558 POSTER

Antitumor activity of brostallicin on human prostatic cancer cells: fundamental role of combination with hypomethylating agents

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Brostallicin preclinical antitumor activity depends on the intracellular levels of glutathione (GSH) and/or glutathione-S-transferase (GST). Among the GST isoenzymes the pi class is the stronger activator of brostallicin.
Almost 90% of human prostatic adenocarcinomas are characterized by the absence of GST-pi protein due to a heavy methylation of the promoter of GSTP1 gene (encoding for GSTpi enzyme), which prevents its transcription. The prostatic cancer cell line LNCaP retains this molecular characteristic and the GST enzymatic activity in these cells is very low; conversely prostatic carcinoma DU-145 cells, which do not present promoter hypermethylation, express GST-pi protein. We used these cell lines to determine the relative sensitivity to the new anticancer agent brostallicin, currently in Phase II clinical evaluation. The drug showed a differential cytotoxicity against these cell lines, being more effective on DU 145 than on LNCaP cells (IC_{50} 38 and >200 ng/ml, respectively).
DNA methylation can be reverted by using demethylating agents, such as the cytidine analogs 5-aza-deoxycytidine (5-aza-dC) and zebularine, or procaine and procanaimide.
The aim of this work was:
- to test the ability of these agents to restore the expression of GSTP1 gene in LNCaP cells;
- to investigate whether re-expression of GST-pi in these cells was associated with an increased activity of brostallicin.

Although 5-aza-dC is a very strong demethylating agent, its use in combination is limited by its very high cytotoxicity; on the contrary, the more stable cytidine analog zebularine is less cytotoxic and combination treatments with brostallicin were performed.
LNCaP cells were treated with zebularine at the concentrations of 50 μM, 75 μM, 100 μM and 125 μM for 96 or 120 hours. GST total activity measurement was performed on cytosolic proteins, by using the substrate 1-chloro-2,4-dinitrobenzene. Results showed a dose-dependent increase of GST activity.
Sequential administration of zebularine and brostallicin resulted in an increased cytotoxicity of brostallicin on LNCaP cells. In vivo experiments are in progress.
The two reported hypomethylating agents, procainamide and procaine did not show, in these cells, strong hypomethylating activity and as a consequence, did not increase the activity of brostallicin.

559 POSTER

The novel DNA cross-linking agent SJG-136 (NSC 694501) exhibits potent, selective and p53-independent cytotoxicity in human chronic lymphocytic leukaemia cells

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SJG-136 (NSC 694501) is a novel chemotherapeutic agent that binds in a sequence-selective manner in the minor groove of DNA. It is structurally distinct from other clinically-used DNA cross-linking agents and has exhibited a unique multi-log differential pattern of activity in the NCI 60 cell line screen (i.e. is COMPARE negative to other cross-linking agents). Given this profile, we undertook a pre-clinical evaluation of SJG-136 in primary tumour cells derived from 34 chronic lymphocytic leukaemia (CLL) patients. SJG-136 induced apoptosis in all the CLL samples tested with mean LD_{50} and LD_{90} values (the concentration of drug required to kill 50% and 90% of the cells) of 9.06 nM and 43.09 nM respectively. SJG-136-induced apoptosis was associated with the activation of caspase-3 and was partially abrogated by the caspase-9 inhibitor Z-LEHD.FMK but not the caspase-8 inhibitor Z-IETD.FMK. Importantly, its cytotoxicity was undiminished in CLL cells derived from previously treated patients, those with unmutated V_H genes and those with p53 mutations (P = 0.17; P = 0.63; P = 0.42 respectively). Furthermore, SJG-136 did not trigger the phosphorylation of p53 or the up-regulation of GADD45 expression in CLL cells whereas the cross-linking agent chlorambucil elicited both of these effects. This indicates that SJG-136 cross-linking adducts are not subject to p53-mediated DNA excision repair mechanisms in CLL cells. Taken together these data demonstrate a novel, p53-independent mechanism of action for SJG-136 that appears to circumvent the effects of poor prognostic markers in CLL. This unique cytotoxicity profile warrants further investigation and supports the use of this agent in phase I clinical trials.

