

# AGEM400(HES), a Novel Erythropoietin Mimetic Peptide Conjugated to Hydroxyethyl Starch with Excellent *In Vitro* Efficacy

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**Abstract:** We developed and tested a compound called AGEM400(HES) that consists of a novel erythropoietin mimetic peptide (EMP) which is produced as a continuous N- to C-linked dimer and is conjugated to biodegradable hydroxyethyl starch (HES). In various *in vitro* assays, AGEM400(HES) demonstrated excellent efficacy, better than the peptide alone, and comparable to the efficacy of erythropoietin (EPO) and Aranesp (Darbepoietin alpha). The assays included survival assays on EPO-responsive cell lines (EC50 below 1 ng/ml peptide) and clonogenic assays on human bone marrow cells (EC50 1 to 10 ng/ml). AGEM400(HES) caused phosphorylation of STAT5 and ERK signalling proteins in UT7/EPO cells in a similar fashion as EPO. AGEM400(HES) replaced EPO from its receptor and the *in vitro* activity of AGEM400 (HES) was inhibited by soluble EPO receptor. Antibodies generated in mice and rabbits against EPO did not recognize AGEM400(HES) peptide, and vice versa. A sensitive ELISA was able to detect AGEM400(HES) at low nanogram per ml concentrations which allows for bioanalytics of AGEM400(HES) serum levels in future *in vivo* studies. As a result, AGEM400(HES) is a promising drug candidate for anemias related to renal insufficiency and/or in oncological settings.

**Keywords:** Erythropoietin, erythropoietin mimetic peptide (EMP), erythropoietin receptor, hydroxyethyl starch (HES).

## INTRODUCTION

Erythropoietin (EPO) is a cytokine synthesized by the kidney that regulates the synthesis of erythrocytes. As a drug, it is used for anemias that result from chronic kidney failure, cancer and anti-cancer treatment. There are currently many different variants of EPO as well as other erythropoiesis stimulating agents (ESAs) on the market. These include variants of EPO with an improved biological half-life, such as Darbepoietin alpha (Aranesp) and continuous erythropoietin receptor activator (CERA) in addition to EPO biosimilars [1-4]. An erythropoietin mimetic peptide (EMP-1), without any sequence homology with EPO, was introduced in 1996 as a chemically synthesized agent with the same biological activity as EPO [5]. An optimized dimeric EPO mimetic peptide (EMP) that is conjugated to polyethylene glycol (PEG) and called Hematide has finished clinical phase II [1, 6]. A dimeric recombinant protein containing the EMP-1 sequence and the Fc portion of human IgG has also been tested in phase I studies under the name CNTO 528 [7].

The cellular mechanism of action of EPO mimetics appears to be identical to that of EPO in that they bind the EPO receptor and thereby cause phosphorylation of signalling proteins such as JAK2, STAT5 and others [8, 9]. Synthetic ESAs are, however, expected to be cheaper to produce than recombinant EPO or variants thereof, mainly due to the complex purification procedures and various controls associated with recombinant production.

Because the sequences of EMPs are different from the EPO protein sequence, antibodies that might develop against EMPs after long-term administration, are not likely to react with endogenous EPO. EPO-associated pure red cell aplasia (PRCA), which is the development of antibodies against recombinant EPO that neutralize the activity of endogenous EPO, is a rare condition in patients being treated with EPO [10, 11]. PRCA has not been observed and is not expected to occur after treatment with EMPs, meaning that in cases where PRCA already developed, EMPs may become the only effective treatment apart from blood transfusions [1].

The biological half-life of recombinant EPO was significantly increased in hyperglycosylated EPO variants such as Darbepoietin alpha although this benefit is partly offset by decreased receptor binding affinities and *in vitro* efficacies [12]. PEGylation has also been used as a means of extending the biological half-life of many drugs, including that of an EPO variant (CERA) and of an EMP (Hematide) [8, 13]. At the same time, PEGylation of recombinant cytokines has often led to decreased receptor binding affinities and decreased efficacies *in vitro*. This was not only the case in early attempts to increase the biological half-life of cytokines by randomly conjugating PEG to lysines [14, 15], but also in recent approaches with ESAs [8, 13].

Hematide, the EMP conjugated to PEG, was effective in healthy volunteers in increasing reticulocytes and hemoglobin, and these effects lasted for one month after a single application [6]. Even though PEGylated cytokines and other PEGylated drugs are used intensively in the clinic [16, 17], there is astonishingly little knowledge on the metabolism of larger PEG moieties, especially those with a molecular weight of 40kD or higher [18, 19]. Although there is no evidence of short or medium term toxicity of Hematide [20, 21],

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the combination of a relatively small peptide being conjugated to a relatively large PEG with chronic use in patients with renal failure (who thus have a compromised ability to eliminate larger PEG molecules) is an unprecedented combination of properties of a PEGylated drug. Hematide clearance in rats with chronic renal injury was much slower than in healthy rats [8]. As a result, we thought the use of a biodegradable macromolecule instead of PEG would be preferable because it would allow even patients with impaired kidney function to degrade and eliminate the macromolecular carrier.

Here, we present AGEM400, a novel dimeric EMP conjugated to hydroxyethyl starch (HES) in a way that up to 5 peptide molecules are coupled to one HES molecule. The conjugate was called AGEM400(HES), and it is the prototype of a new generation of clinically applicable ESAs. HES is already clinically used as an active pharmaceutical ingredient (API) as a plasma volume substitute and plasma expander in high doses (in excess of 10 g/patient). The major fraction of HES is biodegradable by amylase, and its breakdown products are renally excreted [22]. Conjugation of AGEM400 to HES, primarily intended to improve half-life *in vivo*, also led to increased *in vitro* efficacy of the peptide. AGEM400(HES) is shown here to have an *in vitro* efficacy that is superior to that reported for Hematide [8] and competitive to that reported for CNTO 530 [9]. The mechanism of action of AGEM400(HES) appears to be similar to that of EPO and other EPO mimetics, while at the same time AGEM400(HES) appears to be immunologically distinct from EPO.

## MATERIALS AND METHODOLOGY

### Sources of Erythropoietin

Epoetin alfa from Ortho Biotech/Janssen-Cilag (Neuss, Germany, brand name: Erypo, stock solutions 16.8 µg/ml (2000 IE/ml) or 33.6 µg/ml (4000 IE/ml)) was used as a source of EPO in all experiments, except in some cases when Aranesp (Amgen, Munich, Germany, stock solution of 500 µg/ml) was also used.

### Peptides

The sequence of our basic EMP, called BB68, differs at three positions from the published EMP-1 sequence [5, 23], in which both proline residues were replaced by lysines and a tryptophane was replaced by a 1-Naphthylalanine. A C-N linked dimer of BB68, with an addition of a C-terminal tBu-blocked cysteine residue, was synthesized continuously and called AGEM400. Internal disulfide bridges were closed between Cys(6)-Cys(15) and (in AGEM400) between Cys(26)-Cys(35), leading to the peptides depicted in Fig. (1). The structure of the AGEM400 dimer also differs from a published dimeric EMP [24] because those two monomeric units were linked between their N-termini by PEG, while here a continuous dimer was produced with C-N linked peptide bonds.

### Synthesis of Peptides

Peptides BB68 and AGEM400 were synthesized at a scale of 0.25 mmol by microwave assisted solid phase peptide synthesis in an automated Liberty (CEM) unit. The

