INHIBITORY ANTIBodies OF HER3 ACTIVITY

HEMMENDE ANTIKÖRPER DER HER3-AKTIVITÄT

ANTICORPS INHIBITANT L'ACTIVITE DE HER3

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**Description**

**[0001]** The present invention relates to a pharmaceutical composition comprising as an active agent an inhibitor of HER3 activity, namely an anti-HER3 antibody. Further, the use of this composition for the diagnosis, prevention or treatment of hyperproliferative diseases, particularly tumor diseases is disclosed.


**[0003]** HER3 has been found to be overexpressed in several types of cancer such as breast, gastrointestinal and pancreatic cancers. When HER3 is co-expressed with HER2, another member of the EGFR subfamily of receptor protein tyrosine kinases, an active heterodimeric signalling complex is formed.

**[0004]** A monoclonal antibody against HER3 (Rajkumar et al., Br. J. Cancer 70 (1994), 459-456) had an agonistic effect on the anchorage-independent growth of cell lines expressing HER3. On the other hand, anti-HER3 antibodies described in U.S. patent 5,968,511 (corresponding to WO 97/35885) are reported to reduce Heregulin-induced formation of HER2/HER3 heterodimers. Such an activity, however, is only demonstrated for an antibody which increases Heregulin binding to HER3.

**[0005]** Vadlamudi et al. (Oncogenes 18 (1999), 305-314) describe the regulation of the cyclooxygenase (COX-2) pathway by the HER2 receptor. It was found that a specific inhibitor of COX-2 can suppress the mitogenic and invasive action of colorectal cancer cells. Further, it was found that incubation with a Monoclonal anti-HER3 antibody leads to a reduction in the levels HER2/HER3 heterodimers, but results in an only partial reduction of COX-2 expression.

**[0006]** WO 00/31048 discloses a quinazoline derivative which acts as an inhibitor of receptor tyrosine kinases such as EGFR, HER2 and HER4. An inhibition of HER3 is however not disclosed.

**[0007]** WO 00/78347 discloses methods for arresting or inhibiting cell growth, comprising preventing or reducing ErbB-2/ErbB-3 heterodimer formation. For example, the agent may be a combination of an anti-HER2 extracellular domain antibody and an anti-HER3 antibody, e.g. the HER3 antibody H3.105.5 purchased from Neomarkers. It is however not clear which type of anti-HER3 antibody is required to obtain desirable therapeutic effects.

**[0008]** US-patent 5,804,396 describes a method for identifying an agent for treatment of a proliferative disorder, comprising the steps of assaying a potential agent for activity in inhibition of signal transduction by a HER2/HER3 or HER2/HER4 or HER3/HER4 heterodimer. Specific HER3 inhibitors are not disclosed.

**[0009]** We compared the biological properties of Herceptin, an agonistic monoclonal antibody against HER2 with anti-HER3 antibodies, namely (i) α-HER3-EC-D, a murine monoclonal antibody IgG1. Upstate Biotechnology, Cat. No.: 05-471, directed against the Heregulin binding site of HER3, (ii) antibody 1B4C3 from our laboratory and (iii) antibody 2D102 also from our laboratory, in invasive breast cancer cell lines MCR-7 (DKFZ Heidelberg), MDA-MB-468 (ATCC HTB-132) and MDA-MB231 (ATCC HTB-26) expressing different HER2: HER3 ratios. We provide evidence that pretreating the breast cancer cell lines with anti-HER3 antibody prior to α/β-Herceptin (α/β-HRG) stimulation diminished the HER2/HER3 tyrosine phosphorylation content in contrast to Herceptin. In addition, anti-HER3-antibody abrogated HER2/HER3 heterodimerization and also reduced the complex formation of the p85 subunit of PI3-kinase and the adaptor protein SHC with HER3, resulting in decreased PI3-kinase and c-jun-terminal kinase activity (JNK), respectively. In comparison to Herceptin, anti-HER3-antibody was also capable of downregulating extracellular signal-regulated kinase 2 (ERK2) after α/β-HRG stimulation. Furthermore, we demonstrate a significant reduction of the migratory and proliferative properties of the breast cancer cell lines after pretreatment with anti-HER3-antibody. Our data clearly show that an anti-HER3-antibody is more potent in diminishing signal transduction processes after HRG stimulation than Herceptin. Furthermore, in specific cancer types, e.g. melanoma, anti-HER3 antibodies are effective in reducing migratory and proliferative properties while anti-HER2 antibodies do not show any significant effect at all. These data demonstrate the great potential of anti-HER3 antibodies or other HER3 inhibitors for the therapy of breast cancer and other malignancies characterized by hypersignalling through HER3 and its heterodimerization partners.

**[0010]** Thus, the present invention relates to a pharmaceutical composition comprising as an active agent an inhibitor of HER3 activity and pharmaceutically acceptable carriers, diluents and/or adjuvants, wherein binding of said inhibitor to HER3 reduces HER3 mediated signal transduction, wherein said reduction of signal transduction is caused by a down-regulation of HER3, which results in at least partial disappearance of HER3 molecules from the cell surface, wherein said inhibitor is an anti-HER3 antibody. The HER3 inhibitor of the invention is characterized in that binding of the inhibitor to HER3 reduces HER3 mediated signal transduction. According to the invention, the reduction of HER3 mediated signal transduction is caused by a down-regulation of HER3 resulting in at least partial disappearance of
HER3 molecules from the cell surface. For comparison reference is made to reduction of HER3 mediated signal transduction being caused by a stabilization of HER3 on the cell surface in a substantially inactive form, i.e. a form which exhibits a lower signal transduction compared to the non-stabilized form, which is also caused by an anti-HER3 antibody. However, according to the present invention, reduction of HER3 mediated signal transduction is caused as defined in claim 1.

