(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 May 2001 (25.05.2001)

PCT

(10) International Publication Number
WO 01/35899 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/EP00/11444

(22) International Filing Date:
17 November 2000 (17.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

99123042.6	19 November 1999 (19.11.1999)	EP
09/448,013	23 November 1999 (23.11.1999)	US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/35899 A2

(54) Title: INHIBITORS OF HELICOBACTER PYLORI INDUCED GASTROINTESTINAL DISEASES

(57) Abstract: The invention refers to a method for the manufacture of a medicament for treating or preventing Helicobacter mediated diseases in a mammal and a method for treating or preventing Helicobacter mediated diseases.

Inhibitors of Helicobacter pylori induced gastrointestinal diseases

Description

The invention refers to a method for the manufacture of a medicament for treating or preventing Helicobacter mediated diseases in a mammal and a method for treating or preventing Helicobacter mediated diseases.

Helicobacter pylori (H.pylori) is a pathogen which is known to induce gastrointestinal diseases. Recent therapy against H.pylori includes a combination therapy comprising the application of two of the following antibiotics: tetracycline, amoxicillin, clarithromycin or metronidazole combined with an inhibitor of the proton pump like ranitidine or a bismuth salt, e.g. bismuth citrate. However, emerging resistance against metronidazole among Helicobacter strains is already found worldwide and correlates with treatment failure (Alarcon et al., Int. J. Antimicrob. Agents 1 (1999), 19-26).

H.pylori fights the acidic gastric juice of the stomach by releasing urease which converts gastric urea into bicarbonate and ammonia. As a consequence, peripheral regulation of gastric acid secretion is initiated by the release of gastrin from G-cells, employing the gastrin receptor, a G-protein coupled receptor (GPCR). In addition, H.pylori is crucially involved in the upregulation of gastric epithelial interleukin 8 (IL-8) expression. This upregulated epithelial IL-8 secretion and concommitant chemotaxis and activation of immunocompetent cells are discussed to be involved in tissue damage, ulceration and gastric carcinogenesis (Crabtree & Lindley, Eur. J. Gastroenterol. Hepatol. 6 Suppl 1 (1994), 33-38; Crabtree et al., J. Clin. Pathol. 48 (1995), 41-45; Crabtree, Aliment. Pharmacol. Ther. 10 Suppl. 1 (1996), 29-37).

Within H.pylori strains, there is division into two major groups: those having genetic loci coding for immunodominant proteins of uncertain functions (cagA islands; cagA + strains) and those lacking this genetic area (Crabtree et al. (1995), Supra; cagA- strains). This cagA pathogenicity island is supposed to be responsible for the disease induction by H.pylori, as the H.pylori strains, which lack this gene region, are still infectious, but the patients remain asymptomatic (Akopyants et al., Mol. Microbiol. 28 (1998), 37-53).

By hybridizing cDNA arrays with complex cDNA probes of human gastric cancer cell lines infected or uninfected with H.pylori cagA- (TX30a, ATCC 51932)/cagA + (60190, ATCC 49503) strains the following results were obtained: upregulation of cytokines, upregulation of ADAM20, a gene of the ADAM family (proteins containing a disintegrin and metalloprotease domain) as well of members of the epidermal growth factor (EGF) family like heparin-binding EGF (HB-EGF), amphiregulin and transforming growth factor (TGF)-alpha occurred. In sharp contrast to the results of Akopyants et al. our results demonstrate, that not only cagA + strains of H.pylori are responsible for disease development, but also cagA- strains show the same molecular effects as cagA + strains by employing the triple membrane passing signaling cascade published by Prenzel et al., Nature 402 (1999), 884-888.

There are about 500,000 cases of gastric cancer each year in Japan and about 200,000 in Europe (SCRIP No 2467, p.18), many of them possibly caused by chronic H.pylori infections. Therefore we reasoned that interfering with the cascade of events caused by cagA-/cagA + H.pylori strains should inhibit the deleterious chronical effects of the pathogen.

Thus, the present invention refers to a method for the manufacture of a medicament for treating or preventing Helicobacter mediated diseases in a mammal, wherein said medicament comprises as an active ingredient at

least one compound selected from

(a) inhibitors of gastrin/cholecystokinin (CCK)-B receptor,

(b) inhibitors of protein kinase C,

(c) inhibitors of membrane-associated metalloproteinases,

(d) inhibitors of growth-factor receptor activation,

(e) inhibitors of growth-factor receptor kinase activity,

(f) inhibitors of the mitogen-activated protein kinase cascade and

(g) transcription inhibitors.

The active ingredient is preferably a compound capable of inhibiting the"triple membrane passing signal" cascade. By means of this inhibition, a pathogenic stimulation of this cascade caused by H.pylori infection resulting in a pathogenic phenotype will be at least partially suppressed. This leads to the improvement or the disappearance of clinical symptoms associated with H.pylori infections.

The Helicobacter induced diseases to be treated or prevented are preferably selected from gastrointestinal cancer and inflammatory conditions, e.g. chronic non-erosive gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma and intestinal-type adenocarcinoma.

The active ingredient is selected from compounds (a) to (g). The medicament may comprise only one active ingredient or several active ingredients which may be selected from compounds which are directed to the same target or from compounds which are directed to two or more different targets.

The inhibitors of gastrin/cholecystokinin (CCK)-B receptor (a) are preferably selected from nonapeptide CCK-B antagonists, amino acid derivatives, benzodiazepines, dipeptoids, pyrazolidinones, quinazolinones, ureidoacetamides, bicyclic compounds such as dibenzobicyclo [2.2.2]octane, aromatic compounds such as binaphthalene derivatives,

ureidobenzazepines and ureido methylcarbamoyl phenylketones as described by Revel and Makovec (Drugs of the Future 23 (1998), 751-766). Further suitable compounds are described by Crawley (J. Neuroscience 12 (1992), 3380-3391) and Hughes et al. (Proc. Natl. Acad. Sci. U.S.A. 87 (1990), 6728-6732). The disclosure of these documents is herein incorporated by reference.

Specific examples of amino acid derivatives are glutamic acid derivatives such as spiroglumides, e.g. CR-2622 (Makovec et al., J. Med. Chem. 39 (1996), 135-142). Specific examples of benzodiazepines are L-365260 (Bock et al., J. Med. Chem. 32 (1989), 13-16), YM-022 (Satoh et al., Chem. Pharm. Bull. (Tokyo) 43 (1995), 2159-2167) and GR-199114X (Bailey et al., Bioorg. Med. Chem. Lett. 7 (1997), 281-286). Specific examples of pyrazolidinones are LY-288513 and LY-262691 (Helton et al., Pharmacol. Biochem. Behav. 53 (1996), 493-502). A specific example of quinazolinones is LY-247348 (Yu et al., J. Med. Chem. 34 (1991), 1505-1508). Specific examples of dipeptoids are PD-136450, CI-988 and PD-134308 (Boden et al., J. Med. Chem. 36 (1993), 552-565; Maldonado et al., Br. J. Pharmacol. 114 (1995), 1031-1059). Specific examples of ureidoacetamides are RP-69758 and RP-73870 (Pendley et al., J. Pharmacol. Exp. Ther. 273 (1995), 1015-1022). Specific examples of ureidobenzazepines are 5-phenyl-3-ureidobenzazepin-2-ones such as CP-212454 (Lowe III et al., J. Med. Chem. 37 (1994), 3789-3811). A specific example of ureidomethylcarbamoylphenylketones is S-0509 (Hagishita et al., Bioorg. Med. Chem. 8 (1997), 1695-1714). The disclosure of these documents is herein incorporated by reference.

