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Original Paper

Polyethylene Glycol Conjugated Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) Inhibits Growth of Breast Cancer in Athymic Mice

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Insulin-like growth factor (IGF) binding protein-1 (BP-1) inhibits IGF-mediated proliferation of some breast cancer cell lines *in vitro*. Here we examined whether recombinant human wild-type IGFBP-1 (WT-BP-1) and IGFBP-1 conjugated with polyethylene glycol (PEG-BP-1) could inhibit breast cancer growth. Three breast cancer cell lines were used: MCF-7, MDA-MB-231 and MDA-MB-435A (ascites model). The cells were grown in agar with or without the BP-1 conjugates to investigate their effect on colony formation. Both WT-BP-1 and PEG-BP-1 inhibited anchorage-independent growth (AIG) of MCF-7 and MDA-MB-435A cells. AIG of MDA-MB-231 cells was not inhibited by PEG-BP-1, whereas WT-BP-1 significantly stimulated colony number. We also tested both forms of BP-1 in xenograft tumour models. Two solid breast tumour models were studied using MCF-7 and MDA-MB-231 cell lines, and one ascites model using the MDA-MB-435A cell line. PEG-BP-1 inhibited malignant ascites formation in the MDA-MB-435A model. Conversely, PEG-BP-1 did not significantly inhibit MCF-7 xenograft growth. However, the MDA-MB-231 tumour growth curves were significantly different by a constant amount, suggesting that PEG-BP-1 treatment inhibited early tumour growth of this cell line. In contrast, WT-BP-1 was ineffective in the MDA-MB-231 tumours. These data show that anti-IGF strategies can be used to inhibit breast cancer cell growth. Since PEG-BP-1 inhibited the *in vivo*, but not *in vitro*, growth of MDA-MB-231, we speculate that PEG-BP-1 may block host IGF functions required for optimal tumorigenesis. Because PEG-BP-1 has a prolonged serum half-life compared to WT-BP-1, we conclude that improvements in BP-1 pharmacological properties enhanced its antitumour effects *in vivo*. © **1997 Elsevier Science Ltd.**

Key words: insulin-like growth factor, insulin-like growth factor binding protein, human breast cancer cell lines

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INTRODUCTION

INSULIN-LIKE GROWTH factors (IGFs) are among the many growth factors identified that stimulate breast cancer cells by interacting with specific receptors [1, 2]. In addition to IGF mitogenic effects, angiogenic and anti-apoptotic properties may also contribute to growth of breast cancer [3–6]. These findings support the idea that IGF-I receptor stimulation may have important roles in cellular proliferation and maintenance of the transformed phenotype.

IGFs are abundant in breast cancer tissue specimens. Studies examining tissue expressions have shown that IGF-II is often contained in normal stromal cells adjacent to the malignant breast epithelial cell [7–10]. Thus, local expression of IGF-II could constitute an important growth stimulatory signal via its interaction with the IGF-I receptor [8]. Proof of the importance of these IGF mediated effects

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has been demonstrated by an anti-IGF-I receptor strategy using the αIR-3 monoclonal antibody which inhibited xenograft growth of breast tumours in athymic mice [11, 12].

Under certain conditions, IGF binding protiens (BPs) can neutralise IGF-mediated cellular effects by interrupting ligand–receptor interactions. Some BPs bind both IGF-I and -II [13]. Thus far, at least six binding proteins have been identified, which are produced by normal and breast cancer cells. In breast cancer, the production of specific binding protein species is correlated the oestrogen receptor status of the tumour and could allow for regulation of local IGF effects [14–16]. Since the BPs have high affinity for the IGFs, they could also potentially be used as antagonists of IGF action.

We have been interested in BP-1 as a potential therapeutic agent. Unlike the other binding proteins, BP-1 is not commonly found in breast cancer specimens [15, 17], suggesting that its role in regulating local IGF action in breast cancer is negligible. BP-1 binds both IGF-I and -II with high affinity [13, 18]. Given the potential role that both ligands have in IGF-I receptor stimulation, pharmacological concentrations of BP-1 may bind free ligand both in the serum and locally in breast cancer tissue and inhibit IGF effects on breast cancer cells.

