**Discovery of tetrazolo-pyridazine-based small molecules as inhibitors of MACC1-driven cancer metastasis**

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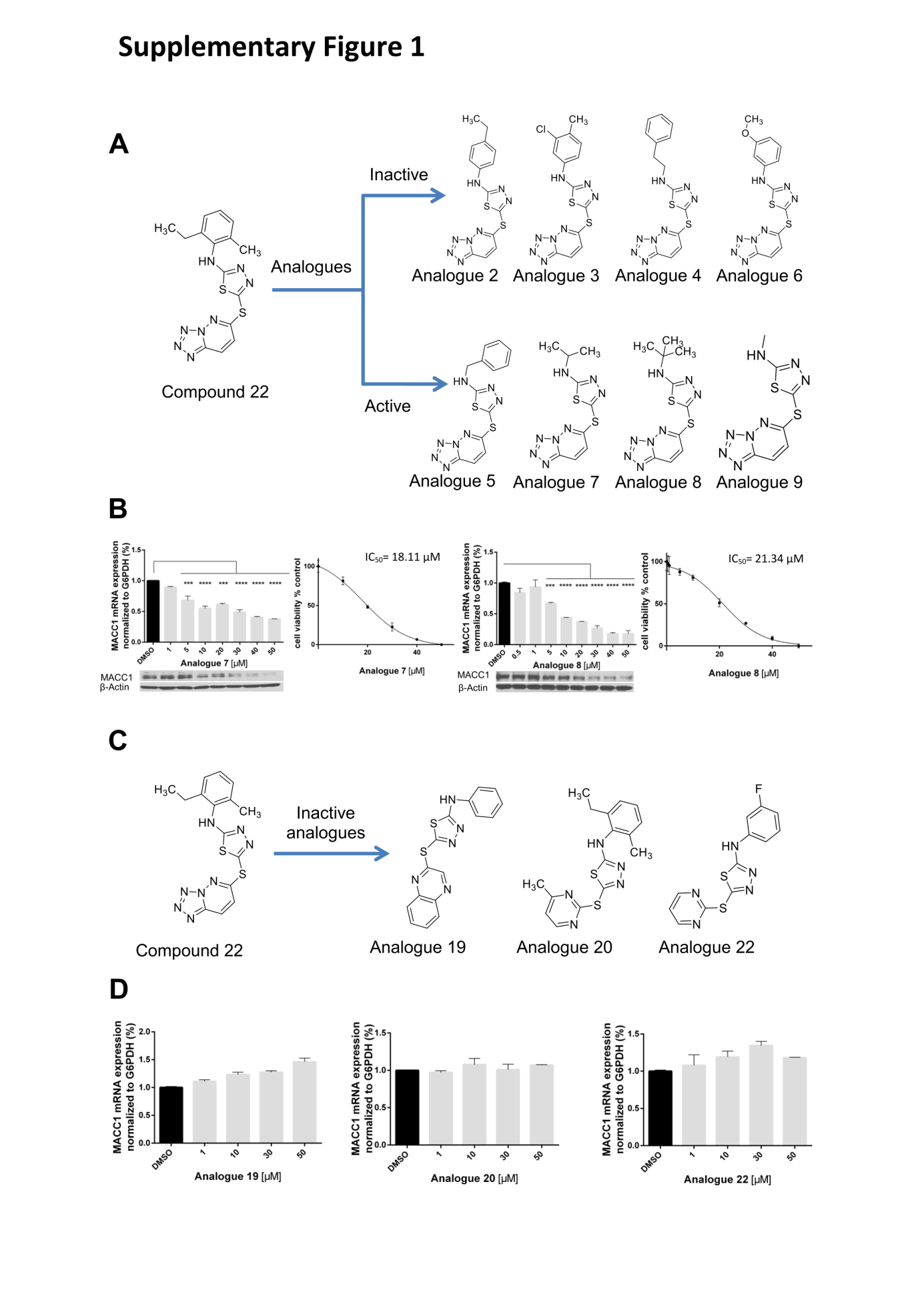
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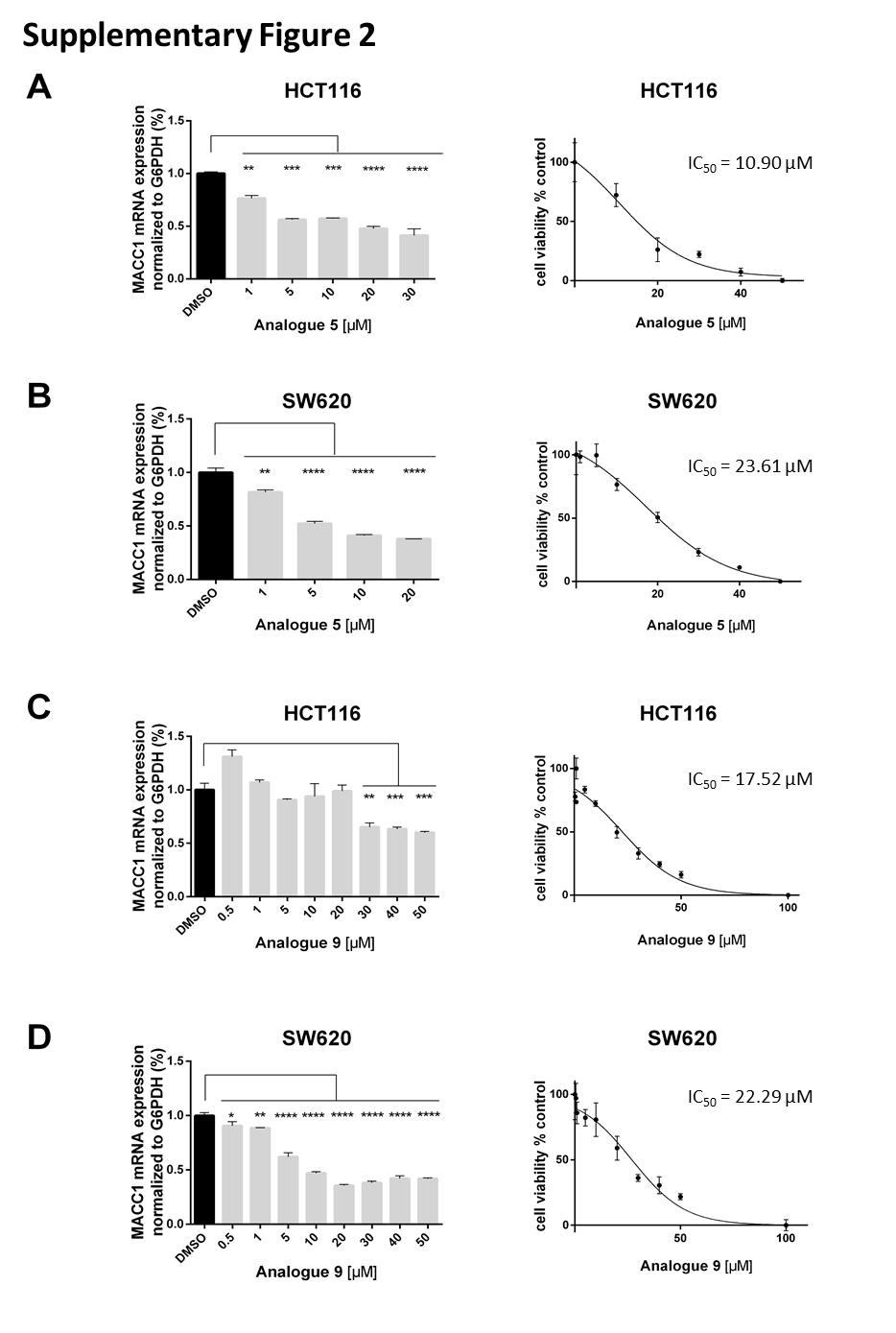
Prof. Ulrike Stein, Experimental and Clinical Research Center, Charité - Universitätsmedizin Berlin, and Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany; ORCID: 0000-0001-7006-282x

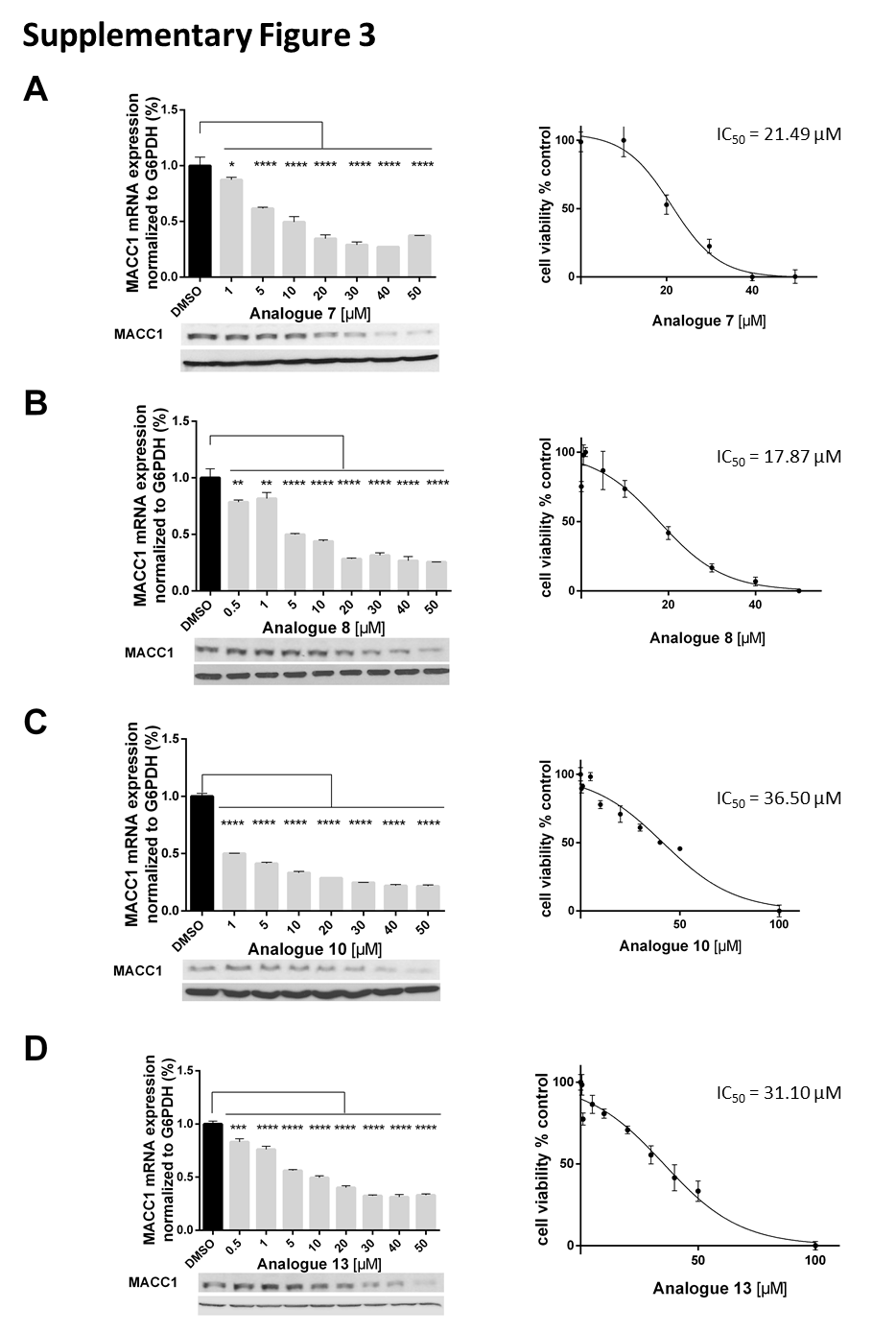
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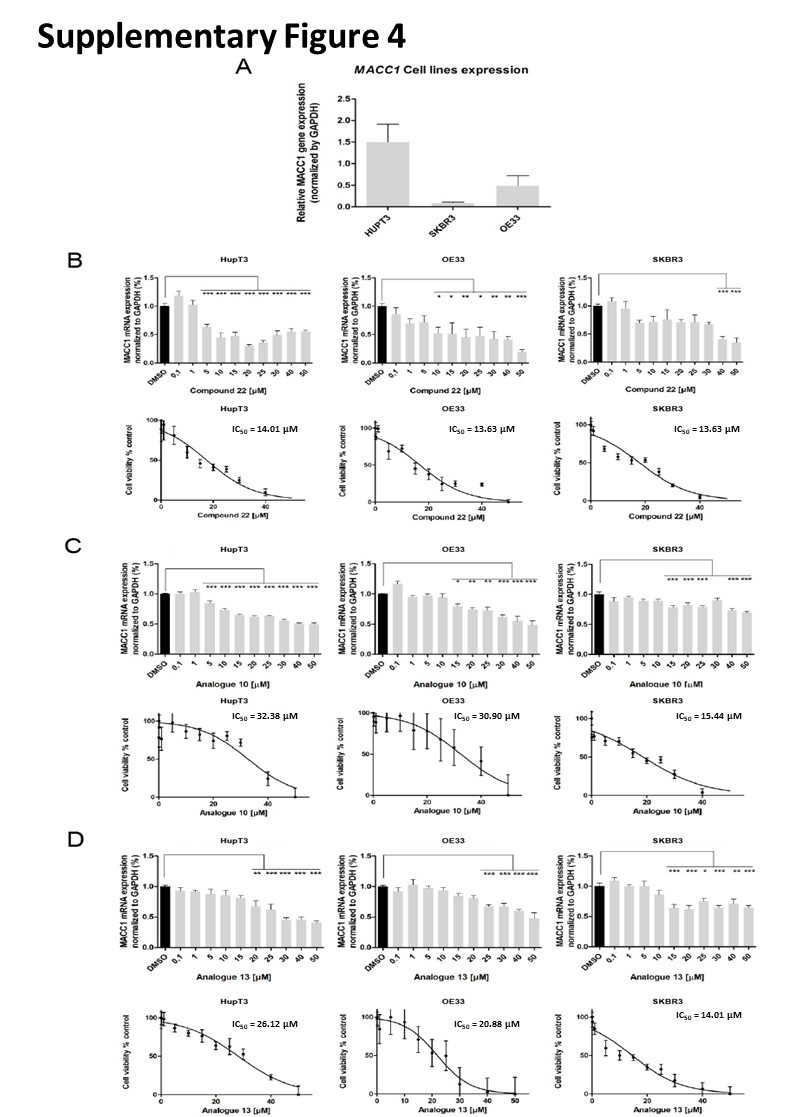
