An Osteoprotegerin-like Peptidomimetic Inhibits Osteoclastic Bone Resorption and Osteolytic Bone Disease in Myeloma

Deborah J. Heath,1 Karin Vanderkerken,2 Xin Cheng,3 Orla Gallagher,1 Matthew Prideaux,1 Ramachandran Murali,1 and Peter I. Croucher1

1Academic Unit of Bone Biology, Division of Clinical Sciences (South), University of Sheffield Medical School, Sheffield, Yorkshire, United Kingdom; 2Department of Haematology and Immunology, Free University Brussels (VUB), Brussels, Belgium; and 3Department of Pathology and Laboratory Medicine, Abramson Family Cancer Research Institute for Cancer Research, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania

Abstract

Multiple myeloma is a B-cell malignancy characterized by the uncontrolled growth of plasma cells in the bone marrow and the development of osteolytic bone disease. Myeloma cells express the receptor activator of nuclear factor κB ligand (RANKL), induce RANKL expression in the bone marrow, and down-regulate expression of the decoy receptor osteoprotegerin, thereby promoting bone resorption. Targeting this system in myeloma has clear therapeutic potential. However, osteoprotegerin also binds tumor necrosis factor–related apoptosis inducing ligand (TRAIL) and prevents TRAIL-induced apoptosis of myeloma cells. Whether or not osteoprotegerin can bind TRAIL and prevent apoptosis in vivo and the relative importance of osteoprotegerin binding to TRAIL and RANKL are unclear. In the present study, we have investigated the ability of an osteoprotegerin-like peptidomimetic (OP3-4), designed to block the RANKL/RANK interaction, to inhibit osteoclastic bone resorption and TRAIL-induced apoptosis in vitro and myeloma bone disease in vivo. OP3-4 inhibited osteoclast formation (P < 0.01) and bone resorption (P < 0.01) in vitro. However, OP3-4 had no effect on TRAIL-induced apoptosis of RPMI 8226 myeloma cells. Treatment of 5T2MM myeloma–bearing mice with OP3-4 decreased osteoclast number and the proportion of bone surface covered by osteoclasts (P < 0.05). Treatment also prevented the tumor-induced decrease in cancellous bone area and the development of osteolytic lesions (P < 0.05). OP3-4 also reduced tumor burden when compared with the control (P < 0.05). These data suggest that OP3-4 and the selective inhibition of RANKL, but not TRAIL activity, are effective in preventing myeloma bone disease and offer a novel therapeutic approach to treating this aspect of myeloma. [Cancer Res 2007;67(1):202–8]

Introduction

Multiple myeloma is a B-cell malignancy characterized by the infiltration and growth of plasma cells in the bone marrow. Patients with myeloma develop osteolytic bone disease predominantly in the skull, ribs, vertebrae, pelvis, and proximal long bones. This is characterized by bone pain, pathologic fractures, and hypercalcaemia, making this a major cause of morbidity. Histomorphometric studies have shown that myeloma-induced bone loss is mediated by an increase in osteoclastic bone resorption (1–3).

One molecule reported to play a key role in osteoclastogenesis in myeloma is the receptor activator of nuclear factor κB ligand (RANKL; ref. 4). RANKL, a tumor necrosis factor (TNF) family member, binds to its receptor RANK on osteoclast precursors to stimulate osteoclastogenesis and bone resorption (5, 6). RANKL also binds a secreted decoy receptor, osteoprotegerin, which prevents RANKL binding RANK and thus inhibits osteoclast formation and bone resorption (7–9). Myeloma cells hijack the RANK/osteoprotegerin system, which is required for normal bone remodeling (10–13). Myeloma cells express RANKL and can promote osteoclastogenesis and bone resorption in a stromal cell–independent manner in vitro (14–16). The myeloma cells can also up-regulate the expression of RANKL in cells found in the bone marrow, including osteoblasts, fibroblasts, stromal cells, and endothelial cells (10, 17), and down-regulate the production of the decoy receptor osteoprotegerin in osteoblasts and endothelial cells (11, 18). These activities allow bone resorption to go unchecked.

