Concentrations of EpCAM ectodomain as found in sera of cancer patients do not significantly impact redirected lysis and T-cell activation by EpCAM/CD3-bispecific BiTE antibody MT110

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Key words: EpCAM, T cell, BiTE antibody, immunotherapy, adenocarcinoma, tumor antigen shedding

Ectodomains of target antigens for antibody-based therapies can be shed from the target cell surface and found in sera of patients. Shed ectodomains of therapeutic targets not only pose the risk of sequestering therapeutic antibodies but, in a multimeric form, of triggering T cell activation by bispecific antibodies binding to CD3 on T cells. Recently, epithelial cell adhesion molecule (EpCAM) has been shown to be activated by release of its ectodomain, called EpEX. Here, we show that only very low amounts of EpEX are detectable in sera of cancer patients. Among 100 cancer patient samples tested, only 17 (17%) showed serum levels of EpEX in excess of 0.05 ng/ml with highest EpEX concentrations of 5.29, 1.37 and 0.52 ng/ml. A recombinant form of human EpEX (recEpEX) was produced to assess its possible effect on redirected lysis and T cell activation by EpCAM/CD3-bispecific BiTE antibody MT110, currently being tested in patients with solid tumor malignancies. RecEpEX had a very minor effect on redirected lysis by MT110 with an approximate IC50 value of 3,000 ng/ml, which is a concentration close to three orders of magnitude higher than the highest EpEX concentration found in cancer patients. Concentrations of 30 ng/ml EpEX in combination with 250 ng/ml MT110 were minimally required to induce a detectable activation of CD4+ and CD8+ T cells. We conclude that soluble EpEX in sera of cancer patients is unlikely to pose an issue for the efficacy or safety of MT110, and perhaps other antibodies binding to N-terminal epitopes of EpCAM.

Introduction

Epithelial cell adhesion molecule (EpCAM) has a long history as a target antigen for antibody-based cancer therapies.1-3 Murine, chimeric, humanized and fully human anti-EpCAM monoclonal antibodies (mAbs) have been tested in various clinical settings with modest success. Only recently, catumaxomab (Removab®, Fresenius Biotech), a trifunctional EpCAM/CD3-specific antibody, gained market approval in Europe for the treatment of malignant ascites in patients with EpCAM-expressing cancers.4 Another EpCAM/CD3-bispecific antibody called MT110 is currently being tested in a Phase 1 dose-escalating study.5 Common to these T-cell-engaging antibodies is a remarkably high capacity for redirected lysis of EpCAM-expressing cancer cells and a potent activation of T cells. Because of their potency, EpCAM specificity, and requirement for clustering of T-cell receptors for activation, these T-cell-engaging antibodies are expected to be very sensitive to soluble antigen in serum.

EpCAM is very frequently expressed on diverse human carcinoma and respective cancer stem cells.6-8 In several cancers, its expression is negatively correlated with overall survival.9 Previous studies have shown involvement of EpCAM in calcium-independent, homophilic epithelial cell adhesion,10 antagonism to E-cadherin-mediated adhesion,11 as well as a role in induction of cell proliferation via transcriptional upregulation of c-myc.12 Only recently, EpCAM was shown to be a signaling molecule that undergoes regulated intra-membrane proteolysis.13,14 Controlled by homophilic association15 and other interacting proteins, EpCAM is cleaved by tumor necrosis factor alpha converting enzyme and a γ-secretase complex containing presenilin-2 to shed its extracellular portion called EpEX, and to release a short intracellular peptide, called EpICD, which can associate with adaptor proteins FHL-2 and β-catenin. As part of a large signaling complex, EpICD migrates to the cell nucleus to recruit transcription factor LEF/TCF and activate c-myc and other genes involved in cancer cell proliferation16 similar to the canonical wnt pathway. EpEX can serve as a soluble ligand for membrane-bound...
EpCAM,19 but its fate and possible further biological functions are poorly understood.