[0011] The inhibitor of the invention may influence the binding of Heregulin to HER3, particularly by decreasing the binding of Heregulin to HER3. In other embodiments, however, the inhibitor may not compete with the binding of Heregulin to HER3.

[0012] The inhibitor is an anti-HER3-antibody. Preferably, the antibody is directed against the extracellular domain of HER3.

[0013] According to the invention, the term "antibody" covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two antibodies and antibody fragments as long as they exhibit the desired activity.

[0014] The antibody may be a monoclonal antibody which may be obtained by the hybridoma method as described by Köhler et al. (Nature 256 (1975), 495) or by recombinant DNA methods (cf. e.g. U.S. Patent 4,816,567). Monoclonal antibodies may also be isolated from phage antibody libraries using techniques described in Clackson et al. (Nature 352 (1991), 624-628) and Marks et al. (J.Mol.Biol.222 (1991), 581-597). The antibody may be an IgM, IgG, e.g. IgG1, IgG2, IgG3 or IgG4.

[0015] Antibody fragments comprise a portion of an antibody, generally the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab)2 and Fv fragments, diabodies, single chain antibody molecules and multispecific antibody fragments.


[0017] In an especially preferred embodiment the antibody is the antibody 1 B4C3 (IgG2a) produced by the hybridoma cell line DSM ACC 2517. Further preferred are antibodies, e.g. chimeric or humanized antibodies or fragments thereof, which have substantially the same biological activity (e.g. as described in the Examples) compared to the antibody produced by the deposited hybridoma cell line, for example, by binding to the same epitope on HER3. The hybridoma cell line DSM ACC 2517 was deposited under the Budapest Treaty for the Deposit of Microorganisms on July 24, 2001 at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, 38124 Braunschweig, Germany. The hybridoma cell line DSM ACC 2527 producing the antibody 1 B4C3 was deposited on August 07, 2001 at DSMZ.

[0018] The antibody of the invention may be coupled to a labelling group, particularly for diagnostic applications. Examples for suitable labelling groups such as radioactive groups, fluorescent groups or other labelling groups are known in the art. Further, particularly for therapeutic applications, the antibody may be coupled to an effector group, e.g. a cytotoxic group such as a radioactive group, a toxin or another effector group as known in the art.

[0019] Further, the present application relates to the use of an inhibitor of HER3 activity, wherein binding of said inhibitor to HER3 reduces HER3 mediated signal transduction, for the manufacture of an agent for the diagnosis, prevention and/or treatment of hyperproliferative diseases, particularly tumor diseases such as breast cancer, gastrointestinal cancer, pancreas cancer, prostate cancer, glioma, melanoma or other HER3 expressing or overexpressing cancers or formation of tumor metastases. The disease may be associated with increased HER3 signal transduction and may be associated with concomitant HER2 expression or lack of HER2 expression. Particularly the disease is associated with increased HER3 phosphorylation and/or increased HER2/HER3 heterodimerization and/or increased Pl3 kinase activity and/or increased c-jun terminal kinase activity and/or AKT activity and/or increased ERK2 activity and/or PYK2 activity.

[0020] Surprisingly it was found that the HER3 inhibitor of the invention, namely an anti-HER3-antibody showing the HER inhibiting activity of the invention, shows a significantly higher efficiency in diminishing signal transduction processes than a HER2 inhibitor such as Herceptin. Particularly, in melanoma cells, the anti-HER3-antibody was effective, while Herceptin did not show a significant effect, even though HER2 was expressed by the melanoma cells.

[0021] Preferably, the HER3 inhibitor of the invention exhibits at least one of the following characteristics:

- decreasing the association of Heregulin (p85) with transactivated HER3, preferably substantially completely inhibiting the binding of p85 with HER3,
- inhibiting the binding of GRB2 to HER2, the binding of HER2 to HER3 and/or the association of GRB2 with SHC,
- inhibiting receptor tyrosin phosphorylation,
- inhibiting AKT phosphorylation,
- decreasing tumor invasiveness, particularly in breast cancer and melanoma,
- inhibiting PYK2 tyrosine phosphorylation and
- inhibiting ERK2 phosphorylation.

Further, the invention relates to the use of an anti-HER3 antibody inhibiting HER3 activity as defined in claim 1 for the manufacture of an agent for diagnosing, preventing or treating a hyperproliferative disease, particularly a tumor disease, comprising administering a subject in need thereof, e.g. a human, an effective amount of an inhibitor of HER3 activity, wherein binding of said inhibitor to HER3 reduces HER3 mediated signal transduction.

The HER3 inhibitor, namely the anti-HER3-antibody may be formulated by mixing the active agent with physiologically acceptable carriers, diluents and/or adjuvants, e.g. in the form of lyophilized formulations, aqueous solutions, dispersions or solid preparations such as tablets, drages or capsules as described in Remington’s Pharmaceutical Sciences.