We have previously shown that IGF-I action can be neutralised by a 2-fold molar excess of purified human BP-1 in MCF-7 breast cancer cells [19]. The recombinant human BP-1 (WT-BP-1) inhibited IGF-I-, oestradiol- and serum-stimulated growth of MCF-7 cells in anchorage-dependent assays [19]. However, in the athymic mouse, the half-life of 2.5 h for this protein limited further *in vivo* tumour studies [20]. To circumvent this pharmacokinetic limitation, recombinant human BP-1 was conjugated to polyethylene glycol (PEG-BP-1), increasing the M_r from 29 kDa to 90 kDa and correspondingly increasing the serum half-life to 13.6 h (data not shown).

In this study, we compared the *in vitro* and *in vivo* effects of PEG-BP-1 and WT-BP-1 in three breast cancer lines. MCF-7 cells respond to the IGFs *in vitro* and we have shown that WT-BP-1 inhibited IGF-I-mediated growth of MCF-7 cells. The MDA-MB-231 cell line was chosen as a solid tumour model since IGF-I receptor targeting by monoclonal antibody αIR-3 has previously demonstrated inhibitory activity [11]. The MDA-MB-435A cell line was chosen to test a breast tumour model that grows as ascites, in the hope of improving PEG-BP-1 tumour distribution with direct intraperitoneal (i.p.) injection.

MATERIALS AND METHODS

Cells and cell culture

MDA-MB-231 human breast cancer cells were obtained from ATCC (American Type Culture Collection, Rockville, Maryland, U.S.A.). MDA-MB-435A human breast cancer cells were kindly provided by Nils Brünner (Finsen Laboratories, Copenhagen, Denmark). MDA-MB-435 cells (Janet Price, M.D. Anderson Cancer Center, Houston, Texas, U.S.A.) were grown in the mammary fat pad of athymic mice. By direct extension an MDA-MB-435 tumour eroded through the abdominal wall and proliferated as an ascites tumour. These cells were re-established in tissue culture. Chromosomal and isoenzyme analysis demonstrated that these cells were of human origin and derived from the parent MDA-MB-435 cells. The cells maintained their capacity to grow as an ascites tumour and were designated MDA-MB-435A. MCF-7 cells were maintained in our laboratories (CKO). Each cell line was maintained in phenol red-free improved minimal essential medium (IMEM) plus insulin and 10% FCS (fetal calf serum) in humidified 5% CO_2 atmosphere.

Materials

WT-BP-1 was synthesised as previously described [19]. Four- to five-week old athymic nu/nu female mice, weighing approximately 20 g each, were purchased from Harlan Sprague-Dawley (Madison, Wisconsin, U.S.A). Mice were maintained in a germ-free laboratory using institutional animal care standards.

Briefly, PEG-BP-1 was produced and conjugated in the following manner. Serine-101, a major site of phosphorylation, in the WT-BP-1 mature protein was changed to cysteine-101 by site-directed mutagenesis. When protein is refolded in *E. coli*, cysteine-101 is free and does not participate in disulphide bonds. Maleimide polyethylene glycol (20 kDa) was attached to cysteine-101, and polyethylene glycol conjugated WT-BP-1 was then purified from unreacted polyethylene glycol and unreacted WT-BP-1.

Anchorage-independent growth (AIG) experiments

Briefly, 0.8 ml of top layer containing a cell suspension of 15×10^3 (MCF-7, MDA-MB-231 or MDA-MB-435A) cells, 5% charcoal stripped serum (CSS), and 0.4% SeaPlaque agarose (FMC Bioproducts Rockland, Maine, U.S.A.), was added to 35 mm dishes with 1 ml of an already solidified bottom layer of 5% CSS and 0.6% SeaPlaque agrarose. Colonies containing 20 or more cells were counted after a 12 day incubation period. Each group was assayed in triplicate dishes. Groups of 5% CSS (control), 80 nM WT-BP-1 and 80 nM PEG-BP-1.