Targeting this system offers the possibility of developing novel therapeutic approaches to treating myeloma bone disease (19–21). Fc-osteoprotegerin has been shown to decrease osteoclast number and prevent the development of lytic bone disease in the 5T2MM murine model of myeloma (22). Fc-osteoprotegerin also decreases tumor burden and increases survival in the 5T33MM murine model for myeloma (23). Furthermore, administration of a recombinant osteoprotegerin construct to multiple myeloma patients resulted in the reduction of biochemical markers of bone resorption (24). However, osteoprotegerin has been shown to bind another TNF family member, the TNF-related apoptosis inducing ligand (TRAIL; ref. 25), and can inhibit TRAIL–induced apoptosis of human myeloma cells (26). Osteoprotegerin produced by osteoblasts has been shown to prevent the actions of TRAIL (26, 27). This raises the possibility that osteoprotegerin not only inhibits osteoclastogenesis but may also play a role in promoting tumor cell survival (27–29). Whether or not osteoprotegerin can bind TRAIL and prevent apoptosis in vivo and the relative importance of osteoprotegerin binding to TRAIL and RANKL are unclear. It is likely that these two different activities may play key roles at different stages in disease development. During tumor initiation and the initial arrival and colonization, osteoprotegerin may function to prevent TRAIL from targeting tumor cells for removal. Whereas, once the tumor is established and is environment independent, down-regulation of osteoprotegerin may allow RANKL-driven osteoclast formation to go unchecked. Developing tools to selectively regulate these two activities is critical.
One mechanism for selectively targeting the RANKL system is through the use of osteoprotegerin-like peptidomimetics. We have designed an osteoprotegerin-like peptidomimetic (OP3-4), modeled on the TNF-α-TNF receptor 1 complex, TRAIL-death receptor 5 complex, and Fas-Fas ligand complex, and showed that it functions by inhibiting the RANKL/RANK system. However, it is unclear whether it will inhibit bone resorption and block the actions of TRAIL. Therefore, in the present study, we have determined the effect of OP3-4 both on bone resorption and TRAIL-induced apoptosis in vitro and on osteoclast formation and myeloma bone disease in vivo.

Materials and Methods

Peptide synthesis. OP3-4 peptide (YCEIEFCYLIR) was designed based on the three-dimensional structure of modeled osteoprotegerin and RANKL receptor complex (Fig. 1; ref. 30). The cyclic OP3-4 peptide was synthesized by the American Peptide Company (Sunnyvale, CA) at a purity of 95% to 98%. A control peptide (FCYISEVEDQCY), which is similar in size and net hydrophobicity to OP3-4, was purchased from the Protein Chemistry Laboratory, University of Pennsylvania (Philadelphia, PA). The purity and identity of the peptides were confirmed by reverse-phase high-performance liquid chromatography and mass spectrometry. Both of the peptides were reconstituted in phosphate buffered saline (PBS; pH 7.5).

Effect of OP3-4 on murine osteoclastogenesis and bone resorption in vitro. Mononuclear cells were isolated from murine whole blood using Histopaque-1077 (Sigma, Poole, United Kingdom) and seeded onto glass coverslips in a 96-well plate at a density of 10^5 cells per well in α-MEM (Life Technologies, Paisley, United Kingdom) containing 10% FCS (Sigma). After 2 h, the coverslips were washed in medium and then transferred to a 24-well plate (three coverslips per well) with 1 mL of medium containing dexamethasone (0.1 nmol/L; Sigma), macrophage colony-stimulating factor (MCSF; 25 ng/mL; R&D Systems, Abingdon, United Kingdom), and recombinant human RANKL (30 ng/mL; Peprotech, London, United Kingdom) in the presence or absence of either PBS, recombinant mouse osteoprotegerin (0.1 μg/mL; R&D Systems), control peptide (5–50 μmol/L), or OP3-4 (5–50 μmol/L). The cells were incubated for 6 days at 37°C. Osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) activity using an acid phosphatase kit (Sigma). The number of TRAP-positive multinucleated osteoclasts was counted manually using light microscopy (Leica Microsystems, Milton Keynes, United Kingdom).

Figure 1. Three-dimensional model of the osteoprotegerin/RANKL complex. The model was built based on the three-dimensional structures of the TNF-α-TNF receptor 1 complex (37), TRAIL-death receptor 5 complex (38), and Fas-Fas ligand complex. The receptor complex is shown as a spherical model. Osteoprotegerin (OPG) is colored according to atom (green, C; blue, N; red, O; orange, S) and, for clarity, the ligand is shown in orange. The critical loop OP3-4 is shown in cyan from which the mimetic used in this study was designed.