Soluble EpCAM, which likely is a product of intramembrane proteolysis, was first described in sera of cancer patients in 2002.17 This result was further supported by a study in 2007,18 showing that soluble EpCAM levels in patients with esophageal cancer were negatively correlated with survival rates (p = 0.0211) and were independently associated with prognosis (p = 0.0074; hazard ratio 7.40). In these studies, serum levels of shed EpCAM in patients were found to be in the low ng/ml range. Biochemical characterization of EpCAM has shown that its extracellular domain tends to aggregate and form multimers, most prominently tetromers.19

Soluble antigens sharing epitopes with membrane-associated antigens have the potential to bind and neutralize therapeutic antibodies before they can exert their biological activity on the surface of a target cell. In particular, the high potency of bispecific antibodies, which function by transiently connecting immune effector cells with cancer target cells, is expected to suffer from high levels of soluble antigen. Furthermore, T-cell-engaging antibodies activate polyclonal T cells by clustering their T-cell receptors via cross-linking of CD3 signaling subunits. While this clustering typically is mediated through antibody presented to T cells on the surface of target cells, multimeric forms of soluble antigen coated with several bispecific antibody molecules may likewise represent a stimulus for T-cell activation. Given the routine clinical use of the anti-EpCAM T-cell-engaging antibody catumaxomab, and in view of the ongoing clinical trials with MT110 and catumaxomab, we considered it important to explore the impact of soluble EpCAM (EpEX) on both the potency of redirected lysis and T-cell activation by an EpCAM/CD3-bispecific antibody.

For this study, we re-examined serum levels of EpEX in cancer patients and healthy donors using a mAb recognizing the same epitope as the bispecific antibody MT110. Serum concentrations of the EpCAM ectodomain (EpEX) were found in a similarly low-ng/ml range as previously reported.17,18 We then tested the impact of recombinant (rec)EpEX on the biological activities of MT110. RecEpEX had a very minor effect on redirected lysis by MT110 with an approximate IC_{50} value of 3,000 ng/ml, which is a concentration close to three orders of magnitude higher than the highest EpEX concentration found in a cancer patient. Concentrations of 30 ng/ml EpEX in combination with 250 ng/ml MT110 were minimally required to induce a detectable activation of CD4+ and CD8+ T cells. We conclude that soluble EpEX in sera of cancer patients is unlikely to pose an issue for the efficacy or safety of MT110, and perhaps other antibodies binding to N-terminal epitopes of EpCAM.

### Results

EpEX is present at very low levels in sera of cancer patients.

We have established a sensitive electrochemiluminescence-based sandwich ELISA assay for the detection of the shed extracellular domain of EpCAM (EpEX). For detection of serum EpEX captured on microtiter plates by polyclonal goat anti-human EpCAM antibodies, mAb 5–10, which recognizes an N-terminal epitope of EpCAM shared with EpEX, was used.20 The same antibody had been used for construction of BiTE antibody MT110.21 The ELISA gave highly reproducible results as shown by a standard calibration curve (Fig. 1): Serum samples of cancer patients were analyzed for levels of EpEX retaining the N-terminal epitope recognized by mAb 5–10 and the corresponding bispecific antibody MT110. Only 17 out of 100 patient samples gave detectable signals for EpEX (Table 1). The three highest EpEX concentrations determined were 5.29, 1.37 and 0.52 ng/ml. While breast and prostate cancer had a similar incidence of EpEX-positive sera (15 and 17%, respectively), colorectal cancer patients showed a trend for a higher incidence (36%). Out of 20 healthy individuals, seven (35%) showed detectable EpEX levels with a similarly high incidence as for colorectal cancer patients. Differences of EpEX levels between indications and normal donors did not reach statistical significance.

Naturally shed and recombinant EpEX are multimeric.

We next analyzed in cell culture experiments the potential effect of EpEX on activities relevant to the efficacy and safety of EpCAM/CD3-bispecific antibody MT110. For this purpose, a recombinant soluble form of EpEX (recEpEX) was produced in stably transfected CHO cells. Secreted recEpEX contained amino acid residues 1–265 of human EpCAM representing the complete ectodomain. When recEpEX was coated to chips for plasmon resonance spectroscopy, MT110 showed binding to immobilized recEpEX with an equilibrium dissociation constant in the range of K_{D} = 10^{-8} M.21

The mobility of recEpEX in SDS gels was compared with that of EpEX naturally shed from cultured carcinoma cell lines HCT-8 (colon), MCF-7 (breast) and FaDU (hypopharynx). Prior immunoprecipitation was required to enrich for shed EpEX.