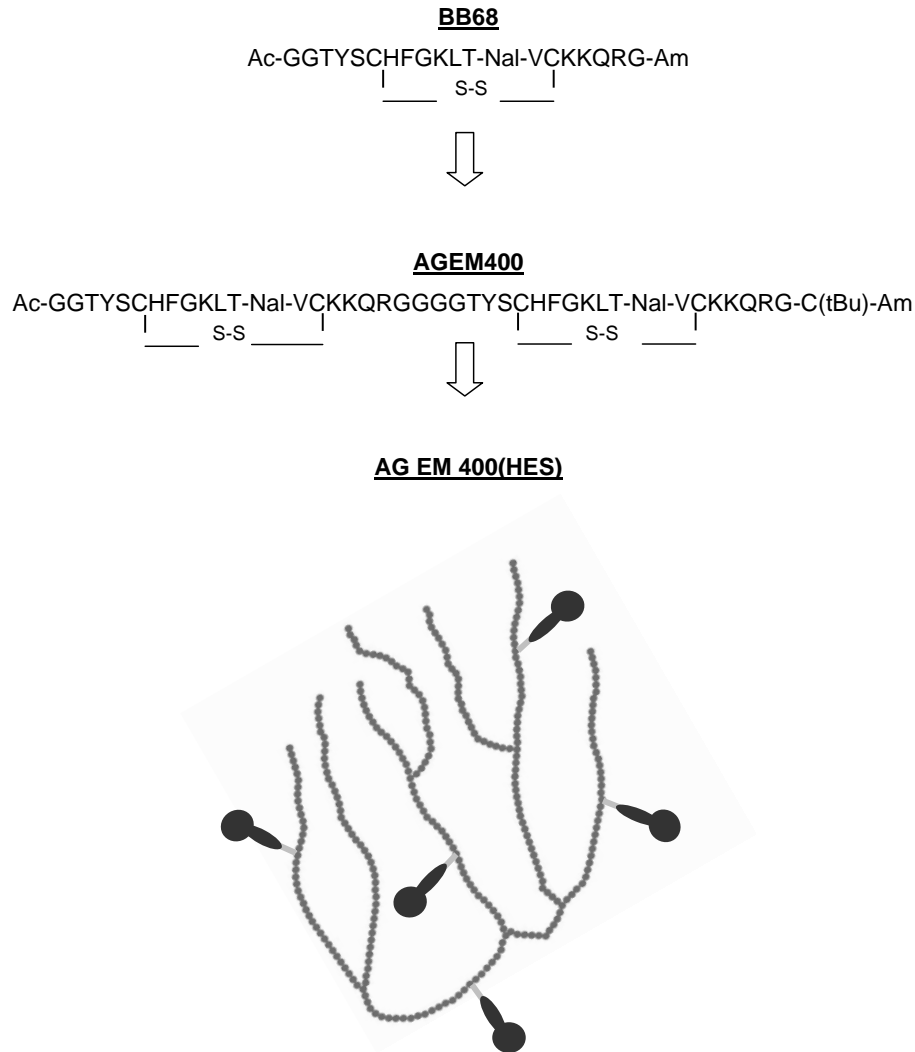
growing peptide chain was assembled on PAL ChemMatrix resin from Matrix Innovation. Deprotection was achieved by adding 10 ml Piperidine (25% in DMF) and irradiation with 65 W for 3 min. Coupling of the next amino acid was achieved using a five fold excess of reagents (amino acid, Pybop, DIEA) in 10 ml DMF and irradiation with 40 W for 5 min. All couplings were done by a double coupling procedure including capping with Z-2-Cl-OSu (40 equivalents) and irradiation with 40 W for 2 min. Acetylation of the peptide was achieved by introducing Ac-Gly-OH as last building block. After washing with DCM, the peptide was cleaved off by adding 40 ml cleavage cocktail (94% TFA, 1.0% TIS, 2.5% H<sub>2</sub>O, 2.5% DODT) and incubating at RT for 3 h. The peptide was then precipitated in cold ether, redissolved in acetonitrile/water (2/1), and directly purified by LCMS using a Nebula (Gilson) purification system. The disulfide bond in BB68, and the first disulfide bond in AGEM400, were created by the use of AplaGen's cyclization reagent AGOX15 [25] using a simple protocol. This reagent has advantages compared with standard oxidation methods (oxygen, iodine) as no workup is needed to remove the reagent prior to purification and as it leads to higher yields. In a typical experiment, 10-20 mg of the peptide was dissolved in 10ml AGOX15 (room atmosphere). After 18-24 h at RT this solution was directly purified by LCMS using a Nebula (Gilson) purification system. To create the second disulfide bond in AGEM400, the monocyclic peptide (20 mg) was dissolved in 40 ml 80% acetic acid. After addition of 0.512 ml 0.1M HCl and 3.424 ml 50 mM iodine solution in acetic acid, the solution was stirred for 2.5 h. Excess iodine was removed by adding ascorbic acid until the solution was colorless. The solution was then diluted with water to a total volume of 400 ml and added onto a C18-SPE-Column. The column was washed with 200 ml water, and the peptide was eluted with 50ml acetonitrile/water (95/5). The crude peptide was directly purified by LCMS using a Nebula (Gilson) purification system. Because the 41-mer was purified 3 times by HPLC, a high purity of the final product could be achieved (above 90%).

### Removal of the Remaining Cys(tBu)-Protection Before Conjugation to HES

Purified bicyclic AGEM400 peptide was transferred into a teflon vessel and anisole was added. The vessel was cooled down with dry ice/acetone and HF was condensed into the vessel (anisole:HF = 1:10). The deprotection was run at 0°C for 75 min. HF was removed *via* recondensation followed by a nitrogen gas stream. The oily residue was precipitated with TBME and removed by centrifugation. The precipitant was then washed 4 times with TBME, taken up with 0.1 % TFA in water and lyophilized over night. The crude peptide was purified by LCMS using a Nebula (Gilson) purification system.

### Conjugation to HES

A hydroxyethyl starch HES200/0.5 was fractionated in order to obtain a size fraction with an Mw = 130±20 kDa. A number of 30±3 µmol/g active groups were introduced in a three step modification process. For the conjugation of the deprotected AGEM400, 80 mg (M = 4657.5 g/mol, 1.2 eq, peptide/active thiol content = 70%) of the peptide were dis-



**Fig. (1).** Schematic of the EPO mimetic peptides used in this study: monomeric EMP BB68; dimeric AGEM400 with a C-terminal tBu-blocked cysteine; and AGEM400(HES), with on average 5 AGEM400 peptides per conjugate. Symbols: Ac: N-terminal acetylation; Nal: 1-naphthylalanine; Am: C-terminal amidation.

solved in 10 ml 50 mM phosphate buffer pH = 6.5. Then 300 mg of Supravalent-Maleimide-HES130/0.5 were dissolved in 20 ml 50 mM phosphate buffer pH 6.5, added to the peptide solution and vigorously stirred for 2h at 37°C. The peptide excess was removed by size exclusion chromatography on a Superdex 200 column (250 x 50mm, 440 ml bed volume) using a 50 mM phosphate buffer pH 6.5 with 500 mM NaCl as mobile phase. The macromolecular fractions were collected and desalted *via* a Sephadex G15 column (500 x 50mm, 880 ml bed volume) with desalted water as mobile phase. The collected and combined product fraction was lyophilized and 265 mg of a white solid product, AGEM400 (HES), were obtained.

#### Cell Lines

Cell lines TF-1 [26] and BA/F3 were obtained from DSMZ (Braunschweig, Germany), and cultured in RPMI medium with Glutamax (Gibco, Invitrogen, Karlsruhe, Germany) and supplemented with: antibiotics (penicillin/

streptomycin and amphotericin B, Invitrogen); fetal calf serum (FCS, Sigma Aldrich, Taufkirchen, Germany): 20% for TF-1, and 10% for BA/F3; and human IL-3 (Peprotech, Hamburg, Germany) or murine IL-3 (Peprotech), respectively. The UT7/EPO cell line, which is a strictly EPO-dependent subline of UT7 [27, 28], was a kind gift of Wolfgang Jelkmann (University of Lübeck, Germany). UT7/EPO cells were cultured in DMEM with Glutamax (Invitrogen) supplemented with antibiotics, 10% FCS, and 3.4 ng/ml EPO. Cells were passaged every two to three days, always keeping cell densities between 200,000 and 1,500,000 cells/ml. A large number of aliquots of the cell lines were frozen as soon as possible after receipt from the providers. Cells used for experiments were always between 2 and 4 weeks from the time that they were thawed. Before performing experiments with TF-1 or UT7/EPO cell lines, they were starved for 48h in culture medium without cytokine (and in case of TF-1 with a reduced FCS content: 5% instead of 20%). BA/F3 cells did not survive such starvation periods,

and therefore were cultured in medium with IL-3 until experiments were started.

### Cell Survival Assays (MTS Assays)

The dependence of cell lines on EPO or EPO mimetics for their survival (by stimulation of proliferation and/or inhibition of cell death) was tested using the CellTiter 96 AQ non-radioactive cell proliferation assay (Promega), as described for TF-1 cells elsewhere [29], and measuring the conversion of MTS in colored formazan by viable cells. Starved TF-1 or UT7/EPO cells, or BA/F3 cells taken directly from culture, were plated in multiwell plates in assay medium (the cell lines' culture medium without cytokine, and in case of TF-1, with only 5% FCS) with differing concentrations of EPO or peptide. UT7/EPO cells were plated at 10,000 cells per well (containing 100  $\mu$ l assay medium per well), TF-1 and BA/F3 cells were plated at 15,000 cells per well. Every substance concentration was tested in triplicate. Assays were incubated for 72h before addition of 20  $\mu$ l of MTS reagent per well, and cultured another 2 to 4 h to develop brown formazan color. Cells were lysed by the addition of 25  $\mu$ l of 10% SDS per well, and absorbance was measured at 492 nm.