Figure 2. OP3-4 inhibits mouse osteoclastogenesis and bone resorption. A, murine peripheral blood mononuclear cells (PBMC) cultured in the presence of RANKL and MCSF formed large multinucleated cells that stained positively for tartrate-resistant acid phosphatase. Osteoprotegerin and OP3-4 (right) completely prevented the formation of TRAP-positive multinucleated cells, which could be seen clearly in the vehicle and control peptide containing cultures (objective magnification, ×10). B, assessment of the number of osteoclasts showed that osteoprotegerin (1.4 nmol/L) and OP3-4 inhibited osteoclast formation. C, osteoclasts generated from murine PBMCs formed resorption pits on dentine slices, which were decreased by osteoprotegerin and OP3-4, whereas the control peptide had little or no effect (pits stained with toluidine blue and arrowed; objective magnification, ×4). D, assessment of the area of dentine slice covered by resorption pits showed that osteoprotegerin (1.4 nmol/L) and OP3-4 (30 μmol/L) completely inhibited osteoclastic bone resorption. **, P < 0.05, compared with the no peptide control (ANOVA-Dunnett’s post test).
In separate experiments, mononuclear cells were cultured on elephant dentine slices (a gift from HM Customs and Excise, High Wycombe, United Kingdom) for 10 days. The cells were removed by incubation with trypsin (Life Technologies) for 10 min, 1 mol/L ammonium hydroxide for 4 h, and then sonication in water for 30 s. The resorption pits were stained with 0.05% toluidine blue solution for 5 min. The proportion of dentine slice covered by resorption pits was determined using light microscopy (Leica Microsystems) and the Leica Qwin software program.

**Effect of OP3-4 on TRAIL-induced apoptosis of human myeloma cells in vitro.** RPMI 8226 human myeloma cells, obtained from the European Collection of Animal Cell Culture (Salisbury, United Kingdom), were cultured in RPMI 1640 (Life Technologies) containing 10% FCS (Sigma). RPMI 8226 cells were seeded into six-well plates at a density of 5 \times 10^3/mL in the presence or absence of TRAIL (20 ng/mL; R&D Systems). To each well, either PBS, human recombinant osteoprotegerin (0.05 μg/mL; R&D Systems), control peptide (5–50 μmol/L), or OP3-4 (5–50 μmol/L) was added. Cells were incubated for 24 h and the proportion of viable cells was determined following trypan blue exclusion. Cells were then fixed in 4% paraformaldehyde, cytospun onto glass slides, stained with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/mL), and visualized under UV light. Apoptotic cells were defined as those with condensed chromatin and the presence of apoptotic bodies. The proportion of apoptotic cells was determined in five fields of view. Apoptosis was also assessed using a fluorescence in situ nick translation assay as previously described (31). The proportion of cells undergoing apoptosis was measured by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, United Kingdom).

**Treatment of 5T2MM myeloma-bearing mice with OP3-4.** The 5T2MM murine model of multiple myeloma originated spontaneously in elderly C57BL/KalwRij mice (32, 33). Thirty-six female C57BL/KalwRij mice (Harlan) were divided into three groups: group 1 (n = 12) were left uninjected (naive group) and groups 2 and 3 (n = 12 each) were injected via the tail vein with 5T2MM cells that were isolated and purified from the bone marrow of disease-bearing mice as previously described (34). Progression of the disease was assessed by measuring the percentage area occupied by tumor cells (10 fields, each field of view was 0.0156 mm^2, starting 0.25 mm from the growth plate). Cancellous bone area was measured in an area 0.5625 mm^2, 0.25 mm from the growth plate on three different tissue levels per bone using the osteomasure analysis software (Osteometrics, Decatur, GA). The number of osteoclasts per millimeter and the percentage surface covered by osteoclasts were measured on 6.5 mm of the corticoendosteal surfaces, starting 0.25 mm from the growth plate. Tumor burden was analyzed by measuring the percentage area occupied by tumor cells (25 fields, each field of view was 0.0625 mm^2, starting 0.25 mm from the growth plate). Apoptotic myeloma cells were analyzed by measuring the proportion of apoptotic tumor cells (10 fields, each field of view was 0.0156 mm^2, starting 0.25 mm from the growth plate). Apoptotic cells were defined by characteristic changes in nuclear morphology, including chromatin condensation and the presence of two or more distinct apoptotic bodies (36).