The formulation may also contain more than one active compound, e.g. inhibitors of other receptor protein tyrosine kinases such as EGFR, HER2, HER4 or vascular endothelial factor (VEGF). Alternatively or additionally, the composition may comprise a cytotoxic agent such as doxorubicin, cis-platin or carboplatin, or a cytokine.

The inhibitor of the invention is also suitable for in vivo diagnostic applications, e.g. in order to determine the expression and/or activity of HER3 on target cells. Such a diagnostic application may be carried out according to known procedures.

Depending on the type and severity of the disease to be treated, about 1 μg/kg to 15 mg/kg of antibody may be administered to a human patient, e.g. by one or more separate administrations or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to about 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition to be treated, the treatment is sustained until a desired suppression of disease symptoms occurs.

Further, the present invention shall be explained by the following figures and examples:

Examples

The present invention is illustrated by anti-HER3-antibodies, which are characterized as outlined in the claims. The anti-HER3-antibody 2D1d12 is incorporated for comparison only.

1. Monoclonal antibody α-HER3ECD decreases receptor tyrosine phosphorylation of HER3 and HER2

Further, we subsequently asked whether α-HER3ECD has an effect on the known substrates of HER3, namely SHC and phosphatidyl-3- OH-kinase (PΙ3-K), which are effector proteins responsible for MAPK cascade activation and lipid signalling, respectively. Therefore, we immunoprecipitated SHC and PΙ3-K under the experimental conditions described above and assessed the tyrosine phosphorylation of these effectors. As shown in Figure 2, α-HER3ECD significantly decreased the tyrosine phosphorylation of SHC in the cell lines MCF-7 and MDA-MB-468 (Figure 2a, b compare lane 13 with 16). Interestingly, the association of SHC was attenuated in MDA-MB-486 cells, which are insensitive to α-HRG, β-HRG was used as stimulus.
by measuring GRB2 binding (Figure 2c). Therefore we performed GST-pulldown assays in MCF-7 cells using GST-GRB2 fusions and the same experimental design as before. Indeed, the reduced tyrosyl-phosphorylation of SHC resulted in strongly decreased binding of GRB2 to SHC (Figure 2c lower panel, compare lanes 5 and 8), and a complete inhibition of its association with HER2 (Figure 2c middle panel, compare lanes 5 and 8). These data clearly show that α-HER3CED inhibits SHC and PL3-K binding to HER3 in MCF-7, but conversely in MDA-MB-486 both proteins associated with HER3 regardless of the phosphorylation status of HER3.

3. α-HER3CED downregulates JNK1 and PL3-K activity

The adaptor protein SHC mediates MAPK signalling pathways downstream of growth-factor receptors, activating ERK2 and JNK, respectively. To investigate the effect of α-HER3CED on ERK2 and JNK, we performed MAPK kinase assays under the same experimental conditions in MCF-7 and MB-486 (Figure 3). We observed a strong decrease of JNK activity in all cell lines, but an equivalent effect of HC on JNK was only detectable in MCF-7 (Figure 3a). ERK2 activity was only slightly but significantly decreased through α-HER3CED, whereas HC had no effect on ERK2 activity (data not shown).

Since an involvement of PL3-K in carcinoma invasion has recently been demonstrated, we investigated the inhibitory properties of α-HER3CED on PL3-K activity carried out PL3-K assays (Figure 3). In MCF-7 and MDA-MB-486 PL3-K activity was strongly reduced in comparison to HRG-treated cells (Figure 3a, b). In MDA-MB-486 HC possessed an even greater effect on PL3-K activity than α-HER3CED. These data suggest that α-HER3CED downregulates JNK and PL3-K activity, respectively, in MCF-7 and MDA-MB-486 cells.

4. α-HER3CED enhances endocytic downregulation of HER3

HER2 and HER3 are endocytosed and recycled after HRG stimulation. We were interested in establishing whether α-HER3CED-mediated inhibition of HER3 tyrosyl-phosphorylation originates from accelerated endocytosis. To gain insight, we performed a time course with MCF-7 cells in the absence or presence of α-HER3CED or HRG, respectively, and stimulated subsequently with HRG (Figure 4). HER3 was then immunoprecipitated after biotinylation of the membrane proteins. We observed that HER3 is endocytosed rapidly after pretreatment with α-HER3CED (Figure 4b upper panel). Applying HRG to the cells had the same effect, with the difference that after two hours HER3 was exported back to the membrane and after three hours it was endocytosed again. As a control, whole cell lysates (WCL) were probed with PY (Figure 4b lower panel). In comparison to HRG-treated cells where the content of tyrosyl-phosphorylated protein was diminished after three hours, accelerated endocytosis of HER3 occurred after one hour of pretreatment with α-HER3CED. To compare α-HER3CED with HC we performed the same experiment with HC and immunoprecipitated HER2 (Figure 4a upper panel). Strikingly, HER2 was not endocytosed after pretreatment with HC at any time point, whereas HRG lead to rapid endocytosis. When endocytosed receptors and whole cell lysates, probed with anti-phosphotyrosine (PY) are compared, it clearly appears that the phosphotyrosine content decreased with α-HER3CED, but not with HC (Figure 4b lower panel). Our data indicate that α-HER3CED downregulates HER3 rapidly through accelerated endocytosis, thus rendering the cell insensitive to HRG stimulation.