Inhibitors of protein kinase C (b) may be ATP-competitive inhibitors, antisense oligonucleotides, peptides and lipids. Particularly preferred are indolocarbazoles, bisindolylmaleimides, balanol analogs, antisense oligonucleotides and alkyl-lysophospholipids (Goekjian and Jirousek, Curr. Med. Chem. 6 (1999), 877-903). The disclosure of this document is herein

incorporated by reference.

Specific examples of indolocarbazole compounds are e.g. the compounds dislosed in EP-A-0328000, EP-A-0370236, EP-A-0410389 and EP-A-0434057 or the compounds disclosed in EP 99116426.0. Specific examples of suitable indolocarbazoles are Gö7612 and CGP41251 (Ikegami et al., Jpn. J. Pharmacol. 70 (1996), 65-72). Specific examples of bisindolylmaleimides are LY333531, GF109203x, Ro32-0432 and Ro31-8220 (Jirousek et al., J. Med. Chem. 39 (1996), 2664-2671). A specific example of balanol analogs is SPC100840 (Goekjian and Jirousek, Supra). A specific example of an antisense oligonucleotide is CGP64128A (Goekjian and Jirousek, Supra). A specific example of alkyl-lysophospholipids is ET-18-OCH3 (Civoli and Daniel, Cancer Chemother. Pharmacol. 42 (1998), 319-326). The disclosure of these documents is herein incorporated by reference.

Inhibitors of membrane-associated metalloproteinases (c), particularly members of the ADAM family, or TACE (Moss et al., J. Neuroimmunol. 72 (1997), 127-129) may be selected from inhibitors of proteinase activity or compounds which block the metalloproteinase's access to its substrate, e.g. by masking the substrate. These inhibitors may be selected from e.g. compounds containing a hydroxamic acid group. Specific examples of hydroxamic acid derivatives are GW9471 and GW9277 (Moss et al., J. Neuroimmunol. 72 (1997), 127-129), BB-94 (Batimastat), BB-2516 (Marimastat) and BB-1101 (Wojtowicz-Praga et al., Invest. New Drugs 15 (1997), 61-75), CT1418 (Epps et al., J. Protein Chem. 17 (1998), 699-712), N-(D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl)-L-3-(2'naphthyl)-alanyl-L-alanine,2-aminoethylamide (Mohler et al., Nature 370 (1994), 218-220), 2,3,4,5-tetrahydro-1H-[1,4]-benzodiazepine-3-hydroxamic acid (WO0044730), sulfonamido bicyclic heteroaryl hydroxamic acids (EP1021413), ortho-sulfonamido heteroaryl hydroxamic acids (US5962481), sulfonamido aryl hydroxamic acids (EP0938471),

arylsulfonamido substituted hydroxamic acids (EP0873312, WO9600214), hydroxamic acid derivatives (US6118001), GI129471 (McGeehan et al., Nature 370 (1994), 558-561) and CGS27023A and RO31-9790 (Lombard et al, Cancer Res 58 (1998), 4001-4007). Especially alkynyl and/or sulfonamide containing thiols and hydroxamic acids have been determined as potent tumor necrosis factor-alpha converting enzyme (TACE) inhibitors (WO0009492). For instance the following thiol and hydroxamic acid derivatives have been used as TACE inhibitors: acetylated ortho-sulfonamido or phosphinic acid amido bicyclic heteroaryl hydroxamic acids (WO0044749), heteroaryl acetylenic sulfonamide or phosphinic acid amide hydroxamic acids (WO0044740), acetylenic sulfonamide thiols (WO0044716), alkynyl containing hydroxamic acids (WO0044713), acetylenic (ß)-sulfonamido or phosphinic acid amide hydroxamic acids (WO0044711), acetylenic aryl sulfonamide and phosphinic acid amide hydroxamic acids (WO0044710), acetylenic alpha-amino acid based sulfonamide hydroxamic acids (WO0044709), beta-sulfonamido hydroxamic acids (US5977408), ortho-sulfonamido bicyclic heteroaryl hydroxamic acids (WO9918076), ortho-sulfonamido heteroaryl hydroxamic acids (WO9816520), beta-sulfonamido hydroxamic acids (WO9816506) or ortho-sulfonamido aryl hydroxamic acids (WO9816503).

Furthermore, orther classes of chemical compounds have been proved to be potent MMP (matrix metalloproteinase) inhibitors, e.g. lactams, alkenyldiarylmethanes, amides and sulfonamides, azepans or carboxylic acids and derivatives thereof. As members of the lactam family have been cited N-carboxymethyl substituted benzolactams (AU1836900) or 3-thio substituted amidolactams (AU1926700). Alkenyldiarylmethanes are mentioned in WO9936384, WO9740072 and GB748400. Suitable amides, sulfonamides or succinamides for MMP inhibiton are N-hydroxyformamide (WO0044712), amidomalonamides (WO0040552), N-hydroxy-2-(alkyl, aryl or heteroaryl sulfanyl, sulfinyl or sulfonyl)-3-substituted alky, aryl or heteroarylamides (CN1252713T), CN1252790T),

thiadiazole amides (WO9748688), amino acid amides of 5-amino-1,3,4-thiadiazones (WO9640745); sterically hindered sulfonamides (US6114568), cyclic sulfonamide derivatives (WO9941246), biphenylsulfonamides (CN1219166); heteroaryl succinamides (EP0937042), N-(amino acid) substituted succinic acid amides (WO9703966), succinamide derivatives (WO9633161). Also azepan derivatives like 1-caboxymethyl-2-oxoazepan derivatives or 3-mercaptoacetylamino-1,5-substituted-2-oxoazepans have been shown to exhibit MMP inhibitor activity. In addition to these classes of chemical compounds carboxylic acids, carboxylic acid derivatives and carboxylic acid group containing substances have been cited as metalloproteinase inhibitors. Cited members of the carboxylic acid family are carboxylic acid derivatives (US6118001), carboxylic acid substituted heterocycles (EP1042297, WO9932452), aminomalonic acid derivatives (WO0002904), butyric acid (US6020366), malonic acid (WO9911608), bile acid (US5646316). The following substances or substance classes have also been mentioned as MMP inhibitors: sulfonated amino acids (AU3022200, EP0757037), phosphono derivatives of amino acids (WO9314096), natural amino acid derivatives (US5643964), N-sulfonylamino derivatives of dipeptides (US5530128), peptidyl compounds (US5981490), US5300501), arylpiperazines (WO0012478), N-hydroxyacylamino compounds (WO0012467), hydroxylamine derivatives (HU9904165), sulfonylaminophosphinic and phosphonic acids (DE19831980), C-terminal ketones (US5985911) macrocyclic compounds (US5952320), aporphinoid (WO9916441), dioxolane (EP075688), mercaptoketones and mercaptoalcohols (EP0818443), mercaptosulfides (US5455262), thiadiazolyl(thio)ureas (WO9740031), sulfoximines and sulfodiimines (US5470834). The disclosure of the above-mentioned patent documents is herein incorporated by reference.

Further examples are members of the TIMP (tissue inhibitor of metalloproteinase) family e.g. TIMP-1 and TIMP-2 (Lombard et al., Supra).

The disclosure of these documents is herein incorporated by reference.

Inhibitors of growth-factor receptors (d) may be inhibitors of members of the epidermal growth factor receptor family, e.g. EGF receptor, HER2, HER3, HER4 or inhibitors of other growth-factor receptors, e.g. TNF-alpha receptor. The inhibitors of growth-factor receptors may be selected from low-molecular weight compounds or from antibodies which inhibit the binding of receptor ligands to their receptors, particularly of receptor ligands of the EGF-like ligand family, which are released from membrane-bound ligand precursors (f.e. HB-EGF). Specific examples are anti-receptor antibodies or fragments thereof, e.g. the antibody Herceptin or Trastuzumab (Goldenberg, Clin. Ther. 21 (1999), 309-318). The disclosure of this document is herein incorporated by reference.