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**Supplementary Figure 1: The effect of Compound 22 analogues on MACC1 expression, cell viability and functional substructure screening.** (**A**) The 5-(tetrazolo[1,5-b]pyridazine-6-ylthio)-1,3,4-thiadiazol-2-amine core represents the essential part for transcriptional MACC1 inhibition, which is dose dependent and reduces MACC1 at mRNA and protein level. 6-(Methylsulfanyl) [1,2,3,4]tetrazolo[1,5-b]pyridazine is the main functional substructure required for MACC1 expression inhibition. Removal of the 2-ethyl-6-methyl residues from the phenol ring, prolongation of ring distance to 1,3,4-thiadiazol-2-amine or alternative residues at the phenol ring lead to loss of inhibitory capacity. (**B**)Analogues ofN,5-dimethyl-1,3,4-thiadiazol-2-amine however retain inhibitory effect on MACC1 expression at mRNA and protein level at reasonable cytotoxicity. (**C**, **D**) The presence of the tetrazolo[1,5-b]pyridazine moiety is critical for MACC1 inhibition, and their replacement leads to loss of activity, shown by qRT-PCR analysis. For all qRT-PCR and MTT assays, HCT116 cells were treated for 24 h with the Compound 22 analogues. MACC1 mRNA levels were normalized to G6PDH mRNA expression and respective DMSO control (black bar). Results for mRNA represent means ± SEM of three independent experiments. By Western blot, one representative example of three independent experiments is shown, β-actin served as loading control. Cell viability was measured independently by MTT assay. Results are shown as mean ± SEM of three independent experiments performed in triplicate. Significant results were determined by one-way ANOVA and multiple comparison was done by Dunnett’s post tests (\* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001, \*\*\*\* = p < 0.0001).