5. α-HER3CED inhibits migratory and proliferative properties of breast cancer cell lines

In order to assess the biological function of α-HER3CED on the migratory and proliferative properties of breast cancer cells, we performed BrdU-incorporation assays in the presence or absence of α-HER3CED and stimulated with HRG. Pretreatment with α-HER3CED decreased proliferation by 28.7% ± 6.18% and 21.1% ± 7.62% in MCF-7 and MDA-MB-486, respectively. The observed inhibition in proliferation correlated with the ERK2 assays, whereas HC had no effect in these cell lines (data not shown). Furthermore, to investigate the effect of α-HER3CED on the migratory properties of breast cancer cells, we conducted chemotaxis experiments with MCF-7 and MDA-MB-486 in the presence or absence of α-HER3CED. We observed a strong decrease in migration of 59.1% (P=0.018) and 55.4% (P=0.00005) in MCF-7 and MDA-MB-486, respectively. In addition, migration could also be inhibited in MDA-MB231 by 35%, but with a lesser significance (P=0.06). Our data clearly show an inhibitory effect of α-HER3CED on proliferation and migration in MCF-7 and MDA-468.

6. Generation of monoclonal antibodies against HER3

We generated then murine monoclonal antibodies against HER3. Balb/c mice were immunized with a human recombinant fusion protein of the extracellular part of HER3 and a C-terminal His-Tag (HER3-6xHis-CT). The immunogen was obtained by transfection, selection with G418 and stable expression of the construct in HEK293 cells; the cell culture supernatant of the clone with the highest expression level was collected and the protein purified after ammonium sulfate precipitation, dialysis and subsequent metal ion affinity chromatography (Ni-NTA). Quality assurance was accomplished by Western blotting (data not shown). Immunization was performed by intraperitoneal injection with 22 μg of HER3-6xHis-CT according to the manufacturer’s protocol (Qiagen ImmunEasy Mouse Adjuvant). Hybridoma cell lines producing monoclonal antibodies were generated using standard methods.
7. Monoclonal antibodies against HER3 preferentially bind to its protein backbone and have different effects on the endocytic processes of HER3

[0036] We identified by FACS analysis three monoclonal antibodies recognizing specifically native HER3 on the cell surface of MCF-7 cells (data not shown). 1 B4C2 and 1 B4C3 are IgG2a isotype antibodies, whereas 2D1D12 is an IgG1 isotype antibody. No cross-reactivity with the other members of the EGFR family was detected (data not shown). We then wanted to determine whether the antibodies bind to glycosylated structures or to the protein backbone of HER3 and which consequences this would have on the endocytic regulation of the receptor. Therefore we pretreated MCF-7 cells in the presence or absence with the antibiotic Tunicamycin for 16 h, which is known to prevent N-linked glycosylation of cell surface proteins. After lysing the cells we immunoprecipitated HER3 with the monoclonal antibodies 2F12 (directed against the intracellular part of HER3), α-HER3ECD (extracellular part of HER3), 1B4C2, 1B4C3 and 2D1D12. Our data show that α-HER3ECD, 1B4C3 and 2D1D12 all bind preferentially to the protein backbone of HER3, whereas 1B4C3 also has an affinity to glycosylated forms of HER3 (Figure 6A).

[0037] To investigate the effect of 1B4C3 and 2D1D12 on the endocytic processes of HER3, we performed a time-course experiment, wherein MCF-7 cells where incubated for various time periods with 1B4C3 or 2D1D12, respectively. The cell surface proteins were biotinylated, precipitated with an antibody against the intracellular domain of HER3 and probed against streptavidin. We observed that 1B4C3 accelerates the endocytosis of HER3 similarly to α-HER3ECD, whereas 2D1D12 stabilized and therefore accumulated HER3 on the cell surface (Figure 6B).

8. Monoclonal antibodies 1B4C3 and 2D1D12 inhibit downstream signals of HER3 and HER2

[0038] We then asked whether 1B4C3 and 2D1D12 could inhibit tyrosine phosphorylation of HER2 and of the HER3 substrate SHC. Since GRB2 binds to HRG-stimulated HER2 and transmits in the same way as SHC mitogenic signals to the MAPK pathway, we immunoprecipitated SHC and in parallel performed a GST-GRB2 pulldown in MCF-7 cells untreated or pretreated with the antibodies and subsequently stimulated with HRG (Figure 6C). This experiment shows that both antibodies inhibit tyrosine phosphorylation of SHC and the association of GRB2 with SHC. Furthermore, the antibodies inhibit the association of SHC with HER3 and the heterodimerization between HER2 and HER3. These data show that downstream signalling is inhibited by 1B4C3 and 2D1D12, albeit with 2D1D12 having an even stronger inhibitory effect than 1 B4C3.

9. Monoclonal antibody 2D1D12 inhibits proliferation of breast cancer cell lines MDA-MB-435S, ZR-75-1 and melanoma cell line Mel Gerlach

[0039] We next set out to explore the biological activity of 1 B4C3 and 2D1D12 in the two breast cancer cell lines MDA-MB-435S (ATCC HTB-129), ZR-75-1 (ATCC CRL-1500) and the melanoma cell line Mel Gerlach (Klinikum Großhadern, Munich). We have chosen the cell lines due to their tumorigenicity in nude mice and their high HER3 expression level. It should be noted that melanoma cells overexpress HER3, since HER3 is critical in the development of melanocytes as well as oligodendrocytes. To test our hypothesis that 1B4C3 and 2D1D12 abrogate mitogenic signals and consequently the proliferative properties of cancer cells, we performed BrdU-incorporation assays in the presence or absence of the antibodies (Figure 7). Proliferation was strongly reduced in all cell lines by 2D1D12, whereas 1B4C3 had only an inhibitory effect in Mel Gerlach. Taken together, our data constitute the first evidence that monoclonal antibodies against HER3 could be potentially regarded as new therapeutic weapons against cancer.