Tissue distribution of PEG-BP-1

Six athymic mice were injected subcutaneously with 1 mg of WT-BP-1. Two of the mice were sacrificed using chloroform and cervical dislocation at each time point: 1, 3 and 7 h. In a similar manner, three MCF-7 tumour-bearing mice treated with PEG-BP-1 were sacrificed 12 h after the last dose of PEG-BP-1. Steady-state trough serum and tissue samples were obtained. Blood was obtained by transecting the axillary artery. Serum and harvested tissue (tumour, liver, brain, muscle, heart and kidney) samples were stored at −70°C until analysed. Prior to weighing and freezing, tissues were blotted and compressed to remove as much blood as possible.

Analysis of PEG-BP-1 levels

MCF-7 tumour and tissue protein was extracted using a buffer containing Tris 50 mM pH 7.2–7.8, EDTA 2 mM, Nonidet-40 1%, NaCl 100 mM, Na orthovanadate 100 mM, leupeptin 100 μg/ml, aprotinin 20 μg/ml, and phenylmethylsulphonyl fluoride (PMSF) 10^{-7} M. PEG-BP-1 serum and tissue concentrations were analysed using IGFBP-1 enzyme-linked immunosorbant assay (ELISA) kit (Alpha Diagnostics, San Antonio, Texas, U.S.A). Samples were diluted 10- to 100-fold for accurate determination on the standard curve, using PEG-BP-1 in buffer when appropriate, ranging from 0–150 μg/l. Each assay was performed in triplicate, and absorbency read at 405 nm. Tissue con-

centrations were expressed as concentration per milligram of protein. Intra-assay variance was <10%.

BP-1 ligand blots and immunblots were performed as previously described [20, 21] with 2.5 ml of serum or 50 μg of tissue protein fractionated by SDS–PAGE gel electrophoresis and transferred to nitrocellulose. Ligand blots were subsequently incubated with radiolabelled ^{125}I-IGF. BP-1 immunoblotting was performed with BP-1 antisera, provided by Phillip D. K. Lee (DSL, Inc, Webster Texas, U.S.A).

WT-BP-1 and PEG-BP-1 in vivo tumour inhibition experiments

MDA-MB-435A cells (5×10^6) were injected into the peritoneum of 10 athymic mice. On the same day, 5 of the mice were subcutaneouusly injected with 750 mg PEG-BP-1 and 5 of the mice received diluent (phoshate buffered saline, PBS) alone. Injections were continued daily for 33 days. Mice were weighed every 3 days as a measure of ascites development. For experiments using MCF-7 cells, six ovariectomised mice were injected subcutaneously into the interscapular region with a 0.25 mg oestradiol sustained-release pellet (Innovative Research, Rockville, Maryland, U.S.A) one day prior to MCF-7 cell injection. The following day, 5×10^6 MCF-7 cells were injected into the mammary fat pad of six mice. Beginning on day 1, 3 mice were injected subcutaneously with 500 μg of PEG-BP-1 twice daily and 3 of the mice were injected with diluent alone for 21 days. In a similar manner 5×10^6 MDA-MB-231 cells were injected into the mammary fat tissue of 10 non-ovariectomised mice. Beginning on day 1, 5 of the mice were injected subcutaneously with 1 mg of PEG-BP-1 and 5 of the mice received PBS diluent alone daily for 30 days. In a separate experiment, animals were injected with either PBS or 1 mg of WT-BP-1 subcutaneously every 12 h for 33 days after inoculation with MDA-MB-231 cells. Tumour growth was measured with calipers every 3 days. Tumour size was expressed as tumour volume (mm^3) and calculated by the formula: volume = (smaller dimension^2 × large dimension)/2. Each experiment was repeated and similar results were obtained. Representative experiments are shown.

