Antitumour and antiangiogenic effects of Aplidin© in the 5TMM syngeneic models of multiple myeloma

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Aplidin© is an antitumour drug, currently undergoing phase II evaluation in different haematological and solid tumours. In this study, we analysed the antmyeloma effects of Aplidin in the syngeneic 5T33MM model, which is representative for the human disease. In vitro, Aplidin inhibited 5T33MMv DNA synthesis with an IC50 of 3.87 nM. On cell-cycle progression, the drug induced an arrest in transition from G0/G1 to S phase, while Western blot showed a decreased cyclin D1 and CDK4 expression. Furthermore, Aplidin induced apoptosis by lowering the mitochondrial membrane potential, by inducing cytochrome c release and by activating caspase-9 and caspase-3. For the in vivo experiment, 5T33MM-injected C57Bl/KaLwRij mice were intraperitoneally treated with vehicle or Aplidin (90 μg kg−1 daily). Chronic treatment with Aplidin was well tolerated and reduced serum paraprotein concentration by 42% (P<0.001), while BM invasion with myeloma cells was decreased by 35% (P<0.001). Aplidin also reduced the myeloma-associated angiogenesis to basal values. This antiangiogenic effect was confirmed in vitro and explained by inhibition of endothelial cell proliferation and vessel formation. These data indicate that Aplidin is well tolerated in vivo and its antitumour and antiangiogenic effects support the use of the drug in multiple myeloma.

Keywords: multiple myeloma; Aplidin; mouse models

Multiple myeloma (MM) is a plasma-cell malignancy characterised by accumulation of monoclonal plasma cells in the bone marrow (BM) and production of large amounts of monoclonal immunoglobulin or paraprotein. Multiple myeloma disease progression has been recognised as the result of different acquired changes in plasma-cell behaviour (self-sufficiency in growth signals, evasion of apoptosis and acquisition of invasive and spreading capacities) combined with an evolving crosstalk between myeloma cells and different cell types within the BM microenvironment (Hanahan and Weinberg, 2000; Mueller and Fusenig, 2004; Sirohi and Powles, 2004). Myeloma cells activate fibroblasts to secrete different growth factors, endothelial cells to initiate an angiogenic response, they stimulate immune and inflammatory cells and finally disrupt the balance between osteoblasts and osteoclasts, which results in osteolysis (Roodman, 2002; De Raeve and Vanderkerken, 2005).

The disease remains incurable for most patients, but advances in transplantation, the introduction of new compounds such as bortezomib (Richardson et al, 2005), thalidomide (Palumbo et al, 2003; Gajate et al, 2003) and lenalidomide (Richardson et al, 2006), and the implementation of supportive agents (erythropoietin, bisphosphonates) have led to significant advances in delaying morbidity and mortality of the disease. Double autologous transplantation clearly provides an overall survival advantage, with an extended disease-free survival, while reduced-dose-intensity allogeneic transplantation has opened the door to many more patients for this treatment option (Kroger et al, 2002; Attal et al, 2003; Harousseau et al, 2005). The recent progress in unravelling the biology of the disease, particularly the intracellular pathways and the interaction with the microenvironment, has resulted in development of novel targeted therapies and therapeutic regimens with more disease specificity and less systemic toxicity (Anderson, 2007).

Aplidin© is such an antitumour drug, which was originally isolated from the marine tunicate Aplidium albicans and currently obtained by total synthesis. Aplidin was initially selected for its enhanced cytotoxicity against different tumour cell lines and its lower myelotoxicity. Aplidin is an extremely potent inducer of apoptosis, with concentrations that caused 50% inhibition of tumour growth (IC50) in vitro in a low nanomolar range, and it also exhibits strong antitumor activity in xenograft models (Urdiales et al, 1996; Erba et al, 2002; Broggini et al, 2003; Biscardi et al, 2005). In different haematological malignancies, such as acute lymphoblastic leukaemia, acute myeloid leukaemia and lymphoma, the drug is cytotoxic on primary human cells and cell lines, even on leukaemic cells carrying cytogenetic abnormalities that have poor prognostic implications (Bresters et al, 2003; Erba et al, 2003; Gajate et al, 2003). In addition to the cytotoxic properties of Aplidin, the molecule exhibits also strong antiangiogenic activity.
The elucidation of direct effect against the VEGF loop has also been studied in several preclinical in vitro and in vivo models (Tarabottelli et al., 2004). The results of these studies show that Aplidin reduces secretion of VEGF from MOLT-4 human leukaemia cells in vitro and has been shown to reduce the expression of VEGFR-1 in the same cell line (Broggini et al., 2003; Biscardi et al., 2005).