Marine compound

560 POSTER

Kahalalide F (KF) induces apoptosis-independent cell death that involves ErbB3 downregulation and inhibition of Akt signalling

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Kahalalide F (KF) is a novel, marine-derived antitumor agent that is currently undergoing phase II clinical trials, however the mechanism of action is not well understood. We show that KF caused rapid and potent cytotoxic effects in the breast cancer cell lines SKBR3 and BT474. Several markers of caspase-dependent apoptosis were negative after KF exposure, including the externalization of phosphatidyl serine, release of cytochrome C out of mitochondria and the cleavage of caspase-3 and PARP. Moreover, molecular and chemical inhibitors of caspases or cathepsins failed to protect against KF-cytotoxicity. These data indicate that KF-induced cytotoxicity is independent from the basic apoptotic machinery, resembling necrotic cell death. Furthermore, the sensitivity to KF in a panel of human tumor cell lines derived from breast (SKBR3, BT474, MCF7), vulval (A431), non-small cell lung (H460, A549, SW1573, H292), and hepatic (Skhep1, HepG2, Hep3B) carcinoma, significantly correlated with protein expression levels of ErbB3 (HER3) but not other ErbB receptors. Downregulation of ErbB3 expression and inhibition of the PI3K-Akt/PKB signaling pathway was observed in KF-sensitive cell lines within 4 h exposure to KF. Conversely, ectopic expression of a constitutively active mutant of Akt protected against KF cytotoxicity in SKBR3 cells. Moreover, a KF-resistant subline of the colon carcinoma cell line HT29 expressed significantly reduced levels of all ErbB receptors (ErbB1–4) relative to the parental cell line. In conclusion, ErbB3 and Akt are major determinants of the cytotoxic activity of KF *in vitro*. The potential impact of the role of ErbB3/Akt as determinant for clinical response to KF should be considered in upcoming phase II studies.

561 POSTER

The marine, anticancerous compound dehydrothyrsiferol affects integrin mediated adhesion of human breast cancer cells

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We have previously shown that dehydrothyrsiferol (DT), a triterpenoid isolated from a marine, red alga, induces dose and cell type dependent apoptosis in human breast cancer cell lines. Within this process, our group observed detachment of entire cell clusters leading to programed cell death. Integrins are transmembrane, heterodimeric receptor molecules which are frequently involved in cell–extracellular matrix (ECM) interactions through which they mediate a wide range of biological processes, including vascularization, differentiation, and apoptosis. Therefore, our previous findings suggest a potential interference of DT in the bidirectional (inside-out and outside-in) integrin signaling complex. In adhesion assays on collagen and fibronectin coated plastic, DT but not the standard chemotherapeutics doxorubicin and taxol reduced the basal adhesion of estrogen receptor negative MDA MB 231 breast cancer cells via the integrins alpha2beta1 (collagen receptor) and alpha5beta1 (fibronectin receptor) in a dose-dependent manner. Maximum inhibition of adhesion (60%) was reached by incubating the cells with 20 myg/ml of DT for 30 min. A cytotoxic effect was excluded by analyzing propidium iodide uptake of cells treated for even 1 h with the same concentrations as used in the adhesion assays. Flow cytometric analysis revealed that the basal cell surface expression levels of both integrins were not altered by the presence of DT, thereby ruling out protein expression changes as the cause for the reduction of cell adhesion to ECM. To examine changes in the binding capability of the integrin alpha2beta1 in MDA MB 231 cells, we established a flow cytometric assay using a fluorescent, soluble collagen. The treatment with 20 myg/ml DT for 30 min caused a significant reduction of 43±12% (n=7) in the basal collagen binding of this cell type. Taken together, these data suggest that the marine compound DT modulates the signaling through integrins in MDA MB breast cancer cells which may explain, at least in part, the previously observed apoptotic effect, converting

DT into a promising new anti-tumor agent of potential interest for further oncological/pharmacological investigation.

