Grb2-SH3 ligand inhibits the growth of HER2+ cancer cells and has antitumor effects in human cancer xenografts alone and in combination with docetaxel.

Brunilde Gril, Michel Vidal, Franck Assayag, Marie-France Poupon, Wang-Qing Liu, Christiane Garbay

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Gril Brunilde [1] **, Vidal Michel** [1] **, Assayag Franck** [2] **, Poupon Marie-France** [2] **, Liu Wang-Qing** [1] **, Garbay Christiane** [1 *]

[1] *PMC, Pharmacochimie Moléculaire et Cellulaire CNRS : FRE2718, INSERM : U648, CNRS : IFR95, Université Paris Descartes - Paris V, Faculté de Médecine 45, rue des Saints-Pères 75270 PARIS CEDEX 06,FR*

[2] *DIGBFC, Dynamique de l'information génétique : bases fondamentales et cancer CNRS : UMR7147, INST CURIE, Université Pierre et Marie Curie - Paris VI, section recherche 26 Rue d'Ulm 75248 PARIS CEDEX 05,FR*

* Correspondence should be adressed to: Christiane Garbay <christiane.garbay@univ-paris5.fr>

Abstract

HER2 represents an important signaling pathway in breast and other cancers. Herceptin has demonstrated clinical activity, but resistance is common. Thus, new therapeutic approaches to the HER2 pathway are needed. Grb2 is an adaptor protein involved in Ras-dependent signaling induced by HER2 receptors. A specific Grb2-SH3 ligand, designed and synthesized in our laboratory, called peptidimer-c, inhibited colony formation in HER2 over-expressing SKBr3 cancer cells. Combined treatment of peptidimer-c with docetaxel further inhibited both colony formation and tumor cell survival compared to docetaxel treatment alone. Efficacy of this combined treatment was correlated with a reduction in the phosphorylation of MAPK and AKT. Finally, peptidimer-c reduced the growth of a HER2$^+$ human breast cancer (BK111) xenograft in nude mice and potentiated the anti-tumor effect of docetaxel in a HER2+ hormone-independent human prostate adenocarcinoma (PAC120 HID28) xenograft. These results validate Grb2 as a new target for the HER2 pathway.

MESH Keywords Amino Acid Sequence ; Animals ; Antineoplastic Agents ; administration & dosage ; metabolism ; pharmacology ; Antineoplastic Combined Chemotherapy Protocols ; therapeutic use ; Apoptosis ; drug effects ; Cell Line, Tumor ; Dose-Response Relationship, Drug ; Drug Synergism ; Female ; GRB2 Adaptor Protein ; metabolism ; Humans ; Ligands ; Male ; Mice ; Mice, Nude ; Molecular Sequence Data ; Neoplasms ; drug therapy ; metabolism ; pathology ; Oligopeptides ; adverse effects ; metabolism ; pharmacology ; Receptor, erbB-2 ; metabolism ; Signal Transduction ; drug effects ; Taxoids ; administration & dosage ; pharmacology ; Time Factors ; Tumor Burden ; drug effects ; Xenograft Model Antitumor Assays ; src Homology Domains

Introduction

Receptors tyrosine kinases (RTK) such as EGFR (Epidermal Growth Factor Receptor) and HER2 (HER: Human EGFR-Related) (ErbB2) are involved in the progression of numerous types of cancer when overexpressed or mutated 1, 2. The oncogene HER2 (erbB-2/Neu), is amplified in 20–30% of breast carcinomas, leading to a highly aggressive disease and a poor prognosis 3, 4. HER2 protein heterodimerizes with other members of the HER super-family (HER1/2/3/4) to activate multiple signaling pathways. Once HER2 proteins are activated, the phosphotyrosine residues serve as docking sites for the Src-homology (SH2) domains of adaptor proteins, such as Shc and Grb2, resulting in the Ras/Mitogen-activated protein kinase (MAPK) and PI3K/AKT pathway activation which induce cell proliferation and mitogenesis 5.

HER2 protein is one of the most studied targets for the development of therapeutic compounds for breast cancer treatment. Trastuzumab is a humanized anti-HER2 antibody that has been approved for treatment of breast cancer in the metastatic and adjuvant settings 6. However, numerous patients who had initial responses to trastuzumab-treatment develop resistance 7. There is an urgent need to identify new targets to disrupt the HER2 signaling pathway in patients who develop metastatic breast cancer. Our laboratory has focused on the HER2 downstream adaptor protein Grb2 (Growth factor receptor bound protein 2) as a new target. Grb2, constituted by one SH2 (Src homology) domain flanked by two SH3 domains, plays a key role in linking the Ras signaling pathway to RTKs. The SH2 domain of Grb2 binds to a phosphotyrosine of the RTK or Shc and its two SH3 domains bind to Sos (Son of sevenless), the guanine nucleotide exchange factor of Ras 8, 9, which can activate Ras and the mitogen-activated protein MAP kinase (ERK1/2) cascade 10, 11. Grb2 over expression has been observed in various breast cancer cell lines 12, 13. Grb2 also plays a key role in the transformation processes. Transgenic mice heterozygous for the ΔGrb2 mutation were shown to be rate-limiting for mammary carcinoma induced by polyomavirus middle T antigen 14. The transfection of NIH3T3 cells expressing activated HER2, with a Grb2 mutant with an altered or deleted amino-terminal SH3 domain, led to a reversion of cell phenotype 15. These findings suggest that Grb2 constitutes a target for the design of new inhibitors 16, 17.

We have designed peptide dimers that specifically bind the two SH3 domains of Grb2 with high affinity (Kd ranging 10^{-8} M) 18. Such a peptide-dimer conjugated with penetratin, ($(VPPPVPPRRR)_2$-K-Aha-RQIKIWFQNRRMKWKK), is referred to as “peptidimer-c”.

It was previously shown to disrupt the Grb2-Sos complex on EGFR-expressing, non tumorigenic ER22 cells and to block downstream ERK1/2 phosphorylation, with no toxic effects. It also showed an anti-proliferative effect on NIH3T3/HER2 cells 18. No in vivo data have been reported for this compound to date.