In the current study, we explored the in vitro and in vivo activity of Aplidin against MM cells. In the past years, our group examined the pathogenesis of MM by using the 5TMM murine myeloma models that were valuable in identification of biological processes and new therapeutic targets. Compared with xenograft models, using subcutaneously implanted cells from human myeloma cell lines that grow in vitro in a stroma-independent manner, these models are characterised by myeloma development in the bone marrow (Caers et al., 2005). This is a clinically important feature as the BM environment is strongly implicated in the development of drug-resistance (Damiano et al., 1999; Dalton, 2003). In addition to in vivo efforts, we also aimed to characterise some of the intracellular processes involved in the antitumor activity of Aplidin.

MATERIALS AND METHODS

Murine 5T33MM myeloma model

The 5T33MM cells originated spontaneously in ageing C57BL/KaLwRij mice and have since been propagated by intravenous transfer of the diseased marrow into young syngeneic mice (Radl et al., 1988). The model was continued by intravenous injection of five 10⁶ diseased BM cells into young (6- to 10-weeks old) syngeneic recipients (Harlan, Horst, the Netherlands) as described previously (Vanderkerken et al., 1997). The mice were housed and treated following the standards required by the UKCCR Guidelines (UKCCR, 1998). The 5T33MMvivo (5T33MMv) cells grow in vitro in a stroma-independent manner, with a survival of only a few days. The 5T33MMvitro (5T33MMv) is a clonally identical variant that originated spontaneously from such an in vitro culture of 5T33MMv cells (Manning et al., 1992). This myeloma cell line grows in vitro in a stroma-independent manner and is cultured and maintained in RPMI-1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% bovine serum (Fetal C0, Sigma-Aldrich) and is cultured and maintained in RPMI-1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% bovine serum (Fetal C0, Sigma-Aldrich) and 2.5 mM pnp benzoate (Sigma-Aldrich). The 5T33MMcells will allow cytoplasmic cytochrome c to diffuse out of the cells. Cells were initially permeabilised and subsequently fixed and incubated with the FITC-conjugated rabbit anti-cytochrome c antibody (BD Biosciences). Mitochondrial membrane integrity was analysed using the fluorescent dye DiIC1 (Invitrogen, Merelbeke, Belgium) following the manufacturers' instructions. Cytochrome c release from mitochondria was finally analysed following a protocol from (Waterhouse and Trapani, 2003). This assay is based on the principle that permeabilisation of cells will allow cytoplasmic cytochrome c to diffuse out of the cells. After a 16-h incubation with different concentrations of Aplidin, cells were harvested and the cell pellets were lysed in lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% NP-40 and 0.25% sodium deoxycholate. The following protease and phosphatase inhibitors were added: 4 mM Na₂VO₄ (Sigma-Aldrich, Bornem, Belgium), 1 mM Na₃P₂O₇ (Sigma-Aldrich), 50 mM NaF (VWR, Leuven, Belgium), 5 mM EDTA (VWR), 1 mM AEBSF (ICN, Costa Mesa, CA, USA), 2 mM 1 M leupeptin (Sigma-Aldrich), 50 mg ml⁻¹ leupeptin (Sigma-Aldrich), 50 mg ml⁻¹ pepstatin A (ICN), 500 mg ml⁻¹ trypsin inhibitor (Sigma-Aldrich), 10 mM benzamidin (Sigma-Aldrich) and 2.5 mM pnp benzoxate (Sigma-Aldrich). The cells were then cleared by centrifugation (5 min, 13 000 g) and sample buffer was added. After boiling, the samples were separated on a 10% SDS–PAGE gel and transferred to PVDF membranes (Bio Rad, Hercules, CA, USA). The membranes were blocked with PBS-containing 5% low-fat milk and 0.1% Tween 20 and probed with the appropriate antibodies, namely anti-caspase-8 and anti-caspase-9 (Cell Signaling Technology, Bioké, Leiden, the Netherlands), anti-cyclin D1 (eBioscience), cyclin D2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin-dependent kinase 2, 4 and 6 (all from Santa Cruz Biotechnology). For measuring total protein levels, the blots were stripped and reprobed with anti-b-actin (Santa Cruz Biotechnology). The horseradish peroxidase (HRP)-coupled secondary antibodies donkey anti-rabbit and goat anti-mouse antibodies were acquired from Amersham. Bands were visualised using the ECL system (Perkin Elmer, Zaventem, Belgium).