**Statistical analysis.** All experiments were done on a minimum of three separate occasions and analyzed using ANOVA-Dunnett’s post test. The histologic and radiographic data from the 5T2MM in vivo experiments were analyzed using the Mann Whitney U test. All results are expressed as the mean ± SD.

**Results**

**OP3-4 inhibits osteoclastogenesis and bone resorption in vitro.** Murine peripheral blood mononuclear cells maintained...
in the presence of soluble RANKL and MCSF formed multinucleated TRAP-positive osteoclasts (Fig. 2A). When cultured on dentine slices, these cells formed resorption pits (Fig. 2C). Assessment of the number of TRAP-positive multinucleated osteoclasts and resorption pits showed that recombinant osteoprotegerin completely inhibited osteoclast formation and bone resorption (Fig. 2B and D, respectively). Addition of OP3-4 also inhibited the formation of TRAP-positive osteoclasts in a dose-dependent manner, with 30 μmol/L OP3-4 being adequate to completely inhibit osteoclast formation (0.0 ± 0.11% versus 67.7 ± 17.0%; P < 0.01). This is consistent with our previous data showing that OP3-4 inhibits the formation of osteoclasts generated from murine bone marrow osteoclast precursors (30). Similarly, 30 μmol/L OP3-4 significantly inhibited bone resorption (3.1 ± 5.6% versus 90.0 ± 36.9%; P < 0.01; Fig. 2D). Control peptide had no significant effect on osteoclast formation or bone resorption. These data show that OP3-4 is able to prevent RANKL-induced osteoclast formation and bone resorption in a similar manner to osteoprotegerin.

OP3-4 does not promote myeloma cell survival in vitro. Osteoprotegerin has been shown to block TRAIL-induced apoptosis; however, it is unclear whether OP3-4 has a similar effect. TRAIL (20 ng/mL) caused an increase in apoptosis of RPMI 8226 human myeloma cells (Fig. 3A). Recombinant osteoprotegerin significantly inhibited TRAIL-induced myeloma cell apoptosis (P < 0.01). In contrast, OP3-4 and the control peptide did not inhibit TRAIL-induced cell apoptosis (Fig. 3B). Assessment of the number of nonviable cells using trypan blue (Fig. 3C) also showed that recombinant osteoprotegerin inhibited TRAIL-induced myeloma cell apoptosis whereas OP3-4 had no effect. These results were also confirmed using the nick translation method (OP3-4 versus vehicle control, 34.0 ± 4.4% versus 47.6 ± 7.5%). These data confirm that OP3-4 at the concentrations examined had no effect on TRAIL-induced apoptosis of myeloma cells.

OP3-4 inhibits osteoclast formation in the 5T2MM model. Injection of 5T2MM cells into C57BL/KaLwRijHsd mice resulted in the development of a serum paraprotein in all animals. Histologic assessment of the tibia showed the presence of TRAP-positive osteoclasts on the corticoendosteal surface of naive mice (Fig. 4A). The presence of 5T2MM cells was associated with a significant increase in the number of osteoclasts per millimeter (Fig. 4B) and the percentage of bone surface covered by osteoclasts (Fig. 4C) as compared with naive mice (9.0 ± 3.1/mm versus 2.2 ± 2.1/mm and 24.9 ± 8.7% versus 4.1 ± 2.7%; P < 0.001). Treatment of the 5T2MM-bearing mice with OP3-4 reduced the number of osteoclasts and the surface covered by osteoclasts as compared with mice treated with vehicle (6.3 ± 3.1/mm versus 9.0 ± 3.1/mm and 17.0 ± 8.4% versus 24.9 ± 8.7%; P < 0.05). These data are consistent with OP3-4 inhibiting 5T2MM-induced osteoclast formation in vivo.