In this paper, the effect of peptidimer-c on clonogenic cell growth, an established marker of malignant potential, is evaluated using HER2-over expressing SKBr3 human cancer cells. An interaction between peptidimer-c and the widely used chemotherapeutic agent docetaxel was observed. Modifications in signaling pathway intermediates were correlated with the anti-clonogenic effects of peptidimer-c and its combination with docetaxel. The anti-tumor effect of peptidimer-c alone was tested in vivo on a model of slowly growing human cancer BK111 xenografts in nude mice and finally, a potentiating effect of peptidimer-c with docetaxel was observed on PAC120 HID28 xenograft, an aggressive hormone-independent prostate cancer.

Materials and Methods

Cell lines and culture

The human breast cancer cell line SKBr3 was obtained from the ATCC (American Type Culture Collection, Manassas, VA. Cat No. HTB-30). Cells were cultured in McCoy glutamax medium supplemented with 10% (v/v) FBS and 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco Invitrogen Corporation, UK). PC3 cells were cultured in DMEM glutamax (supplemented as above). Cells were maintained at 37°C in an atmosphere containing 5% $CO_{2,}$ in a humidified incubator.

Antibodies and reagents

Docetaxel (Taxotere®) was kindly provided by Sanofi Aventis, France. Grb2-SH3 inhibitor conjugated to penetratin and peptide binding Mona SH3 domain (sequence: PPPVNRNLKPGRKSRPPPLD-Aha-penetratin) were synthesized by Fmoc chemistry as described in Cussac et al. 18. ERK1/2 antibody, Phosphorylated-ERK1/2 (p42/44 MAP kinase) (Thr 202/Tyr204) antibody, AKT antibody, phosphorylated-AKT (Ser473) antibody, phosphorylated-Shc (Tyr 239/240) antibody and Shc antibody were purchased from Cell Signaling Technology Inc. (Beverly, MA). Cleaved PARP antibody was purchased from BD Pharmingen. Actin antibody and Grb2 antibody were obtained from Santa Cruz Biotechnology (California).

Cell treatment and western blot analysis

SKBr3 cells were seeded in 6-well plates at a density of 2.5×10^4 cells/well, in 2 mL of medium supplemented with 10% FBS. They were incubated for 48 h and then treated for 24 h with peptidimer-c (30 μM) or the vector penetratin (30 μM). 10 nM docetaxel was subsequently administered to peptidimer-c or the vector treated or untreated cells. Signal transduction markers were tested after cell treatment with peptidimer-c for 48 h and docetaxel for 24 h. Apoptosis was studied by treating cells with peptidimer-c for 72 h and docetaxel for 48 h. Cells were washed with PBS (phosphate-buffered saline) and lysed with HNTG buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF and 1 X protease inhibitor cocktail from Roche Diagnostics (Manheim, Germany)). The lysate was centrifuged (12,000g for 10 min at 4°C) and the protein concentration in the supernatant determined using the Bradford method. Samples were separated on 7.5% or 10% polyacrylamide gels by SDS-PAGE, and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech. UK) by standard procedures. Membranes were blocked by incubation for 1 h at room temperature with 5% BSA (bovine serum albumin) in TBS (Tris buffered saline), 0.1% Tween 20 (TBS-T) and then incubated overnight at 4°C with the appropriate antibody diluted 1:1000 in 5% BSA in TBS-T. Membranes were washed several times in TBS-T and incubated for another 1 h at room temperature with 1:10,000-diluted anti-rabbit (or anti-mouse) IgG coupled to horseradish peroxidase. Proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech. UK). Membranes were then stripped in TRIS-HCl buffer with 100 mM β-mercaptoethanol and 2% SDS for 30 min at 50°C. The membranes were washed 3 or 4 times with water and another 2 times with TBS and incubated in a new blocking buffer before incubation with anti-actin antibody as a protein loading control. Each band was quantified and the signal was reported to the corresponding level of actin. After this signal treatment, each signal was reported to the correspondent control (not treated) and was expressed as the percentage compared to the control. 100% corresponds to the intensity of the control. For the apoptosis study, films were scanned and quantified. All quantizations were determined with “QuantityOne” software.

Clonogenic assay

2×10^3 SKBr3 cells or 40 PC3 cells were used per well to seed six-well plates, with 2 mL of medium/well. Cells were cultured for 24 h at 37°C, and were then treated with various doses of either peptidimer-c, a peptide binding Mona SH3 domain, penetratin vector (between 0.01 and 10 or 30 μM) or docetaxel (0.01 to 10 nM). For experiments using combination of docetaxel and peptidimer-c, peptidimer-c was added 24 hours before docetaxel (0.01 to 3 nM). For PC3 cells, colonies were allowed to grow for 10 days, and for SKBr3 cells, colonies were allowed to grow for 21 days in the incubator. Cells were then washed with PBS buffer and fixed in 10% formaldehyde for 30 min. They were washed again with PBS, stained with crystal violet (2 g in 100 mL ethanol, then 2 mL in 100 mL H_2 O) for 30 min and finally rinsed with water. Colonies were then counted.

Tumor engraftment in mice, drugs, protocols and in vivo treatments

We used 8- to 10-week-old Swiss nu/nu male or female mice from the animal facilities of the Curie Institute (Paris, France). PAC120 was xenografted by the serial subcutaneous grafting of cancer tissue into male mice 19 and BK111 xenografts and BK130JAM xenograft were established by subcutaneous implantation of tumor samples into female mice under anesthesia (0.5 ml of avertine, consisting of tribromoethanol i.e. 1g in 1 mL/100 mL isoamylic alcohol/sterile water, given i.p.). Tumors were maintained by successive passages in mice, as described 19. Mice were grafted with tumor fragments of approximately 5 mm^3 in volume. Mice bearing growing tumors with a volume of 60–100 mm^3 were individually identified and randomly assigned to the control or treated group, and treatment was initiated. For ethical reasons, xenografted animals were killed when tumor volume reached 2000 mm^3. As BK111 tumors and BK130JAM xenograft are hormone-dependent, mice grafted with these tumors were given estrogen in water. The PAC120 hormone-independent variant HID28 was obtained in PAC120-bearing mice, after bilateral orchidectomy and was selected and used after the 4^{th} passage.