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Figure 1. Characteristics of an EpEX-specific ELISA. An electrochemiluminescence-based ELISA was established. EpEX in serum samples is bound to a MULTI-ARRAY™ streptavidin-coated microtiter plate pre-coated with biotinylated polyclonal goat-anti-human EpCAM antibodies. Bound EpEX is detected by murine-anti-human EpCAM monoclonal antibody 5–10, which is specific for the N-terminus of EpEX. Bound mAb 5–10 is then detected by goat-anti-mouse IgG antibodies conjugated to SULFO-TAG™. A standard calibration curve is shown with respective assay specifications. CV, coefficient of variance; LLoD, lower limit of detection; LLoQ, lower limit of quantitation. (Fig. 1)
Recombinant human EpEX has a marginal inhibitory effect on redirected target cell lysis by MT110. MT110 can redirect T cells contained in human PBMC samples for highly efficient lysis of EpCAM-expressing target cells.5,21 Here, we investigated whether this activity is impacted by increasing concentrations of recEpEX. As shown in Figure 3A, the two donor PBMC tested led to half maximal lysis of human MDA-MB-453 breast cancer cells at MT110 concentrations of 0.496 and 0.588 ng/ml in the absence of recEpEX. Only at the two highest concentrations of 4 and 20 μg/ml recEpEX, an impact on redirected lysis by recEpEX became noticeable as a shift of the dose response curve to the right. EC50 values for half maximal lysis at 4 and 20 μg/ml recEpEX were 1,564 ng/ml and 4,799 ng/ml MT110 for donor 1, and 1,037 ng/ml and 3,339 ng/ml MT110 for donor 2, respectively. A reduction of redirected lysis with increasing concentrations of recEpEX was further analyzed by computation of the dose response curves. This revealed that half maximal inhibition of MT110 with the two donor PBMC was at recEpEX concentrations of 2.9 and 2.8 μg/ml, respectively (Fig. 3B).

Impact of recombinant human EpEX on T-cell activation by MT110 in the absence of target cells. Upon redirected lysis of target cells, MT110 can strongly induce activation of engaged T cells leading to upregulation of activation markers CD69 and CD25, and to T-cell proliferation.5,21 Here, we studied the expression of the immediate-early marker CD69 on both CD4+ and CD8+ T cells in response to an incubation with recEpEX. Concentrations of both MT110 and recEpEX up to 250 ng/ml were tested. The lower MT110 concentration of 5 ng/ml is likely to be reached in sera of patients, whereas the higher concentration of 250 ng/ml was selected to eventually force a positive effect in the assay. In the presence of 250 ng/ml MT110 alone, the background activation of T cells was slightly increased when compared to 0 or 5 ng/ml MT110 (Fig. 4 and first three bars in upper
leading up to 90% CD69-expressing, activated CD4+ and CD8+ T cells as tested with two PBMC donors. The extent of T-cell activation of 15–20% as seen in the presence of 250 ng/ml of both MT110 and recEpEX (Fig. 4A) was therefore rather modest compared to that seen in the presence of EpCAM+ target cells (Fig. 4B).

Discussion

The use of EpCAM as a tumor-associated target antigen for newly developed antibody-based therapies and the discovery of proteolytic activation of EpCAM in cancer cells13,14 prompted our interest in studying the potential impact of shed EpEX on one such therapy. This study confirms findings of two earlier studies17,18 showing that a shed version of the class I transmembrane protein EpCAM can be found in detectable levels in sera of a subgroup of cancer patients and normal donors. Abe et al.17 used two mAbs in their sandwich ELISA that could independently bind to recombinant EpEX as shown by BiaCore analysis. One of the antibodies recognized a 6 kDa N-terminal fragment of EpEX. Approximately 10% of patients with various cancers (n = 236) were found to have detectable EpEX serum levels ranging from 2–78 ng/ml. Kimura et al.18 used a commercial sandwich ELISA that was also based on two mAbs. Their publication described EpCAM levels as units/ml. According to the manufacturer of the ELISA, 1 U corresponded to 70–80 pg/ml EpCAM, which means that maximum serum levels of EpEX did not exceed 10 ng/ml in the 60 samples from esophageal cancer patients analyzed in that study. In healthy volunteers, maximum serum levels reached approximately 2 ng/ml. Both studies noted higher levels in cancer patients compared to those of healthy donors, reaching significance in the esophageal cancer patient set (p = 0.0221). Our own data, based on an electrochemiluminescence-based sandwich ELISA using a mAb binding very close to the N-terminus of EpCAM, are well aligned with those from the two earlier studies with regard to EpCAM serum levels. We could not, however, observe a difference in EpEX serum levels between our cancer patients and healthy donors. This may relate to a difference in the assay procedure, antibody reagents, or study sample collectives.