[0040] The hybridoma cell lines producing antibodies 1B4C3 and 2D1D12 were deposited on August 07, 2001 and July 24, 2001, respectively, at DSMZ.

10. Effect of HER3 antibodies on signal transduction

10.1. Methods

[0041] MDA-MB-435S were obtained from ATCC (HTB-129) and Mel-Juso were obtained from Cell Lines Service (CLS) (0282-HU). GST-p85 (a.a 333-430) was obtained from Santa Cruz. GST-GRB2 was purified as described previously. Phospho-AKT (P-Ser 473) was from New England Biolabs (NEB). HER2 antibody was purified as described from the hybridoma culture supernatant (ATCC CRL-10463). For GST-pull-down assays 1.25 µg bait protein was used. BrdU-incorporation and invasion assays were performed as previously described. All experiments were performed at least two times.

10.2. Results and Discussion

[0042] In order to examine the surface expression of HER2 and HER3 receptors in MDA-MB-435S and Mel-Juso, we additionally determined their expression level by FACS analysis (Fig. 8A, B). We observed HER2 and HER3 expression in these cell lines and went on to dissect the molecular mechanism by which HER3 antibodies act on Herregulin (HRG) mediated signal transduction. Therefore, we performed GST-pull-down assays in the human breast carcinoma cell line MDA-MB-435S and melanoma cell line Mel-Juso (Fig. 8). Quiescent cells were pretreated with HER3 antibodies 1 B4C3, 2D1D12, the control anti-HER2 antibody and with PI(3)K inhibitor.
LY294002 and were subsequently stimulated with β-HRG. After cell lysis, protein levels were normalized and since HER3 has six potential p85 binding sites a GST-pull-down assay with GST-p85 (a.a. 333-430) as bait was performed. Western blot against phosphotyrosine (PY), reveals that anti-HER2 and 1B4C3 equally decrease p85 association with transactivated HER3, while LY294000 (negative control) has no inhibitory effect on p85 binding in MDA-MB-435S (Fig. 8C). However, 2D1D12 almost completely abrogates binding of p85 with HER3 in MDA-MB-435S (Fig. 8C).

[0043] In the human melanoma cell line Mel-Juso 1B4C3 and 2D1D12 equally decrease p85 association with HER3, while anti-HER2 exhibits a more pronounced decrease in receptor association of p85 (Fig. 8D). Again, LY294002 showed no inhibitory effect on p85 binding (Fig. 8D). Moreover, we observed some prominent tyrosine phosphorylated bands in the phosphotyrosine blot at 125kDa and 66kDa in MDA-MB-435S (Fig. 8D). Furthermore, we investigated the activation status of AKT, which is a downstream target of PI(3)K and has an important role in cell survival. We observed that anti-HER2, 1B4C3 and 2D1D12 markedly inhibited AKT phosphorylation in Mel-Juso melanoma cells (Fig. 10B upper panel). In MDA-MB-435S breast cancer cells both HER2 and HER3 antibodies significantly decreased AKT phosphorylation (Fig. 10C). LY294002 served as the positive control. This observation is of major importance, since breast cancer patients with markedly increased expression of activated AKT are more prone to relapse with distant metastasis resulting in poor clinical outcome (Perez-Tenorio G et al. British Journal of Cancer, 86, 540-545 (2002)).

Monoclonal antibodies 1B4C3 and 2D1D12 inhibit proliferation and migration of breast cancer cell lines MDA-MB-435S and melanoma cell line Mel-Juso

[0048] To evaluate the inhibitory function of 1B4C3 and 2D1D12 on cell cycle progression and tumor invasion, we performed BrdU-Incorporation and invasion assays (Fig. 11).

[0049] We saw a robust decrease in β-HRG stimulated BrdU-incorporation in MDA-MB-435S and Mel-Juso cells pretreated with 2D1D12 (Fig. 11A). Invasion assays revealed that anti-HER3 antibodies 2D1D12 and 1B4C3 substantially decreased invasiveness of MDA-MB-435S breast cancer and Mel-Juso melanoma cells. Surprisingly HER2 antibody 4D5 only showed an inhibition in MDA-MB-435S but not in the melanoma cell line Mel-Juso although the receptor is expressed at the cell surface (Fig. 11 B, C and Fig. 8A, B). Our results suggest the use of anti-HER3 antibodies for the treatment of breast cancer and melanoma.

Monoclonal antibody 2D1D12 inhibits Heregulin-stimulated phosphorylation of PYK2

[0050] We previously demonstrated that the intracellular tyrosine kinase PYK2 associates with and is phos-
phorylated by HER3, suggesting that PYK2 functions as a mediator of HER3 activities. Consistent with this, dominant-negative PYK2 inhibited HRG-mediated invasion of glioma cells. Therefore, we wanted to explore the effect of anti-HER3 antibodies on HRG-induced PYK2 tyrosine phosphorylation. We pretreated quiescent SF767 human glioma cells with anti-HER2, 1B4C3 and 2D1D12 and subsequently stimulated the cells with α-HRG. After lysis and normalising for equal protein amounts, we immunoprecipitated PYK2 and blotted against phosphotyrosine (PY). We observed that whereas anti-HER2 and 1B4C3 had no inhibitory effect on tyrosine phosphorylation of PYK2, 2D1D12 markedly decreased PYK2 tyrosine phosphorylation (Fig. 12A). Thus, anti-HER3 antibodies are effective in inhibiting HRG-induced tyrosine phosphorylation of PYK2.