Assessment of Angiogenesis by rat aortic ring assay

The rat aortic ring assay was performed as previously described (Van Valckenborgh et al., 2002). Briefly, thoracic aortas were removed from rats and sectioned into aortic rings of 1-mm long. The ring-shaped explants were then embedded in a rat-tail matrix and cultured on a tissue culture plate in 10% FCS and 1% Penicillin/streptomycin. The rings were treated with Aplidin for 72 h. The proliferation was assessed by measuring DNA synthesis in a [3H]-labelled thymidine incorporation assay. The cells were kept in serum-free medium RPMI-1640 and were treated with Aplidin at different concentrations for 17 h, while the vehicle solution was used as negative control. At 16 h before harvesting, cells were pulsed with 1 μCi (methyl-³H) thymidine (Amersham, Buckinghamshire, UK). Cells were harvested on paper filters (Filtermat A; Wallac, Turku, Finland) using a cell harvester (Inotech, Wohlen, Switzerland). The filters were dried for 1 h in an oven heated to 60 °C and sealed in sample bags (Wallac) containing 4 ml of Optiscint Scintillation Liquid (Wallac). Radioactivity was counted using a 1450 Microbeta Liquid Scintillation Counter (Wallac). Results are expressed as percentages of the relative DNA synthesis: the amount of radioactivity (c.p.m.) of the vehicle treated cells was set to 100% and the decrease of the radioactivity of the treated cells compared with this is shown.

Apoptosis assays

Apoptosis was assessed by FACS analysis of annexin V binding, caspase-3 cleavage, mitochondrial membrane integrity and cytochrome c release. To demonstrate the involvement of caspase activation in mediating the apoptotic effects of Aplidin, 5T33MMvt cells were preincubated for 30 min with the broad-spectrum caspase inhibitor, Boc-D-FMK (Merck Biosciences, Darmstadt, Germany) prior to treatment with Aplidin. After overnight incubation with Aplidin (20, 10 and 5 μM) cells were harvested and fixed for 10 min in 4.5% formaldehyde and 2% (v/v) aceton in FACSCluding aortic rings, male Wistar rats were used and also treated according to the standards required by the UKCCCR (2004). The results of these studies show that Aplidin reduces secretion of VEGF from MOLT-4 human leukaemia cells in vitro and has been shown to reduce the expression of VEGFR-1 in the same cell line (Broggini et al., 2003; Biscardi et al., 2005).

In vivo

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interstitial collagen (type 1) gel (Collagen B; Serva, Heidelberg, Germany) and allowed to polymerise in cylindrical agarose wells. Aortic rings (triplicates) were kept in culture at 37 °C in 6 ml of medium conditioned for 48 h by 5T33MMvv cells in the presence of vehicle or 2.5 and 1.25 nM Aplidin. After 10 days, photomicrographs were captured using a Leica microscope and pictures taken with an AxioCam cold camera using AxioVision software. Images were recorded in triplicate. Image analysis was performed using the software Photoshop CS. After generation of a binary image, the number of microvessels, the maximal microvessel length, and the total number of branchings were determined manually (Van Valckenborgh et al, 2002).