Control mice consisted of 5 mice not injected with tumour cells but receiving 750 mg i.p. of PEG-BP-1 daily. Six mice which did not receive either tumour cells or injections served as controls for normal weight gain.

Statistical analysis

In vitro data were analysed in the following manner. Each experiment was performed in triplicate. Data were transformed using the natural logarithm to equalise variances and compared using one-way ANOVA. Experimental groups were compared to control *t*-test with Bonferroni corrected *P*-values. *In vivo* data were analysed using a repeated measures ANOVA of weight change, as a measurement of MDA-MB-435A ascites development, and log-transformed tumour volumes (mm^3) for the MCF-7 and MDA-MB-231 model.

RESULTS

Effects of WT-BP-1 and PEG-BP-1 on in vitro growth

Figure 1 demonstrates representative *in vitro* experiments which were analysed using one-way ANOVA as a method for multiple comparison. The AIG assays demonstrated that

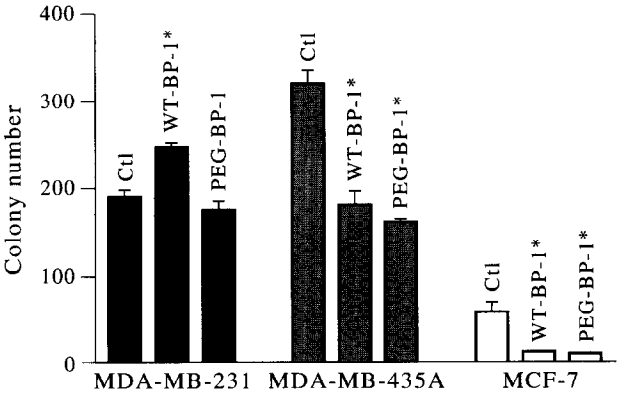

Figure 1. Anchorage-independent growth assay. WT-BP-1 and PEG-BP-1 inhibition of MDA-MB-231 (solid bars), MDA-MB-435A (shaded bars) and MCF-7 (open bars) human breast cancer cell lines in the AIG assay. Each cell line consisted of three groups: 5% CSS alone as control, 80 nM WT-BP-1 and 80 nM PEG-BP-1. Each group was carried out in triplicate and graphically illustrated as the mean colony number. A representative experiment is shown. WT-BP-1 significantly increased colony number ($P = 0.02$), whereas PEG-BP-1 had no effect on colony number ($P = 0.46$) of MDA-MB-231 cells. Conversely, WT-BP-1 and PEG-BP-1 both significantly inhibited MDA-MB-435A ($P = 0.002$, $P = 0.0008$, respectively) and MCF-7 colony number ($P = 0.0022$, $P = 0.0012$, respectively). Error bars represent standard error of the mean. Asterisks represent results statistically significantly different from control.

PEG-BP-1 had comparable *in vitro* activity to WT-BP-1 in MDA-MB 435A cells. WT-BP-1 inhibited colony growth to 44% of control ($P = 0.002$) while PEG-BP-1 inhibited growth to 50% of control ($P = 0.0008$). The MCF-7 colony number was significantly inhibited by WT-BP-1 to 17% of control ($P = 0.0022$) and by PEG-BP-1 to 14% of control ($P = 0.0012$). However, *in vitro* growth of MDA-MB-231 cells were not significantly inhibited by the presence of either BP-1 compounds; PEG-BP-1 resulted in a non-significant change in growth to 91% of control ($P = 0.46$), whereas, WT-BP-1 significantly stimulated colony growth to 30% more than control ($P = 0.02$).