562 POSTER

The combination of ET-743 and Irinotecan is active in preclinical models in rhabdomyosarcoma

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Background: ET-743 is a marine natural product that has shown activity in early clinical trials in adults. Response were observed in soft tissue sarcoma and ovarian cancer refractory to previous chemotherapy. This study was designed to investigate the effect of the combination ET-743 with the topoisomerase I inhibitor Irinotecan in the rhabdomyosarcoma cell line TE671 and in xenografts derived from this cell line.
Methods: ET-743 was combined with either Irinotecan or its active metabolite SN38 in the sequential schedule ET-743 (1 h exposure) followed by Irinotecan or SN38 (24 h exposure) and in the reverse sequence. Cell growth inhibition exerted by each drug and by their combination was assessed utilising the clonogenic assay. Dose-effect relationships were analysed by the combination index-isobologram method. We also investigated the antitumoral effects of the combination of ET-743 and Irinotecan in TE671 xenograft bearing mice. ET-743 and Irinotecan were administered at the doses of 0.2 mg/kg/d and 20 mg/kg/d respectively and drug treatments were delivered every 4 days for 3 times.
Results: The sequence ET-743 followed by Irinotecan was synergistic at the IC70 and IC50 doses, additive at lower doses (IC30). The reverse sequence (Irinotecan followed by ET-743) was synergistic at the IC70 dose and additive at lower doses. Averaging the results of independent experiments, the combination of ET-743 followed by Irinotecan resulted slightly more effective than the reverse sequence (combination Index at IC50:0.89 vs 1.07), but both were close to the additivity level. Similar results were obtained when SN38 was used in combination with ET-743. In vivo studies showed that ET-743 and Irinotecan as single agent were only marginally active, whereas the combination produced a significant antitumor effect: tumor weight inhibition (TWI) and Log Cell Kill (LCK) were significantly higher (TWI:85%, LCK: 0.91) after the combination of the two drugs than after each drug given alone (40% and 0.321 for ET743 and 47% and 0.521 for Irinotecan, respectively). No difference in weight loss was observed after treatment with the combination compared with the individual agents.
Conclusions: These results suggest that the combination of ET-743 and Irinotecan should be assessed in clinical trials for the treatment of rhabdomyosarcoma. Supported by AIRC, FOP, CNR, Italian Ministry of Health

563 POSTER

Distinct effects of proteasome inhibition by a novel inhibitor in lymphoid cells

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The 20S proteasome represents a novel target for cancer therapy because it controls the degradation of numerous key substrates including cell cycle regulatory proteins and apoptosis related proteins. Strategies to inhibit the 20S proteasome have been successful in multiple myeloma, using the recently FDA approved compound, bortezomib (Velcade). NPI-0052 is a novel, orally active, non-peptide small molecule proteasome inhibitor, which was discovered during the fermentation of a new marine Gram-positive actinomycete, *Salinospora* sp. Unlike bortezomib which only inhibits the chymotrypsin-like activity of the proteasome, NPI-0052 inhibits the chymotrypsin-like as well as the trypsin-like and caspase-like activities of human erythrocyte derived 20S proteasomes with EC_{50} values in the picomolar to nanomolar range. NPI-0052 is also a potent regulator of NF-kB activation and TNF-α synthesis in a murine macrophage-like cell line. In the present study we examined apoptosis induction by NPI-0052 in leukemia cell lines and patient specimens. NPI-0052 demonstrated varying degrees of apoptosis in three leukemia cell lines representative of AML, (ML-1), ALL, (Jurkat), and CML, (K562). All three cell lines underwent DNA fragmentation as measured by propidium iodide staining and subsequent FACS analysis upon addition of NPI-0052 doses ranging between 1 nM to 10 μM. In addition, treatment of Jurkat cells with NPI-0052 resulted in a dose related activation of caspase-3 and the cleavage of poly ADP-ribose polymerase (PARP). Closer examination of the mechanism of apoptosis induction revealed that lymphocytes may be more susceptible to modulation by caspase-8 and pro-oxidant production. When combined with an inhibitor specific for caspase-8 (IETD-fmk), Jurkat cells were protected against NPI-0052 induced apoptosis whereas ML-1 and K562 cells were not. Similarly, the antioxidant, N-acetyl cysteine (NAC), elicited a cell line dependent protection against apoptosis when combined with NPI-0052. NAC dramatically protected the Jurkat cells from apoptosis in combination with NPI-0052, but surprisingly, ML-1 were not protected by NAC. Direct examination of ROS production revealed increases in peroxide and superoxide production following a four hour exposure to NPI-0052 in cell lines of lymphocytic origin. CLL and Ph+ ALL patient material confirmed that lymphocytes from these patients are sensitive to antioxidant inhibition of NPI induced apoptosis. In the Ph+ ALL patient, differential Ficoll was conducted to separate lymphocytes and polymorphonuclear cells. Subsequent experiments on these cell populations within the same patient revealed that lymphocytes were more sensitive to NPI-0052 induced apoptosis and that apoptosis induction relied on oxidant production, whereas non-lymphocytes did not. These preliminary findings suggest that NPI-0052 induces apoptosis in leukemia cells, however the mechanisms governing apoptosis induction vary in cells of nonlymphoid versus lymphoid lineage.