Inhibitors of growth-factor receptor kinase activity (e) may be selected from phenylaminoquinazolines, substituted pyrimidines comprising pyrido-, pyrrolo-, pyrazolo-, pyrimido- and phenylaminopyrimidines, tyrphostins, lavendustins and dianilino-phthalimides (Traxler and Lydon, Drugs of the Future 20 (1995), 1261-1274), the disclosure of which is herein incorporated by reference.

Specific examples of phenylaminoquinazolines are PD153035 (Fry et al., Science 265 (1994), 1093-1095), ZD1839 (Woodburn et al., Proc. Am. Assoc. Cancer Res. 38 (1997), 633) and CP-358,774 (Proc. Am. Assoc. Cancer Res. 38 (1997), 633). Specific examples of substituted pyrimidines are PD158780 (Rewcastle et al., J. Med. Chem. 39 (1996), 1823-1835), PD166285 (Panek et al., J. Pharmacol. Exp. Ther. 283 (1997), 1433-1444), CGP59326, CGP60261 and CGP62706 (Traxler et al., J. Med. Chem. 40 (1997), 3601-3616). Specific examples of tyrphostins are AG1478, RG13022 and AG825 (Levitzki and Gazit, Science 267 (1995), 1782-1788). A specific example of lavendustins is Lavendustin A (Onoda et al., J. Nat. Prod. 52 (1998), 1252-1257). A specific example of

dianilino-phthalimides is CGP54698 (Buchdunger et al., Clin. Cancer Res. 8 (1995), 813-821). The disclosure of these documents is incorporated herein by reference.

Inhibitors of the mitogen-activated protein cascade (f) are preferably selected from inhibitors of MAPKKK (Raf), inhibitors of MAPKK (Mek) and inhibitors of MAPK (Erk). Specific examples are PD098059 (Dudley et al., Proc. Nat. Acad. Sci. U.S.A. 92 (1995), 7686-7689; He et al., Cell Growth Differ. 10 (1999), 307-315), U0126 (Favata et al., J. Biol. Chem. 273 (1998), 18623-18632) and SB203580, L167307, RWI68354, SK&F86002, SC102, SB226882, VK19911 and RWI67657 (Bhagwat et al, DDT 4 (1999), 472-479). The disclosure of these documents is incorporated herein by reference.

Transcription inhibitors (g) may be general transcription inhibitors or transcription inhibitors which have a selectivity for inhibiting the transcription of the c-fos gene, e.g. antisense oligonucleotides.

The administration of compounds (a) to (g) may be part of a combination therapy wherein several compounds belonging to the same inhibitor class and/or belonging to different inhibitor classes are employed. Furthermore, the therapy may comprise the administration of a further active ingredient which is effective against Helicobacter infections. This further active ingredient may be selected from antibiotics, e.g. tetracycline, amoxicillin, pantoprazole, clarithromycin or metronidazole, proton pump inhibitors such as ranitidine, lansoprazole, omeprazole, rabeprazole, benzimidazoles or bismuth salts and H.pylori vaccines, e.g. recombinant urease or urease subunit vaccines. The further active ingredient may also be an immunogenic adjuvant, e.g. a compound which provides a general or unspecific immune stimulation, e.g. an immune adjuvant such as aluminum hydroxide or particularly a CpG-motif containing adjuvant (cf. WO96/02555; WO98/40100; WO99/51259) or a cytokine. It should be

noted that the respective active ingredients of a combination medicament may be administered together or separately.

The dosage and type of administration depend on the degree of the disease and on the medicament administered each and or combination of medicaments administered each. In this context it is referred to the documents referenced above. Usually the medicaments are administered over a period of several days and several weeks, respectively, in an effective amount, with the treatment intervals being repeated several times, if necessary.

Further, a method for identifying new medicaments for treating or preventing Helicobacter mediated diseases in a mammal is provided. This method comprises a screening assay for compounds of the classes (a) to (g) as defined above. This screening assay is based on an identification of modulators of targets molecules selected from CCK-B receptor, protein kinase C, membrane-associated metalloproteinases, growth-factor receptors and members of the mitogen-activated protein kinase cascade and c-fos. The screening method may be a cellular assay, e.g. an assay wherein the effect of test compounds on cells overexpressing a target molecule as specified above is determined, or a molecular assay, wherein the effect of test compounds on the target molecule is determined in a cell-free system, and wherein the target molecule may be present in a substantially purified and isolated form.

The invention shall be further illustrated by the following Examples.

Example 1

1. Materials and methods:

1.1 RNA Preparation for hybridizing cDNA arrays: In principle, gastric cancer cell lines were washed after removal of medium with 15 ml ice-cold phosphate-buffered saline (PBS), trypsinized and pelleted in a 15 ml flacon tube for 5 min at 4°C and 2000 rpm. After centrifugation the supernatant was removed and the cells were lysed in 1 ml of TRIZOL reagent per 1.5 x 10^6 cells. The TRIZOL/cell lysate was transferred to an eppendorff tube and centrifuged for 15 min at 4°C and 13000 rpm. The supernatant was transferred into a new eppendorff tube and 0.1 ml of BCP (1-bromo-3-chlorpropane) for each ml of TRIZOL was added. The samples were vortexed for 15 seconds and incubated for 5 min at room temperature. Then the samples were centrifuged for 15 min at 4°C and 13000 rpm. The resulting upper aqueous phase was transferred into a new eppendorff tube, 0.5 ml of isopropanol for each ml of TRIZOL was added; vortexed; and incubated for additional 8 min at room temperature. After centrifugation for 10 min at 4°C and 13000 rpm the supernatant was completely aspired off, the precipitated RNA was washed twice with ice-cold 75% ethanol and air-dried. The RNA pellet was resuspended in 50 µl Tris-HCl pH 7.5. The amount of the RNA was monitored by UV-spectroscopy and the quality was determined via gel electrophoresis on a formaldehyde-containing 1.2% agarose gel.When RNA for Northern Blotting was prepared, the protocol and reaction buffers included with the "RNeasy® Mini Kit" (QIAGEN) were used.

1.2 Preparation of cDNA: 10 µg of total RNA was reverse transcribed into first strand cDNA by adding 1 µg of oligo-dT primer. In a final reaction volume of 12 µl, the mixture was incubated for 5 min at 60°C, and quickly cooled for 2 min on ice, followed by a 30 second

centrifugation at 13000 rpm. 4 µl 5x first strand buffer (250 mM Tris/HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, GibcoBRL, Superscript) 2 µl 0.1 M DTT, 1 µl 10 mM dNTP and 1 µl Superscript II (=200 U) reverse transcriptase were added, incubated first for 10 min at room temperature and then for additional 60 min at 38°C. The reaction was stopped by addition of 5 µl 0.5 M EDTA and 25 µl 0.6 N NaOH.

1.3 Purification of the cDNA reactions using ProbeQuant Sephadex G-50 columns ™ (Amersham): After vortexing the column, the bottom closure was snapped off, the column placed in a 2 ml tube and centrifuged for 1 min at 735 x g. Then the column was placed in a new 1.5 ml eppendorff tube without cap and the sample was pipetted carefully on the center of the preformed resin. After centrifugation for 2 min at 735 x g, the resulting flow-through containing the cDNA was precipitated by adding 1/25 volume 5 M NaCl (final concentration 0.2 M), 1 µl polyacryl carrier, 2.5 volumes 100% ethanol, vortexed, incubated for 10 min on ice and pelleted by centrifugation for 10 min at 4°C with 13000 rpm. Finally the supernatant was removed, the cDNA-pellet washed with 80% ethanol, air-dried and resuspended in 30 µl Tris/EDTA (10 mM/0.1 mM).