[0051] By probing immunoblots of WCL with phospho-ERK antibodies we observed that pretreating the cells with anti-HER2, 1B4C3 and 2D1D12 inhibited α-HRG activated ERK2 phosphorylation (Fig. 12B middle panel). Reproofing with ERK antibodies confirmed equal amount of loaded protein (Fig. 12B lower panel). Again, our data show that HER3 antibodies downregulate HRG-mediated signaling events in MDA-MB-435S, Mel-Juso and SF767. Furthermore, our analysis suggests that antibodies directed against ectodomains of HER2 and HER3 modulate differential signaling, leading to distinct responses of downstream effector proteins.

Claims

1. A pharmaceutical composition comprising as an active agent an inhibitor of HER3 activity and pharmaceutically acceptable carriers, diluents and/or adjuvants, wherein binding of said inhibitor to HER3 reduces HER3 mediated signal transduction, wherein said reduction of signal transduction is caused by a down-regulation of HER3, which results in at least partial disappearance of HER3 molecules from the cell surface, wherein said inhibitor is an anti-HER3-antibody.

2. The composition of claim 1, wherein the inhibitor does not compete with the binding of Heregulin to HER3.

3. The composition of claim 1, wherein said antibody is a monoclonal antibody or a fragment thereof, which causes downregulation of HER3.

4. The composition of claim 1, wherein said antibody is a recombinant antibody or antibody fragment, which causes downregulation of HER3.

5. The composition of claim 4, wherein said recombinant antibody or antibody fragment, which causes downregulation of HER3, is selected from chimerized antibodies, humanized antibodies, single chain antibodies and fragments thereof.

6. The composition of claims 1-5, wherein said antibody is coupled to a labelling group or an effector group, wherein the labelling group is a radioactive group or a fluorescent group and the effector group is a cytotoxic group.

7. The composition of claims 1-6, wherein the antibody is 1B4C3 produced by the hybridoma cell line DSM ACC 2527, fragments thereof or recombinant derivatives thereof, which cause downregulation of HER3.

8. The composition of any one of claims 1-7 comprising a further active agent, wherein the active agent is selected from inhibitors of other receptor tyrosine kinases or cytotoxic agents.

9. The composition of any one of claims 1-8 for diagnostic applications.

10. The composition of any one of claims 1-8 for therapeutic applications.

11. Use of an anti-HER3 antibody inhibiting HER3 activity as defined as in any one of claims 1-7 for the manufacture of an agent for the diagnosis, prevention or treatment of tumour diseases, wherein HER3 activity is inhibited by reduction of signal transduction caused by a down regulation of HER3, which results in at least partial disappearance of HER3 molecules from the cell surface.

12. The use of claim 11, wherein the antibody is 1B4C3 produced by the hybridoma cell line DSM ACC 2527, fragments thereof or recombinant derivatives thereof, which cause downregulation of HER3.

13. The use of any one of claims 11 or 12 for the diagnosis, prevention or treatment of breast cancer, gastrointestinal cancer, pancreas cancer, prostate cancer, glioma, melanoma or formation of tumor metastases.

14. The use of any one of claims 11-13, wherein said disease is caused by HER3 expression or overexpression and associated with increased HER3 phosphorylation with respect to normal cells.

15. The use of any one of claims 11-14, wherein said disease is caused by HER3 expression or overexpression and associated with increased HER2/HER3 heterodimerization with respect to normal cells.

16. The use of any one of claims 11-15, wherein said
disease is caused by HER3 expression or overexpression and associated with an increased activity of PI3-kinase, c-jun-terminal kinase, AKT, ERK2 and/or PYK2 with respect to normal cells.

17. An in vitro method for identifying a novel agent for diagnosing, preventing or treating a hyperproliferative disease, particularly a tumour disease comprising assaying if a candidate compound is capable of inhibiting HER3 activity, wherein binding of said inhibitor to HER3 reduces HER3 mediated signal transduction, wherein said reduction of signal transduction is caused by a down-regulation of HER3, which results in at least partial disappearance of HER3 molecules from the cell surface.

18. Hybridoma cell line DSM ACC 2527 and cell line derived therefrom producing antibodies which inhibit HER3 activity, wherein HER3 activity is inhibited by reduction of signal transduction caused by a down regulation of HER3, which results in at least partial disappearance of HER3 molecules from the cell surface.

19. Antibody produced by a cell line of claim 18 which inhibits HER3 activity, wherein HER3 activity is inhibited by reduction of signal transduction caused by a down regulation of HER3, which results in at least partial disappearance of HER3 molecules from the cell surface.

Patentansprüche

1. Pharmazeutische Zusammensetzung umfassend als Wirkstoff einen Inhibitor von HER3-aktivität und pharmazeutisch annehmbare Träger, Verdünnungsmittel und/oder Adjuvanzien, wobei die Bindung des Inhibitors an HER3-HER3-vermittelte Signaltransduktion verringert, wobei die Verringerung der Signaltransduktion verursacht wird durch eine Abregulierung von HER3, welche zum wenigstens teilweisen Verschwinden von HER3-Molekülen von der Zelloberfläche führt, wobei der Inhibitor ein Anti-HER3-Antikörper ist.