Natural products

564 POSTER

In vitro cytotoxic activity of NBT-272, a novel quassinoid analog

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Seventy-six proprietary quassinoid analogs were designed and screened for cytotoxic potency in order to improve upon the cytotoxic effects of the quassinoid bruceantin, which selectively inhibits peptidyl transferase, polyribosomes, peptide chains, and protein synthesis. In turn, these effects are associated with decreased expression of C-myc, (Cuendet, M. *et al* Clin Ca Res 2004), diminished DNA synthesis, and reduced cell cycling beyond the G1/S transition point. Bruceantin, paclitaxel, and the quassinoid analogs were screened in the neuroblastoma cell line SK-N-AS which lacks N-myc over-expression, MDR-1, and which permits reproducible drug responses. Twenty-four hours after adding 4000 SK-N-AS cells into each well of a 96 well plate 0.01–1.0 ug/ml of analog was added to sextuplicate wells. After 1 or 24 hours, cells were washed with fresh medium and incubated for 5 days in drug-free medium. Cell viability was assayed with MTT. Cytotoxicity was calculated by comparing the mean O.D.s of sextuplicate wells containing treated or control cells. The most potent analog from the initial screening, NBT-272, was evaluated in other cell lines and in combination at ED_{50} levels with paclitaxel (PTX). NBT-272 was 2 and 10 fold more potent than bruceantin in C-myc positive intestinal cancer (LS174T) cells and in N-myc normal SK-N-AS cells respectively. It exceeded PTX potency in 7 of 10 cell lines including NCI-AR, an MDR-1 adriamycin resistant MCF-7 variant. Subtractive effects of 28–60% were observed with concomitant NBT-272 and PTX exposure. These persisted when taxane exposure preceded NBT-272 by 4 or 24 hours, and vice versa. Cytotoxic effects in both C-myc over-expressing LS174T and N-myc normal SK-N-AS cells suggest that myc inhibition is not the only intracellular target. The subtractive effects of combined NBT-272 and PTX remain unexplained. NBT-272 should have clinical utility in tumors where the cytotoxicity data show that it is more potent than paclitaxel. These include those expressing a neuronal phenotype such as desmoplastic small round cell tumor, neuroblastoma, GI tract, breast, and lung cancers.

Cancer	Cell line	ED_{50} in ug/ml 1 hour exposure		Relative potency NBT-272 vs PTX	Cytotoxicity after co-administration of PTX + NBT-272
		NBT-272	PTX		
Desmoplastic	JPNSRLT	0.03	0.3	10	
Duodenal	HUTU-80	0.04	0.08	2	
Neuroblastoma	SK-N-AS	0.05	0.09	1.8	−60%
Breast	MCF-7	0.07	0.1	1.4	
Squamous cell	SCC-25	0.1	0.09	0.9	−28%
Squamous cell	FADU	0.1	0.05	0.5	
Pancreas	PL-45	0.2	0.1	0.5	−38%
Fibroblast	BNR-11*	0.4	4.0		