OP3-4 inhibits the development of osteolytic bone disease in the 5T2MM model. X-ray analysis of the long bones of 5T2MM-bearing mice showed the presence of osteolytic lesions (Fig. 5A). Lesions were present in 5T2MM-bearing mice but not in naive mice (7.5 ± 4.1 versus 0.4 ± 0.5; Fig. 5B). Treatment of 5T2MM-bearing mice with OP3-4 resulted in a significant reduction in osteolytic lesions when compared with mice treated with vehicle (3.7 ± 2.4 versus 7.5 ± 4.1; P < 0.05). Histologic analysis of the tibia showed the presence of osteolytic lesions in the cortex of 5T2MM-bearing mice but not in naive mice (1.9 ± 1.9 versus 0.0 ± 0.0; P < 0.01; Fig. 5C and D). OP3-4 significantly reduced histologically detectable osteolytic lesions in 5T2MM-bearing mice as compared with mice treated with vehicle (0.4 ± 0.7 versus 1.9 ± 1.9; P < 0.05). Histomorphometric analysis of cancellous bone area showed the presence of cancellous bone in naive mice (Fig. 6A). Cancellous bone was significantly reduced in the 5T2MM-bearing mice as compared with naive mice (0.5 ± 0.6% versus 2.5 ± 0.9%; P < 0.001; Fig. 6B). Treatment of 5T2MM tumor-bearing mice with OP3-4 partially prevented 5T2MM-induced cancellous bone loss (1.4 ± 1.9% versus 0.5 ± 0.6%);
Discussion

The bone destruction associated with multiple myeloma is a major cause of morbidity. Myeloma cells seem to hijack the RANKL/osteoprotegerin system in favor of RANKL, promoting increased osteoclastic bone resorption. Inhibiting RANKL represents a novel strategy for targeting this aspect of the disease. In support of this, we have shown that recombinant osteoprotegerin prevents myeloma bone disease and decreases tumor burden in murine models of myeloma (22, 23). Furthermore, osteoprotegerin has been shown to reduce biochemical markers of bone resorption in patients with myeloma (24). However, osteoprotegerin can bind another TNF family member, TRAIL, to prevent TRAIL-induced apoptosis, which may confound the effect of osteoprotegerin.

The present study showed that OP3-4 prevents osteoclast formation and bone resorption in a functional osteoclast assay. These data are consistent with the observation that osteoprotegerin also prevents osteoclast formation and bone resorption in the same system. In contrast, OP3-4, at the same concentration, had no effect on TRAIL-induced apoptosis of myeloma cells, whereas osteoprotegerin was effective at preventing TRAIL-induced apoptosis. These data suggest that the structural determinants of osteoprotegerin binding to RANKL and osteoprotegerin binding to TRAIL may differ. A detailed understanding of the three-dimensional structure of these two receptor complexes may allow the rational design of inhibitors that can selectively block the activity of these two different interactions.

Because OP3-4 was able to inhibit osteoclastic bone resorption, but had little effect on TRAIL in vitro, studies were undertaken to determine its effects on osteoclast bone resorption in vivo. 5T2MM murine myeloma cells were injected into C57BL/KaLwRijHsd mice. The tumor cells promoted a significant increase in osteoclastic bone resorption. Treatment with OP3-4 significantly decreased the surface covered by osteoclasts. This is consistent with a significant inhibitory effect on osteoclast formation. The inhibitory effect of OP3-4 on osteoclast formation partially prevented tumor-induced cancellous bone loss. The partial effect may reflect the fact that the treatment started when serum paraprotein was detected and significant bone loss may have already occurred. However, treatment was able to significantly decrease the presence of lytic bone lesions. These data are consistent with the demonstration that OP3-4 prevents bone loss in ovariectomized mice (30). However, OP3-4 was not as effective at decreasing osteoclast formation and the surface covered by osteoclasts as we observed with a recombinant Fc-osteoprotegerin fusion protein (22). In this previous study, Fc-osteoprotegerin completely inhibited osteoclast formation and the surface covered by osteoclasts as we observed with a recombinant Fc-osteoprotegerin fusion protein (22). In this previous study, Fc-osteoprotegerin completely inhibited osteoclast formation, which contrasts with the partial inhibition seen in the present study. The reason for this is unclear. The concentration of OP3-4 required to inhibit osteoclast formation in vitro was at least 1,000-fold greater than osteoprotegerin. This suggests that the...
relative affinity of OP3-4 for RANKL is likely to be much lower than osteoprotegerin for RANKL. This may reflect a requirement for the full-length molecule to bind and position the OP3-4 sequence for optimal interaction with RANKL. Alternatively, the design of the OP3-4 peptide may not be the most favorable. Indeed, the three-dimensional structure of osteoprotegerin is not known, and the OP3-4 peptide was designed by modeling the osteoprotegerin/RANKL interaction based on the three-dimensional structures of the TNF-β-TNF receptor 1 complex (37), TRAIL-death receptor 5 complex (38), and Fas-Fas ligand complex. Peptidomimetics designed on the basis of the structure of the osteoprotegerin/RANKL complex when it is solved may allow improved peptide design. In addition, OP3-4 may have been less effective than osteoprotegerin simply due to its small size/surface area and, therefore, OP3-4 was only able to modify the RANKL/RANK interaction, rather than completely blocking activity. However, these data are consistent with the demonstration that Fc-osteoprotegerin can reduce osteolytic bone disease in the same model (22) and that soluble RANK-Fc constructs also block bone disease in vivo (10, 39).