**Aplidin treatment in the 5T33MMvv myeloma model**

Three groups of 10 mice were given intravenous injections of 5T33MM cells; one group of 10 naive mice were included as negative control. Groups of 10 tumor-bearing mice received daily intraperitoneal treatment with either 90 or 60 μg kg−1 Aplidin. A total of 90 μg kg−1 was earlier found to be the maximum-tolerated dose by CS7BL/KaLwRij mice. A similar group was treated with vehicle alone (cremophor/ethanol/water, dissolved in PBS). The treatment schedule consisted of 5 days of treatment, followed by 2 days of rest and was started in the 5T33MM model from injection of tumour cells onwards. Mice were weighed daily. When the vehicle controls showed signs of morbidity, all of them were killed. From one femur, BM cells were isolated to determine tumour load by FACS staining and cytospin staining with May–Grünwald Giemsa; the other femur was fixed for immunohistochemical staining. Blood was harvested to determine serum paraprotein concentrations by capillary zone electrophoresis (Vanderkerken et al, 2005). To determine the effect of Aplidin, on survival, a similar experiment was performed. Two groups of 12 mice were given injections of 5T33MM cells: one group was treated with vehicle and the last group was daily treated with Aplidin, 90 μg kg−1, intraperitoneally. Treatment continued until each animal showed signs of morbidity, namely, hind-limb paralysis, at which point they were killed. Tumour load was confirmed on BM samples.

CD31 and Ki-67 staining on paraffin-embedded bone sections

The contralateral tibias and femora were incubated in zinc fixative (0.1 M Tris, 3 mM calcium acetate, 0.27 M zinc acetate, and 0.037 M zinc chloride), decalified in FE10 (0.27 M EDTA, 0.3 M NaOH, 2% formalin), embedded in paraffin and 5 μm sections were cut. Sections were stained for the presence of CD31 (PECAM-1) to identify the presence of microvessels or for Ki-67 to identify the proliferative cells (De Raeye et al, 2004). For CD31 retrieval, sections were incubated in trypsin to promote antigen retrieval and blocked with normal goat serum for 30 min. Sections were then incubated with a rat anti-CD31 antibody (PECAM-1; BD Biosciences), or an appropriate isotype control, at 4 °C overnight. The sections were washed and incubated with a goat anti-rat antibody conjugated with biotin (1:100 dilution; BD Biosciences). The presence of bound antibody was detected with streptavidin-HRP conjugate in combination with tyramide signal amplification (NEN Life Science Products, Boston, MA, USA). Diaminobenzidine was used as substrate. The number of blood vessels and sinusoids (per 0.22 mm²) were counted in a tumour-infiltrated area with the highest microvessel density (hot spot). To measure proliferative activity of myeloma cells, immunohistochemical stainings with Ki-67 was used. Ki-67 antigen is expressed during the G1, S, G2 and M phases of the cell cycle, but is not expressed during the G0 (resting) phase. As Ki-67 antigen has a short half-life, it can be used as a marker of actively proliferating cells (Schulter et al, 1993). Sections from embedded tibia were incubated with a polyclonal anti-Ki-67 antibody (DakoCytomation A/S, Glostrup, Denmark) and subsequently visualised using the Envision + detection systems (K4011, Rabbit DAB +; DakoCytomation). Ki-67-positive cells were counted at high magnification in an area with the highest proliferative activity. The percentage of Ki-67-positive cells was subsequently determined in this area at ×200 magnification by counting at least 400 nuclei.

**Statistical analysis**

For statistical analysis of the in vitro data, Mann–Whitney test was used. For in vivo (antitumour) data, Student’s t-test was used. For survival study, Kaplan–Meier analysis was performed. P-values smaller as 0.05 were considered significant. The IC₅₀ concentrations were calculated using nonlinear regression analysis on the results obtained from the proliferations assay. The Statistical Package for the Social Sciences (SPSS) software v15.0 (SPSS Inc., Chicago, IL, USA) was used.

**RESULTS**

We first investigated the effect of Aplidin on the growth of 5T33MMvt and 5T33MMvv cells in vitro by measuring 3H-labelled thymidine uptake. Treatment of these cells with Aplidin induced a dose-dependent decrease in cell proliferation compared with proliferation of the respective untreated cells. The mean Aplidin concentrations that caused 50% inhibition of growth (IC₅₀) after 17 h of treatment were, respectively, 7.10 nM (95% confidence interval (CI) = 6.87–9.17 nM) for 5T33MMvt cells and 3.87 nM (95% CI = 0.021 to 3.90 nM) for 5T33MMvv cells (Figure 1A).

