., and Aktories, K. (2004) Action of *Pasteurella multocida* toxin depends on the helical domain of Galphaq. *J Biol Chem* **279:** 34150–34155.

Orth, J.H., Aktories, K., and Kubatzky, K.F. (2007) Modulation of host cell gene expression through activation of STAT transcription factors by *Pasteurella multocida* toxin. *J Biol Chem* **282:** 3050–3057.

Orth, J.H., Fester, I., Preuss, I., Agnoletto, L., Wilson, B.A., and Aktories, K. (2008) Activation of Galphai and subsequent uncoupling of receptor-Galphai signaling by *Pasteurella multocida* toxin. *J Biol Chem* **283:** 23288–23294.

Orth, J.H., Preuss, I., Fester, I., Schlosser, A., Wilson, B.A., and Aktories, K. (2009) *Pasteurella multocida* toxin activation of heterotrimeric G proteins by deamidation. *Proc Natl Acad Sci USA* **106:** 7179–7184.

Pekarsky, Y., Koval, A., Hallas, C., Bichi, R., Tresini, M., Malstrom, S., *et al*. (2000) Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. *Proc Natl Acad Sci USA* **97:** 3028–3033.

Preuss, I., Kurig, B., Nurnberg, B., Orth, J.H., and Aktories, K. (2009) *Pasteurella multocida* toxin activates Gbetagamma dimers of heterotrimeric G proteins. *Cell Signal* **21:** 551–558.

Rozengurt, E., Higgins, T., Chanter, N., Lax, A.J., and Staddon, J.M. (1990) *Pasteurella multocida* toxin: potent mitogen for cultured fibroblasts. *Proc Natl Acad Sci USA* **87:** 123–127.

Sabri, A., Wilson, B.A., and Steinberg, S.F. (2002) Dual actions of the Galpha(q) agonist *Pasteurella multocida* toxin to promote cardiomyocyte hypertrophy and enhance apoptosis susceptibility. *Circ Res* **90:** 850–857.

Saris, C.J., Domen, J., and Berns, A. (1991) The pim-1 oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. *EMBO J* **10:** 655–664.

Seo, B., Choy, E.W., Maudsley, S., Miller, W.E., Wilson, B.A., and Luttrell, L.M. (2000) *Pasteurella multocida* toxin stimulates mitogen-activated protein kinase via G(q/11)-dependent transactivation of the epidermal growth factor receptor. *J Biol Chem* **275:** 2239–2245.

Stein, R.C., and Waterfield, M.D. (2000) PI3-kinase inhibition: a target for drug development? *Mol Med Today* **6:** 347–357.

Testa, J.R., and Bellacosa, A. (2001) AKT plays a central role

in tumorigenesis. *Proc Natl Acad Sci USA* **98:** 10983–10985.

Van Raamsdonk, C.D., Bezrookove, V., Green, G., Bauer, J., Gaugler, L., O'Brien, J.M., *et al.* (2009) Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature* **457:** 599–602.

Wang, Z., Bhattacharya, N., Weaver, M., Petersen, K., Meyer, M., Gapter, L., and Magnuson, N.S. (2001) Pim-1: a serine/threonine kinase with a role in cell survival, proliferation, differentiation and tumorigenesis. *J Vet Sci* **2:** 167–179.

White, E. (2003) The pims and outs of survival signaling: role for the Pim-2 protein kinase in the suppression of apoptosis by cytokines. *Genes Dev* **17:** 1813–1816.

Williamson, E.A., Ince, P.G., Harrison, D., Kendall-Taylor, P., and Harris, P.E. (1995) G-protein mutations in human pituitary adrenocorticotrophic hormone-secreting adenomas. *Eur J Clin Invest* **25:** 128–131.

Wilson, B.A., and Ho, M. (2006) *Pasteurella multocida* toxin. In *The Comprehensive Sourcebook of Bacterial Protein Toxins*, 3rd edn. Alouf, J.E., and Popoff, M.R. (eds). Amsterdam: Elsevier, pp. 430–447.

Yanamadala, V., Negoro, H., and Denker, B.M. (2009) Heterotrimeric G proteins and apoptosis: intersecting signaling pathways leading to context dependent phenotypes. *Curr Mol Med* **9:** 527–545.

Yoshida, S., Kaneita, Y., Aoki, Y., Seto, M., Mori, S., and Moriyama, M. (1999) Identification of heterologous translocation partner genes fused to the BCL6 gene in diffuse large B-cell lymphomas: 5′-RACE and LA-PCR analyses of biopsy samples. *Oncogene* **18:** 7994–7999.