cells were harvested in lysis buffer, RNA from each plate was extracted and analyzed using the Human Cancer PathwayFinderTM RT2 Profiler PCR Array (SuperArray Bioscience Corp). This array profiles the expression of 84 genes representative of 6 biological pathways involved in transformation and tumorigenesis.
Results: A total of 84 genes were analyzed, representing key signaling molecules in (1) apoptosis and cell senescence, (2) adhesion, (3) signal transduction, (4) angiogenesis, (5) invasion and metastasis, and (6) cell cycle control and DNA damage repair. While a number of genes in each category were modulated by SylA and/or GlbA, the strongest response to SylA was a 13-fold upregulation of IL-8 and a 6-fold downregulation of S100A4, while the strongest response to GlbA was a 19-fold upregulation of TEK and a 62-fold downregulation of E2F1 (Table 1). Interestingly, no effects on E2F1 were observed with SylA. Overall, GlbA induced stronger changes in gene expression than SylA.

Table 1

Gene name	Fold change	
	Syringolin A (SylA)	Glidobactin A (GlbA)
IL-8	+13	+15
S100A4	−6	−10
TEK	+7	+19
E2F1	0	−62

Conclusions: The recently discovered molecules SylA and GlbA, which form a new structural class of proteasome inhibitors (syrbactins), induce biologically important pathways in human neuroblastoma and regulate key molecules in all 6 functional cancer gene groupings, with strongest effects on genes that play a role in angiogenesis as well as cell cycle control and DNA repair.

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Combined therapeutic effects of bortezomib and fenretinide on Neuroblastoma cell growth, apoptosis, and angiogenesis

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Background: The proteasome inhibitor bortezomib inhibited cell growth and angiogenesis in neuroblastoma. Bortezomib has been shown to induce synergistic activity when combined with other antineoplastic agents. Here, we assayed a putative increased antitumour activity of bortezomib if delivered to neuroblastoma cells together with fenretinide, a synthetic retinoid used as potential therapeutic agent in a variety of cancers, including neuroblastoma.
Methods: Different neuroblastoma cell lines were tested for sensitivity to bortezomib and fenretinide, given alone or in different dose-and time-dependent combination schedules. Cell proliferation, cell viability and apoptosis were evaluated by measuring 3H-thymidine incorporation, trypan blue staining, DNA fragmentation and western-blot analysis. Angiogenesis was assessed by the chick embryo chorioallantoic membrane (CAM) assay. An orthotopic neuroblastoma mouse model was used to examine in vivo sensitivity.
Results: Isobologram analysis showed that treatment of neuroblastoma cells with bortezomib plus fenretinide caused a synergistic inhibition of cell growth. This inhibition was associated to marked G1 and G2/M cell cycle arrest with nearly complete depletion of S phase by the combined treatment. Neuroblastoma cell death occurs with apoptosis features via ER stress by the activation of specific genes. Tumour-bearing mice treated with fenretinide plus bortezomib lived statistically significantly longer than mice treated with each single drug. Histological evaluation and CAM analysis of primary tumors evidenced that the combined therapeutic effects were due to both increased antitumour and antiangiogenic activities.
Conclusions: Our findings provide the rationale for design a new therapeutic strategy to treat neuroblastoma based on this pharmacological combination.

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Bortezomib and Flavopiridol combination induces down regulation of Mcl-1, mitochondrial permeabilization and cell death in ALCL cells

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Bortezomib is the first proteasome inhibitor to have shown anti-tumour activity in both solid and haematological malignancies and to be approved for clinical use. Anaplastic Large Cell Lymphoma (ALCL) is a high-grade T-cell non Hodgkin lymphoma, characterized by high mitotic index. We previously demonstrated that Bortezomib (BZ), in a concentration range between 20 and 100 nM, leads ALCL cells to apoptosis, despite the up-regulation of the anti-apoptotic protein Mcl-1.
Here we demonstrated that citostatic concentrations of the pan-cyclin-dependent-kinase (cdk) Flavopiridol (FP), in combination with non toxic concentrations of BZ (3.75 to 5 nM) induces cell death in all three ALCL cell lines examined.
Changes in cell cycle progression were measured by FACS and immunoblotting analyses. Apoptosis was measured by assessing mitochondrial activity at a biochemical level (MTT test, annexinV, DiOC6), and homeostasis by protein regulation at a transcriptional (QRT-PCR) and post-transcriptional levels (immunoblotting, immunofluorescence).
We found that when used alone, FP inhibited cdk-dependent phosphorylation of retinoblastoma protein and induced G1 cell cycle arrest at 24 h, together with p21WAF and cyclin D3 up-regulation, while BZ downregulated S phase and increased expression of p27KIP cdk-inhibitor. However, only when used in combination, FP and BZ, induced apoptosis in ALCL cells, which increased over time ($\geqslant$60% at 48 h) together with reduced DiOC6 up-take into mitochondria (~60% of control cells). This was confirmed by the release of cytochrome-C from mitochondrial membrane inner space and the activation of pro-apoptotic Bax protein 24 h after co-administration of FP and BZ. Loss of mitochondria membrane potential correlated with reduction of Mcl-1 protein expression and transcription, most likely because of FP-dependent inhibition of RNA Polimerase II. Furthermore, Mcl-1 down-regulation caused activation of pro-apoptotic Bak, normally bound to Mcl-1 in inactive state.
In conclusion, we demonstrated that combined administration of FP and BZ in ALCL cells, at subtoxic concentrations, caused cell cycle arrest with a significant reduction of cells in S phase at early time points, followed by induction of intrinsic apoptosis through activation of Bax, release of cytochrome-C and down-regulation of anti-apoptotic protein, Mcl-1.