2. Die Zusammensetzung nach Anspruch 1, wobei der Inhibitor nicht mit der Bindung von Heregulin an HER3 konkurriert.

3. Die Zusammensetzung nach Anspruch 1, wobei der Antikörper ein monoklonaler Antikörper oder ein Fragment davon ist, welcher eine Abregulierung von HER3 verursacht.

4. Die Zusammensetzung nach Anspruch 1, wobei der Antikörper ein rekombinanter Antikörper oder ein Antikörperfragment ist, welcher eine Abregulierung von HER3 verursacht.


6. Die Zusammensetzung nach einem der Ansprüche 1-5, wobei der Antikörper gekoppelt ist an eine Markierungsgruppe oder an eine Effektorgruppe, wobei die Markierungsgruppe eine radioactive Gruppe oder eine fluoreszierende Gruppe ist und die Effektorgruppe eine zytotoxische Gruppe ist.

7. Die Zusammensetzung nach einem der Ansprüche 1-6, wobei der Antikörper 1 B4C3, erzeugt durch die Hybridomzelllinie DSM ACC 2527, Fragmente davon oder rekombinante Derivate davon ist, welche eine Abregulierung von HER3 verursachen.

8. Die Zusammensetzung nach einem der Ansprüche 1-7, umfassend einen weiteren Wirkstoff, wobei der Wirkstoff ausgewählt ist aus Inhibitoren anderer Rezeptortyrosinkinasen oder zytotoxischen Mitteln.


12. Die Verwendung nach Anspruch 11, wobei der Antikörper 1 B4C3, erzeugt durch die Hybridomzelllinie DSM ACC 2527, Fragmente davon oder rekombinante Derivate davon ist, welche eine Abregulierung von HER3 verursachen.


14. Die Verwendung nach einem der Ansprüche 11-13, wobei die Erkrankung verursacht wird durch HER3-
1. Composition pharmaceutique comprenant comme
agent actif un inhibiteur de l’activité de HER3 et des vecteurs, diluants et/ou adjuvants pharmaceutiques, où la liaison dudit inhibiteur à HER3 réduit la transduction de signaux à médiation par HER3, où ladite réduction de la transduction de signaux est causée par une régulation négative de HER3 qui

5. Composition selon la revendication 1 où l’inhibiteur n’entre pas en compétition avec la liaison de l’héréguline avec HER3.

10. Composition selon la revendication 1 où ledit anticorps est un anticorps monoclonal ou un fragment d’anticorps qui provoque une régulation négative de HER3.

15. Composition selon la revendication 4 où ledit anticorps recombinant ou fragment d’anticorps qui provoque une régulation négative de HER3 est choisi parmi les anticorps chimérisés, les anticorps humannisés, les anticorps à une seule chaîne et leurs fragments.

20. Composition selon les revendications 1-5 où ledit anticorps est couplé à un groupe marqueur ou un groupe effecteur où le groupe marqueur est un groupe radioactif ou un groupe fluorescent et le groupe effecteur est un groupe cytotoxique.

25. Composition selon les revendications 1-6 où l’anticorps est 1B4C3 produit par la lignée cellulaire d’héridrome DSM ACC 2527, des fragments de celui-ci ou des dérivés recombinants de celui-ci, qui provoquent une régulation négative de HER3.

30. Composition selon l’une quelconque des revendications 1-7 destinée à des applications thérapeutiques.

35. Composition selon l’une quelconque des revendications 1-8 destinée à des applications diagnostiques.

40. Composition selon l’une quelconque des revendications 1-8 destinée à des applications thérapeutiques.

45. Utilisation d’un anticorps anti-HER3 inhibant l’activité de HER3 selon l’une quelconque des revendications 1-7 pour la fabrication d’un agent pour la diagnostics, la prévention ou le traitement des maladies tumorales, où l’activité de HER3 est inhibée par une réduction de la transduction de signaux causée par une régulation négative de HER3, qui conduit à la disparition au moins partielle des molécules de HER3 de la surface cellulaire.
12. Utilisation selon la revendication 11 où l’anticorps est 1B4C3 produit par la lignée cellulaire d’hybridome DSM ACC 2527, des fragments de celui-ci ou des dérivés recombinants de celui-ci, qui provoquent une régulation négative de HER3.


14. Utilisation selon l’une quelconque des revendications 11-13 où ladite maladie est causée par l’expression ou la surexpression de HER3 et associée avec une phosphorylation de HER3 accrue par rapport aux cellules normales.

15. Utilisation selon l’une quelconque des revendications 11-14 où ladite maladie est causée par l’expression ou la surexpression de HER3 et associée avec une hétérodimérisation HER2/HER3 accrue par rapport aux cellules normales.

16. Utilisation selon l’une quelconque des revendications 11-15 où ladite maladie est causée par l’expression ou la surexpression de HER3 et associée avec une activité de PI3-kinase, kinase c-jun-terminale, AKT, ERK2 et/ou PYK2 accrue par rapport aux cellules normales.

