**Supplementary Methods**

## *1. Cell culture*

Cell lines HCT116 and SW620 (both human colorectal cancer) were from ATCC (Manassas, VA, USA), HupT3 (human pancreatic adenocarcinoma), OE33 (human esophageal adenocarcinoma) and SKBR3 (human breast adenocarcinoma) cells were from DSMZ (Braunschweig, Germany). HCT116, HupT3 and OE33 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Bio & Sell, Feucht, Germany). SW620 and SKBR3 cells were maintained in DMEM with 10% FBS, at 37°C and 5% CO2 in a humidified incubator. Cells were regularly tested to exclude mycoplasma contamination (MycoAlert Mycoplasma detection kit, Lonza, Basel, Switzerland).

## *2. Generation of MACC1-promoter reporter cell lines*

To identify transcriptional inhibitors of MACC1, HCT116-MACC1p-Luc cells were generated, expressing the human MACC1-promoter (-992 to -18 bp upstream of the MACC1 transcriptional start site)-driven luciferase reporter gene (1). Luciferase expression was regularly controlled by the Steady-Glo Luciferase Assay System (Promega).

## *3. High throughput screening (HTS)*

The largest academic library of Germany, EMBL, Heidelberg, Germany was used. Selected compounds were obtained from Enamine, Akos and Mcule (Supplementary Table 1).

For the screens, 4×103 HCT116-MACC1p-Luciferase CRC cells were seeded into 384-well plates. Compound concentration was 0.8 nM to 50 µM (serial 3-fold dilutions; triplicates for each concentration). After 24 h of incubation, 11 dose-response-curves of different concentrations were measured (Infinite 200 PRO microplate reader, Tecan, Austria). Most effective compounds were screened for inhibition of MACC1 mRNA expression by qRT-PCR in correlation to their cytotoxicity. Compounds were stored according to the compound provider’s instructions. Stock solutions were prepared freshly. DMSO was used as compound solvent and as control.

## *4. Steady-Glo® Luciferase assay*

For validation of compound activity, 1.5×104 HCT116-MACC1p-Luc cells were seeded into 96-well plates (triplicates for each concentration) and incubated for 24 h. The prepared compound concentration of 10 µM was diluted according to the respective final concentration. Luciferase assay was performed according to the manufacturer’s instructions (Promega) and measured (Infinite 200 PRO, Tecan).

## *5. MTT cell viability assay*

## 7.5×103 cells/well were seeded in a 96-well plate (triplicates for each concentration). After incubation for 24 h, cells were treated with compounds for another 24 h. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Carl Roth, Germany) was prepared at a concentration of 5 mg/ml, added to each well (final concentration 0.5 mg/ml) and incubated for 2 h. Medium was removed and 150 μl DMSO was added to each well for 10 min to dissolve the purple formazan crystals. Absorbance was measured at 560 nm (Tecan Infinite 200 PRO). Results are expressed as percent viable cells compared to solvent-treated controls.

## *6. Quantitative real-time reverse transcription PCR (qRT-PCR)*

For MACC1 gene expression analyses, 7×104 cells/well were seeded in a 24-well plate and incubated for 24 h. Drug treatment was conducted the next day followed by 24 h of incubation. Total RNA was isolated by using GeneMATRIX Universal RNA Purification Kit (Roboklon, Berlin, Germany) according to the manufacturer´s instructions. RNA samples without compound treatment were used as standard. 50 ng RNA was taken from each sample for reverse transcription (RT) (reaction mix: 25 μM hexamer primer, 200 U/μl reverse transcriptase, 40 U/μl RNase inhibitor, 5× synthesis buffer, dNTP mix, PCR grade water; all from Biozym). RT was done at 30°C 10 min, 50°C 40 min and 99°C 5 min with cooling at 4°C for 5 min. After RT, samples were diluted 1:1 with PCR grade water. Glucose-6-phosphate dehydrogenase (G6PDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as housekeeping genes. The primers for human MACC1, G6PDH and GAPDH (1,2) were used for qPCR amplification of cDNA (LightCycler 480 II, Roche Diagnostics, Switzerland). Data were analyzed with the LightCycler 480 Software release 1.5.0 SP3 (Roche Diagnostics). The same qPCR protocol was used for analyses of animal tissues. Average values of repeated samples were taken and each mean value of the expressed genes was normalized using G6PDH and GAPDH. All expression analyses were performed three times independently.

## *7. Detection of human satellite DNA in mouse liver*

Frozen mouse liver tissues were sliced with thickness of 10 µm. All tissues were put into pre-cooled tubes at -20°C. After adding lysis buffer and 5 pulse sonications at 50% for cell lysis, DNA isolation and qPCR were carried out as previously described (1, 3).