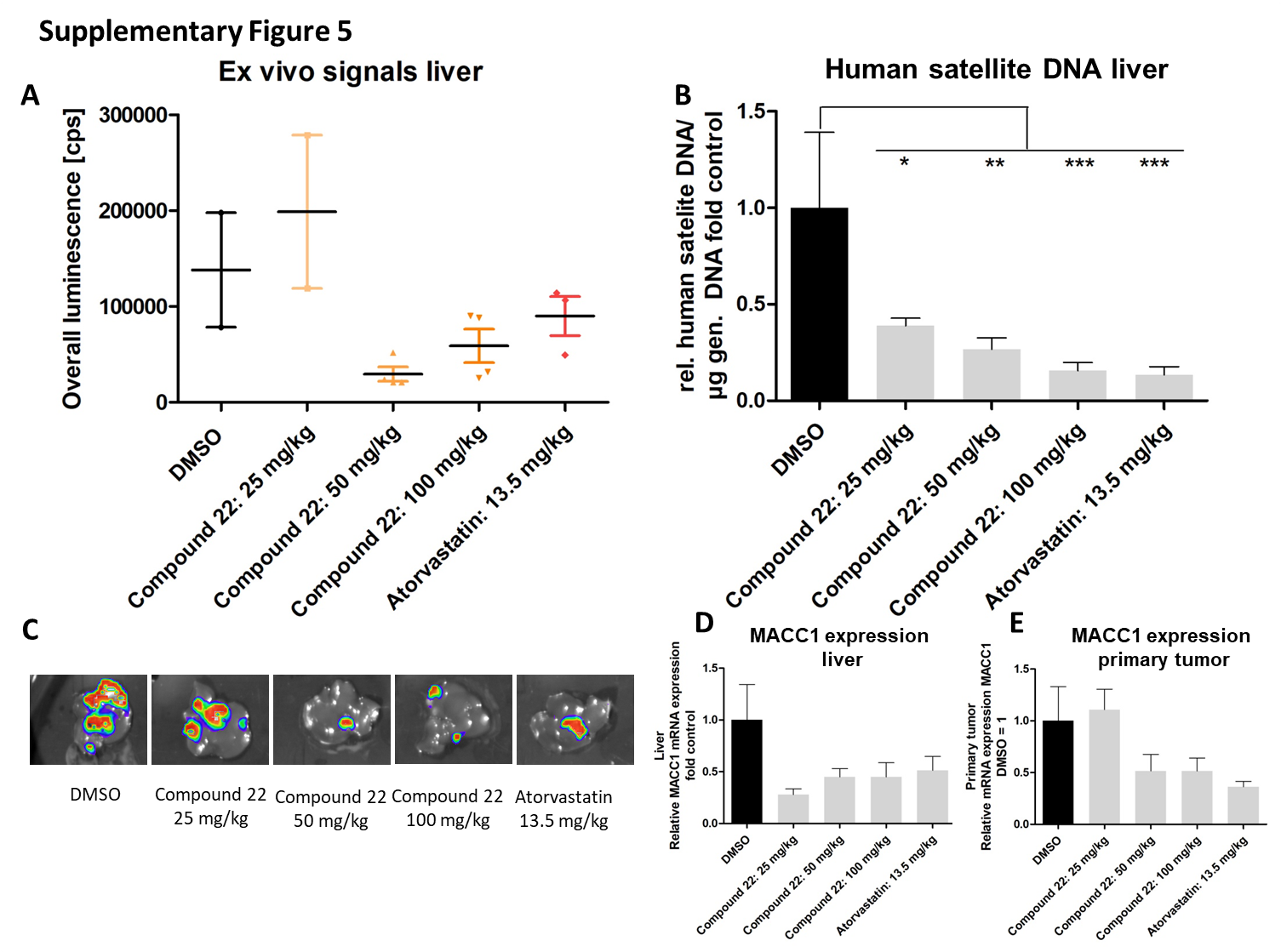
**Supplementary Figure 2: The effect of Compound 22 analogues 5 and 9 on MACC1 expression and cell viability in HCT116 and SW260 CRC cells.** The 5-(tetrazolo[1,5-b]pyridazine-6-ylthio)-1,3,4-thiadiazol-2-amine core represents the essential part for transcriptional inhibition of MACC1, which is dose dependent and reduces MACC1 at mRNA and protein level. MACC1 inhibition is shown at mRNA and at protein level in HCT116 and SW620 cells treated with increasing concentrations of (**A, B**) Analogue 5, (**C, D**) Analogue 9. The left panels show the respective IC50 values for the two analogues in HCT116 and SW620 cells.

**Supplementary Figure 3: The effect of Compound 22 analogues 7, 8, 10 and 13 on MACC1 expression and cell viability in SW620 CRC cells.** The 5-(tetrazolo[1,5-b]pyridazine-6-ylthio)-1,3,4-thiadiazol-2-amine core represents the essential part for transcriptional inhibition of MACC1, which is dose dependent and reduces MACC1 at mRNA and protein level. MACC1 inhibition is shown at mRNA and at protein level in SW620 cells treated with increasing concentrations of (A) Analogue 7, (B) Analogue 8, (C) Analogue 10 and (D) Analogue 13. The left panels show the respective IC50 values for the four analogues.