OP3-4 also significantly reduced tumor burden in the 5T2MM model. Further analysis showed that OP3-4 treatment did not affect myeloma cell apoptosis in vivo. This suggests that changes in rates of apoptosis do not contribute to the reduction in tumor burden. The mechanism responsible for the reduction is unclear; however, a similar reduction was seen in separate studies when bone resorption was inhibited with either Fc-osteoprotegerin or a bisphosphonate (23, 40). In this previous study, Fc-osteoprotegerin was shown not to have a direct antmyeloma effect. These data are consistent with the hypothesis that the reduction in tumor burden is likely to reflect an indirect effect on tumor growth via changes in the bone microenvironment caused by the inhibition of bone resorption. The use of OP3-4 in established disease (i.e., once a serum paraprotein had been detected) allowed study of the effect of the peptide on osteoclast biology and the development of bone disease, but not on initiation and colonization in bone.

We hypothesize that osteoprotegerin plays a different role in different stages of disease development. In the early stage of tumor development (initial arrival/colonization), osteoprotegerin may function to prevent TRAIL from targeting tumor cells for removal. However, once the tumor is established and becomes microenvironment independent, osteoprotegerin is down-regulated and plays a role in the development of myeloma bone disease. The question of whether osteoprotegerin binding to TRAIL in vivo is of biological significance is key to this hypothesis and is being addressed in separate studies. However, we believe that the current study provides data to show not only that small-molecule mimetics can prevent myeloma bone disease but also that this approach may be of value in distinguishing these two activities.

Taken together, these data suggest that OP3-4 and the selective inhibition of RANKL, but not TRAIL, activity are effective in preventing myeloma bone disease. Refining the design of such peptides may improve efficacy and allow their use in both dissecting the relative importance of osteoprotegerin/RANKL interactions versus osteoprotegerin/TRAIL interactions. This may lead to improved therapeutic approaches for treating myeloma and the associated bone disease.

Acknowledgments

Received 4/13/2006; revised 9/14/2006; accepted 10/19/2006.

Grant support: Leukaemia Research Fund and the Multiple Myeloma Research Foundation (D.J. Heath, O. Gallagher, M. Prideaux, and P.I. Crouch), Onderzoeksraad Vrije Universiteit Brussel and Multiple Myeloma Research Foundation (K. Vanderkerken), and Abramson Family Institute for Cancer Research Foundation, and National Cancer Institute (X. Cheng and R. Muraili).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Angelo Willems for his expert technical assistance.


An Osteoprotegerin-like Peptidomimetic Inhibits Osteoclastic Bone Resorption and Osteolytic Bone Disease in Myeloma

Deborah J. Heath, Karin Vanderkerken, Xin Cheng, et al.


**Updated version**  Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/67/1/202](http://cancerres.aacrjournals.org/content/67/1/202)

**Cited articles**  This article cites 39 articles, 16 of which you can access for free at: [http://cancerres.aacrjournals.org/content/67/1/202.full.html#ref-list-1](http://cancerres.aacrjournals.org/content/67/1/202.full.html#ref-list-1)

**Citing articles**  This article has been cited by 7 HighWire-hosted articles. Access the articles at: [http://cancerres.aacrjournals.org/content/67/1/202.full.html#related-urls](http://cancerres.aacrjournals.org/content/67/1/202.full.html#related-urls)

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.