1.4 Random primed labeling of cDNA using Random Primers Labeling System (GibcoBRL): 50 ng of cDNA template were denatured for 5 min at 95°C and quickly chilled on ice for 5 min. After spinning down the sample the following ingredients were added to a final volume of 50 µl: 2 µl 0.5 mM dCTP, 2 µl 0.5 mM dGTP, 2 µl 0.5 mM dTTP, 5 µl P^{32}-α-dATP (50 µCi) and 1 µl Klenow-enzyme (3 U/µl). The sample was incubated for 60 min at 25°C and the reaction was stopped by adding 5 µl 0.5 M EDTA, pH 8.0.

1.5 Removal of unincorporated P^{32}-α-dATP-: After vortexing the column, the bottom closure was snapped off, the column placed in a 2 ml tube and centrifuged for 1 minute at 735 x g. Then the column was placed in a new 1.5 ml eppendorff tube without cap and the radioactive sample was pipetted carefully on the center of the preformed resin. Centrifugation for 2 min at 735 x g removed the unincorporated P^{32}-α-dATP nucleotides.

1.6 Prehybridization, preassociation, hybridization and filterwash: Filters were incubated for 5 min at room temperature with 25 ml TE buffer in roller bottles. Following incubation TE solution was replaced by 8 ml prewarmed hybridization-solution (5 x SSPE, 10 x Denhardt's solution, 50% formamide, 1% SDS, 100 µg/ml denatured and fragmented salmon sperm-DNA). Before hybridization, the radioactively labelled probe was pre-associated in a solution containing yeast RNA (final concentration 0.7 µg/µl), polyA (0.7 µg/µl), Cot DNA (0.07 µg/µl), SDS (1%) and 5 x SSPE. Denaturation occured for 5 min at 95°C and was followed by a pre-association step for 60 min at 65°C. Then the preassociated probe was added to the filters and hybridization reaction was performed for 2 days at 42°C. After hybridization, the filters were washed twice with 2 x SSC for 10 min at room temperature, twice with preheated 2 x SSC/0,5% SDS for 30 min at 65°C and finally, twice with preheated 0,5 x SSC/0,5% SDS for 30 min at 65°C. Filters were exposed up to 3 days on a Phosphoimager-screen (Fuji).

1.7 Northern Blot Analysis: 5 µg of total RNA were size-fractionated on a 1.2% formamide containing agarose gel (0.8 mA/cm^2). After visualization of bands corresponding to the 28S RNA and 18S RNA, using ethidium bromide (1µg/ml), the gel was equilibrated for 30 min in 2 x SSC and subjected to capillary transfer of the RNA to a nitrocellulose membrane using 20 x SSC as the transfer buffer. After

transfer was completed, RNA was immobilized to the filter using UV-light for crosslinking. Subsequently filters were hybridized to specific cDNA probes for the following genes: heparin-binding epidermal growth factor (HB-EGF, Genebank accession no. M60278), Amphiregulin (AR, Genebank accession no. M30704), tumor necrosis factor α converting enzyme (TACE/ADAM17, Genebank accession no. U69611), ADAM20 (Genebank accession no. AF029899) and glyceraldehyde phosphate dehydrogenase (GAPDH, Genebank accession no. AF261085) or β-Actin (Genebank accession no. NM_001101).

25 ng of the respective cDNA template were denatured for 5 min at 95°C and quickly chilled on ice for 5 min. After spinning down the sample, the following ingredients were added to a final volume of 50 µl: 1 µl 0.5 mM dCTP, 1 µl 0.5 mM dGTP, 1 µl 0.5 mM dTTP, 5 µl P^{32}-α-dATP (50 µCi) 20 µl random primer solution (125 mM Tris/HCl pH 6.8, 12.5 mM $MgCl_2$, 25 mM 2-mercaptoethanol, 50 µg/ml random octamer primer) and 1 µl Klenow-enzyme (40 U/µl). The sample was incubated for 10 min at 37°C and the reaction was stopped by adding 2 µl 0.5M EDTA, pH 8.0.

Unincorporated P^{32}-α-dATP nucleotides were removed as follows: After vortexing a ProbeQuant Sephadex G-50 column ™ (Amersham), the bottom closure was snapped off, the column placed in a 2 ml tube and centrifuged for 1 minute at 735 x g. Then the column was placed in a new 1.5 ml eppendorff tube without cap and the radioactive probe was pipetted carefully on the center of the preformed resin. Centrifugation for 2 min at 735 x g removed the unincorporated P^{32}-α-dATP nucleotides. Before starting the hybridization, the nitrocellulose filter was prehybridized for 4 hours at 42°C in the following solution: 50% formamide, 5 x SSC, 5 x Denhardt's solution, 0,1% SDS. After prehybridization, the

respective radioactively labeled probe was added after denaturation at 100°C for 10 min together with 20 µg/ml salmon sperm DNA to the filters. Hybridization was performed for 16 hours at 42°C. Then the filters were washed twice for 10 min with 2 x SSC/0.1% SDS at 42°C and twice for 10 min in 0.2 x SSC/0.1% SDS at 42°C. Hybridization signals were detected using Kodak BioMax MS films.

1.8 Western Blot analysis of tyrosine phosphorylation content of the EGFR: For the investigation of the EGF-receptor tyrosine phosphorylation induced by the cagA- and cagA+ H.pylori strains, 24 hours prior to the incubation experiments, gastric cancer cells (e.g. MKN-1, MKN-28) were transferred to RPMI 1640 medium without fetal calf serum (FCS) (starvation phase). Incubation with the cagA- and cagA+ H.pylori strains occured for 0, 30, 90, 150 and 210 min, respectively. Cells were then lysed with 420 µl of lysis buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 µg/ml Aprotinin, 1 mM orthovanadate) on ice. Lysed cells were cleared from debris by centrifugation (15 min, 13000 rpm, 4°C). Cleared lysates were subjected to immunoprecipitation (40 µl Protein A/G sepharose, 4 µl anti-human EGF antibodies 108.1 per lysate) for 3 hours, 4°C. After 3 consecutive washings in 750 µl of HNTG (20 mM Hepes, pH 7.5; 150 mM NaCl, 10% glycerin; 0.1% Triton X-100), the immunoprecipitations were dissolved in 40 µl of 3 x Laemmli buffer, denatured for 5 min at 100°C and submitted to SDS-PAGE (gradient gel 7%-12%). Gels were blotted onto nitrocellulose filters (Amersham) for 3 hours (0.8 mA/cm^2) at 4°C and the EGF-receptor specific phosphorylation was detected with the 4G10 (UBI #05-321) antibody using the ECL Kit (Amersham).

1.9 Quantitative determination of interleukin 8 (IL-8) concentrations in

gastric cancer cell lines culture supernatants by enzyme linked immunosorbant assay (ELISA).