Tumor growth was assessed by measuring two perpendicular diameters with a caliper twice a week. Tumor volume (V) was calculated as $V = (a^2 \times b)/2$, where a is the width of the tumor in mm and b is the length of the tumor in mm. Individual relative tumor volume (RTV) to initial size and mean relative tumor volume ± SD per group were calculated. Tumor growth inhibition was calculated as the ratio of RTV in the treated to control group x 100 at a given time.

For PAC120 HID28 xenografted mice, Docetaxel, (Taxotere®) was prepared immediately before use, injected i.p. according to doses and protocols detailed in the Results section. For BK111 xenograft treatments, peptidimer-c was administered i.p. at doses of 1.5 mg/kg, three consecutive days each 2 weeks for four weeks. For BK130JAM xenograft treatments, peptidimer-c was administered i.p. at doses of 1.5 mg/kg, five consecutive days/week for three weeks. Each mouse was xenografted by only one tumor.

“Penetratin vector used as a control was given following similar protocols at the dose of 0.75 mg/kg (corresponding to the same molar concentration as peptidimer-c).”

Statistical analysis

Data are expressed as means +/− SD. The significance of differences between control and treated groups was evaluated using Student’s t-test. Differences were considered significant if $p < 0.05$.

Ex vivo experiments

Mice were grafted with BK111 tumor fragments as described above. Peptidimer-c was administered i.p. at doses of 1.5 mg/kg and vector penetratin was administered at the same molar concentration. 3 mice were used in each group (peptidimer-c and penetratin vector). Before treatment, animals were anesthetized and a sample of each tumor was removed to be used as a control. Two days after treatment, animals were killed. Tumor removed from the mice were mechanically disrupted and frozen in liquid nitrogen for subsequent protein extraction. Cell lysis and Western blot analysis of pERK1/2 were performed as described in the previous paragraph.

Results

Effects of peptidimer-c on HER2 over-expressing cells in a clonogenic assay

The effects of the peptidimer-c treatment were investigated on clonogenic cell growth using two different tumor cell lines: the HER2 over-expressing human breast cancer line SKBr3 and, as a negative control, a cell line which does not express HER2, the human prostate carcinoma PC3 (Figure 1A). In SKBr3 cells, peptidimer-c reduced clonogenic growth with an IC50 of approximately 0.1 μM. Colony formation decreased by 80% at maximal peptidimer-c concentrations of 0.3 μM (Figure 1B). Peptidimer-c had no effect on PC3 cells (Figure 1C). The vector penetratin was tested in a large range of concentrations on both cell lines (Figure 1D and E), and showed no toxicity up to a concentration of 30 μM.

As an additional control, a peptide that interacts with the SH3 domain of Mona protein 20, 21, coupled to penetratin, was tested (Figure 1F). This peptide exhibits a Kd value for Grb2 of 120 μM (measured by fluorescence). It is essential to note that the slight effects observed in SKBr3 cells in figure 1F, occur at only high concentrations of the peptide binding Mona (30 μM) relative to the effective dose of peptidimer-c (0.03 μM). As expected, this irrelevant peptide did not show any inhibition of clonogenic SKBr3 growth even at high concentrations.

Prior treatment with peptidimer-c increases docetaxel cytotoxicity in HER2 over-expressing cells in a clonogenic assay

Since Trastuzumab, which specifically targets HER2, is often used in clinical treatment combined with docetaxel, we decided to test for a potential interaction between peptidimer-c and docetaxel. Isobologram analysis was performed to evaluate the potential interactions of the drugs, using a clonogenic assay. SKBr3 cells were treated with various concentrations of docetaxel (0-0.03-0.3 nM) alone and in combination with peptidimer-c (0-0.03-0.3 μM) or its penetratin vector (0-0.03-0.3 μM). 24 hours prior treatment with peptidimer-c

sensitized the cells to docetaxel and this was not observed with vector treatment (Figure 2A). Plate scans (Figure 2B) illustrate this increase in docetaxel effect with peptidimer-c pretreatment. Using combined treatment of 0.03 μM peptidimer-c and 0.03 nM docetaxel, a significant decrease (70–80%) in colony formation (Figures 2A and B) was observed. This represents an approximate 4-fold increased sensitivity to peptidimer-c over single agent data (Figure 1). Interestingly, no interaction was observed with PC3 cells, which do not express HER2 (Figures 2C and D).

Signaling pathway changes in HER2 over-expressing breast cancer cells (SKBr3) after peptidimer-c treatment alone or in combination with docetaxel

Since at least two major signalling pathways (Ras and PI3K/AKT) are induced by HER2 oncoprotein 22, we investigated the phosphorylation status of proteins involved in these pathways when SKBr3 cells were treated with docetaxel, peptidimer-c or combinations. Signalling pathways were studied using 30 μM and 10 nM peptidimer-c and docetaxel, respectively. Levels of phosphorylated and total Shc, ERK1/2 and AKT were determined by western blotting. Treatment of SKBr3 cells for 24 hours with peptidimer-c, followed by 8 hours of docetaxel, resulted in no changes in PI3K or Ras signaling (data not shown). Figure 3 diagrams a longer treatment schedule in which cells were cultured for five days, receiving peptidimer-c for the last two days and docetaxel for the last day of culture. For each western blot, densitometric values were graphed to the right of each membrane (for quantization see materials and methods). Phosphorylated-Shc levels increased after 24 hours of docetaxel incubation (Figure 3B), two-fold by densitometry. As expected, peptidimer-c treatment did not affect the docetaxel-induced increase in pShc levels, since Grb2, the target of peptidimer-c, is located downstream protein Shc.

Levels of phosphorylated-ERK1/2 (p42 and p44) decreased after 48 hours with peptidimer-c treatment (Figure 3C), whereas the vector had no significant effect on phosphorylation levels. The phosphorylation of both p42 and p44 decreased after docetaxel treatment, which might reflect docetaxel cytotoxicity. Finally, treatment with the peptidimer-c/docetaxel combination led to a decrease in both p42 and p44 phosphorylation (P+D) as compared to control (V or V+D).