To determine whether the reduction in proliferation of 5T33MM cells was accompanied by induction of apoptosis, we used annexin V-staining. 5T33MMvt cells were treated with different concentrations Aplidin (20, 10 and 5 nM) for 18 h and stained with annexin V for activated caspase-3. A total of 56.7% (s.d.: 15.4) of the cell population treated with 20 nM Aplidin was annexin V-positive (i.e. apoptotic), compared with 24.7% (s.d. 11.34) of cells cultured in control culture conditions. An increase in apoptotic rate could also be observed at 10 and 5-nM concentrations (Figure 1B). Induction of apoptosis may involve activation of aspartate-specific cysteine proteases or caspases. 5T33MMvt cells treated with different concentrations of Aplidin were stained for activated caspase-3. As shown in Figure 1, caspase-3 was activated in a dose-dependent manner (Figure 1C). Preincubation with a broad-spectrum caspase inhibitor Boc-D-FMK resulted in inhibition of the apoptotic effects of Aplidin (Figure 1B), which proves the involvement of caspase activity in mediating the effects of Aplidin. Western blotting (Figure 1D) showed appearance of cleavage products of both caspase-8 and caspase-9 after incubation with Aplidin, indicating involvement of both the intrinsic and extrinsic apoptotic pathway in mediating the effects of Aplidin on apoptosis induction. As caspase-9 is activated through the intrinsic pathway, initiated by the disruption of the mitochondrial membrane and cytochrome c release, we evaluated mitochondrial membrane function and release of cytochrome c in Aplidin-treated 5T33MMvt myeloma cells. After incubation with the mitochondrial binding probe DiIC1, the percentage of cells with a lower fluorescence was higher in Aplidin-treated cells, indicating altered mitochondrial membrane potential (Figure 2B). After staining with an antibody recognising cytochrome c, the mean fluorescence (MF) of Aplidin-treated cells was lower compared with the MF of non-treated cells (Figure 2A).

On cell-cycle progression, Aplidin exposure resulted in an increase in the percentage of 5T33MMvt cells in G0/G1 phase (65.6 vs 57.5%) and a decrease in the S and M phases (33.3 vs 23.1%, results not shown). Knowing that the transition from G1 to S phase is regulated by the cyclin D1, cyclin D2 and cyclin-dependent...
Aplidin inhibits 5T33MM cell proliferation at low nanomolar concentrations by causing arrest in G0/G1 by affecting kinase 2 (CDK2), CDK4 and CDK6 complex, we performed Western blot of these proteins. Aplidin exposure resulted in a decrease in protein expression of both cyclin D1 and CDK4, while concentrations of actin (as control protein) remained unaffected (Figure 3). CDK2 was affected only slightly. These data indicate that Aplidin inhibits 5T33MM cell proliferation at low nanomolar concentrations by causing arrest in G0/G1 by affecting the expression of cyclin D1 and CDK4. Cell-cycle progression was also monitored in vivo by using immunohistochemical staining for Ki-67. As in the human disease (Witzig et al., 1999), murine 5T33MMvivo have a low proliferative index, which yielded in a low percentage of Ki-67-positive myeloma cells. Intrapertioneal treatment with Aplidin decreased the number of Ki-67-positive cells (Figure 4B), indicating an in vivo confirmation of the in vitro effects of proliferation and cell-cycle progression.
Following in vitro studies, in vivo intraperitoneal treatment with maximum tolerated doses of Aplidin resulted in decreased tumour load. In Figure 4A, the effect of Aplidin on paraprotein and tumour load in the BM is shown. Mice treated with Aplidin at 90 \( \mu \)g kg\(^{-1}\) showed a 42% reduction in serum paraprotein concentrations and a 35% reduction in the percentage of 5T33MM idiotype-positive cells in the BM (\( P < 0.001 \)). In this model, MM cells also accumulate in both the spleen and liver. Treatment with Aplidin reduced overall splenomegaly by 60% and hepatomegaly by 40% (\( P \)-values < 0.001). As Aplidin treatment inhibited tumour progression and angiogenesis, we next studied its effect on the overall survival of the mice by Kaplan–Meier analysis (Figure 4B). The mice were treated either with the vehicle or with Aplidin. The Aplidin treated mice had a prolonged survival (51.5 days as compared with 42.9 days treated with vehicle only, \( P < 0.05 \)) according to Kaplan–Meier analysis. In the past years, other agents have been administrated to 5TMM-bearing mice to study their potential effect on survival. Most agents (zoledronic acid, the CDK4/6 inhibitor PD 0332991, the IGF-1R tyrosine kinase inhibitor picropodophyllin and recombinant osteoprotegerin) showed similar effects (1.2 to 1.5 \( \times \) prolongation) in survival studies, as the results obtained with Aplidin (Croucher et al, 2003; Vanderkerken et al, 2003; Menu et al, 2006, 2007). Only the survival rates obtained with the p38 MAP Kinase inhibitor SCIO-469 (which were three times increased) were superior to these results (Vanderkerken et al, 2007).