## *8. Gene expression analysis after TNF-α stimulation*

1×105 cells/well were seeded in a 24-well plate and incubated for 24 h. The next day compound treatment (10 µM) was conducted with or without 10 ng/ml or 100 ng/ml TNF-α following 24 h incubation. The next day, qRT-PCR was performed.

## *9. Western blot analysis*

3×105 cells per well were seeded in a 6-well plate. After compound treatment, RIPA buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40; supplemented with protein and phosphate inhibitor cocktail tablets, Roche Diagnostics) was used for cell lysis on ice for 30 min. Protein concentration was quantified with the Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific).

For Western blot, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used. After electrophoresis, transfer to the polyvinylidene difluoride (PVDF) membrane was performed. Membranes were blocked with 5% milk powder in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h at room temperature, washed with TBS-T and incubated with MACC1 or β-actin (as loading control) primary antibody (MACC1 antibody, Sigma, 1:3000; β-actin antibody, Sigma, 1:20,000; all prepared with albumin bovine fraction V; Serva, Heidelberg, Germany) at 4°C overnight. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG, Promega, 1:10,000; anti-mouse IgG, Thermo Fisher, 1:40,000; prepared with TBS-T) at room temperature for 1 h, and washed. Detection was performed with WesternBright (Advansta, Menlo Park, CA, USA), then exposed to Fuji medical X-ray film SuperRX (Fujifilm, Tokyo, Japan).

## *10. Transwell migration assay (Boyden chamber assay)*

Cells were starved in serum-free medium for 6-8 h before seeding. A 96-well plate (REF 3384, Corning, USA) with an insert membrane pore size of 8 µm was pre-soaked with RPMI-1640 medium for 30 min (no FBS). Four replicates were made for each sample. 5×104 serum-starved cells in 100 µl of compound-containing RPMI-1640, 0.5% FBS were seeded in the upper chamber and, 235 μl of compound-containing RPMI-1640, 10% FBS was added to the lower chamber for each well. DMSO served as control. The number of living cells migrated after 24 h incubation to the lower chamber was detected by cell Titer-Glo Reagent (Promega) and measured with the Infinite Pro multi-plate reader (Infinite 200 PRO, Tecan, Austria).

## *11. Wound healing assay*

On day 0, 1.1×105 cells per well were seeded into a 96-well ImageLock plates (Essen Bioscience, USA). After 6-8 h of incubation, the wound was established using the IncuCyte Woundmaker Tool (Essen Bioscience, USA). Compound or solvent was added and incubated in the IncuCyte Zoom (Essen Bioscience, USA) to record wound closure every 2 h. Data were analyzed after 72 h (4).

## *12. Immunohistochemistry*

## Frozen mouse livers were sliced with thickness of 5 µm, fixed in 4% paraformaldehyde/PBS solution at room temperature and quenched with 0.1 M glycine for 20 min. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide buffer for 10 min at room temperature. After washing three times with PBS, sections were immersed for 2 min in PBS containing 0.2% TritonX-100. Slides were blocked with 5% albumin-free IgG for 1 h at room temperature, rinsed in PBS and incubated with anti-human cytokeratin 19 (CK19) rabbit monoclonal antibody (1:200; DB103-0.2, DB Biotech, Slovakia) overnight at 4˚C in a humidified chamber. The slides were washed five times with PBS and incubated with anti-rabbit HRP secondary antibody (1:500; HRP-conjugated antibody, Promega) for 1 h in a humidified condition at room temperature. CK19 detection was performed with 3,3'-diaminobenzidine (DAB) and counter-stained with hematoxylin.

## *13. Prediction of NF-κB transcription factor binding sites of the MACC1 promoter*

To predict binding sites of transcription factors, the online tool PROMO 3.0 (5) was used. The promoter sequence of MACC1 (GeneBank accession code JN544571, 6) from -1992 to -18 bp upstream of the TSS (+1) was used for the prediction. Only human NFκB-factors and human sites were analyzed by the online tool for c-Rel, RelA (p65), NF-kappaB (p50), NF-kappaB1 (p52).

*14. ADME/TOX methods*

ADME/TOX studies were carried out by the Lead Discovery Center GmbH (LDC; Dortmund, Germany). Dilution to 10 mM stocks in DMSO were used for all assays with subsequent dilution.

*14.1 Physical and chemical properties*

Compound identity and purity was confirmed by UPLC (Ultra Performance Liquid Chromatography)-MS/MS. Kinetic solubility (SolRank) of compounds was determined by spectrophotometric measurement of a 500 µM compound solution in HEPES buffer, pH 7.4 compared to the organic solvent acetonitrile after 90 min of vigorous shaking at room temperature. Prior to analysis, precipitated material was removed by filtration.