Human gastric cancer cell lines (KatoIII, MKN-1, MKN-28) were cultured in RPMI 1640 medium supplemented with 10% FCS at 37°C and 7% CO_2. 24 hours prior to the incubation experiments with cagA- or cagA+ H. pylori strains, medium was changed and cells were cultivated inRPMI 1640 medium without FCS (starvation phase). For the IL-8 induction experiments, the following compounds were tested:

- PD 134,308 (endconcentration 1 μM; inhibitor of gastrin/chole-cystokinin (CCK)-B receptor)
- Batimastat (BB94; endconcentration 1μM, inhibitor of matrix metallo-proteinase enzymes)
- [Glu^{52}]-Diphtheria Toxin (CRM197, a non toxic mutant of diphteria toxin which inhibits strongly and specifically the mitogenic activity of HB-EGF; Mitamura et al., J. Biol. Chem. 270, 1015-1019 (1995); endconcentration 5 μg/ml)
- Tyrphostin AG1478 (endconcentration 250 nM; inhibitor of EGF-receptor phosphorylation)
- PD 098,059 (endconcentration 10 μM; inhibitor of MEK1)

Cells were preincubated with these inhibitors for 30 min at 37°C and 7% CO_2 followed by 24 hours incubation at a concentration of 10^7/ml H. pylori bacteria in the presence or absence of the specific inhibitors. After incubation period, cell supernatants were collected and cleared from bacterial debris by low speed centrifugation (5 min, 3000 rpm) and ELISA detection for IL-8 was performed according to the manufacturer´s recommendation (R&D Systems) as follows: 96 well polystyrene microtiter plates were coated with a murine monoclonal antibody against human IL-8. 100 μL of an assay diluent buffer with blue dye was added to each well. Then 50 μL of cell culture supernatants were added to the plate, followed by adding of

100 µL per well of a polyclonal antibody against IL-8 conjugated to horseradish peroxidase. Test samples were incubated for 2.5 hours at room temperature. After incubation time, wells were aspirated and washed. Then 200 µL of substrate solution containing equal parts of hydrogen peroxide and tetramethylbenzidine were added per well and incubated for 30 min at room temperature. Finally, 50 µL of 2 N sulfuric acid were added per well to stop the reaction. The determination of the optical density of each well was done using a microplate reader set to 450 nm.

2. Results

Expression data of our array studies showed, that H.pylori infection of human gastric cancer cell lines MKN-1 or MKN-28 interferes with the mRNA expression level of members of the "triple membrane passing signal" (TMPS) cascade published by Prenzel et al. (1999).

In brief, this signal transduction pathway involves a G-protein coupled receptor (GPCR), a still unknown signaling element(s) leading to the activation of members of the ADAM gene-family of proteases. Activation of the protease is followed by the processing of membrane-bound ligands of the EGF-like family of ligands of the EGFR. After being processed they can stimulate in an autocrine or paracrine manner EGFR-activity, reflected by increased tyrosine phosphorylation of this receptor.

First, we confirmed our expression data from the cDNA arrays with Northern blot analysis. In a time course experiment, MKN-1, ST42 and MKN-28 cells were incubated for 0, 45 and 180 min with cagA- (TX30a, ATCC 51932) and cagA+ (60190, ATCC 49503) H.pylori strains. As shown in Fig.1, within 45 min a dramatic increase in the expression level of HB-EGF- and ADAM20-mRNA species was observed. In addition, inspection of the later time point clearly demonstrates an even more

pronounced increase in the appropriate mRNA expression levels.

This observation demonstrates, that incubation of human gastric cancer cell lines with cagA-/cagA+ H.pylori strains upregulates the expression level of members of TMPS cascade, which is a prerequisite for the establishment of H.pylori-induced diseases like peptic ulcers or gastric cancer involving activation of the EGFR.

To test whether tyrosine phosphorylation of the EGFR is induced upon incubation of human gastric cancer cell lines with TX30a and 60190 H.pylori strains, MKN-1 and MKN-28 cells were incubated for distinct time points (0, 45, 90, 150 min) with the above mentioned H.pylori strains at a concentration of $1x10^7$ bacteria per ml. Both H. pylori strains induced increased tyrosine phosphorylation of the EGFR (Fig.2). This observation parallels the increase in HB-EGF and ADAM20 mRNA expression levels.

If EGFR tyrosine phosphorylation is mediated via TMPS cascade, inhibition of one of the involved signaling members should abrogate this activation. To investigate this possibility, MKN-1 and ST42 cells were preincubated for 30 min with CRM197, the specific inhibitor of HB-EGF. Both cells lines were then stimulated for 150 min with H.pylori strain 60190 at a conc. of 1×10^7 cells per ml in presence or absence of CRM197. As seen in Fig.3, H.pylori-induced tyrosine phosphorylation of the EGFR is completely abrogated in the presence of CRM197 in comparison to untreated cells. The experiment clearly demonstrates that H.pylori-induced EGFR activation involves, at least by part, TMPS cascade.

To explore the involvement of TMPS cascade on H.pylori-induced IL-8 production (a well characterized cellular response for this pathogen) MKN-1 and MKN-28 gastric cancer cell lines were incubated with 60190 and TX30a strains of H.pylori in the presence or absence of specific inhibitors of members of TMPS cascade. IL-8 release was measured from the

supernatant of cells using the IL-8 Immunoassay (ELISA). The following compounds were used:

- an inhibitor of gastrin/cholecystokinin (CCK)-B receptor (PD134308)
- an inhibitor of matrix metalloproteinase enzymes (batimastat = BB-94; interferes with TMPS signaling)
- an inhibitor of proHB-EGF (CRM197, a non toxic mutant of diphteria toxin which inhibits strongly and specifically the mitogenic activity of HB-EGF; Mitamura et al., J. Biol. Chem. 270, 1015-1019 (1995); interferes with TMPS signaling)
- an inhibitor of epidermal growth factor receptor (Tyrphostin AG1478; interferes with TMPS signaling)
- an inhibitor of MEK1 (PD098059)

The respective inhibitors were added prior to incubation with H. pylori cagA- and cagA+ strains. The presence of PD134308 had a minor effect on H.pylori-induced IL-8 production (inhibition in average of approx. 10%), while the effect of batimastat and CRM197 was more pronounced (inhibition in average of approx. 25%). In the case of AG1478, the observed inhibition was between 30-70% and PD098059 led to a dramatic decrease of about 60-75% of the H. pylori-induced IL-8 response in human gastric cancer cells (c.f. Fig.4). In repeated experiments we confirmed the inhibitory effect of PD134308 at 1μM, of BB-94 at 5 μM, of CRM197 at 5μg/ml, of AG1478 at 250 nM and of PD098059 at 1 μM.

We therefore conclude that the following compounds interfere with H. pylori induced gastrointestinal diseases: BB-2516, BB-1101, BB-94, GI129471, 2-Aminoethyl-amide, CT1418, RO31-9790, CGS27023A, PD134,308, CAM-1028, L-365,260, spiroglumide CR2622, YM-022, RB210, RB213, LY-288,513, LY-262,691, DA-3934, RP-73870, S-0509, CP-212,454, CI-1015, YF-476, L-740,093, Lavendustin A, AG 112, AG 183, AG 1478, PD 153035, PD 158780, ZD1839, CP-358,774, CGP 59326, CGP 60261, CGP 62706.

In an additional experimental setup we investigated the influence of the above mentioned inhibitors on the expression of HB-EGF mRNA in gastric cancer cell lines MKN-1 and MKN-28, which is a major player in TMPS signaling. As seen in Fig.5, H.pylori strain 60190 induced increased expression of HB-EGF and mRNA in the cell lines tested. If BB-94, CRM197, the combination of BB-94 and CRM197 or AG1478 was present during infection, the pathogen-induced increase of mRNA accumulation of HB-EGF was significantly reduced. It was nearly completely abolished when PD098059 was used as inhibitor. In an additional experiment we could demonstrate that not only mRNA expression level of HB-EGF is sensitive to inhibitors of TMPS signaling, but also ADAM20 mRNA expression (Fig.6). Both mRNA expression levels are significantly reduced in the presence of BB-94 and CRM197 in comparison to untreated cells. These results clearly demonstrate, that key players of TMPS signaling are induced by H.pylori infection of gastric cancer cell lines, leading to the establishment of an autocrine signaling cascade which is responsible of disease development.