Next to analysing the antimumoma effects of Aplidin, we also analysed the antiangiogenic effects of Aplidin. We have previously shown that ST33MM cells stimulate angiogenesis in vivo, as assessed by quantifying the MVD (Van Valkenborgh et al, 2002). Immunohistochemical staining for CD31 (Figure 5A and B) demonstrated the increased MVD in tumour-bearing mice. When mice were treated with Aplidin, the MVD was reduced back to the mean of 10 mice in each treatment group. The experiment was performed in duplicate with similar results on endpoints. Aplidin treatment also prolonged the survival of ST33MMvv mice (B): the mean survival of vehicle-treated mice was 42.9 days, compared with 51.5 days for Aplidin-treated mice (\( P < 0.05 \)). In vivo, cell-cycle progression and myeloma cell proliferation were analyzed by Ki-67 staining. Aplidin treatment decreased the nuclear expression of Ki-67, suggesting a lowered proliferative index and less cells in cell-cycle progression (\( ** P < 0.05 \)).

On the different tumor parameters, we noted significant effects on paraprotein concentrations in peripheral blood, bone marrow plasmacytosis and spleen and liver mass (A). \( ** P < 0.001 \)). The results shown are the mean of 10 mice in each treatment group. The experiment was performed in duplicate with similar results on endpoints. Aplidin treatment also prolonged the survival of ST33MMvv mice (B): the mean survival of vehicle-treated mice was 42.9 days, compared with 51.5 days for Aplidin-treated mice (\( P < 0.05 \)). In vivo, cell-cycle progression and myeloma cell proliferation were analyzed by Ki-67 staining. Aplidin treatment decreased the nuclear expression of Ki-67, suggesting a lowered proliferative index and less cells in cell-cycle progression (\( ** P < 0.05 \)).

**Figure 3** (A) Western blots of cell-cycle regulators affected by Aplidin. (B) Optical densities of bands seen in Panel A. Aplidin causes an arrest in G0/G1 phase by decreasing the expression of cyclin D1 and CDK4. Western blotting of the different proteins involved at cell-cycle progression revealed decreased expression of cyclin D1 and CDK4. These effects were concentration dependent, as higher concentrations further decreased the expression. *\( P < 0.05 \).

**Figure 4** (A) Effect of Aplidin on different myeloma parameters of treated mice. (B) Effect of Aplidin on the survival of ST33MMvv diseased mice. (C) Quantification of Ki-67-positive ST33MMvv cells in the treatment groups. Aplidin treatment reduced tumour load in the ST33MMvv diseased mice and decreased proliferation in vivo. ST33MMvv diseased mice were treated according to the schedules described under Materials and Methods. On the different tumor parameters, we noted significant effects on paraprotein concentrations in peripheral blood, bone marrow plasmacytosis and spleen and liver mass (A). *\( P < 0.001 \)). The results shown are the mean of 10 mice in each treatment group. The experiment was performed in duplicate with similar results on endpoints. Aplidin treatment also prolonged the survival of ST33MMvv mice (B): the mean survival of vehicle-treated mice was 42.9 days, compared with 51.5 days for Aplidin-treated mice (\( P < 0.05 \)). In vivo, cell-cycle progression and myeloma cell proliferation were analyzed by Ki-67 staining. Aplidin treatment decreased the nuclear expression of Ki-67, suggesting a lowered proliferative index and less cells in cell-cycle progression (C). *\( ** P < 0.05 \)).
DISCUSSION

In the treatment of MM disease, an increasing number of therapeutic agents are becoming available, with the potential for significant symptom palliation, induction of disease responses and prolongation of disease-free survival. These different therapeutic approaches, including peripheral-blood stem cell transplantation and newer agents (thalidomide, bortezomib, and lenalidomide), have pronounced antimyeloma effects; but, unfortunately, MM disease often relapses. These approaches have only a modest impact on global patient overall survival in several randomised trials, and, as a consequence, there is still much need for improved antimyeloma therapies.