Pharmacokinetic analysis of PEG-BP-1

Tissue distribution studies further characterised the predicted properties of the PEG conjugated BP-1 compared to the smaller WT-BP-1 molecule. Previous studies [20] of WT-BP-1 tissue distribution were used to compare the properties with the PEG compound. Direct comparison was not possible, since WT-BP-1 samples were peak concentrations after a single 1 mg dose in non-tumour burdened mice and PEG-BP-1 samples were 12 h steady state trough concentrations in tumour burdened mice. Despite the limited comparisons, tissue distribution of the PEG-BP-1 determined by ELISA appeared substantially less than WT-BP-1. For example, the mean 1-h peak liver concentration of WT-BP-1 was over 10-fold higher than the mean 12-h trough steady-state liver concentration of PEG-BP-1 (1617 μg/l/mg versus 130 μg/l/mg, respectively). Most importantly, we found that PEG-BP-1 tumour concentrations per milligram of protien were 0.3% of the corresponding serum concentration. In comparison, the WT-BP-1 tissue levels were 1.33% of the corresponding serum level. Ligand blot and immunoblot analysis of PEG-BP-1 tissue and serum

samples confirmed the ELISA results by the presence and intensity of bands at approximately 90 kDa for PEG-BP-1 and WT-BP-1, respectively (data not shown). Thus, it appears that PEG-BP-1 is retained for longer periods in the vascular space, and the distribution to tissue is less than that of WT-BP-1.

Effect of BP-1 on in vivo *solid tumour growth*

Figure 2a shows that tumour growth curves for treated and control mice bearing MCF-7 tumours were not significantly different after 21 days of treatment. In contrast, MDA-MB–231 tumours grew more rapidly than MCF-7 tumours and the two-sample *t*-test comparison of the log-transformed tumour volumes on day 30 indicated that treated mice had smaller tumours (Figure 2b) compared to control mice ($P = 0.049$, geometric means were 1737 mm^3 and 3108 mm^3, respectively). Repeated measures ANOVA of the log-transformed tumour volumes revealed that the growth rates over the course of the experiment did not differ ($P = 0.77$), but that very early tumour growth was significantly slowed in the treated group ($P = 0.0008$). PEG-BP-1 delayed MDA-MB-231 early stage tumour growth of development by 4.2 days, thus resulting in the difference in final tumour size after 30 days. Not included in the analysis was one mouse from the treatment group that failed to develop any tumour during the 30 day observation period. Figure 2c shows that MDA-MD-231 tumours were not affected by twice daily injection of WT-BP-1. Although slight differences in tumour size were evident at the end of the experiment, the growth curves were not significantly different.