Without wishing to be bound to theory, our results lead to the establishment of the following model (Fig.7): H.pylori infection leads via stress-induced signaling pathways to a specific cellular defense program, which involves the expression of members of the TMPS cascade leading to EGFR-mediated downstream signaling events. Depending on the severeness of H.pylori infection prior to eradication, an auto-paracrine signaling cascade is manifested which essentially contributes to H.pylori-induced disease development. In this model, H.pylori-induced pH changes will also lead to the activation or even chronic stimulation of GPCR-signaling as response to pH change. This disregulated signaling event involving GPCRs and TMPS cascade, will contribute as well to the establishment of the pathogenic phenotype.

Claims

1. A method for the manufacture of a medicament for treating or preventing Helicobacter mediated diseases in a mammal, wherein said medicament comprises as an active ingredient at least one compound selected from

   (a) inhibitors of gastrin/cholecystokinin (CCK)-B receptor,

   (b) inhibitors of protein kinase C,

   (c) inhibitors of membrane-associated metalloproteinases,

   (d) inhibitors of growth-factor receptor activation,

   (e) inhibitors of growth-factor receptor kinase activity,

   (f) inhibitors of the mitogen-activated protein kinase cascade and

   (g) transcription inhibitors.

2. The method according to claim 1, wherein the Helicobacter mediated diseases are selected from gastrointestinal cancer and inflammatory conditions.

3. The method according to claim 1 or 2, wherein the inhibitors of gastrin/cholecystokinin (CCK)-B receptor are selected from peptides, amino acid derivatives, benzodiazepines, dipeptoids, pyrazolidinones, quinazolidinones, bicyclic heteroaromatic compounds, ureidoacetamides, ureidobenzazepines and ureidomethylcarbamoylphenylketones.

4. The method according to claim 1 or 2, wherein the inhibitors of protein kinase C are selected from indolocarbazoles, bisindolylmaleimides, balanol analogs, alkyl lysophospholipids and antisense oligonucleotides.

5. The method according to claim 1 or 2, wherein the inhibitors of membrane-associated metalloproteinases are selected from compounds containing a hydroxamic acids group and members of the TIMP-family.

6. The method according to claim 1 or 2, wherein the inhibitors of growth-factor receptor activation are selected from anti-receptor antibodies or fragments thereof.

7. The method according to claim 1 or 2, wherein the inhibitors of growth-factor receptor kinase activity are selected from phenylamino-quinazolines, substituted pyrimidines, tyrphostins, lavendustins and dianilinophthalimides.

8. The method according to claim 1 or 2, wherein the inhibitors of the mitogen-activated protein kinase cascade inhibitors are selected from inhibitors of MAPKKK (Raf), inhibitors of MAPKK (Mek) and inhibitors of MAPK (Erk).

9. The method according to claim 1 or 2, wherein the inhibitors of transcription inhibitors are selected from general transcription inhibitors and c-fos specific transcription inhibitors.

10. The method according to claim 1 or 2, wherein said medicament comprises at least two active ingredients selected from compound classes (a) to (g).

11. The method according to claim 1, 2 or 9, wherein said medicament comprises a combination of at least one active ingredient selected from compounds (a) to (g) and a further active ingredient which is effective against Helicobacter infections.

12. The method according to claim 11, wherein said further active ingredient is selected from antibiotics and proton pump inhibitors.

13. The method according to claim 11, wherein said further active

ingredient is selected from H. pylori vaccines.

14. The method according to claim 1, 2 or 9, wherein said medicament comprises a combination of at least one active ingredient selected from compounds (a) to (g) and an immunogenic adjuvant.

15. The method according to any one of claims 1-14 wherein the Helicobacter is a H. pylori cagA + strain.

16. A method for identifying a medicament for treating or preventing Helicobacter mediated diseases comprising an assay of test compounds for molecular targets (a) - (g) as defined in claim 1.

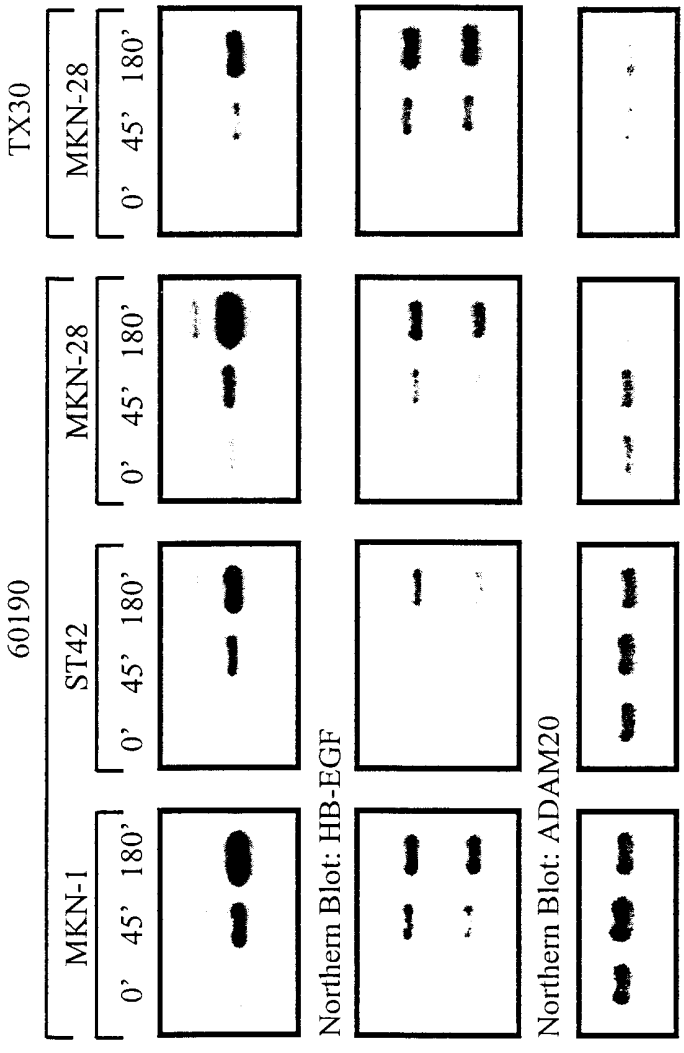

Fig.1: H. pylori 60190 (cagA+) and TX30 (cagA-) strains induce HB-EGF and ADAM20 expression