In the discovery of these therapies, preclinical mouse models may be exploited to accelerate drug development. They not only provide the opportunity for rapid validation of potential drug targets, but also help to derive critical variables such as achievable concentration of drug dose, routes of administration, frequency of dosing and toxicity profile. In our laboratory the syngeneic STMM models have been used in several studies to identify different biological processes in MM disease and validate the antimyeloma effects of different compounds (Menu et al, 2006; Vanderkerken et al, 2007). As Aplidin has pronounced anticancer activities in vitro, we were interested in testing the compound in this model, with the hope of identifying some of the intracellular pathways involved and help define optimal dosing schedules in our model.

The present report indicates that Aplidin has both in vitro and in vivo antimyeloma effects in the murine ST33MVM model. Studies involving the mechanism of action of Aplidin indicate that this compound acts through a complex number of events that affect proliferation, cell-cycle progression and apoptosis (Erba et al, 2002; Garcia-Fernandez et al, 2002). In ST33MMV cells, Aplidin induces a G0/G1 cell-cycle arrest. Analyses of the proteins implicated in the control of cell-cycle progression showed decreases in the expression of both cyclin D1 and CDK4. Translocation of the cyclinD1 gene to the immunoglobulin heavy-chain locus (11;14) and overexpression of cyclin D1 RNA have been implicated in cell-cycle dysregulation in MM (Bergsagel and Kuehl, 2001). The group of Chen-Kiang, however, showed that in the transition from G1 to S phase, retinoblastoma becomes phosphorylated by the exclusive pairing of CDK4 with cyclin D1 or CDK6 with cyclin D2 (Ely et al, 2005). The decreased expression of both CDK4 and cyclin D1 by Aplidin thus explains the arrest seen in cell-cycle progression.

Apart from affecting proliferation and cell-cycle progression, Aplidin also induces apoptosis in ST33MM cells. This was characterised by annexin V staining cleavage of caspase-3, -8 and -9 and decreased mitochondrial membrane potential. The
latter is often accompanied by increased permeability of the outer mitochondrial membrane, with release of intermembranous material to the cytosol, including several regulators of apoptosis. Flow cytometry demonstrated a significant release of cytochrome c from the mitochondrial to the cytosol of ST33MM cells exposed to Aplidin. Released cytochrome c is known to participate, together with procaspase-9 and apoptotic protease-activating factor, in the assembly of active apoptosomes, that in turn trigger the proteolytic cleavage of procaspase-3, with its ensuing activation (Riedl and Salvesen, 2007). In fact, evidence showing activation of caspase-3 and caspase-9 were present, indicating activation of the endogenous caspase-dependent cell death route. To confirm whether caspase activity really was required for cell death induced by Aplidin, the pan-caspase inhibitor BOC-D-FMK was used to block apoptosis. These data shown indicate that Aplidin indeed induced BOC-D-FMK-sensitive cell death.

Previous works have demonstrated that activation of intrinsic apoptotic pathways through mitochondria is one of the hallmark activities of Aplidin (Garcia-Fernandez et al, 2002; Gajate et al., 2003). In the subsequent studies, the mitochondrial changes were shown to be caused by different interactions. The sustained activation and phosphorylation of c-Jun N-terminal kinase (JNK) by reactive oxygen species (ROS) is recognised as one of the crucial mechanisms in the effects of Aplidin on breast cancer cells (Garcia-Fernandez et al., 2002; Cuadrado et al., 2004; Gonzalez-Santiago et al., 2006; Suarez et al., 2006). This ROS induction was found to be caused by an alteration in the glutathione homeostasis and by a rapid activation of the small GTP-binding protein Rac (Cuadrado et al., 2003; Gonzalez-Santiago et al., 2006). Next to phosphorylation of JNK, Aplidin also causes cleavage of Bid, a proapoptotic Bcl-2-family member that connects the Fas/CD95 cell death receptor (DR) to the mitochondrial apoptotic pathway, in human leukaemic cell lines (Gajate et al., 2003). Phosphorylation of JNK is currently being studied as in vivo pharmacodynamic marker of Aplidins’ mechanisms of action. In this study, maximal activation of JNK was seen in tumour derived from Aplidin-treated animals 4–12 h post treatment, while phospho-JNK levels were lower at earlier and later times (24–48 h) (Muñoz et al., 2007).