After docetaxel treatment, alone or in combination with peptidimer-c or vector, phosphorylated-AKT levels decreased (Figure 3D), whereas no modification was observed without docetaxel treatment. Thus, it appears that docetaxel treatment decreased both AKT and MAPK pathway activation. Peptidimer-c, decreasing MAPK activation, re-enforced the docetaxel effect.

Prior treatment with peptidimer-c enhances docetaxel-induced apoptosis in SKBr3 breast cancer cells

SKBr3 cells treated with docetaxel (10 nM) or peptidimer-c (30 μM), or their combination, were investigated for apoptotic activity following drug treatment. Apoptosis was estimated by densitometry of immunoblotting to determine cleaved PARP (poly ADP ribose polymerase) levels (Figure 4 right panel). Peptidimer-c treatment alone for 72 h did not affect PARP cleavage. In contrast, docetaxel treatment for 48 h clearly induced apoptosis as shown by a 70% increase in cleaved PARP bands. Previous treatment with peptidimer-c for 24 h before docetaxel treatment resulted in higher levels of cleaved PARP production (94% of cleaved PARP). These results are consistent with the ability of peptidimer-c to potentiate the effect of docetaxel in reducing clonogenic growth in SKBr3 cells.

Anti-tumor activity in xenograft models

For measurement of the in vivo activity of peptidimer-c, alone or in combination with docetaxel, we chose HER2-expressing cancer tumors which were already established as transplantable xenografts in nude mice in the laboratory. The first model was nude mice xenografted with $HER2^+$ BK111 cells, established from a human breast tumor. The second one was nude mice xenografted with a hormone-independent, $HER2^+$ human prostate adenocarcinoma, PAC120 HID28, a fast-growing aggressive tumor model described in a previous study 19. A human HER2-independent breast cancer cell line (BK130 JAM) xenografted in mice was used as a negative control. The high HER2 expression level of PAC120 HID28 tumor was identified by real time semi-quantitative RT-PCR by Oudard et al. 23. The level of HER2 expression in BK111 and BK130JAM xenografts was analyzed by the same method. BK111 strongly expressed HER2 protein, while BK130JAM did not (data not shown). Mice were grafted with tumor fragments of approximately 5 mm^3 in volume. Mice bearing growing tumors with a volume of 60–100 mm^3 were randomly assigned to different treatment groups, and treatment was initiated. Tumor growth was assessed by caliper measurement.

We have tested only two doses of peptidimer-c: 1.5 mg/kg and 15 mg/kg. With the two doses tested, similar effects on tumor growth were obtained. BK111 model constitutes a tumor which is sensitive to herceptin®. Therefore, as a first attempt, we chose a starting concentration for the peptidimer that was one order of magnitude higher than herceptin (the dose of 1.5 mg/kg), and ten-fold higher (15 mg/kg). In fact, 1.5 mg/kg of peptidimer-c was the lowest active dose in vivo, thus we present here the results with only this dose.

We first tested two of the most common schemes used in clinical studies for herceptin®: 3x/week (on consecutive days) and 5x/week. Peptidimer-c showed no significant difference between these conditions. Moreover, even at the dose of 15mg/kg 3x/week (on consecutive days), no toxicity was observed on mice.

Peptidimer-c inhibited the growth of xenografted BK111 breast tumors in mice treated with 1.5 mg/kg, 3 days a week, for 4 weeks (Figure 5A). There is no rational explanation for the peculiar growth curve and the tumor size slowly decreases with a frequency of approximatively 15 days. This « wave growth » is a cyclic and highly reproducible phenomenon for this tumor.

Tumors had doubled in volume after 40 days in mice treated with peptidimer-c, whereas they were five times larger in vehicle control mice. No effect was observed with penetratin vector alone (dashed lines on figure 5A).

Since all molecules were injected i.p., in order to verify that peptidimer-c reached the tumor, we tested if HER2 signalling was targeted. The level of phosphorylated ERK1/2 after peptidimer-c treatment was evaluated on the BK111 xenograft, which was the model that showed the more significant effect of peptidimer-c treatment alone. As expected, a significant decrease in phosphorylated ERK1/2 appeared after two days of peptidimer-c treatment (Figure 5B, lane 2 compared to lane 1 on blot), while no difference was observed in mice treated by the penetratin vector alone (Figure 5B, lane 4 compared to lane 3). The rate of actin was constant in all cases, as shown by corresponding western blot. The western blot in the figure 5B shows the pERK1/2 levels before and after treatment of the tumor from only one mouse, but this data is representative of analysis of three mice. The quantization is the average of pERK1/2 level in the tumors of three different mice, and was normalized to actin protein levels. We did not detect any significant difference in level of pERK1/2 between the non-treated sample and penetratin vector-treated sample.

We tested the peptidimer-c on the human HER2-independent breast cancer cell line (BK 130 JAM) xenografted mice, used as a negative control (Figure 5C). Even if the experiment on BK130 JAM was performed during 23 days, while BK111 were treated during 39 days, it is essential to note that BK130 JAM shows a slower and more regular growth. At day 23, control BK 130 JAM tumors are at a level of growth equivalent to that obtained with BK111 at 25 days (relative tumor volume equal to 3–4). As the control tumors had reached the ethically acceptable size, it was necessary to sacrifice the animals at day 23 for BK130 JAM and day 39 for BK111. No significant effect was observed with the peptidimer-c on the BK 130 JAM cell line xenografted mice. In all cases no adverse effect was observed in the groups of mice treated with peptidimer-c.