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NPI-0052 (a 2nd generation proteasome inhibitor) Phase 1 study in patients with lymphoma and solid tumors

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Background: NPI-0052 is a novel 20S proteasome inhibitor that is being evaluated in clinical trials as a single agent and in combination with other oncology therapies in patients with solid tumors, leukemia, lymphoma and myeloma. NPI-0052 has a novel structure and preclinical data suggest the following:
– unique proteasome inhibition profile
– unique signal transduction profile
– improved toxicology profile
– improved therapeutic ratio
Materials and Methods: Patients with solid tumor or lymphoma were treated with NPI-0052 administered weekly, for 3 weeks in 4-week cycles in this 3+3 design dose escalation study. The dose of NPI-0052 was escalated in 50–100% increments. In addition to regular safety monitoring, proteasome inhibition (PI) (baseline, D1−2, D8, D15−17, D22 and D29) and PK (D1 and D15) were assayed in blood. Once a Recommended Phase 2 Dose (RP2D) is identified, an RP2D cohort of up to 20 patients (10 lymphoma and 10 solid tumor) will be enrolled.
Results: A total of 29 patients have been enrolled at doses ranging from 0.0125 mg/m^2 to 0.375 mg/m^2 for up to 12 cycles without reaching an MTD. The most common adverse events include fatigue, nausea, constipation and back pain. SAEs reported as potentially related include MRSA sepsis

and renal failure in one patient treated at 0.1 mg/m^2 and Grade 4 neutropenia of 3 days duration in one patient treated at 0.112 mg/m^2, otherwise drug related adverse events have been unremarkable at the highest dose level tested. Preliminary pharmacokinetic data indicate an elimination half-life of approximately 2−5 minutes with clearance of 5.5−15 mL/min and volume of distribution of 16.5–103.5 L. In all clinical trials with NPI-0052, proteasome inhibition has been assayed in whole blood from patients treated at 0.0125 mg/m^2 through 0.45 mg/m^2 demonstrating inhibition of CT-L, increasing with time and dose, up to 92%. Seven patients (24%) have had stable disease for at least two months (8 weeks; 2 months), including patients with hepatocellular carcinoma (6 cycles) adenoid cystic carcinoma (4 & 5 cycles), melanoma (4 cycles), CRC (6 cycles), ovarian (4 cycles) and cervical carcinoma (12 cycles).
Conclusions: NPI-0052 produces dose and time-dependent pharmacologic effects into the predicted efficacious range at well tolerated doses. Chronic dosing can be maintained with prolonged stable disease without toxicity emerging. Safety and clinical benefit continues to be evaluated to further characterize the significance of the initial findings. Based on these results, clinical trials have been initiated in combination with other cancer therapies.

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Preclinical and clinical monitoring of cell-and circulating plasma specific proteasome biomarkers after treatment with the proteasome inhibitor, NPI-0052