In addition to the mitochondrial pathway, the DR pathway regulates apoptosis in myeloma cells. Binding of its ligands, such as Fas ligand, TNF-α, and TNF-related apoptosis-inducing factor, to DR on the plasma membrane activates its adaptor proteins and finally triggers proteolytic activation of caspase-8 (Oancea et al., 2004). It was earlier shown that adding an anti-Fas-blocking antibody partially inhibited the Aplidin-induced apoptotic response, suggesting that the apoptotic response induced by Aplidin in leukaemic cells is partially mediated through Fas/CD95 binding (Gajate et al., 2003). Aplidin acts by clustering of CD95, but also by additional DRs and membrane-bound Fasl, together with downstream signalling molecules, into aggregated lipid rafts (Gajate and Mollinedo, 2005). Ocio et al. (2007) recently showed that, in addition to JNK phosphorylation, Aplidin induces a translocation of Fas and CD95 translocation to these lipid rafts in human myeloma cells. Our results show both cleavage of caspase-8 and caspase-9, and thus indicate an involvement of both the intrinsic and extrinsic apoptotic pathways in mediating the effects of Aplidin.

Daily administration of Aplidin in mice did not influence body weight suggesting that the agent is well tolerated when administered on a daily basis. The schedule consisted of daily administration of Aplidin and resulted in a 42 and 35% reduction in paraprotein levels and BM plasmacytosis, respectively, in the ST33MM model. These results are in the same range as the results obtained with a single treatment with melphalan (4 mg kg⁻¹) in the same model. This treatment decreased paraprotein levels with the 44% and BM plasmacytosis with the 47% schedule. Also, when treatment was started after the first signs of disease manifestation in the ST2MM model (in casu appearance of an M-spike in serum electrophoresis), Aplidin treatment significantly (P = 0.030) decreased tumour burden, as measured by serum paraprotein concentrations in the ST2MM model. Mean paraprotein concentration of diseased mice was 0.62 g dl⁻¹ (s.d.: 0.44) and 0.19 g dl⁻¹ (s.d.: 0.11) in vehicle- and Aplidin-treated mice, respectively (results not illustrated).

Of particular importance in MM disease is the lack of toxicity on haematopoiesis. Alkylating chemotherapies are part of induction treatments in myeloma, but affects the haematopoietic stem cells, which might interfere with an autologous BM transplantation, which is crucial to obtain longer remission times in MM patients. Albella and co-workers showed that Aplidin inhibits colony formation by human BM cells at high concentrations (150 – 530 nm) in vitro. These concentrations are 20-fold higher than the maximal drug concentrations found in the plasma of patients, given the maximal tolerable dose and 15-fold higher than the concentrations required to inhibit cancer growth. The clinical toxicological data obtained in phase-I and phase-II studies do not show BM toxicity in the different treatment schedules used (Gomez et al., 2003).

Aplidin has previously been described as an angiogenic compound (Broggini et al., 2003; Biscardi et al., 2005). In vitro and in vivo, Aplidin inhibited the neovascularisation associated with MM disease. These results support the earlier hypothesis proposed by others that Aplidin exerts a potent antiangiogenic activity by having a direct effect on endothelial cells, in addition to inhibiting production of angiogenic factors (Taraboletti et al., 2004). As seen in this study, Aplidin inhibited, in our experiments, both the physiological as the ST33MMvv-conditioned media-induced angiogenesis seen in a rat aortic ring assay.

In conclusion, administration of Aplidin resulted in strong antымyeloma activity in this synergistic in vivo model. Aplidin clearly demonstrated activity against the various endpoints evaluated in this model (e.g. paraprotein, BM invasion, angiogenesis and so on). Besides the previously reported antitymoma and antiangiogenesis effect, the present study indicates an additional direct effect on cell-cycle progression. Further studies are needed to define the relative effect of Aplidin on, respectively, the tumour compartment and endothelial cell compartment, and to identify optimal schedule and doses, to better exploit its multifaceted antineoplastic potential.

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