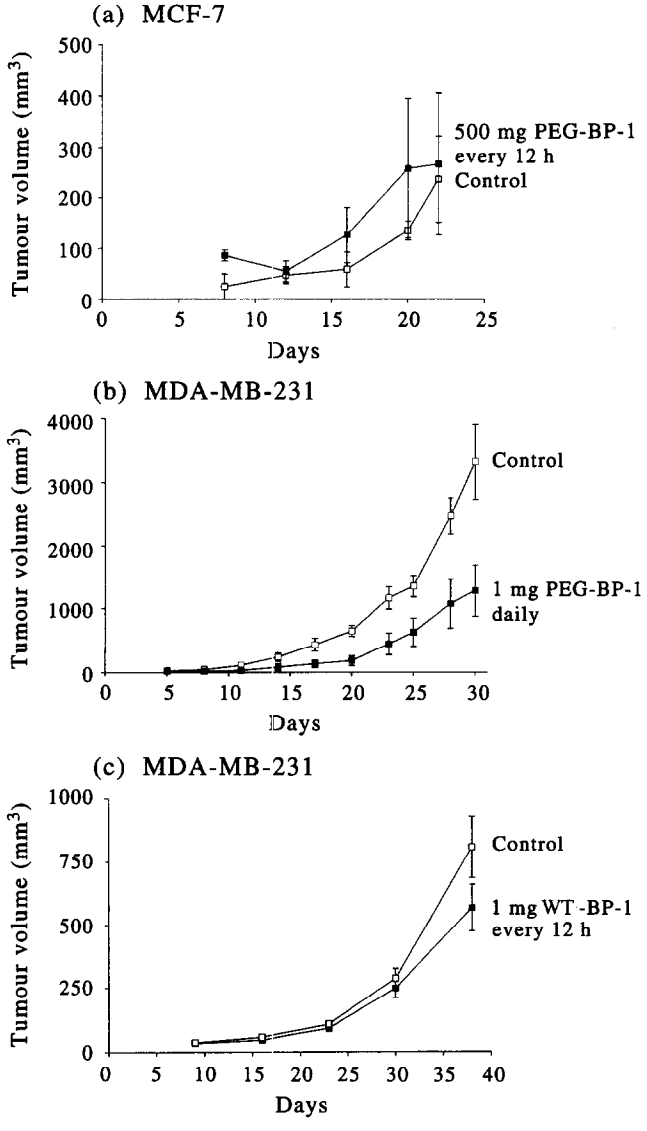

Figure 2. Effect of PEG-BP-1 and WT-BP-1 on xenograft tumour growth. (a) Six ovarectomised estrogen-supplemental mice were subcutanenously implanted with 5×10^6 MCF-7 cells into the mammary fat pad. No difference in tumour growth was noted over 3 weeks between controls and those receiving PEG-BP-1 twice daily (b) MDA-MB-231 cells were injected into the mammary fat tissue of 10 athymic mice. Daily PEG-BP-1 treatment resulted in significantly smaller tumours in mice with MDA-MB-231 tumours compared to control mice ($P = 0.049$). (c) MDA-MB-231 cells were inoculated as in (b) and treated every 12 h with 1 mg WT-BP-1. No significant differences in growth was noted.

Effect of PEG-BP-1 on in vivo *ascites growth*

Figure 3 demonstrated that mice injected intraperitoneally with MDA-MB-435A tumour cells and treated with PBS progressively gained weight especially from day 20 onwards. By day 33, four of these mice became moribund and were sacrificed. The remaining mouse continued to gain weight until sacrificed on day 40. In contrast, mice injected with tumour cells but treated with PEG-BP-1 experienced slower weight gain ($P = 0.001$, by repeated measures ANOVA, days 1–28). Overall control animals gained an average of 7.1 g compared to 3.9 g in the treated group. Two mice treated with PEG-BP-1 were sacrificed on

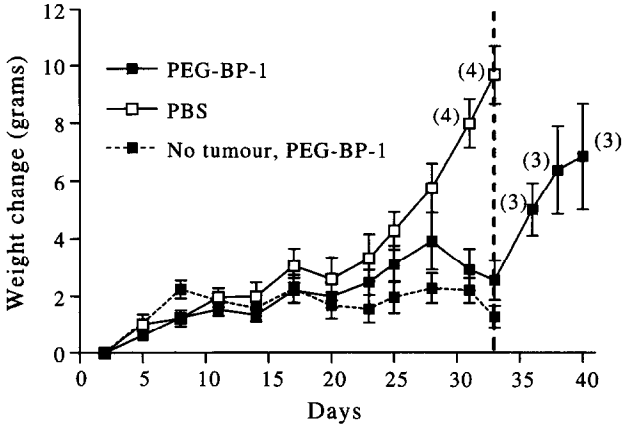

Figure 3. Effect of PEG-BP-1 on MDA-MB-435A ascites growth. MDA-MB-435A cells (5×10^6) were injected into the peritoneal cavity of 10 athymic mice. On the same day, 5 tumour-bearing (closed box, solid line) and 5 non-tumour bearing (closed box, dotted line) mice were injected with 750 μg of PEG-BP-1, and the other 5 tumour-bearing (open box, solid line) mice were injected with the same volume containing diluent alone. Injections were continued daily for 33 days then remaining mice were observed for further weight gain without injections (vertical dotted line). Mice were weighed every 3 days as a measure of ascites development and each time point represents 5 mice unless number on graph indicates otherwise. Mice injected with MDA-MB-435A tumour cells and treated with PEG-BP-1 experienced slower weight gain compared to control mice receiving diluent alone ($P = 0.001$, days 1–28). When treatment was stopped at day 33, the animals' weight increased. Non-tumour bearing animals did not experience significant change in weight over the course of the experiment.