Nuclear translocation of EpICD as well as loss of EpEX at the plasma membrane were shown recently to represent prognostic markers that correlated with worse prognosis and an aggressive phenotype in thyroid cancers.23 The rather low levels of EpEX in serum reported in the studies by Abe et al.17 Kimura et al.18 and here confirmed in our own investigation are somewhat unexpected given that many normal tissues also express EpCAM and that many tumor tissues overexpress EpCAM and use its proteolytic cleavage for signaling.13,14 This may indicate that EpCAM cleavage and signaling are highly controlled processes that do not occur with high frequency in EpCAM-expressing normal and cancer tissues in vivo. Even in esophageal cancer patients, where EpCAM shedding has been shown to correlate with poor prognosis, mean EpEX levels are only slightly elevated for most of the patients and do not exceed 10 ng/ml. Another explanation may be that, due to its tendency to form multimers, the majority of EpEX remains associated on the cell surface bound to
marker in serum and a tumor cell-associated surface antigen targeted by therapeutic antibodies. While certain BiTE antibodies were sensitive to soluble CEA, other CEA-specific BiTE antibodies were insensitive to serum concentrations up to 1 μg/ml soluble CEA. It appears that MT110 is also a BiTE antibody that is insensitive to soluble antigen. This property may relate to the bispecific mode of BiTE action. When both arms of a BiTE molecule simultaneously bind their respective antigens in a forming synapse, the avidity gain allows for biological activity (i.e., redirected lysis and T-cell activation) at EC50 values orders of magnitude below the binding constants of the single arms. At such low but highly active BiTE concentrations, a soluble antigen cannot sequester much BiTE antibody unless it is present at very high concentrations. We nevertheless described BiTE antibodies that were remarkably sensitive to soluble CEA, suggesting that the binding constants and epitope specificity of the BiTE antibody rather than the nature of the soluble antigen determine IC50 values. Insensitivity towards soluble antigen will

Table 1. EpEX serum levels in 100 cancer patients and 20 normal human donors

| Disease            | Patient samples tested [N] | Sera positive for EpEX [N] (%) | EpEX serum concentration of positive samples [ng/ml] |
|--------------------|---------------------------|--------------------------------|--------------------------------------------------|
| Colorectal Cancer  | 14                        | 5 (35.7)                       | 0.41, 0.23, 0.23, 0.21, 0.17                      |
| Lung Cancer        | 3                         | 0 (0)                          | >0.05                                            |
| Gastric Cancer     | 3                         | 0 (0)                          | >0.05                                            |
| Prostate Cancer    | 28                        | 4 (14.3)                       | 0.52, 0.39, 0.16, 0.13                            |
| Breast Cancer      | 52                        | 8 (15.3)                       | 5.29, 1.37, 0.29, 0.17, 0.12, 0.1, 0.09, 0.07     |
| All                | 100                       | 17 (17)                        |                                                   |
| Healthy Individuals| 20                        | 7 (35)                         | 1.64, 0.7, 0.31, 0.15, 0.12, 0.09, 0.07           |

The assay described in Figure 1 was used for determination of EpEX serum levels.
hence be a further criterion to select new BiTE antibodies from larger panels of BiTE candidates that differ by binding kinetics and epitope specificity.

The impact of the shedding extracellular domain of human epidermal growth factor receptor 2 (HER-2) on treatment outcome with monoclonal antibody trastuzumab has been studied in great detail. As recently concluded, there is insufficient evidence from the published literature to support determination of serum levels of HER-2 as a predictor of response or a marker for early recurrence. The high serum levels typically reached and maintained by monoclonal antibody therapies will exceed levels of circulating antigen, which may explain why there is no clear impact on therapeutic outcome.