We searched for a more aggressive $HER2^{+}$ xenograft and selected the PAC120HID28 model. Tumors were implanted and treated with 1.5 mg/kg peptidimer-c or vehicle control by i.p. injection at days 1, 2, 3, 21, 22 and 23. Control tumors doubled in volume in three days (Figure 6), versus twenty days for BK 111 xenografts (Figure 5A). Figure 6 shows a modest inhibitory effect of peptidimer-c on tumor growth as compared to control, not statistically significant. However, it is noteworthy that, for days 11–25 of the experiment, no control mice survived, while the peptidimer-c mice remained alive. As shown on figure 6, docetaxel alone at the dose of 20 mg/kg inhibited the growth of the tumors from the first injection. Combined treatment with docetaxel and peptidimer-c induced a rapid tumor mass regression and tumors were hardly detectable from day 8 onward. Docetaxel treatment inhibited tumor growth during the first days, but tumors re-started to grow on day 31. Tumor volume had tripled on day 49 in mice treated with docetaxel alone whereas tumor mass regression was still observed with the combined treatment after the same period of time. Treatment with a combination of docetaxel and peptidimer-c was significantly more efficient than treatment with docetaxel alone according to a Student's t test (stars above docetaxel curve are for the significant difference between docetaxel treatment and combined treatment).

Docetaxel injected at a sub-active dose (15mg/kg) weakly inhibits tumor growth (Figure 6, black squares on dashed line) as peptidimer-c alone does. The combined treatment (Figure 6, white squares on dashed lines) induces an interesting and significant tumor regression. At day 30, the size of the tumors had not reached the size observed for each treatment alone at day 16.

These results suggest that Grb2 inhibitor might constitute a potential candidate for HER2-dependent tumor treatment especially in association with a cytotoxic agent.

Discussion

Despite the groundbreaking advances made by the discovery and characterization of HER2 and Herceptin, many patients eventually relapse. Consequently, there is a need for additional therapeutics directed to the HER2 signaling pathway. This manuscript investigates the Grb2-Sos portion of the HER2 pathway using a peptide antagonist, peptidimer-c. Using a series of cell lines and transplantable xenografts, we report in vitro and in vivo evidence that peptidimer-c exhibits activity as a single agent and in combination with docetaxel.

In vitro activities of peptidimer-c included effects on clonogenic growth, HER2 signaling and apoptosis. Decreased ERK1/2 activation, but comparable AKT activation was observed after treatment of SKBr3 cells with peptidimer-c. This result is in accordance with literature that suggests a major role of the MAP kinase cascade in HER2-induced cell transformation 24, 25. In the case of non-tumor ER22 cells, which over-express EGFR, peptidimer-c decreased ERK1/2 phosphorylation without cytotoxic effect 18 showing the specificity of this compound for tumor cells.

In our experiments, we have tested only HER2 positive or negative tumor cells. However, the Grb2 SH3 inhibitor may also disrupt other signalling pathways in which Grb2 is involved, such as those induced by PDGFR, EGFR or BcrAbl. It is likely that other signalling

pathways would be targeted by this inhibitor. Therefore, Grb2 inhibitor could be tested not only in breast or prostate cancer but in other systems, for example, in CML (Chronic Myeloid Leukaemia) cells for BcrAbl signalling suppression.

In addition of ERK1/2, using other Grb2 inhibitors, several other signaling mechanisms have been proposed to be functionally involved. Grb2 SH2 inhibitors were reported to induce expression of two cell-cycle regulators, p27 and p21 in MDA-MB-468 cells 26, suggesting that the levels of p21 and p27 may be important in determining the proliferative response in cells with deregulated RTKs. Thus, multiple effects of Grb2 signaling antagonists may be operative.

An interaction between peptidimer-c and docetaxel was correlated with the inhibition of two essential cellular survival pathways: the decrease of both AKT and ERK 1/2 phosphorylation by docetaxel and decrease of ERK1/2 pathway by peptidimer-c and docetaxel. The effect of docetaxel on microtubules may be responsible of the inhibitory effect on MAPK proteins which bind to microtubules 27. We also showed that docetaxel treatment increases phosphorylation of Shc. This increase is in accordance with previous studies that had already reported that taxanes, such as paclitaxel, induced tyrosine phosphorylation of Shc and the Shc-Grb2 complex formation in mouse cells 28

However, these results contrast with those of Lim et al. 29, who reported that the inhibition of Grb2 in HER2-expressing cells led to an inhibition of AKT phosphorylation, with no effect on ERK pathway. Nevertheless, Lim et al. used an antisense approach to suppress the Grb2 effect and different cell types. Moreover, there is still some debate concerning the pathways activated by HER2. Indeed, one recent article presented clinical results on a breast tumor responding to trastuzumab (Herceptin®), with no evidence of Ras-Raf-MAPK and PI3K-Akt pathway modification 30. These different HER2 downstream signaling pathways are probably cell type dependent.

Investigation of the cellular mechanisms of SH3 domains has been hampered by a paucity of reported inhibitors that are effective in cell based assays, or more importantly, in animal tests. We have generated a Grb2 SH3 inhibitor that is capable of blocking tumor growth due to an oncogenic RTK such as HER2 and we described here the first in vivo activity of Grb2 SH3 inhibitors. Human tumor cell lines without HER2 overexpression were included in the analysis to evaluate the specificity of a Grb2 SH3 inhibitor for oncogenic signaling due to another mechanism (p53 mutation for PC3 cells). Different cell or tumor types were used for in vitro and in vivo experiments because we did not succeed in inducing tumor in nude mice with cells used in vitro. We therefore used the transplantable xenografts BK111, BK130JAM and PAC120 HID28 tumor, which were previously established in the laboratory. These cell types only grew as a tumor in animal environment and can not be cultured in plastic plates. The anti-tumor activity of peptidimer-c alone was shown in HER2 dependent BK111 xenografted mice. Peptidimer-c also potentiates the anti-tumor activity of docetaxel, by increasing the survival of nude mice grafted with the fast-growing $HER2^{+}$ human prostate tumor PAC120 HID28. The lack of anti-tumor effect of peptidimer-c on BK130JAM tumor, which did not over-express HER2 argues for the specificity of peptidimer-c on HER2-dependent tumors. Thus, our results suggest that Grb2-SH3 inhibitors should be considered as a tool for new treatment strategies, in association with conventional chemotherapy. Moreover, the observed potent effects with docetaxel suggest that Grb2-SH3 inhibitors may have lowered the resistance of tumors to docetaxel, and might make it possible to decrease the dose of docetaxel administered in combination.