### Methylcellulose Cultures

Development of erythroid colonies and/or erythroid cells from bone marrow cells was studied in methylcellulose media (from StemCell Technologies, Grenoble, France) containing a cocktail of human cytokines (Methocult H4535: with hSCF, hGM-CSF, hIL-3, hIL-6, and hG-CSF). EPO or peptide dilutions were prepared in 20 x stocks in IMDM with 2% FCS and supplemented with antibiotics, and added as 150  $\mu$ l volumes to Methocult aliquots. Cryopreserved unfractionated human bone marrow cells were obtained from StemCell Technologies. Cryopreserved cynomolgus bone marrow cells were obtained from Covance (Münster, Germany). After thawing and washing, cells were added to aliquots of Methocult in 1/20 volume of IMDM (Invitrogen) with 2% FCS. Human bone marrow cells were plated at 20,000 cells per well, primate bone marrow cells at 40,000 cells per well. In cases where colonies derived from human bone marrow cells were enumerated, each concentration of EPO or peptide was prepared in 3 ml aliquots, and plated in duplicate: two 1.1 ml volumes were plated in 6-well plates. After 12-14 days, the numbers of CFU-E colonies (less than 200 red cells per colony) or BFU-E colonies (more than 200 red cells per colony) were counted using a microscope. In experiments testing primate bone marrow cells, and in assays in which human progeny cells were also tested by flow cytometry, each concentration was plated only once: from each 1.4 ml aliquot, 1.1 ml was plated. For subsequent evaluation of erythroid cell formation by flow cytometry, excess liquid RPMI medium with 10% FCS was added to the methylcellulose cultures, which were then liquidified at 4°C. A fixed number of PE-labeled QuantiBrite beads (BD Biosciences) was added to each sample. After pelleting and washing beads and cells in PBS, cells were stained for 30 min. at RT in PBS containing 5% FCS and 5% human serum with FITC-labeled anti-CD36 antibody (diluted 1:10, PeliCluster, Amsterdam) and PE-labeled anti-Glycophorin A antibody (diluted 1:10, Research Diagnostics Inc., Flanders, NJ), or with control mIgG1-FITC (diluted 1:20) and mIgG2a-PE (diluted 1:40,

both from Sigma Aldrich). After staining, 0.4 ml PBS was added, and cells were analysed in a FACScalibur flow cytometer. Cellular debris was gated out in the FSC/SSC plot. The numbers of positive cells in the negatively stained samples were subtracted from the marker-positive cells, and normalized against the number of PE-labeled QuantiBrite beads measured in each sample, to yield relative numbers of marker-stained cells.

### Phosphorylation of STAT5 and ERK Proteins

Starved TF-1 or UT7/EPO cells were pelleted and resuspended in medium without cytokine or with given amounts of EPO or peptide, and incubated at 37°C for a given period of time. All subsequent steps were performed on ice. Cells were then pelleted, washed with PBS, and lysed for 30 min. on ice in 100  $\mu$ l per 1 million cells lysis buffer: 150mM NaCl, 50mM Tris/HCl pH8, 1% Triton-X-100, protease inhibitor cocktail (Complete mini, Roche, Mannheim, Germany), and 1mM orthovanadate. Insoluble material was pelleted, and supernatants were electrophoresed. Lysates were supplemented with reducing Laemmli buffer containing DTT (Invitrogen). Samples were boiled for 5 min. before application to SDS-PAGE gels and electrophoresis. Proteins were electroblotted to PVDF membranes (Invitrogen). Blots were washed, blocked, and incubated with antibodies in Tris-buffered saline with 0.05% Tween 20 (TBST). After blocking with 3% skim milk powder (blocking buffer), primary and peroxidase-conjugated secondary antibodies were also diluted in blocking buffer. Detection was performed using the ECL detection kit (GE Healthcare, Freiburg, Germany) and exposure of X-ray films (ECL hyperfilm, GE Healthcare). Antibodies used were: monoclonal mouse anti-P-STAT5 (Cell Signalling Technology, Beverly, USA; 9356) diluted 1:10,000; monoclonal mouse anti-P-Erk (Cell Signalling 9106) diluted 1:10,000; polyclonal rabbit anti-STAT5 (Cell Signalling 9310) diluted 1:5000; polyclonal rabbit anti-Erk (Cell Signalling 9102) diluted 1:25,000; rabbit-anti-mouse-HRP (Dako, Hamburg, Germany, P0260) diluted 1:30,000; donkey-anti-rabbit-HRP ECL (GE Healthcare, NA934V) diluted 1:10,000.

### Radioligand Binding Assay of EPO to Immobilized EPO Receptor

Competitive binding of 40 pM <sup>125</sup>I-rhEPO (R&D systems) to Fc-tailed soluble EPO receptor (expressed in NSO cells, R&D systems) immobilized to SPA beads, in potassium buffer, pH 7.4, was carried out by adding rhEPO, AGEM400 peptide or AGEM400(HES) in various concentrations. These assays were performed by MDS Pharma (Taipei, Taiwan). The percent inhibition of binding was plotted against concentration of competitor, and concentrations at which half-maximum inhibition was reached (IC50) were calculated using a non-linear, least squares regression analysis. Similarly, the potential of AGEM400 to inhibit binding of 27 different ligand/receptor systems was tested in radioligand binding assays, also from MDS Pharma. For more information on the radioligand binding assays, please check the MDS website (<https://discovery.mdsp.com/Catalog/>).

### Immunizations Against EPO and EMPs

Rabbits (n=2 per antigen) were immunized at Eurogentec (Seraing, Belgium) with: 1) EPO conjugated to keyhole lim-

pet hemocyanin (KLH) *via* amines (lysines); 2) AGEM400 peptide conjugated to KLH *via* free thiol on the terminal cysteine; or 3) KLH alone. Titers were determined by ELISA against EPO, peptide or KLH ten days after each boost, to determine the optimal time point of the final bleed. Titers of antibodies against KLH were always at least 100 fold higher than those against EPO or peptide. Hematocrit values were determined every two weeks beginning at the first immunization. One out of two rabbits immunized with EPO-KLH developed anemia after the second boost, leading to a hematocrit value of 20%. This animal (SA4893) was sacrificed ten days after the second boost. Hematocrits remained in the normal range for all other rabbits immunized, and these animals were sacrificed ten days after the third boost. Mice (n=4) were immunized at Eurogentec with AGEM400 conjugated to bovine serum albumin (BSA). After three boosts, antibody titers against AGEM400 were still relatively low (near 1:100). Serum was collected from mice sacrificed after the third boost, while hybridomas were also produced with splenocytes of one of the mice. However, no monoclonals were found with a detectable reactivity against AGEM400. Sheep (n=2) were immunized at Charles River (Sulzfeld, Germany) with AGEM400-KLH. Final bleeds were undertaken after the third boost. One of the sheep anti-AGEM400 antisera was affinity purified on an AGEM400 column at Squarix (Marl, Germany).

#### Western Blot Analysis on Cross-Reactivity of Anti-EPO and Anti-EMP Antibodies

EPO, BB68 and AGEM400 were electrophoresed and blotted to PVDF membranes, which were incubated with either rabbit antiserum raised against EPO, diluted 1:5000, rabbit antiserum raised against AGEM400, diluted 1:200, or mouse antiserum raised against AGEM400, diluted 1:500. This was followed by incubation with donkey-anti-rabbit-HRP ECL (GE Healthcare, NA934V) diluted 1:10,000 or rabbit-anti-mouse-HRP (Dako, Hamburg, Germany, P0260) diluted 1:30,000, and development with ECL detection kit (GE Healthcare, Freiburg, Germany).

#### Inhibition of Biological Activity by Soluble EPO Receptor or by Rabbit Antisera

MTS assays testing the *in vitro* effect of EPO or AGEM400(HES) were performed with TF-1 or UT7/EPO cells as described above. In addition, however, soluble recombinant EPO receptor (Sigma Aldrich), or antisera were added. Soluble EPO receptor was added to 2 µg/ml. Rabbit anti-EPO antiserum SA4893 or pre-immune serum from the same animal was added to 1%.

#### Sandwich ELISA to Detect EMP

Multiwell (96-well) plates (Greiner Bio-One 655161, Kremsmünster, Austria) were shortly equilibrated in 100 µl PBS, and then coated with 100 µl 5 µg/ml affinity-purified sheep-anti-AGEM400 antibody diluted in (Dulbecco's) PBS for 1h at RT. Wells were washed three times with 100 µl PBS. Dilutions of AGEM400 or AGEM400(HES) were added in 50 µl volumes, and incubated for 1h at RT. Each dilution was tested in triplicates. Wells were washed three times with PBS. Blocking solution (PBS with 2% skim milk powder (Merck, Darmstadt, Germany) was added in 200 µl volumes, and incubated for 1h at RT. After removal of

blocking solution, rabbit-anti-AGEM400 antiserum, diluted 1:1000 in PBS was added in 100 µl volumes. The solution was then incubated with antiserum for 1h at RT. In the meantime, secondary antibody (donkey-anti-rabbit-HRP ECL, GE Healthcare, NA934V) was pre-incubated as a 1:1000 dilution in blocking solution with 10% pooled human serum for 45 min. at RT to reduce background reactivity with serum proteins. After the incubation with primary antibody, wells were washed three times with PBS, after which pre-incubated secondary was added in 100 µl volumes, and incubated for 1h at RT. Wells were washed three times with PBS, and 100 µl volumes of TMB reagent (s(HS)TMB, SDT, Baesweiler, Germany) were added, and incubated until blue color development became apparent. The reaction was stopped by adding 100 µl/well of 1 N HCl, and absorbance was measured at 450 nm.