T-cell activation by BiTE antibodies MT110 and MT103 (blinatumomab) has been shown to be strictly dependent on the presence of target antigen-expressing cells. BiTE-decorated targets exposing multiple anti-CD3 binding arms are thought to serve as an activation matrix for attached T cells leading to clustering and activation of T-cell receptors, while BiTE antibodies monovalently binding to CD3 have no activating effect. A similar situation as with target cells could occur with aggregated soluble target antigen binding several BiTE antibodies at a time. This study provides evidence for such a mechanism in that 15–20% of CD8+ and CD4+ T-cells did upregulate CD69 expression in the presence of recEpEX, T-cell activation by recEpEX, however, required very high doses of MT110 and recEpEX. This may relate to the rather low affinity of MT110 for EpEX in the range of 10⁻⁸ M, which in turn requires high concentrations of the BiTE antibody to form EpEX/MT110 complexes that bind in a multivalent fashion to CD3 on T-cells. Because clinically applied MT110 doses are unlikely to exceed serum levels of 5 ng/mL, the observation of modest T-cell activation at a combination of 250 ng/mL MT110 with >30 ng/mL EpEX may have no bearing for the development of T-cell-mediated side effects as a result of soluble EpCAM in serum.

Materials and Methods

Human serum samples. All analyses described here were performed under a protocol that received approval of the ethics committee of the Medical Faculty of the Ludwig-Maximilians-University Munich, Germany. All patients who donated blood samples for analysis signed an informed consent form. All procedures were carried out in compliance with the 1964 Declaration of Helsinki.

Cell lines and culture. MDA-MB-453 human breast carcinoma cell line were obtained from the German Collection of Microorganism and Cell Cultures (DSMZ, ACC65) and cultured in L15 Leibovitz medium containing L-glutamine and L-amino acids (Invitrogen, 11410049) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ chamber. HCT-8 (colon), MCF-7 (breast), FaDu (hypopharynx) and HeLa (cervix) carcinoma cell lines were cultured in DMEM with 10% fetal calf serum in a 5% CO₂ chamber.

Quantification of EpCAM by an electrochemiluminescence-based ELISA. A biotinylated goat-anti-human EpCAM/Trop-1 antibody (R&D Systems, DAF960) was bound to a MULTI-ARRAY® streptavidin-coated microplate (MSD, L155A). After blocking with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA), serum samples, quality control samples and serially diluted recEpEX spiked in human serum for creating calibration standard curve, all diluted 1:5 in PBS, were added and incubated 1 h at room temperature. The monoclonal mouse anti-human EpCAM antibody 5–10 (Micromet AG, Munich, Germany) was then bound to the captured recEpEX and detected via a goat anti-mouse antibody conjugated to SulfoTag™ (MSD, R32AC-5). After addition of reading buffer (MSD, Gaithersburg, MD) to each well the plate was read with a Sector Imager 2400 instrument (MSD, R92TC-2). Soluble EpCAM concentration of unknowns was back-calculated from a standard curve by a point-to-point analysis integrated into GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Characterization of recombinant and naturally shed EpEX. For immunoblot analysis of EpEX, all cell lines were grown to confluency. Supernatants were collected and precleared (100,000g, 15 min). Samples were loaded on SDS-PAGE gels either directly (native), mixed with SDS-PAGE loading buffer (25 mM Tris-HCl pH 7, 5% glycerin, 1% SDS, 2% β-mercaptoethanol, bromophenol blue), or after immunoprecipitation with EpEX-specific antibody HO.3. After separation and transfer onto a PVDF membrane, soluble EpEX was detected using EpEX-specific antibody HO.3 in combination with horseradish peroxidase (HRP)-conjugated secondary antibody and the enhanced chemiluminescence (ECL) reagent (Pierce, 32106).

Immunoprecipitation and immunoblotting of recEpEX. For immunoblot analysis of EpEX, all cell lines were grown to confluency. Supernatants were collected and precleared (100,000g, 15 min). Samples were loaded on SDS-PAGE gels either directly (native), mixed with SDS-PAGE loading buffer (25 mM Tris-HCl pH 7, 5% glycerin, 1% SDS, 2% β-mercaptoethanol, bromophenol blue), or after immunoprecipitation with EpEX-specific antibody HO.3. After separation and transfer onto a PVDF membrane, soluble EpEX was detected using EpEX-specific antibody HO.3 in combination with horseradish peroxidase (HRP)-conjugated secondary antibody and the enhanced chemiluminescence (ECL) reagent (Pierce, 32106).