day 33 due to dehydration and jaundice, while treatment was stopped in the remaining mice. During the no treatment phase, after day 33, these mice underwent accelerated weight gain, became moribund and were sacrificed on day 40. Non-tumour bearing mice receiving PEG-BP-1 daily experienced no obvious toxicities and were also sacrificed on day 40. Necropsy of non-tumour bearing animals revealed smaller uteri in PEG-BP-1 treated mice. The mean uterine weight of mice receiving PEG-BP-1 (0.044 g) was less than placebo control mice (0.0714 g). Since the uterine weight of only a few representative mice were measured, this difference was not statiscally significant ($P = 0.15$).

DISCUSSION

Recent evidence has established the importance of the IGF ligand–receptor system to many cancer types [1] and interference with IGF action could be an attractive therapeutic target. IGF effects may be inhibited by interfering with three different targets: IGF ligand–receptor interactions, IGF-I receptor gene expression, or IGF-I receptor biochemical function. Although blocking IGF-I receptor with monoclonal antibody, antisense RNA, or competitive antagonists may inhibit IGF action, these approaches are inherently complicated. Therefore, the use of BP-1 to inhibit IGF action appears a relatively simpler approach. Because of its lack of expression in breast cancer specimens and its high affinity for both IGF-I and IGF-II, we believe that BP-1 can be used to test the potential therapeutic effects of neutralising IGF action in breast cancer model systems. The remaining five binding proteins may have local effects on cancer cells, which have yet to be clarified. For example, in patients with axillary lymph node-negative breast cancer and tumours >2 cm, the presence of low tumour levels of BP-4 showed improved long-term survival, compared to patients with tumours containing high BP-4 levels [22]. Similarly, the potential use of BP-3 therapeutically is unlikely since BP-3 can stimulate cellular proliferation by enhancing IGF-I effects [23, 24]. Less is known about the roles of BP-2, -5, and -6 in IGF-mediated effects in breast cancer.

Given the IGF-I receptor inhibitory effects of BP-1 on both IGF-I and -II, we hypothesised that BP-1 would inhibit IGF-mediated proliferation of cancer cells. *In vitro* data show that WT-BP-1 and PEG-BP-1 had similar properties and inhibited IGF-I stimulated growth of breast cancer cells. The effect of WT-BP-1 and PEG-BP-1 in *in vitro* colony growth of the three cell lines demonstrated the greatest inhibition on the MCF-7 cell line and moderate inhibition on the MDA-MB-453A cell line.

The MDA-MB-231 cell line was affected differently by the two BP-1 compounds *in vitro*. The MDA-MB-231 colony number was not altered by PEG-BP-1 treatment, whereas WT-BP-1 treatment actually increased colony number. This cell line is the only example of a differential effect that we have observed between the two BP-1 compounds. BP-1 has previously been shown to have stimulatory effects in MDA-MB-231 cells by other investigators [26]. This cell line has characteristics that further confuse the interpretation of our results. Although MDA-MB-231 cells possess IGF-I receptors, biochemical activation of the receptor is difficult to detect and cellular proliferation is only minimally stimulated by IGF-I [26]. Furthermore, the MDA-MB-231 cells can produce IGF-II and BP-1 [9, 14]. However, none of these characteristics easily explain the difference in effect between the WT-BP-1 and PEG-BP-1 compounds on *in vitro* growth in this cell line.