*14.2 Thermodynamic solubility (miniaturized shake flask solubility)*

Miniaturized shake flask assay was used to determine the solubility of compounds at room temperature in aqueous buffer at pH 7.4. In brief, compound powder was dissolved in PBS, pH 7.4 (4 mg/ml) and equilibrated with shaking overnight at room temperature (>16 h). Solutions were centrifuged, supernatant was filtered and concentration (µg/ml) of test compounds was determined by LC-MS/MS.

Chemical stability of test compounds was determined in aqueous buffer pH 1, 7.4 and 9 at 37°C for 24 h at a final concentration of 2 µM. After selected time-points, samples were analyzed by LC-MS/MS and relative degradation (% remaining) was calculated based on peak area under curve (AUC) values.

*14.3 Plasma stability and protein binding*

Plasma stability was measured by LC-MS-based determination of percentage of remaining selected compounds at 5 µM after incubation in 50% plasma in PBS (obtained from humans) at 37°C for 1 h. Plasma protein binding was determined by equilibrium dialysis. Plasma containing 5 µM test compound was allowed to equilibrate with the buffer compartment at 37°C for 6 h. Compound concentrations on both sides of the semi-permeable membrane were analyzed by LC-MS/MS and % bound was calculated.

*14.4 Metabolic stability*

Metabolic stability under oxidative conditions (microsomal stability phase I) was measured using NADPH-supplemented human liver microsomes. Compound depletion was analyzed by LC-MS/MS at 1 µM over time up to 50 min at 37°C. Based on compound half-life t1/2, in vitro intrinsic clearance CLint was calculated. , with V = assay volume and mg = amount of microsomal protein.

Metabolic stability under conjugative conditions (microsomal stability phase II) was measured in the glucuronidation assay by LC-MS-based determination of % remaining of selected compounds at 5 µM in incubations with human liver microsomes supplemented with UDPGA for 1 h at 37°C.

For the redox assay, test compounds were incubated in the presence of dithiothreitol (DTT) and H2O2 generated through redox cycling was detected using horseradish peroxidase (HRP) mediated phenol red (PR) oxidation.

*14.5 Permeability*

Permeability through artificial membranes assay (PAMPA) was performed at an initial compound concentration of 500 µM in the donor compartment. After 20 h incubation, absorption of the receiver wells was measured by spectrophotometry. Permeation was calculated by normalization of the compound flux across a blank filter.

Cellular permeability was determined using polarized Caco-2 or MDCKII-MDR1 cell monolayers seeded on 96-well transwell plates (Corning). Bi-directional transport of the compounds from the apical to basolateral (A → B) or basolateral to apical (B → A) side across the cell monolayers was analyzed by LC-MS/MS and apparent permeability (Papp) was calculated.

*14.6 In vitro toxicity*

Inhibition of five major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4) was determined using specific substrates in human liver microsomes. A decrease in the formation of the metabolites compared to the vehicle control is used to calculate an IC50 value using XLfit for Microsoft Excel.

The Predictor™ hERG Fluorescence Polarization Assay (Thermo Fisher Scientific) was used according to manufacturer’s instructions to determine hERG binding (Cardio toxicity assay). 8-point concentration series were tested for each compound. Fluorescence polarization was measured with a Wallac EnVision 2103 Multilabel Reader (Perkin Elmer). Data was analyzed using XLfit for Microsoft Excel.

*14.7 CellTiter-Glo® (Promega) luminescent cell viability assays for primary hepatocytes and human PBMCs*

PBMCs were isolated from buffy coats (DRK-Blutspendedienst West, Ratingen) using PanColl lymphocyte separating medium (PAN Biotech). Assay medium was RPMI 1640 (PAN Biotech) with 10% HyClone FBS (heat-inactivated, Thermo Scientific) and 1% L-Glutamine (PAN Biotech). Cells were seeded into 384-well plates. After 24 h, compounds were transferred by an Echo520 (Labcyte) (8-point concentration series).

Alternatively, pooled cryopreserved murine (male ICR/CD-1) or human hepatocytes (mixed gender) were seeded into collagen-coated 384-well plates using a Multidrop Combi (Thermo Scientific). Hepatocytes were obtained from Bioreclamation IVT and handled according to the manufacturer’s instructions.

After 72 h incubation in hepatocytes or PBMCs, IC50 values of tested compounds were determined with the CellTiter-Glo® (Promega) Luminescent Cell Viability Assay according to the manufacturer’s instructions. Luminescence values were measured with a Victor X5 2030 Multilabel reader (Perkin Elmer). IC50 values were calculated using XLfit for Microsoft Excel.

## *15. In vivo tolerability for MACC1 inhibitors*

For Maximum Tolerated Dose (MTD), 6-week-old female severe combined immunodeficiency (SCID beige) mice (Charles River, Sulzfeld, Germany) were randomly assigned to 4 groups (2 animals/group). Dosing of Compound 22 was 25 mg/kg, 50 mg/kg, 100 mg/kg and 200 mg/kg. The compound was applied orally, body weight was measured, potential side effects were monitored and gross necropsy was performed.