Recent papers are consistent with our work showing the efficiency of blocking SH231, 32 or SH3 domain interaction for growth tumor inhibition. Lee et al showed that inhibition of SH3 domain interaction, other than Grb2 SH3 domain, inhibits tumor growth in vivo 33. Another paper reported that actinomycin D inhibited tumor growth of xenografts and this inhibition was explained by actinomycin D effect on blocking Shc-Grb2 SH2 domain interaction 34.

Grb2 inhibitors provide a new example for the proof of concept that pharmacological agents might be obtained by disrupting deregulated transduction pathways at the level of protein/protein interaction. In the same manner, antagonism of protein/protein complexes containing either Crk or the apoptotic proteins Bcl-2, MDM2 or XIAP, by small molecules have already been successfully reported 35, 36. In conclusion, the data validate the inhibition of Grb2 protein/protein interactions as a target in the design of anti-HER2 agents.

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Figure 1

Peptidimer-c inhibits anchorage-independent growth in two types of HER2 over-expressing SKBr3 cells. A- Two different cell lines were used, SKBr3 cells which over-express HER2 and PC3 which do not express HER2 as shown on the western blot. B-Peptidimer-c decreases colony formation of SKBr3 cells by 80% at 0.3 µM. C- No inhibitory effect of peptidimer-c was obtained in HER2-independent PC3 cells. D and E- The penetratin vector has no effect on the two cellular models. F- A peptide which does not interact with Grb2 SH3 domain but which binds to Mona SH3 domain, and referred to as irrelevant peptide, has no significant effect on HER2-dependent cell colony formation.

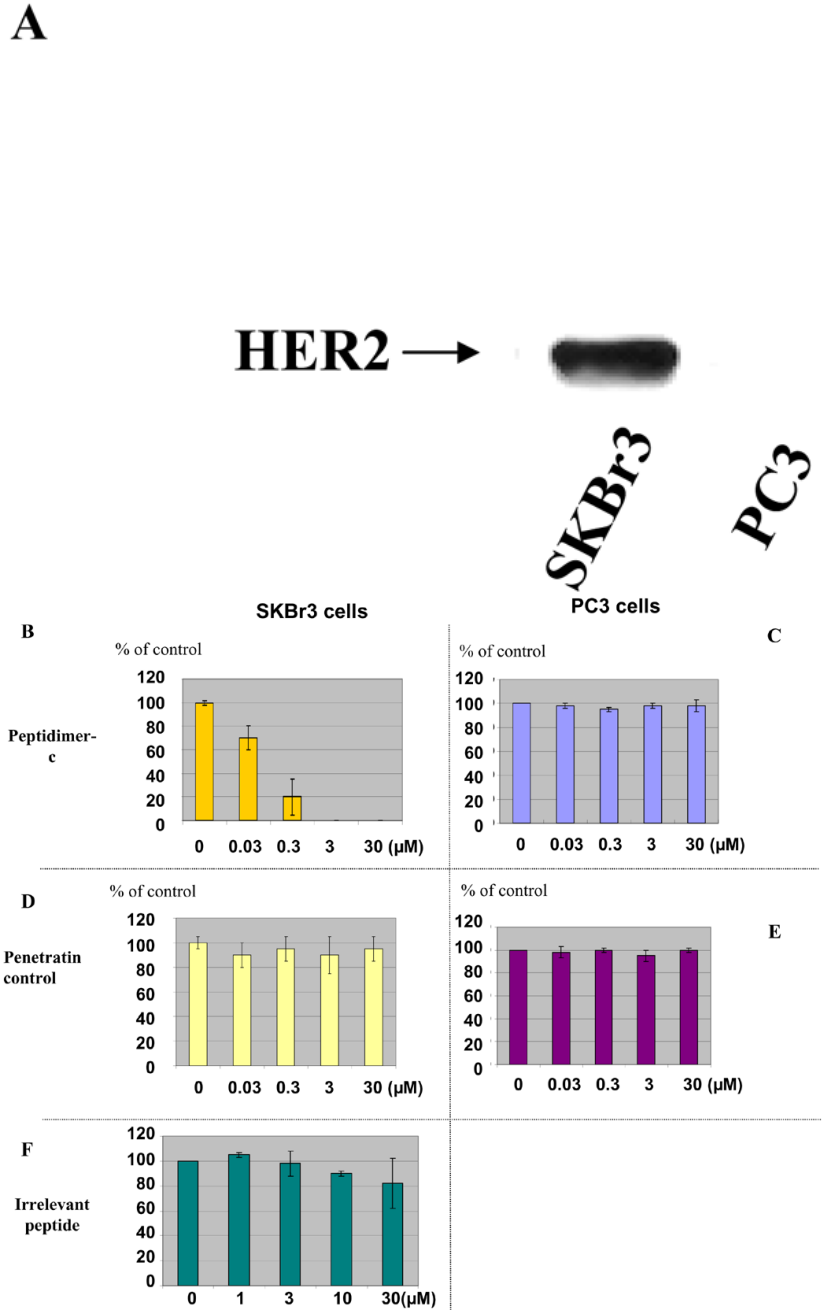


Figure 2

Peptidimer-c inhibits anchorage-independent growth and increases colony formation inhibition by docetaxel in HER2 over-expressing SKBr3 cells (A, B) while it has no additive effect on PC3 cells (C, D). The cells were treated with various concentrations of peptidimer-c and 24 hours later, with various concentrations of docetaxel. **A, B: SKBr3 cells.** The IC_{50} of docetaxel was found around 0.1 nM. Peptidimer-c had very little effect on colony formation at a concentration of 0.03 µM (Figure 1) and 0.03 nM Docetaxel concentration inhibited colony formation by only 20%. Using combined treatment, colony formation decreased significantly. No interaction was obtained using docetaxel-penetratin vector combined treatment. **C, D: PC3 cells.** No interaction was observed using the combined treatment docetaxel-peptidimer-c. On figure 2C, the values without docetaxel correspond to 100%. The results shown in figures A and C are representative of four independent experiments. The results shown in figures B and D are scans of a representative clonogenic assay.