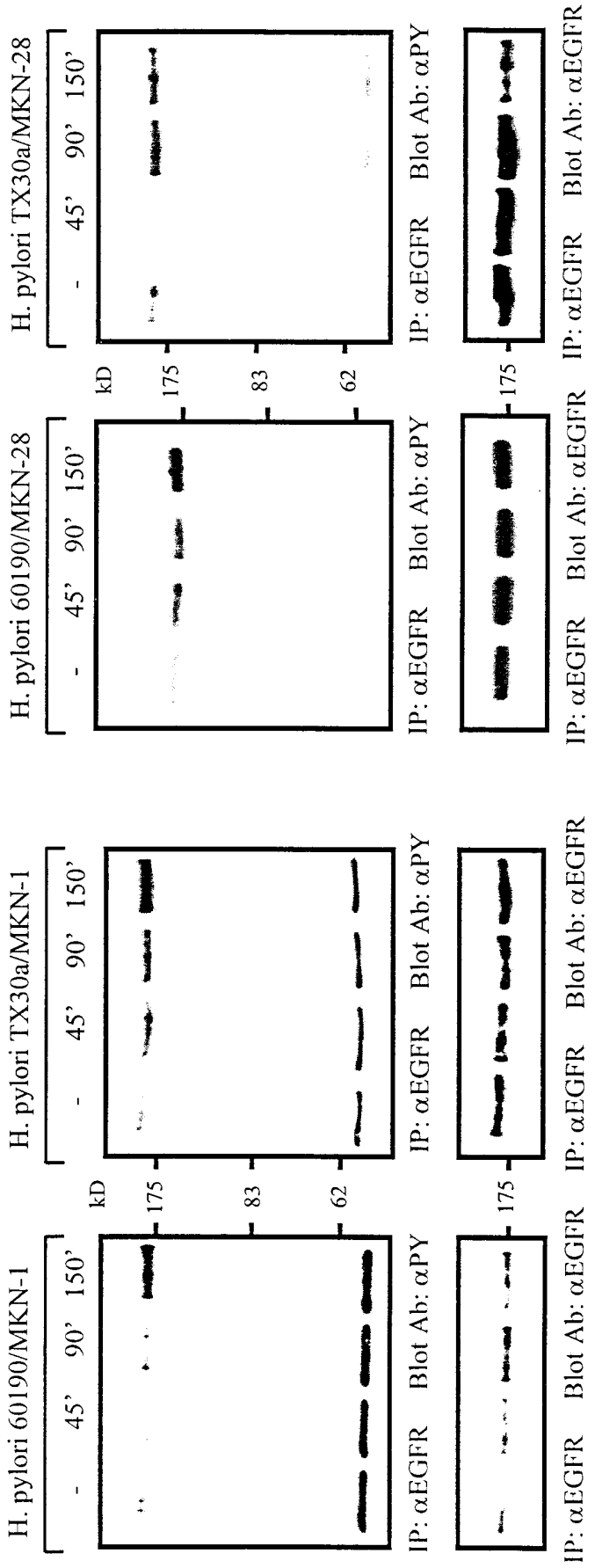

Fig.2: cagA-/cagA+ strains of H. pylori induce increase of EGFR Y-phosphorylation in MKN-1 and MKN-28 cells

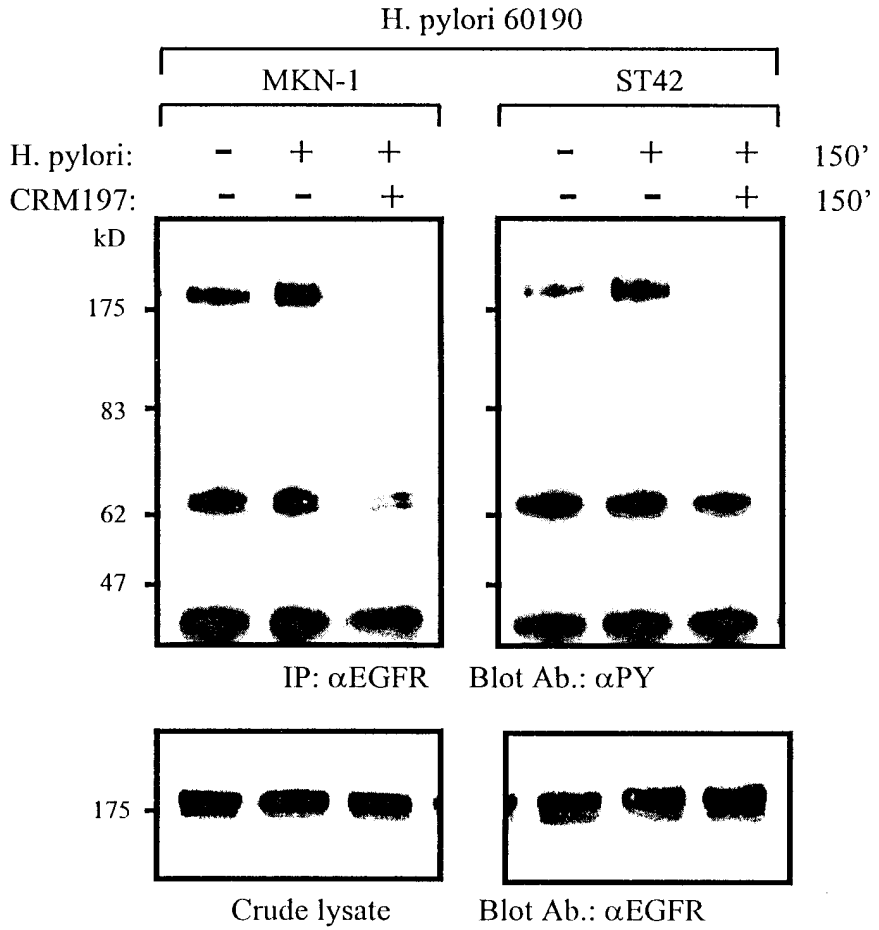

Fig.3: H.pylori induced increase of EGFR Y-phosphorylation in MKN-1 and ST42 cells is CRM 197 sensitive

MKN-1

strain + inhibitor	value measured	value-bgrd	% induction	% inhibition
minus/minus	0,04			
60190/minus	2,04	2	100%	0%
60190/PD134308 *				
60190/BB-94	1,406	1,366	68%	32%
60190/CRM197	1,285	1,245	62%	38%
60190/AG1478	1,148	1,108	55%	45%
60190/PD098059	0,717	0,677	34%	66%
minus/minus	0,01			
TX30a/minus	1,996	1,956	100%	0%
TX30a/PD134308	1,882	1,842	94%	6%
TX30a/BB-94	1,76	1,72	88%	12%
TX30a/CRM197	1,609	1,569	80%	20%
TX30a/AG1478	1,108	1,068	55%	45%
TX30a/PD098059	0,569	0,529	27%	73%

MKN-28

minus/minus	0,02			
60190/minus	1,516	1,476	100%	0%
60190/PD134308	1,241	1,201	81%	19%
60190/BB-94	1,4	1,36	92%	8%
60190/CRM197 *				
60190/AG1478	1,06	1,02	69%	31%
60190/PD098059	0,621	0,581	39%	61%

minus/minus	0,02			
TX30a/minus	0,81	0,77	100%	0%
TX30a/PD134308	0,746	0,706	92%	8%
TX30a/BB-94	0,408	0,368	48%	52%
TX30a/CRM197	0,639	0,599	78%	22%
TX30a/AG1478	0,218	0,178	23%	77%
TX30a/PD098059	0,221	0,181	24%	76%

Fig.4: IL-8 response of MNK-1 and MKN-28 cells upon infection with H.pylori strains 60190 (cagA+) and TX30a (cagA-).