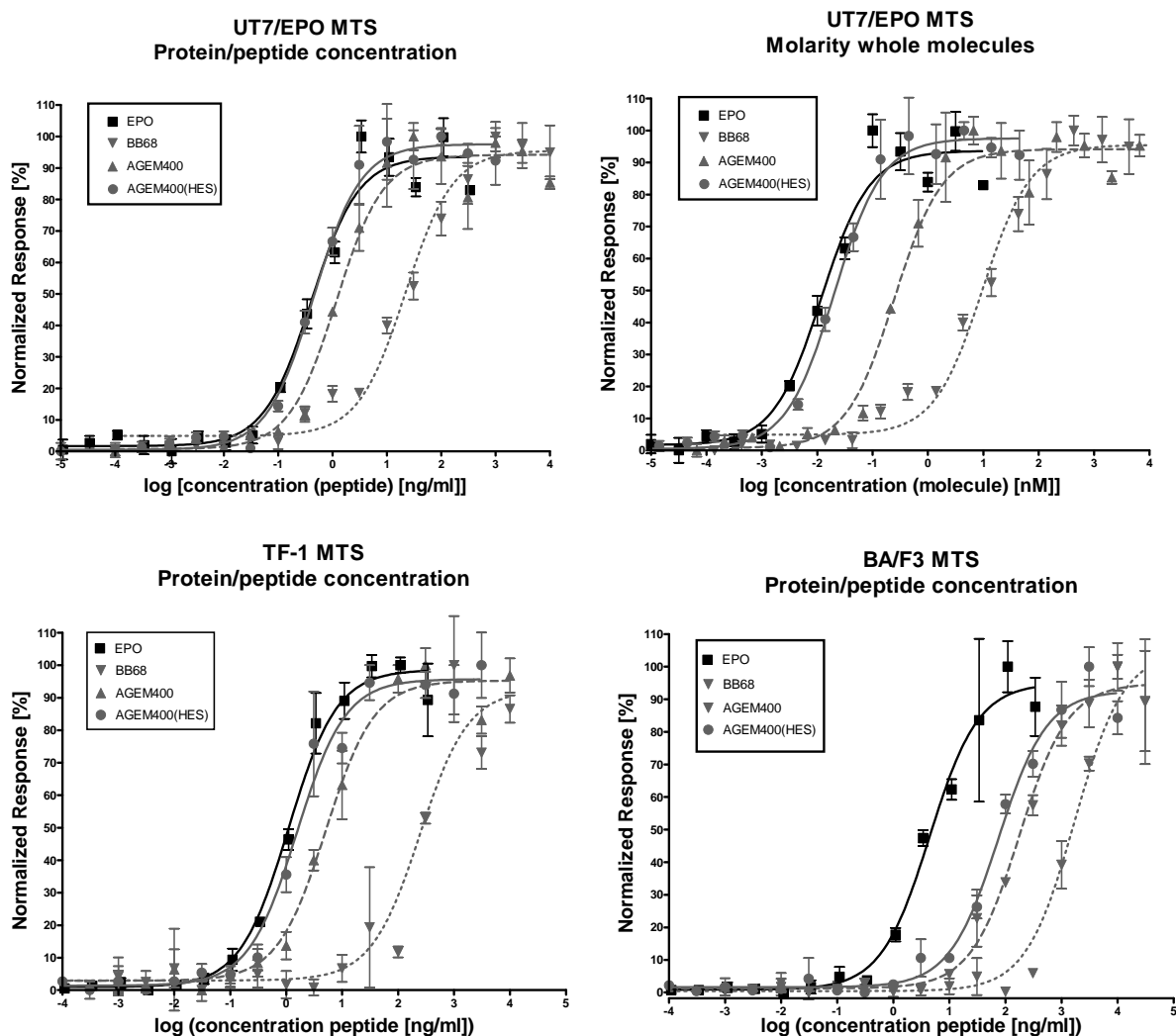
## RESULTS

### Optimization of the EMP

The sequence of the final monomeric EMP, BB68, was chosen after testing several hundreds of different 20-mer peptides for their efficacy in the TF-1 MTS assay. BB68 was among the most potent peptides, and was chosen also because of its clear sequence differences with the published EMP-1 sequence [5, 23]. BB68 contains lysines instead of prolines in positions 10 and 17; it also contains a 1-naphthalalanine instead of a tryptophane in position 13. The dimeric version of the peptide, AGEM400, was synthesized as a continuous 41-mer, in which the C-terminus of one monomer was connected by a peptide bond to the N-terminus of the other monomer. This dimerization strategy differs from those published elsewhere [24, 30], where coupling of both N-termini or of both C-termini and linkers to obtain dimeric peptides were used. The C-terminus of AGEM400 contains an additional of a tBu-protected cysteine which, after deprotecting, allows for conjugation to maleimide-activated macromolecules such as HES. A scheme of monomeric, dimeric, and HES-conjugated peptide is presented in Fig. (1).

### Comparison of Efficacy of Monomeric, Dimeric and HES-Conjugated EMP *in Vitro*

Tested on the human EPO-responsive cell lines TF-1 and UT7/EPO, dimeric peptide AGEM400 showed an efficacy that was about 100-fold higher than that of monomeric peptide BB68, leading to EC50 values for AGEM400 close to 1 ng/ml, and close to that of EPO, in both assay systems (Fig. 2, Table 1). Conjugation of AGEM400 peptides to HES led to a further small, but reproducible, improvement of the peptide's efficacy. In the TF-1 assay, the average EC50 decreased by a factor of 4, while in the UT7/EPO assay, the average EC50 decreased by a factor of 1.6. The EC50 values of AGEM400(HES) in both assay systems went down to 0.66 and 0.45 ng peptide/ml, respectively (Table 1), corresponding to 140 - 100 pM dimeric peptide (MW 4.6 kDa). When molarity of the AGEM400(HES) conjugate was considered (mean molecular mass of AGEM400(HES) was 150,000 Da), the EC50 in the TF-1 or UT7/EPO assays was between 20 and 30 pM conjugate, which was similar to the molar EC50 values found for EPO (see also Fig. (2) right hand upper panel for this graphical representation). It should



**Fig. (2).** Assays comparing the efficacy of EPO and EPO mimetics in stimulating cell survival. Human UT7/EPO cells (both top panels), human cell line TF-1 (left hand bottom panel), or murine BA/F3 cells (right hand bottom panel) were incubated with varying concentrations of agents (usually shown as ng/ml protein or peptide, considering the peptide content of AGEM400(HES) to be 10% w/w). In the right hand top panel, molar concentrations of whole molecules are shown, whereby it should be noted that 1 mole of AGEM400 contains 2 moles of monomeric BB68, while 1 mole of AGEM400(HES) contains on average 5 moles of AGEM400. Values represent means and standard deviations of triplicate measurements. The EC50 values, the numbers of experiments performed with each substance and in each cellular model are given in Table 1. Absorbance values measured were normalized to values between 0 and 100% response, to facilitate calculation of EC50 values. ■, black continuous line: EPO; ▼, gray dotted line: BB68 (monomer); ▲, gray interrupted line: AGEM400 (dimer); ●, gray continuous line: AGEM400(HES). Note that both dimerization of the peptide, and conjugation to HES, increased the efficacy per ng peptide, in each of the assay systems.

be noted, however, that one mole of HES contained, on average, 4-5 moles of AGEM400 peptide. Representation of the efficacy of AGEM400(HES) in molar concentrations can therefore easily lead to confusion if it is not specified whether conjugate molarity or peptide molarity is being described. The assays using murine cell line BA/F3 yielded an efficacy for EPO that was much lower (EC50 about 10-fold higher) than in the assays using human cells, and the efficacy of peptide and AGEM400(HES) was also poorer, even in comparison to that measured with EPO. The increase in efficacy due to dimerization of the peptide was only about 10-fold in the murine assay, while a 2.4 fold increase in efficacy was obtained by conjugation to HES (Fig. 2, Table 1). The average EC50 of AGEM400(HES) was 75.5 ng peptide/ml

in the BA/F3 assay, which was more than 10 fold higher than that of EPO.