17. Procédé in vitro pour identifier un nouvel agent pour le diagnostic, la prévention ou le traitement d’une maladie hyperproliférative, en particulier une maladie tumorale comprenant un test pour déterminer si un composé candidat est capable d’inhiber l’activité de HER3, où la liaison dudit inhibiteur à HER3 réduit la transduction de signaux à médiation par HER3, où ladite réduction de la transduction de signaux est causée par une régulation négative de HER3 qui conduit à la disparition au moins partielle des molécules de HER3 de la surface cellulaire.

18. Lignée cellulaire d’hybridome DSM ACC 2527 et lignée cellulaire dérivée de celle-ci produisant des anticorps qui inhibent l’activité de HER3, où l’activité de HER3 est inhibée par une réduction de la transduction de signaux causée par une régulation négative de HER3 qui conduit à la disparition au moins partielle des molécules de HER3 de la surface cellulaire.

19. Anticorps produit par une lignée cellulaire selon la revendication 18 qui inhibe l’activité de HER3, où l’activité de HER3 est inhibée par une réduction de la transduction de signaux causée par une régulation négative de HER3 qui conduit à la disparition au moins partielle des molécules de HER3 de la surface cellulaire.
Figure 2
Htun van der Horst et al.

A

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220 kDa —
116 kDa —
97 kDa —
66 kDa —
42.5 kDa —
29 kDa —

WB: α-PY
Figure 1
Htun-van der Horst et al.
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**Figure B**

- 116 kDa
- 97 kDa
- 66 kDa
- 42.5 kDa
- 29 kDa

**WB:** α-PY

**WB:** α-HER2
Figure 1
Htun-van der Horst et al.
Figure 1
Htun-van der Horst et al.
Figure 2
Htun-van der Horst
Figure 2
Htun-van der Horst
Figure 2
Htun-van der Horst et al.
Figure 2
Htin-van der Horst et al.

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WB: α-PY
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Figure 3

Htun-van der Horst et al.

A

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WB: α-PY

WB: α-SHC

WB: α-JNK1 (p46)

← p52 SHC

← p52 SHC

← IgG

← p46 JNK1

← 32P-GST-c-jun
Figure 3
Htun-van der Horst et al.
Figure 3

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WB: α-PY

p52 SHC

WB: α-SHC

α-JNK1

WB: α-JNK1 (p46)

IgG

p46 JNK1

32P-GST-c-jun
Figure 3
Htun-van der Horst et al.
Figure 3
Htun-van der Horst et al.
Figure 4
Htun-van der Horst et al.
Figure 4
Htun-van der Horst et al.
Figure 5

Htun-van der Horst et al.

A

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α-HER3ECD:

IP: α-HER3

WB: α-Strep.

WCL:

WB: α-PY

Lane: 1 2 3 4 5 6 7 8 9 10

HER3
Figure 5
Htun-van der Horst et al.

B

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α-HRG

α-HER2

WB: α-Strep.

WCL:

WB: α-PY

Lane: 1 2 3 4 5 6 7 8 9 10

HER2
Figure 6

Hun van der Horst et al.

A

Tunicamycin [10 μg/ml]:

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HER3 (glycos.)

HER3 (deglycos.)

WB: α-HER3

B

T[h]:

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IP: α-HER3

HER3

WB: α-Streptavidin
Figure C

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116 kDa —
97 kDa —
66 kDa —

WB: α-PY
WB: α-SHC

← HER3
← SHC
Figure 7
Htin van der Horst et al.

Proliferation-assay (BrdU-Inc.) - Mel Gerlach

Absorption 450/690

NS  a-HRG  b-HRG  #2  a-HRG  #2 b-HRG  #3  a-HRG  #3 b-HRG
Figure 7
Htun van der Horst et al.
Figure 8
Myc (control) MDA-MB-435S
Htun van der Horst et al.

Figure 8
Myc (control) Mel Juso
Figure 8
Supplementary Data
Htun van der Horst et al.
Figure 8
Supplementary Data
Htun van der Horst et al.
Figure 8
Supplementary Data
Htun van der Horst et al.
Figure 8
Supplementary Data
Htun van der Horst et al.
Figure 9
Supplementary Data
Htin van der Horst et al.

β-HRG

LY294002
RTF8
1B4C3
2D1D12

- - - - + + + +

GST-GRB2

= p185
p160

- - - - - - -

= p66

WB α-PY
Figure 9
Supplementary Data
Htun van der Horst et al.
Supplementary Data

Figure 9

β-HRG

LY294002
RTF8
1B4C3
2D1D12

GST-GRB2

LY294002
RTF8
1B4C3
2D1D12

WB \( \alpha \)-PY

- p185

- p160

- p66
Figure 9
Supplementary Data
Htun van der Horst et al.
Figure 10
Supplementary Data
Htun van der Horst et al.

A

WCL

β-HRG

- - - - - + + + + - - - - + + + + + + +

WB α-PY

Mel Juso Lane 1-8
MDA-MB-435S Lane 9-16

WB α-P-ERK (T202/Y204)

WB α-ERK

= β44

= β42

= β44

= β42
Figure 10
Supplementary Data
Htun van der Horst et al.
Figure 10
Supplementary Data
Htun van der Horst et al.
Figure 11
Supplementary Data
Htun van der Horst et al.

A

**Mel Juso**

- Control
- Anti-HER3

**MDA-MB-435S**

- Control
- Anti-HER3

- Unstimulated
- Heregulin
Figure 11
Supplementary Data
Htun van der Horst et al.
Figure 11
Supplementary Data
Htun van der Horst et al.
Figure 12
Supplementary Data
Htun van der Horst et al.
REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description