## *16. In vivo pharmacokinetic study*

## Six-week-old female CD-1 mice (Charles River, Sulzfeld, Germany) were used for studying the pharmacokinetics of Compound 22. In brief, mice were treated with a single dose of Compound 22 (i.v.: 1 mg/kg; p.o.: 50 mg/kg) and blood was drawn at specific time points, with 2 different animals per timepoint. Time points i.v.: 5, 10, 15, 30, 60 min, 2, 4, 24 h. Time points p.o.: 15, 30, 60 min, 2, 4, 8, 24 h. A standard 10 mM Compound 22 solution was used to calibrate the mass spectrometer, before analyzing the concentration of Compound 22 in the plasma. The terminal halflife (t1/2) was calculated using Microsoft Excel.

## *17. In vivo testing of inhibitors of metastasis in the HCT116 CRC model*

Six-week-old female SCID beige mice (Charles River, Sulzfeld, Germany) were used for intrasplenic transplantation of 3×105 HCT116-CMVp-Luc cells. Meloxicam (Boehringer Ingelheim, Ingelheim, Germany) was administered subcutaneously (3.5 mg/kg) for analgetic treatment 30 min before anesthesia. Anesthesia was carried out with intravenous propofol (30 mg/kg; Braun, Melsungen, Germany) injection.

Mice were randomly assigned to 4 groups (10 animals/group). For therapy, 50 mg/kg Compound 22, Analogues 10 and 13 was applied each day, orally. Control animals received solvent. Non-invasive bioluminescence imaging (BLI) imaging system NightOWL LB 981 (Berthold Technologies, Bad Wildbad, Germany) was used to monitor tumor growth and metastasis formation in the liver in mice anesthetized with isoflurane (Baxter, Unterschleißheim, Germany). For BLI, mice received intraperitoneally 150 mg/kg D-luciferin (Biosynth, Staad, Switzerland) in PBS. Tumor growth and metastasis formation was imaged and quantified by WinLight (Berthold Technologies) and ImageJ (version 1.53 J8, National Institutes of Health, USA). After termination, spleens (transplantation site) and livers (target organ for metastasis) were removed, imaged and cryopreserved in liquid nitrogen.

## *17.1 Dose escalation experiment*

Six-week-old female SCID beige mice (Charles River, Sulzfeld, Germany) were used for intrasplenic transplantation of 3×105 HCT116-CMVp-Luc cells. Meloxicam and propofol were administered for analgetic treatment and anesthesia as described in 17.

The mice were randomly assigned to 5 groups (4 animals/group). For therapy, 25, 50 and 100 mg/kg Compound 22 and 13.5 mg/kg Atorvastatin was applied each day, orally. Control animals received solvent. Non-invasive bioluminescence imaging (BLI) was carried out to monitor tumor growth and metastasis formation as described above in 17.

After termination, spleens (transplantation site) and livers (target organ for metastasis) were removed, imaged and cryopreserved in liquid nitrogen for further analyses.

*17.2 In vivo testing of inhibitors for metastasis in the SW620 CRC model*

Six-week-old female SCID beige mice (Charles River, Sulzfeld, Germany) were used for intrasplenic transplantation of 1×106 SW620-CMVp-Luc cells. Meloxicam and propofol were administered for analgetic treatment and anesthesia as described in 17.

The mice were randomly assigned to two groups (8 animals/group). For therapy 50 mg/kg Compound 22 was applied each day, orally. Control animals received solvent. Non-invasive bioluminescence imaging (BLI) was carried out to monitor tumor growth and metastasis formation as described above in 17. After termination, spleens (transplantation site) and livers (target organ for metastasis) were removed, imaged and cryopreserved in liquid nitrogen.

All animal experiments were performed according to the United Kingdom Coordinating Committee of Cancer Research (UKCCCR) guidelines and in cooperation with Experimentelle Pharmakologie & Onkologie Berlin-Buch GmbH (EPO GmbH, Berlin, Germany). The State Office of Health and Social Affairs, Berlin, Germany granted the animal experiments under the permit Reg 0010/19.

## *18. Statistical analysis*

GraphPad Prism 6.0 was used for statistical analysis. Comparison between two groups was done by t-test, and comparison of three or more groups was done by one-way analysis of variance (ANOVA) and Bonferroni post hoc multiple comparisons. IC50 and EC50 values were calculated by the sigmoidal dose-response inhibition curve fit of x=log(x) transformed data. All significance tests were two-sided with a confidence interval of 95% (\* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001, \*\*\*\* = p < 0.0001).

# References

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