* experiment failed due to contamination

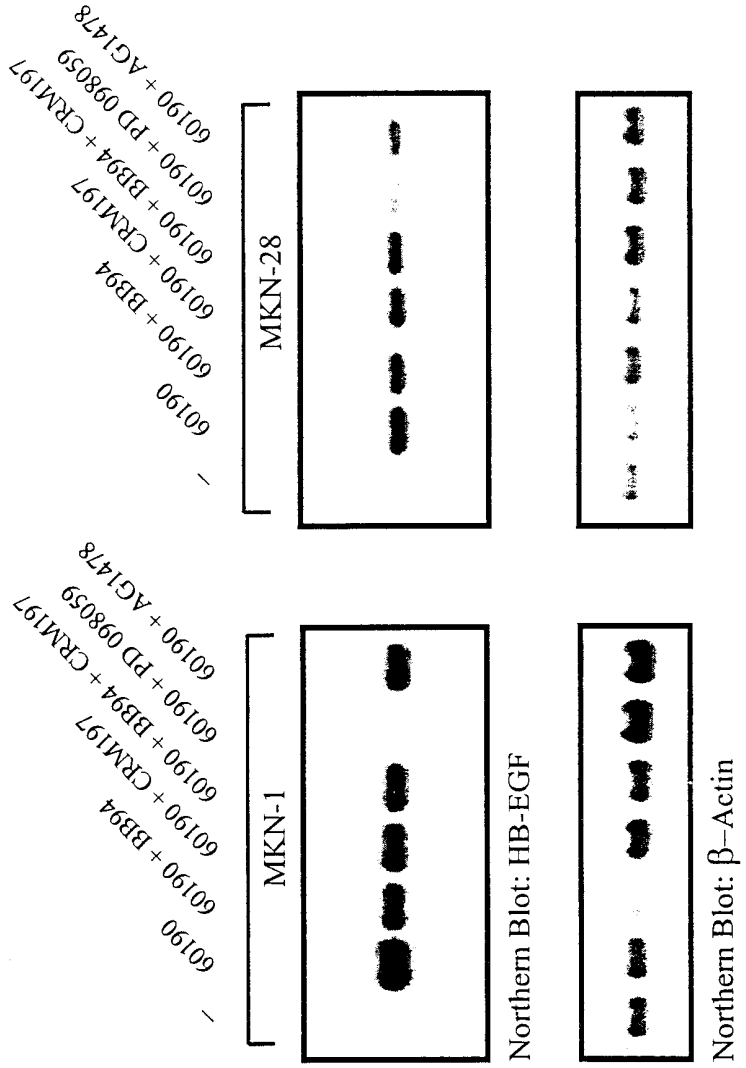

Fig.5: H.pylori induced HB-EGF expression is BB94, CRM197 and AG1478 sensitive and completely abolished by PD098059

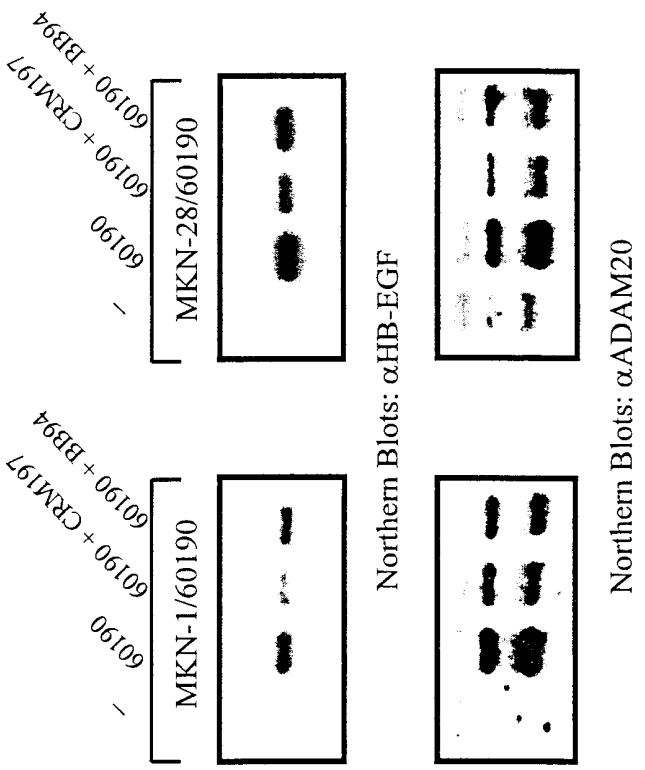

Fig.6: H.pylori induced HB-EGF and ADAM20 expression is BB94 and CRM197 sensitive

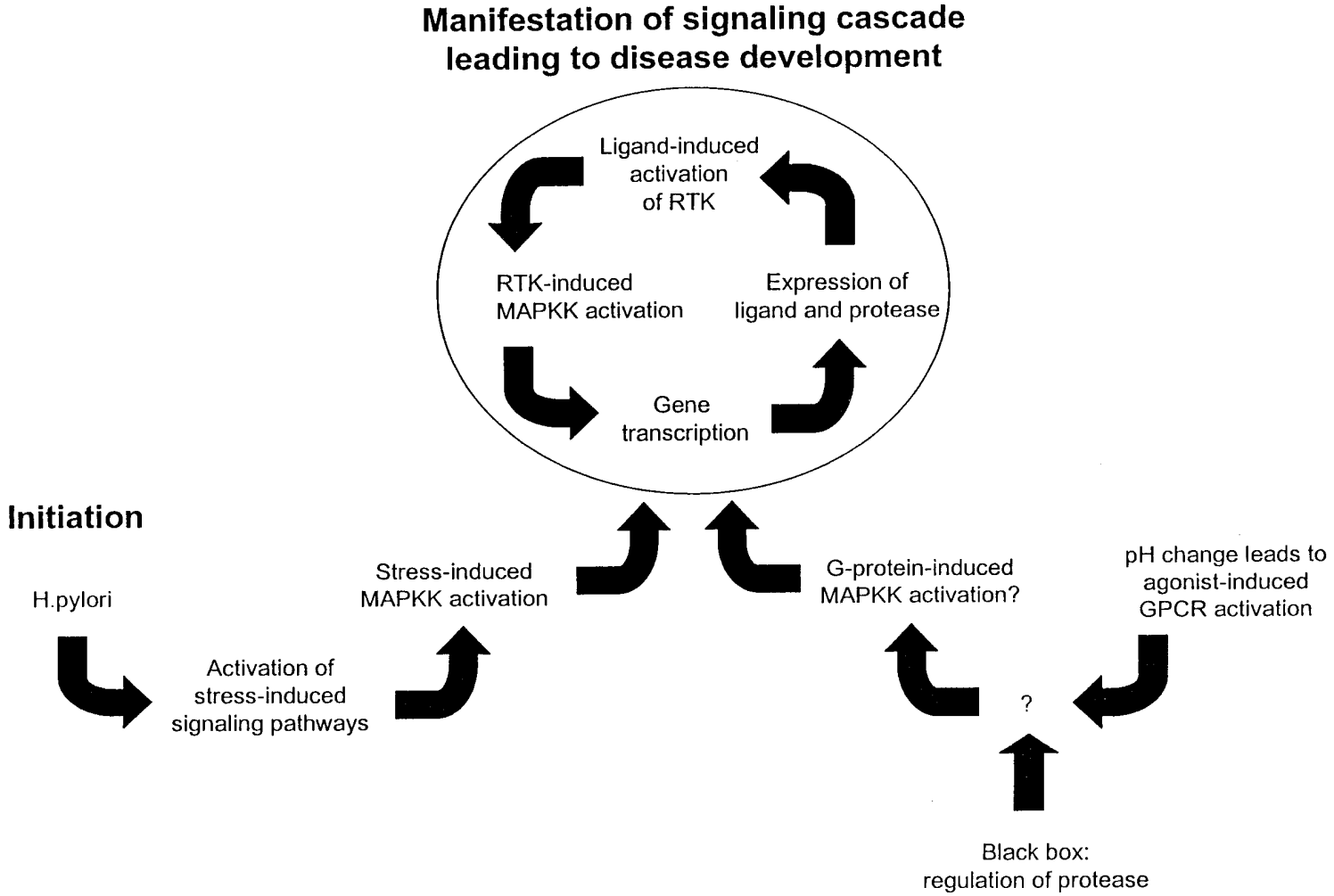

Fig.7: H. pylori induces expression of EGF-like ligands and metalloproteases which leads to the manifestation of a disease process