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Background: Pharmacodynamic profiling of proteasome inhibitors has focused on monitoring activities in packed whole blood (PWB), rather than peripheral blood mononuclear cells (PBMC) or the tumor. Proteasome activities in plasma of cancer patients may correlate with prognosis suggesting they are tumor derived. Therefore studies were performed to evaluate changes in proteasome activities during preclinical studies and Phase I trials with NPI-0052, a novel proteasome inhibitor that produces prolonged inhibition of all three proteasome 20S proteolytic activities.
Material and Methods: Chymotrypsin-(CT-L), trypsin-(T-L) and caspase-like (C-L) activities were measured before and after NPI-0052 treatment by monitoring the production of AMC from specific fluorogenic peptides and plasma proteasome levels by ELISA.
Results: Treatment of mice with NPI-0052 inhibited CT-L, T-L and C-L activities in PWB in a dose-dependent manner which recovered significantly by day seven. Since PWB contains mainly RBCs that do not regenerate proteasomes, the effects of NPI-0052 were evaluated in PBMCs. CT-L activity in PBMCs markedly recovered within 2−3 days after NPI-0052 treatment. In Phase I trials, inhibition of CT-L activity in PWB after treatment at 0.45 mg/m^2 reached ~60% after the first (day 1) and ~90% after the third treatment (day 15). A similar profile was also obtained in PBMC. In a patient with cervical carcinoma with stable disease for ~12 months, treated at 0.025 mg/m^2 and 0.05 mg/m^2, significant inhibition of all three activities was observed in PWB. When proteasome levels and enzymatic activity were compared in plasma of patients treated with low (0.025 or 0.05 mg/m^2) and higher-doses (0.075 or 0.112 mg/m^2), there were significant changes in median proteasome levels at 1 h and 4 h. Therefore the plasma proteasome activity was normalized to proteasome levels. We demonstrate significant changes in the median normalized levels of CT-L, C-L and T-L activity at 1 h in low-dose and higher-dose groups that remained detectable at 4 h in the higher-dose for CT-L and C-L but not the low-dose groups. Normalized T-L activity returned to pre-therapy level at 4 h in both groups.
Summary: The data indicate a complex and dynamic interaction between proteasome inhibitors, proteasome levels and activities in tissues. We are evaluating these parameters at higher clinical doses to determine whether they provide a prognostic value to assess disease progression and treatment response.

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Phase I clinical trial of the 2nd generation proteasome inhibitor NPI-0052 in patients with advanced malignancies with a CLL RP2D cohort

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Background: NPI-0052 is a novel proteasome inhibitor that produces prolonged inhibition of all three catalytic activities of the 20S proteasome. NPI-0052 has a novel structure leading to a unique proteasome inhibition and signal transduction profile. Preclinical models suggest NPI-0052 may demonstrate an improved therapeutic ratio and activity in hematologic (myeloma, lymphoma, leukemia) and solid tumor models. Secondary to these findings, clinical trials are currently being conducted in patients with myeloma, lymphomas, leukemias and solid tumors.
Materials and Methods: Patients with solid tumor, lymphoma or leukemia were treated with NPI-0052 administered weekly, for 3 weeks in 4-week cycles in this 3+3 design dose escalation study. The dose of NPI-0052 was escalated in 50–100% increments dependent on observed adverse events (AE). In addition to regular safety monitoring, proteasome inhibition (PI) (baseline, D1 & D15) and PK (D1 & D15) were assayed in blood. Once a Recommended Phase 2 Dose (RP2D) is identified, a RP2D cohort of patients with CLL will be enrolled. **Preliminary Results:** 22 patients have been treated at doses ranging from 0.1 mg/m^2 to 0.55 mg/m^2 without reaching an MTD. The AE profile has been tolerable with fatigue, transient peri-infusion site arm discomfort and lymphopenia being commonly ascribed to NPI-0052. Preliminary PK data indicate T1/2 of 2−5 min, a mean clearance of 12.6–22.8 L/min and Vss of 72.7–176.3 L. There is a linear relationship between AUC and Cmax. PI has been assayed in blood, indicating a dose:response relationship with inhibition of up to 92% observed. A total of 5 patients (23%) have had stable disease for at least 2 cycles (8 weeks; 2 months), including two with melanoma (4 cycles), one each with mantle cell lymphoma (4 cycles), Hodgkin's lymphoma (4 cycles), follicular lymphoma (4 cycles) and sarcoma (5 cycles).
Conclusions: NPI-0052 produces dose-dependent pharmacologic effects into the predicted efficacious range at doses below the MTD. Enrollment continues to identify a RP2D based on safety, efficacy and pharmacodynamics. These data have supported additional studies being initiated in combination with other targeted agents.

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Leaving groups prolong the duration of 20S proteasome inhibition and enhance the inhibition profile of salinosporamides

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Background: The 26S proteasome, a multicatalytic enzyme complex that degrades intracellular proteins, has emerged as an important target in cancer chemotherapy. Its 20S proteasome core particle contains three pairs of proteolytic subunits with chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (C-L) activities. NPI-0052 (salinosporamide A) is a mono-chlorinated natural product in clinical trials for cancer that exhibits potent and prolonged duration inhibition of all three proteolytic activities (IC50 CT-L < T-L $\ll$ C-L). The crystal structure of NPI-0052/yeast 20S proteasome complex indicated that the chlorine acts as a leaving group (LG) within the proteolytic sites. In this study, we investigated the mechanism whereby the LG enhances the duration and potency of 20S proteasome inhibition and developed analogs that more effectively inhibit all three sites.
Materials and Methods: Analogs with a range of LG potentials were prepared by semi- or total synthesis and evaluated for: (i) stability and hydrolysis product identity by LC-MS; (ii) inhibition of isolated rabbit 20S proteasomes before and after attempted removal of inhibitors by dialysis; (iii) cytotoxicity and inhibition of CT-L activity in human multiple myeloma