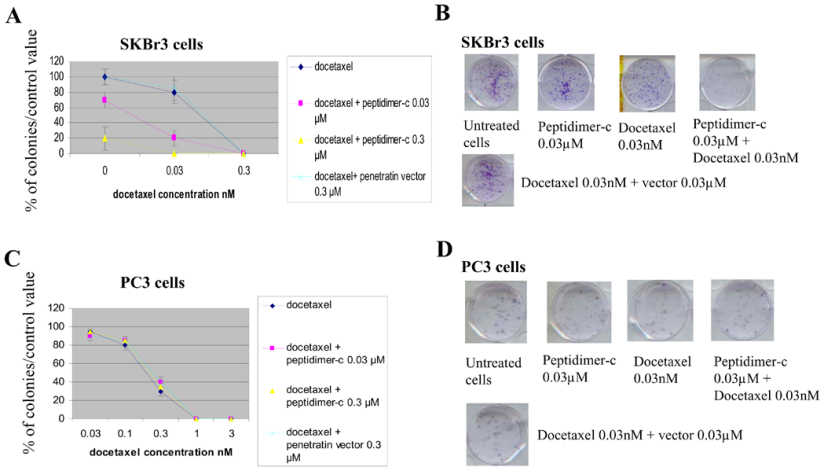


Figure 3

A-SKBr3 cells were treated for 24 hours by docetaxel D and 48 hours by peptidimer-c P or penetratin vector V. Therefore, in the combined treatment P+D and V+D, peptidimer-c (30µM) or the vector (30µM) were added 24 hours before 10 nM docetaxel addition. Total and phosphorylated form of Shc (B), MAPK ERK (C) and AKT (D) were analysed by western blot. Phospho-Shc levels were increased by docetaxel treatment. pERK1/2 (42–44 kDa) levels were decreased by peptidimer-c treatment, docetaxel treatment and combined treatment. pAKT (60 kDa) levels decreased 24 hours after docetaxel treatment. The penetratin vector displayed no toxicity. Signal corresponding to total Shc, total ERK or total AKT were not altered by any treatment. Actin (E) was used as a loading control. Each membrane was stripped and reblotted with anti-actin antibody. Only one representative film is shown in the figure. All experiments were repeated three times and the graph represents the mean quantization of the three experiments (+/− SD). Each band was quantified and the signal was reported to the corresponding level of actin. After this signal treatment, each signal was reported to the control (not treated) and was expressed as the percentage compared to the control. 100% corresponds to the intensity of the control.

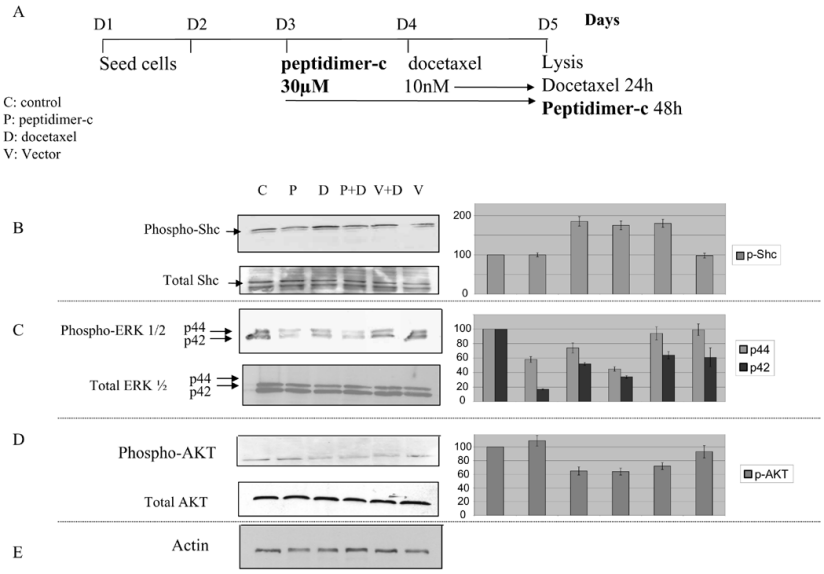
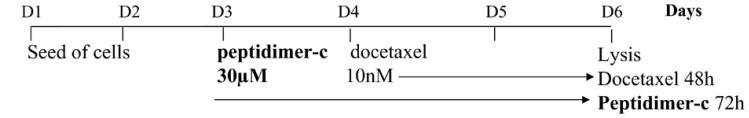


Figure 4

Peptidimer-c sensitized HER2 over-expressing breast cancer cells to docetaxel-induced apoptosis, as shown by the increase in cleaved PARP protein levels. SKBr3 cells were treated with 10 nM Docetaxel alone, 30 μM peptidimer-c alone or 10 nM docetaxel for 48 h and then with 30 μM peptidimer-c overnight (72 h). 25 μg of protein was separated by 10% SDS-PAGE and immunoblotted for PARP (116 kDa) and cleaved PARP (85 kDa). Apoptosis levels were estimated by quantifying the ratio of signals for the total PARP bands to the cleaved PARP band. Prior treatment with peptidimer-c increased the level of docetaxel-induced apoptosis. We carried out three independent experiments and actin blot was used as a control.



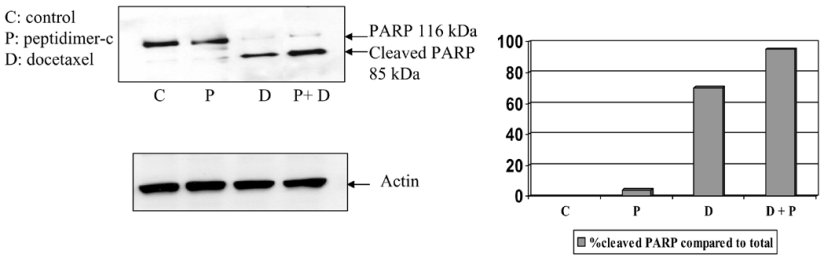


Figure 5

A In vivo experiments on BK111 xenografts. Peptidimer-c significantly inhibited the growth of human breast cancer BK111 xenografts in nude mice while penetratin vector alone did not. Mice were treated three consecutive days a week with 1.5 mg/kg or penetratin vector 0.75 mg/kg, for three weeks. B Ex vivo experiments on BK111 xenografts. Phosphorylation of ERK1/2 proteins decreases after peptidimer-c treatment on BK111 tumor-bearing mice. We analyzed phosphorylation of ERK1/2 on BK111 xenografts established in mice, before treatment and two days after peptidimer-c or penetratin vector treatment. The western blot is a representative results of pERK1/2 level after analysis of tumor from only one mouse. Quantization represents the average of pERK1/2 level of three pooled tumors from three different mice, and was normalized to actin level. A decrease in pERK1/2 was observed two days after peptidimer-c treatment. We did not detect any significant difference in level of pERK1/2 between the non-treated sample and penetratin vector-treated samples. C In vivo experiments on BK130JAM. No significant effects of peptidimer-c alone in BK130JAM, human HER2-independent breast carcinoma xenografts in nude mice. Mice were treated with 1.5 mg/kg, five consecutive days a week for three weeks.