#### Stimulation of Erythropoiesis from Bone Marrow Cells in Methylcellulose

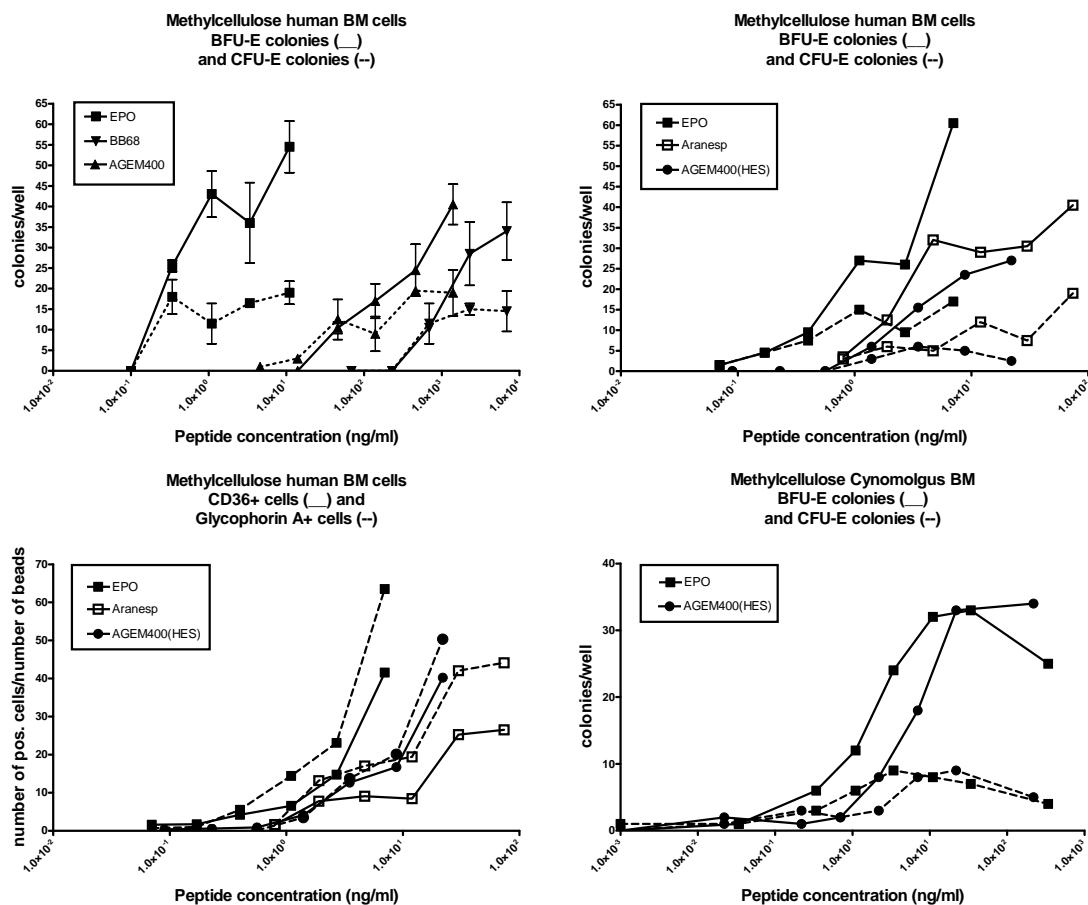
The stimulation of red colonies (CFU-E and BFU-E) or erythroid cells from bone marrow precursor cells in methylcellulose assays is a process that includes stimulation of proliferation, inhibition of apoptosis as well as stimulation of erythroid differentiation. Both EPO and EMP stimulated the development of CFU-E and BFU-E colonies dose-dependently. Dimeric AGEM400 and AGEM400(HES) were more efficient stimulators than monomeric BB68, also in this assay type (Fig. 3). EPO started to stimulate the development

of CFU-E and BFU-E colonies from human bone marrow cells at concentrations below 1 ng/ml. BB68 started to show activity at concentrations between 100 and 1000 ng/ml, while AGEM400 required concentrations of at least 10 ng/ml. AGEM400(HES) worked at concentrations between 1 and 10 ng/ml peptide and had an efficacy similar to that of Aranesp (Fig. 3). Similar dose-effect relationships were found for EPO and AGEM400(HES) when using bone mar-

row cells derived from cynomolgus monkeys (Fig. 3). Quantitative measurement of erythropoiesis from human bone marrow cells was also performed by flow cytometry, after liquidification of the methylcellulose media and staining of erythroid cells with antibodies against erythroid cell markers CD36 and glycophorin A (GPA). Dose-effect curves after flow cytometry showed similar shapes as those obtained after counting of colonies (Fig. 3).

**Table 1. Mean EC50 Values. EC50 Values (in ng/ml Peptide), and Standard Deviations Thereof in n Experiments are Shown, of EPO, BB68 (Monomeric Peptide), AGEM400 (Dimeric Peptide) and AGEM400(HES) in MTS Assays on TF-1 cells, UT7/EPO Cells and BA/F3 Cells**

	TF-1			UT-7/EPO			BA/F3		
	EC50 [ng/ml]	SD	n	EC50 [ng/ml]	SD	n	EC50 [ng/ml]	SD	n
EPO	0.874	0.527	66	0.543	0.354	20	6.78	4.59	53
BB68	248.9	145.8	66	49.80	16.93	11	1909	1151	55
AGEM400	2.650	1.120	54	0.699	0.299	18	181.2	111.3	38
AGEM400(HES)	0.660	0.232	15	0.449	0.305	27	75.53	46.18	6



**Fig. (3).** Clonogenic assay to measure development of red colonies from human bone marrow cells (or cynomolgus bone marrow cells: right hand bottom panel) in methylcellulose medium under the influence of erythropoies stimulating agents. Red colonies (CFU-E, dotted lines; and BFU-E, continuous lines) were enumerated microscopically, or cells were recovered from liquidified media, stained with antibodies against erythroid markers (CD36: continuous lines; glycophorin A: dotted lines), and analysed by flow cytometry (left hand bottom panel). ■: EPO; □: Aranesp; ▼: BB68 (monomer); ▲: AGEM400 (dimer); ●: AGEM400(HES). The experiment shown in the left hand top panel shows means and standard deviations from duplicate measurements, the other data points (without error bars) reflect single determinations. Typical examples are shown of experiments performed at least three times. Human cells were from different donors each time.

### Signalling Through STAT5 and ERK in UT7/EPO

Phosphorylation of signalling molecules such as STAT5 and ERK in UT7/EPO cells occurs with similar efficacy and kinetics by EPO, AGEM400 or AGEM400(HES). STAT5 phosphorylation was tested after 30 minute incubations with increasing concentrations of these agents. Maximum signals were obtained with 3.4 ng/ml or more EPO, 4.6 ng/ml or more AGEM400 and 2.2 ng peptide/ml or more AGEM400(HES) (Fig. 4, upper panel). Incubation of UT7/EPO cells for different periods of time with 3.4 ng/ml EPO or 10 ng peptide/ml AGEM400(HES) showed the strongest P-STAT5 signals after 30 minutes, which decreased upon prolonged incubation. After 24 h of incubation, P-STAT5 signals caused by EPO or AGEM400(HES) were much weaker than the signals observed after 30 minutes, but still equally strong after incubation with EPO or with AGEM400(HES) (Fig. 4, bottom panel). ERK (MAPK p42/p44) was also phosphorylated equally strong by EPO or AGEM400(HES) after 30 minutes, but P-ERK signals disappeared much faster than the P-STAT5 signals, making P-ERK difficult to detect after 4 h or 24 h of incubation.

### Evidence for Binding of AGEM400(HES) to the EPO Receptor

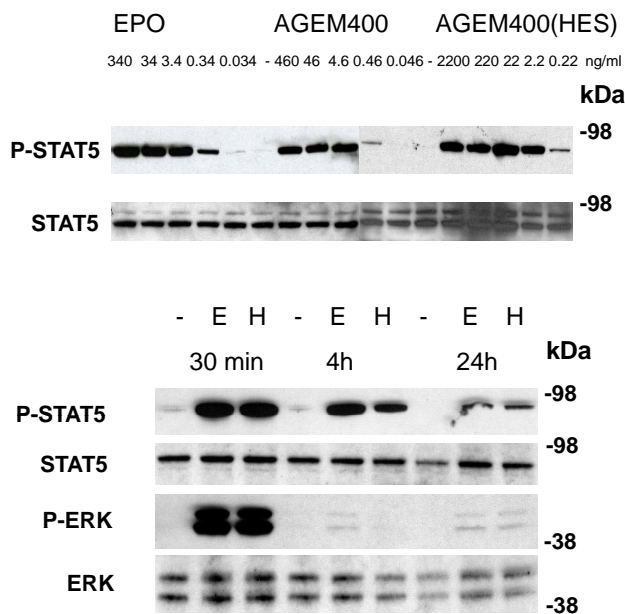
Addition of 2 µg/ml soluble recombinant EPO receptor (sEPO-R) to TF-1 or UT7/EPO MTS assays inhibited the *in vitro* effects of both EPO and AGEM400(HES), clearly shown in Fig. (5) (top and central panel) by right shifts of the dose-effect curves. In contrast, sEPO-R did not influence the dose-effect curve of IL-3 in the TF-1 MTS assay (Fig. 5, central panel). This data indicates binding of both EPO and AGEM400(HES) to the soluble EPO receptor. Radioligand binding assays, in which binding of iodine-labeled EPO to

recombinant EPO receptor was competed for by increasing concentrations of EPO, AGEM400 or AGEM400(HES), showed concentration-dependent inhibition of binding caused by all three agents at similar efficacies. While EPO had an IC<sub>50</sub> of close to 10 ng/ml, AGEM400 and AGEM400(HES), under the same circumstances, showed IC<sub>50</sub> values of 18 ng/ml and 13.5 - 16 ng peptide/ml, respectively (Table 2). The increased efficacy of AGEM400 conjugated to HES compared to AGEM400 as a free dimeric peptide in the *in vitro* assays shown in Fig. (2) was thus not reflected in an increased potency of AGEM400(HES) to replace EPO from its receptor compared with peptide alone.