Because the half-life of PEG-BP-1 is prolonged *in vivo*, we expected to see similar growth inhibitory effects in the animal models. Surprisingly, the results we obtained *in vivo* conflicted with the *in vitro* results. For example, MCF-7 cells were mostly inhibited by PEG-BP-1 *in vitro*, but their growth was not affected in the *in vivo* model of tumorigenesis. One interpretation of these results is that PEG-BP-1 did not display antitumour effects because of relatively poor tumour distribution. Our data support this hypothesis, as we found that PEG conjugation reduced measurable tissue levels by more than 10-fold compared to unconjugated WT-BP-1. An alternative hypothesis is that increased host production of IGF-I or IGF-II in response to prolonged PEG-BP-1 offsets the potential growth inhibitory effect. This seems less likely, as we did observe a trend towards inhibition of uterine growth after prolonged PEG-BP-1 administration. Since IGF-1 has been suggested to be the mediator of oestradiol-induced proliferation [27–29], inhibition of IGF in uterine tissue by PEG-BP-1 would explain these results. Finally, our results with MCF-7 must be interpreted with caution. We had only limited amounts of PEG-BP-1, and during the course of treatment, only limited tumour growth was measured. Prolonged treatment with PEG-BP-1 will be needed to analyse fully the growth inhibitory effects of this strategy on MCF-7 cells. However, it is clear that PEG-BP-1 did not inhibit MCF-7 xenograft establishment.

Another paradoxical result was the inhibition of MDA-MB-231 tumour growth *in vivo* but not *in vitro* by PEG-BP-1. Since MDA-MB-231 cells do not seem to be responsive to IGF-I, a possible explanation for these results is the inhibition of tumour host responses required for tumorigenesis. In support of this concept is the observation that IGF-I is a stimulator of endothelial cell growth and angiogenesis [3, 4]. Since it appears that the antitumour effect of PEG-BP-1 was observed only during the first few days of tumorigenesis, this seems a plausible explanation. Since one animal did not form a tumour at all, perhaps this anti-IGF effect on the host could be further exploited to inhibit the initial steps of tumour information. Of note is the failure of WT-BP-1 to inhibit MDA-MB-231 tumour growth. Since the serum half-life of WT-BP-1 is short [20], it is clear that twice a day injection does not provide sustained BP-1 levels. In contrast, high levels of PEG-BP-1 was easily measurable at the end of the dosing interval, suggesting that prolongation of the half-life of BP-1 results in improved therapeutic effects.

The MBA-MB-435A *in vitro* results were similar to the *in vivo* results. PEG-BP-1 inhibitory activity was demonstrated against the MDA-MB-435A cell line in the mouse ascites model. Intraperitoneal injection of PEG-BP-1 directly into the compartment of tumour growth greatly favoured effective inhibitory concentrations of PEG-BP-1. However, inhibition of ascites formation, as measured weight gain, is only an indirect measurement of tumour growth. Actual inhibition of tumorigenesis is better measured directly in the flank model. In addition, the aetiology of jaundice and dehydration in the treated mice is unclear. Only animals receiving both tumour and PEG-BP-1 developed these side-effects. Thus, further investigation into the aetiology of the observed toxicity will be required.

Our data suggest that the current BP-1 conjugate escapes the intravascular compartment poorly and may not be able to inhibit IGF action directly on the tumour cell. Nonetheless, inhibition was seen in both flank and intraperitoneal models of tumour growth, suggesting that anti-IGF effects on the host may be important in reducing tumour growth. This result is in agreement with Arteaga and associates [30] who demonstrated that blockade of the type I IGF receptor could inhibit MDA-MB-231 tumour growth *in vivo*. Moreover, this antibody was not effective at reducing the tumour growth of MCF-7 cells. Reduction of the size of the conjugated BP-1 PEG molecule may improve its tumour distribution while providing a therapeutically acceptable serum half-life. We plan to characterise smaller BP-1 PEG molecules in a similar manner as described for PEG-BP-1 and to study BP-1 activity on both antitumour and host-mediated effects using additional strategies to investigate the importance of the IGF system in breast cancer.

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