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Cytotoxicity assay. To investigate the extent to which the bioactivity of MT110 was affected by increasing concentrations of recEpEX, a MT110-specific cytototoxicity assay was performed using human peripheral blood mononuclear cells (PBMC) as effector and MDA-MB-453 human carcinoma cells as target cells. PBMC were isolated from healthy donors by Ficoll density gradient centrifugation using standard procedures. Cells were then resuspended in RPMI 1640 culture medium (Invitrogen, 52400-025) supplemented with 10% FBS, and cell numbers adjusted to 8 x 10⁶ cells/ml. If not indicated otherwise, target cell numbers were adjusted to 4 x 10⁵ cells/ml in RPMI 1640...
medium supplemented with 10% FBS. Equal volumes of target and effector cell suspension were then mixed and 50 μl transferred to each well of a 96-well round bottom plate. A serial dilution of MT110 ranging from 250 ng/ml to 4 pg/ml was added to appropriate wells. To analyze the influence of soluble recEpEX, cytotoxicity assays were performed in the presence of 20 μg/ml, 4 μg/ml, 0.8 μg/ml, 0.16 μg/ml and 0.032 μg/ml recEpEX, or citrate buffer alone. The cytotoxicity reaction was allowed for 16 h at 37°C in a 5% CO₂ humidified incubator.

Cytotoxic activity of MT110 was further monitored by determination of adenylate kinase released into the cell culture supernatant (ToxiLight kit, Lonza Rockland Inc., LT07-517, determination of adenylate kinase released into the cell culture medium supplemented with 10% FBS. Equal volumes of target cytotoxicity assays were performed in the presence of 20 μg/ml, 4 μg/ml, 0.8 μg/ml, 0.16 μg/ml and 0.032 μg/ml recEpEX, or citrate buffer alone. The cytotoxicity reaction was allowed for 16 h at 37°C in a 5% CO₂ humidified incubator.

T-cell activation assay. Expression of CD69 on CD4⁺ and CD8⁺ T cells was determined on a FACSCalibur™ instrument (Becton Dickinson, Heidelberg, Germany). After washing cell pellets once with PBS, 1% FCS, 0.05% NaN₃, cells were incubated in a suspension of a FITC-conjugated anti-human CD4 (clone RPA-T4, BD Biosciences, 555346), a PE-conjugated anti-human CD 8 (clone RPA-T8, BD Biosciences, 555368), and a PE-conjugated anti-human CD69 antibody (clone FN50, BD Biosciences, 555351) for 1 h at 4°C. As a negative control, irrelevant, isotype-matched FITC-, PECy5- and PE-conjugated antibodies were used. After incubation cells were washed twice with PBS, 1% FCS, 0.05% NaN₃, and resuspended in 200 μl PBS, 1% FCS, 0.05% NaN₃. Data interpretation was done using the CellQuestPro software (BD Biosciences, Heidelberg, Germany).

For T-cell activation assay, PBMC from human blood were prepared as described above and seeded in a 96-well round bottom plate at a concentration of 8 x 10⁶ cells/ml. RecEpEX at concentrations of 100, 10, 1 or 0 ng/ml and MT110 at concentrations of 250, 5 or 0 ng/ml was added, respectively. As a positive control, PBMC effector cells and MDA-MB-453 target cells were cultivated in the presence of 250, 5 and 0 ng/ml MT110. Plates were incubated for 18 h at 37°C in a 5% CO₂ humidified incubator. After incubation time CD69 expression was determined by fluorescence-activated cell sorting (FACS) analysis.

Acknowledgements

The authors would like to thank Maren Voges (Micromet AG) for editing the manuscript and for logistics of submission and Dr. Eva Vieser (Micromet AG) for discussions.

Conflict of Interest and Sponsorship Statement

S.P., D.R., S.L. and P.B. are all full-time employees of Micromet. O.G. is a consultant of Micromet. The study was sponsored in full by Micromet.