AGEM400 peptide also competed for binding of 8 other ligands to their respective receptors, but only with relatively high IC<sub>50</sub> values of between 1.2 µg/ml and 34.8 µg/ml. AGEM400 peptide was unable to inhibit to 50% or more the binding of 19 more ligand/receptor systems at 10 µM (47 µg/ml). This data is listed in Table 3.

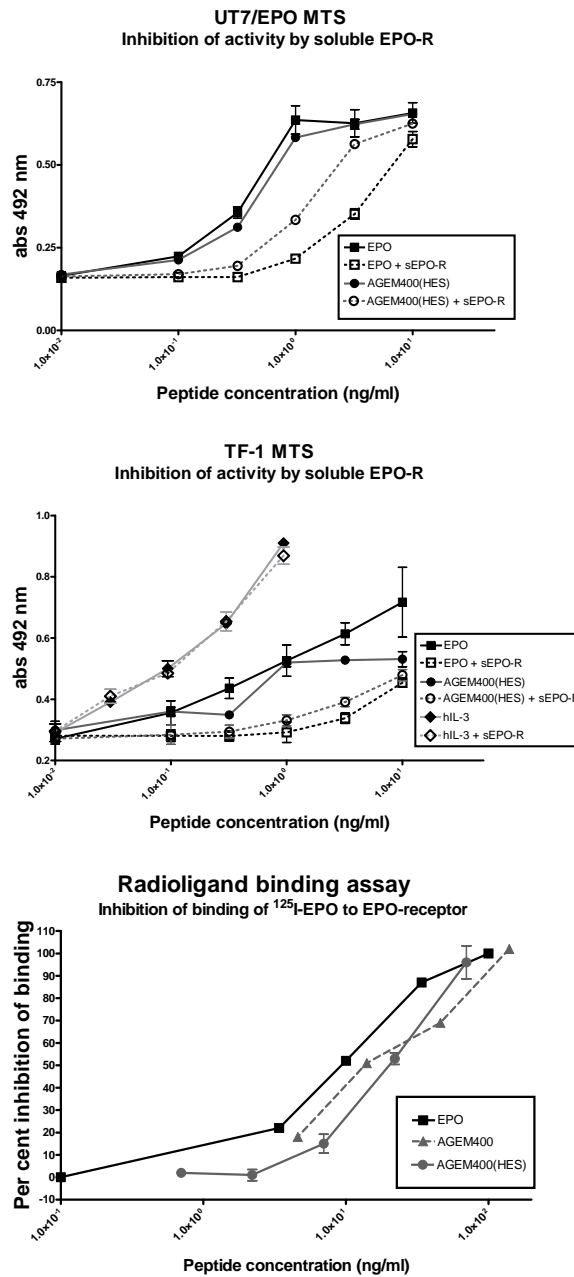
### No Cross Reaction Between Antibodies Against EPO or Against AGEM400

During the generation of rabbit polyclonal antibodies against EPO, one of the animals immunized with EPO-KLH became anemic. The procedure was ceased when this rabbit reached a hematocrit of 20%. The serum of this rabbit (SA4893) appeared to contain neutralizing anti-EPO antibodies, because addition of 1% of this serum to TF-1 MTS assays completely abolished the activity of up to 3.4 ng/ml EPO. Pre-immune serum from the same animal did not inhibit the activity of EPO (Fig. 6, upper panel). However, the neutralizing anti-EPO antiserum did not inhibit the activity of AGEM400(HES) at all (Fig. 6, central panel). This indicates that these neutralizing anti-EPO antibodies do not neu-



**Fig. (4).** Western blot analysis studying STAT5 phosphorylation and/or ERK phosphorylation in UT7/EPO cells. **Top:** Concentration-dependency of STAT5 phosphorylation: starved UT7/EPO cells were incubated for 30 min. at 37°C with EPO, AGEM400 peptide, or AGEM400(HES) at various concentrations (denoted in ng/ml protein/peptide), or no stimulus at all (-). **Bottom:** Persistence of phospho-STAT5 and phospho-ERK signals after continuous incubation of UT7/EPO cells with 3.4 ng/ml EPO (E), 10 ng/ml (peptide concentration) AGEM400(HES) (H), or no stimulus (-) for 30 min., 4h, or 24h. Blots containing electrophoresed cell lysates were probed with antibodies against phospho-STAT 5 (P-STAT5), total-STAT 5 (STAT5), phospho-ERK (P-ERK), or total-ERK (ERK). Typical examples are shown of experiments performed at least twice.





**Fig. (5).** Evidence for binding of AGEM400 and AGEM400(HES) to the EPO receptor. **Top:** UT7/EPO MTS assay showing the dose-dependent effects of EPO (■, black lines) and AGEM400(HES) (●, gray lines) on cell survival, and the inhibition of these effects (right shift of dose-effect curves) by addition of 2 μg/ml soluble EPO receptor (sEPO-R, open symbols and dotted lines). **Center:** TF-1 MTS assay showing the dose-dependent effects of EPO (■, black lines), AGEM400(HES) (●, gray lines) and IL-3 (◇, bright gray lines) on cell survival, and the inhibition of the effects of EPO and AGEM400(HES) – but not of IL-3 – by addition of 2 μg/ml soluble EPO receptor (sEPO-R, open symbols and dotted lines). Data points in the MTS graphs reflect means of triplicate measurements and error bars reflect the standard deviations. Experiments were performed twice with similar outcomes. **Bottom:** Radioligand binding assay performed on bead-immobilized dimeric Fc-tailed recombinant soluble EPO receptor (R&D systems). Binding of radiolabeled rhEPO (R&D systems; 40 pM) was competed for by adding different concentrations of either rhEPO (■, black line), AGEM400 (▲, gray discontinuous line) and AGEM400(HES) (●, gray line). Inhibition of binding was plotted as a percent value against the concentration of competitor (in ng protein or peptide per ml). Inhibition of binding by AGEM400(HES) was measured three times within this experiment, of which means and standard deviations are depicted. IC50 values and statistics of n experiments are listed in Table 2.

tralize EMP. Rabbit-anti-EPO antiserum stained EPO but not BB68 or AGEM400 peptide on Western blots (Fig. 6, bottom panel). Likewise, polyclonal rabbit-anti-AGEM400 antiserum and polyclonal mouse-anti-AGEM400 antiserum

stained BB68 and AGEM400 peptide, but not EPO. These results indicate that there is no cross-reactivity between antibodies against EPO or against AGEM400, at least in the animal models tested here.

**Table 2. Inhibition Efficiencies of Binding of Radiolabeled EPO to EPO Receptor. Inhibition Efficiencies are Expressed as the IC50 Values (in ng Protein or Peptide/ml), with Standard Deviations in n Experiments. For rhEPO (Molecular Mass 34 kDa) and AGEM400 (Molecular Mass 4.6 kDa) only Inter-Assay Variability is Given of Assays in which Each Substance was Measured once. For AGEM400(HES) Both Inter-Assay Variability (2 Experiments) and Intra-Assay Variability (Triplicate Measurement within an Experiment) are Given**

Inhibition of Binding of Radiolabeled EPO to EPO Receptor			
Agent	IC50 [ng/ml]	SD	n
rhEPO	9.69	1.43	4
AGEM400	17.99	3.73	2
AGEM400/HES (interassay)	16.05	3.60	2
AGEM400/HES (intraassay)	13.52	0.63	3

**Table 3. Efficiencies of AGEM400 Peptide to Inhibit Binding of Radiolabeled Ligands to Their Respective Receptors. If Inhibition Efficiencies could be Quantified, These are Expressed as the IC50 Values (in ng Peptide/ml), with the Standard Error of Means of Duplicate Measurements. On the Left Hand Side, 8 Ligand/Receptor Systems are Listed in which AGEM400 Peptide Inhibited Quantitatively. The Three Right Hand Columns List 19 Ligand/Receptor Systems in which AGEM400 Peptide was not Able to Inhibit More Than 50% of the Binding at a Concentration of 47 µg/ml**