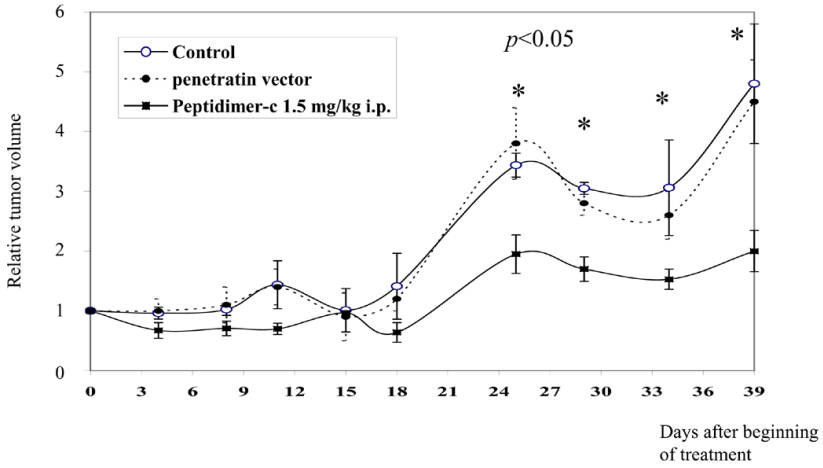


Figure 5A

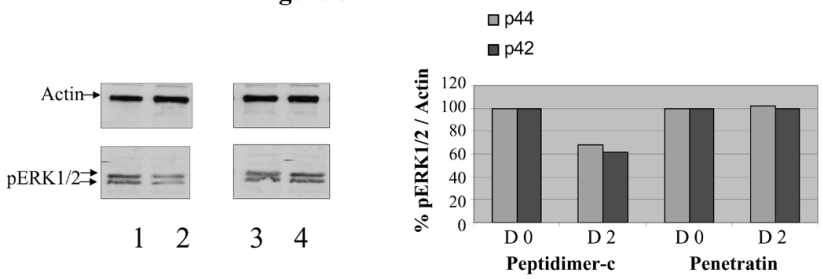


Figure 5B

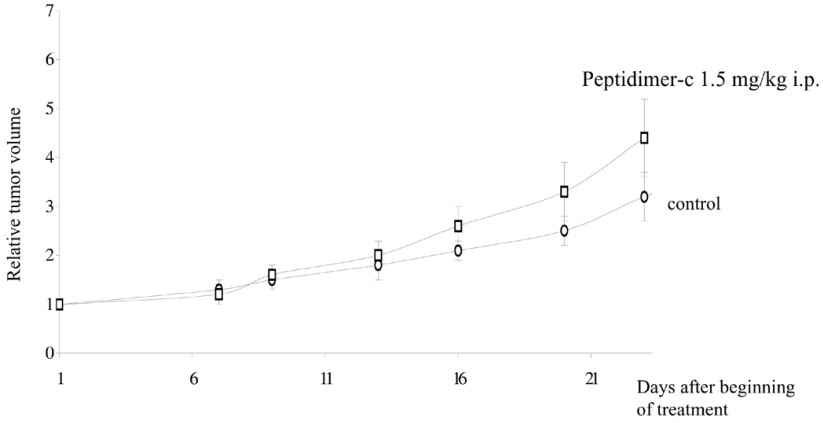


Figure 5C

Figure 6

In vivo combination therapy experiments. Effects of peptidimer-c alone, or in combination with docetaxel in fast-growing PAC120 HID28, human hormone-independent prostate adenocarcinoma xenografts in nude mice. Tumor-bearing mice were treated with 1.5 mg/kg of peptidimer-c (black diamonds on dashed line) at days 1, 2, 3, 21, 22 and 23, and with 20 mg/kg (black squares on full line) or 15 mg/kg (black squares on dashed line) of docetaxel, given as a single dose at the beginning of the treatment and 3 weeks later, or with combined treatment (white squares).

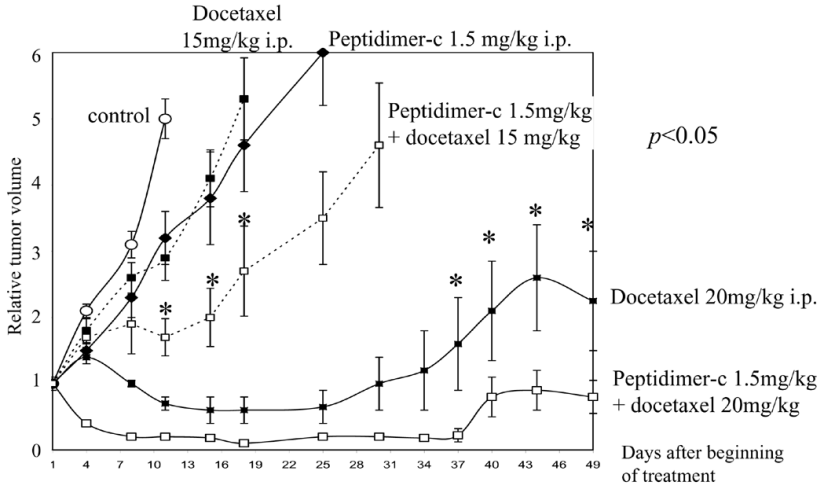


Figure 6