Inhibition of Binding of			No Inhibition of Binding	No Inhibition of Binding	No Inhibition of Binding
Specific Ligand/Receptor System	IC50 [µg/ml]	SEM [µg/ml]			
Bombesin BB1	1.19	0.09	Adenosine A1	Glucocorticoid	Serotonin 5-HT2A
Bradykinin B1	3.61	0.48	Adrenergic α 1D	Histamine H1	Tachykinin NK1
Melanocortin MC1	4.21	0.59	Adrenergic beta 1	IL-2	VEGF
Somatostatin sst1	3.92	0.23	Dopamine D1	IL-6	
Vasopressin V1B	20.4	1.3	Endothelin ETA	Leukotrine	
Chemokine CCR1	16.7	3.3	EGF	Melatonin MT1	
Neuromedin U NM U1	30.7	5.1	GABA B1A	Muscarinic M1	
Angiotensin AT1	34.7	6.8	Galanin GAL1	FPR1	

### Bioanalytics of AGEM400(HES)

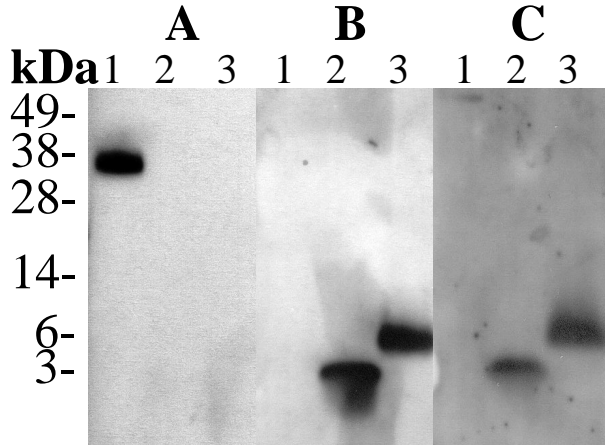
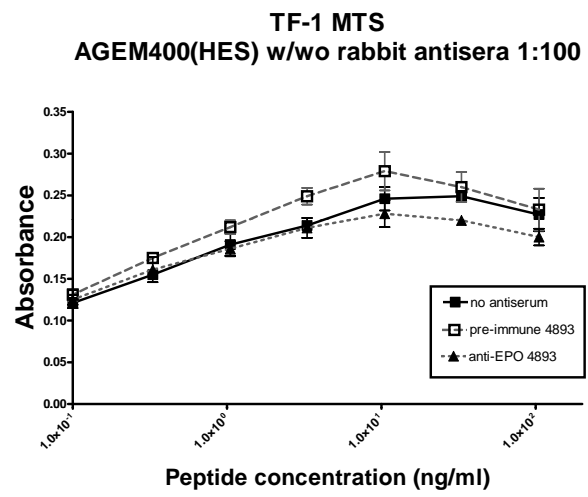
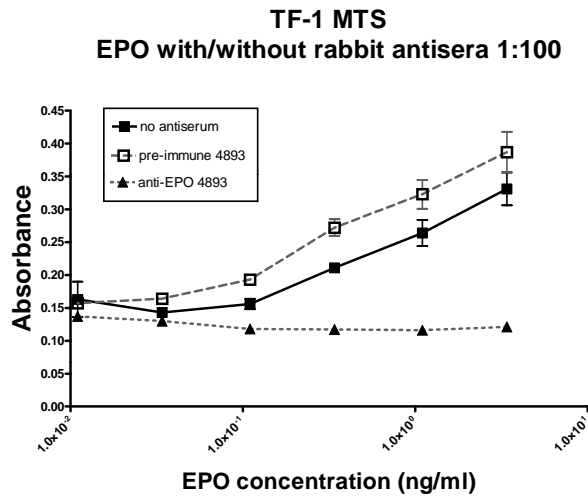
A sandwich ELISA was developed with a limit of detection close to 1 ng/ml peptide for AGEM400 conjugate, and in the low ng/ml range for AGEM400 peptide on its own (Fig. 7). AGEM400 peptide or AGEM400(HES) could be diluted in various buffers, media or sera, and were effectively detected in all of them, albeit with varying levels of detection.

### DISCUSSION

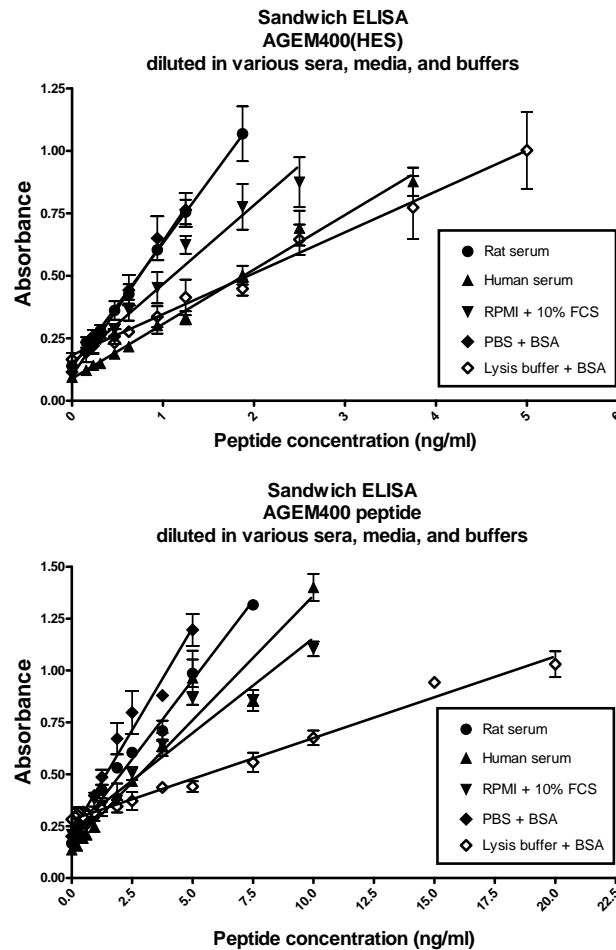
We present here the *in vitro* effects, and a partial characterization of the working mechanism of a novel EMP in its monomeric (BB68) and dimeric forms (AGEM400), and of a conjugate of multiple AGEM400 peptides to HES (AGEM400(HES)).

The sequence of BB68 differed in three positions from EMP-1 [5], and these three exchanged residues (Pro10, Trp13 and Pro17) were considered part of the critical core of EMP-1 [23]. However, AGEM400(HES) is more than just another EMP with a sequence a little different from that of

other EMPs, and dimerized in a different fashion than used for other EMPs [9, 24, 30]. The AGEM400 peptide was conjugated to HES in such a way that on average 5 dimeric peptides were bound to one HES molecule. The primary purpose of the conjugation to HES was to increase molecular mass and *in vivo* half life. However, it is shown here that conjugation to HES also increased the efficacy of the AGEM400 peptide by a factor of between 1.6 and 4, depending on the assay system in which it was tested. A likely explanation for the observed increase in efficacy of AGEM400 peptide due to conjugation to HES is the so-called multivalency principle: once one peptide has bound to the EPO receptor, other peptides present on the same macromolecule will find and bind consecutive EPO receptors faster, while multiple peptides on one macromolecule binding to multiple receptors will have a lower off-rate as a whole than the same number of free peptides. This principle has been described for other EPO receptor-binding peptides in Vadas *et al.* [31]. However, we were not able to demonstrate more efficient replacement of EPO from the EPO receptor by AGEM400 conjugated to HES than by AGEM400 peptide on its own. In



**Fig. (6).** Immunological distinction between EPO and AGEM400. **Top:** Inhibition of the survival-promoting effect of up to 34 ng/ml (1 nM) EPO on TF-1 cells in the MTS assay by addition of rabbit anti-EPO antiserum (▼, dotted gray line) but not by 1% pre-immune serum from the same rabbit (□, discontinuous gray line). **Middle:** No inhibition of the survival-promoting effect of up to 100 ng/ml (peptide concentration) AGEM400(HES) on TF-1 cells in the MTS assay by addition of 1% rabbit anti-EPO antiserum (▼, dotted gray line) or 1% pre-immune serum from the same rabbit (□, discontinuous gray line). ■, black continuous lines: no rabbit serum added. The higher absorbances observed with the pre-immune serum are probably caused by endogenous EPO in this rabbit serum (which is neutralized in the anti-EPO antiserum). Data points in the MTS graphs reflect means of triplicate measurements and error bars reflect the standard deviations. Experiments were performed twice with similar outcomes. **Bottom:** Western blot demonstrating the reactivity of several antisera with either EPO (13 ng in lanes 1), monomeric peptide BB68 (750 ng in lanes 2) or AGEM400 (750 ng in lanes 3). Blots were stained with a rabbit antiserum raised against Erypo, diluted 1:5000 (A); rabbit antiserum raised against AGEM400, diluted 1:200 (B); or mouse antiserum raised against AGEM400, diluted 1:500 (C), followed by incubation with an appropriate secondary antibody conjugated to horseradish peroxidase (HRP), and development with a chemiluminescent substrate. Typical examples are shown of experiments performed at least twice.



**Fig. (7).** Sandwich ELISA demonstrating efficient detection of AGEM400 peptide (upper panel) or AGEM400(HES) (concentrations depicted in ng peptide/ml; bottom panel). Substances were diluted in various relevant dilution media: rat serum (●); human serum (▲); RPMI + 10% FCS (▼); PBS + 1% BSA (◆); lysis buffer + 1% BSA (◇). Shown are means and standard deviations of triplicate measurements. Typical examples are shown of experiments performed at least twice.

fact, IC<sub>50</sub> values were similar for AGEM400 peptide and for AGEM400 HES conjugate. Finding the cause for the improved efficacy of AGEM400(HES) as compared with AGEM400 peptide will be a topic for further study. Interestingly, coupling of a dimeric EMP to PEG [8] led to a substance, Hematide, with a five-fold decreased receptor binding affinity as well as decreased *in vitro* efficacy, as compared to peptide only.

A further difference between peptide conjugation to PEG or to HES is that high molecular weight PEG is not biodegradable while the majority of high molecular weight HES is hydrolysed by amylase and degradation products are renally excreted (reviewed in Boldt [22]). While HES is used as active ingredient in plasma expander solutions on daily basis in many clinical settings worldwide, high-molecular weight PEG is not suited as plasma expander due to the lack of biodegradability and accumulation. The biodegradability of HES, and connected with this its plasma clearance and its elimination half-life, is primarily controlled by the C<sub>2</sub>/C<sub>6</sub> degree of substitution [32, 33]. Use of HES as plasma expander is a high-dose application involving daily doses up to tens of grams of HES. Under these circumstances, there are some reports on accumulation of HES in various organs, especially of those HES grades with high ratios of substitu-

tion [33, 34]. Repeated HES administration as high-dose plasma expander can also lead to impaired renal function, hemostatic problems, itching, and anaphylactic reactions [22, 35], but HES with low degrees of substitution do not show significant accumulation and are generally well tolerated, even after repeated administration [22, 36-38]. All the reports of side effects refer to applications that use HES doses more than 1000 times higher than those expected to be effective for a HES-conjugate like AGEM400(HES). It should be noted that pharmacokinetic and toxicology studies with HES were performed in rats, pigs and humans in single or repeated doses of 0.5 to 1 gram/kg, while AGEM400(HES) is anticipated to be effective against anemia in doses well below 1 mg/kg. Therefore, there are no concerns that the HES component of AGEM400(HES) would accumulate in the body – even in a dialysis patient – avoiding any long term exposure to this macromolecule. Thus, HES-dependent toxicology is not expected at the dose range expected useful for HES bioconjugates.

AGEM400(HES) appears to have superior efficacy *in vitro* in comparison with Hematide, and competitive efficacy compared with many other ESAs. The efficacy of AGEM400 (HES) in various human cell-based assay systems was either similar to that of EPO (TF-1 and UT7/EPO cells) or similar

to that of Aranesp (clonogenic assays on human bone marrow cells) when based on ng/ml peptide concentration. Average EC50 values of AGEM400(HES) in the TF-1 and UT7/EPO assays were 140 pM and 100 pM peptide, respectively, which were lower than those reported for Hematide (900 pM and 460 pM, respectively [8]). In a recently published patent by Affymax [39] the peptide-PEG conjugate having the best efficacy (peptide 1, likely to represent Hematide) had an EC50 of 165 pM in murine BA/F3 cells transfected with the human EPO receptor. This is closer to the efficacy measured here for AGEM400(HES), but the cell model overexpressing EPO receptor may not be comparable with UT7/EPO or TF-1 cells. The EC50 of CNTO 530, a dimeric recombinant protein containing EMP-1 sequences, was close to 100 pM in an UT7/EPO proliferation assay [9], which resembles the efficacy of AGEM400(HES) very well. Also, the monomeric peptide BB68 was more efficient than an analog of EMP-1, upon which the peptide sequence CNTO 530 and CNTO 528 are based [7, 9]. BB68 had an average EC50 in the TF-1 assay of 249 ng/ml (108 nM), while a peptide with the same sequence as EMP-1 [5, 23] in our laboratory showed an average EC50 of 2.8 µg/ml (1200 nM) in TF-1 MTS assays (n = 13 assays; data not shown). In contrast, EMP-1 had potencies of 400 nM or 100 nM in proliferation assays using FDCP-1 cells expressing human recombinant EPO receptor [5, 23], which may, however, not be comparable to the assays using TF-1 cells, because the EPO receptor is probably overexpressed in transfected cells.

The efficacy of AGEM400(HES) in an assay based on murine cells (BA/F3) was much lower than in assays using human models, and about 10-fold lower than that of EPO. The sequence of BB68/AGEM400 was optimized for use on human cells and was not intended for use on murine cells or in other species. The data obtained with BA/F3 cells indicate that the efficacy of AGEM400(HES) in future *in vivo* experiments in mice might underestimate its efficacy in humans. Efficacy of AGEM400(HES) in clonogenic assays on cells of either human or cynomolgus origin, was similar: AGEM400(HES) induced increasing numbers of erythroid cells and colonies at concentrations between 1 ng/ml and 10 ng/ml peptide. Translating this data to the *in vivo* situation, this would mean that efficacies of AGEM400(HES) measured in cynomolgus monkeys in future *in vivo* experiments might well be extrapolated to humans.

In future experiments, pharmacokinetics and pharmacodynamics of AGEM400(HES) in various animal models will be tested extensively. A sensitive sandwich ELISA for AGEM400(HES) is available which can detect down to 1 ng/ml peptide in animal or human sera. Concentrations of 1 to 10 ng AGEM400(HES) peptide/ml were active in various *in vitro* assays, including that monitoring erythropoiesis from human bone marrow cells, so it is likely that the levels that can be detected by sandwich ELISA are clinically relevant levels. This ELISA will thus enable the study of the pharmacokinetics of AGEM400(HES) in animal and clinical studies. These experiments will allow comparisons of the *in vivo* characteristics of AGEM400(HES), Hematide and CNTO 528. The latter two have already been tested extensively *in vivo* in preclinical [8, 9, 20, 21] and clinical [6, 7] settings.

The mechanism of action of AGEM400(HES) is probably similar to that of EPO, Hematide, and CNTO530 since

these agents also bind to the EPO receptor with high affinity, cause phosphorylation of STAT5 in UT7/EPO cells, and stimulate survival/proliferation of cytokine-starved UT7/EPO cells [8, 9].

Because both AGEM400(HES), Hematide and CNTO 528 have no sequence similarity with EPO, it is very unlikely for these EPO mimetics to induce EPO-neutralizing antibodies. In humans, neutralizing anti-EPO antibodies can cause pure red cell aplasia (PRCA). It was already shown extensively that antibodies against EPO and against Hematide did not cross-react, and that Hematide was able to correct anti-EPO antibody induced anemia in rats, a rat PRCA model [40]. Titering of antibodies during the immunizations against AGEM400 peptide indicated that AGEM 400 or AGEM400(HES) might be weak immunogens: immunizations of rabbits and mice with AGEM400-KLH conjugate generated much higher titers of antibodies against KLH than against AGEM400. Still, when applied *in vivo*, especially in a chronic fashion, there is a chance that AGEM 400(HES) will induce anti-AGEM400 antibodies. We found no cross-reactivity between antibodies generated in rabbits and mice against AGEM400 and EPO. Even though this absence of cross-reactivity must be still be proven to be true in humans, it is not likely that anti-AGEM400 antibodies that would arise in humans treated with AGEM400, would react with endogenous EPO or to induce PRCA, In cases in which anti-EPO antibodies have already caused PRCA, AGEM400 (HES) would probably still be able to correct the anemia associated with this state.

## CONCLUSION

In conclusion, AGEM400(HES) is a novel synthetic EPO mimetic that holds a promise in becoming a competitive ESA and drug candidate in the near future.

## AUTHOR'S CONTRIBUTIONS

AG and CK performed research, analyzed and interpreted data, and contributed equally to this work. BB performed research. UH, AR and ME provided and analysed vital reagents and contributed vital ideas. AP designed studies and wrote the paper. HGF designed studies and reviewed the paper.

## ABBREVIATIONS

CERA	=	Continuous erythropoietin receptor activator
EMP	=	Erythropoietin mimetic peptide
EPO	=	Erythropoietin
ESA	=	Erythropoiesis stimulating agent;
HES	=	Hydroxyethyl starch
PEG	=	Polyethylene glycol
PRCA	=	Pure red cell aplasia

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