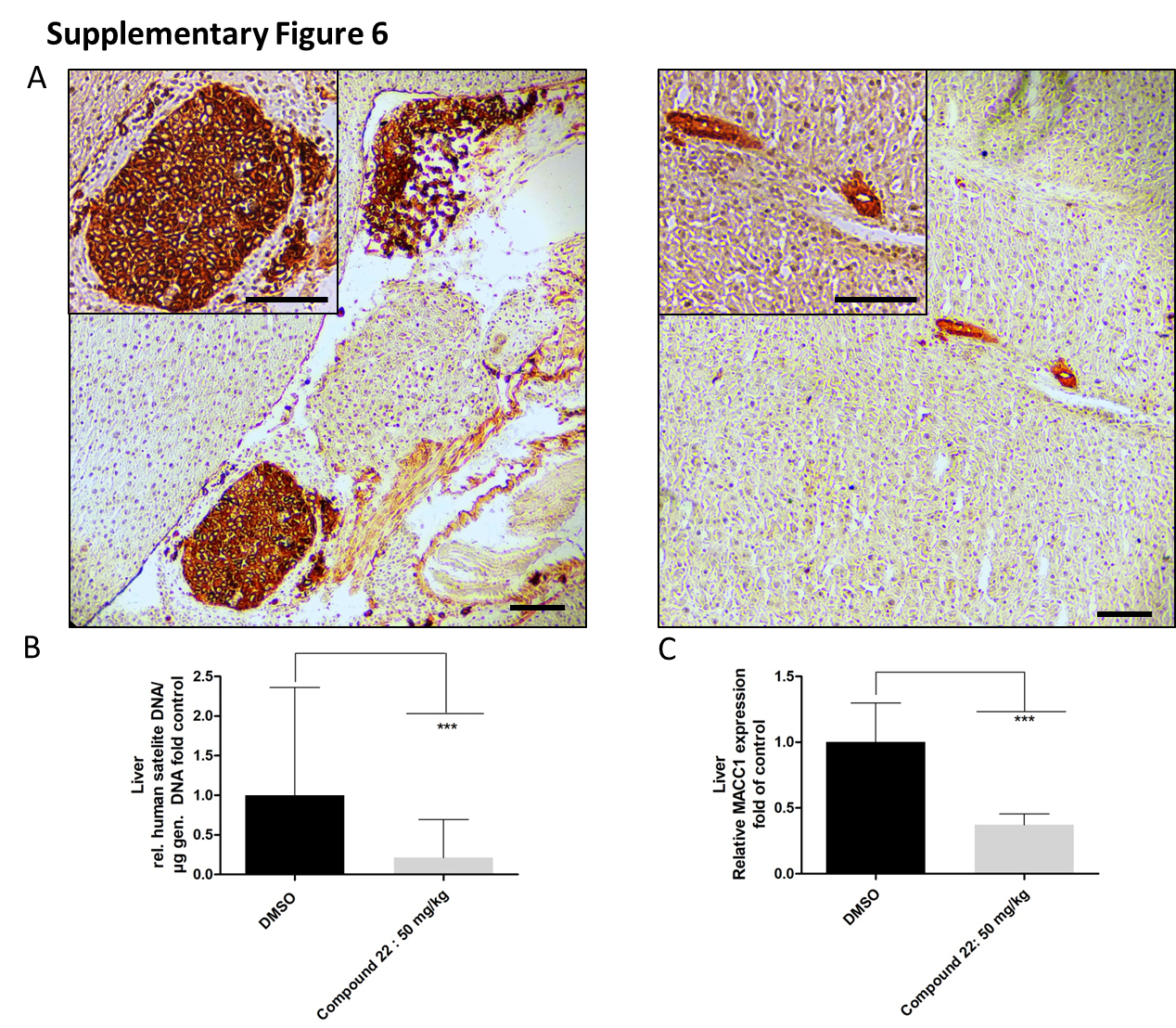


**Supplementary Figure 4: The effect of Compound 22 and analogues 10 and 13 on MACC1 expression and cell viability in cell lines of   
pancreatic (HupT3), esophageal (OE33) and breast cancer (SKBR3) cell lines.**

(A) Three cell lines of three different solid cancer entities with different MACC1 expression levels were used. Compound 22 (B) and the analogues 10 (C) and 13 (D) were able to reduce MACC1 mRNA expression in a dose-dependent manner. The lower panels show the toxicity data measured by MTT.

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**Supplementary Figure 5:** Dose escalation in vivo experiment. (A) Overall luminescence signals of the liver of Mice treated with DMSO control or increasing Compound 22 concentrations (25, 50,  
100 mg/kg) and Atorvastatin control (13.5 mg/kg). (B) One representative picture of ex vivo liver bioluminescence signals. (C) MACC1 expression levels of the primary tumor (spleen). (D) MACC1 expression levels of liver metastasis. (E) Detection of human satellite DNA from the liver. All expression levels were normalized to DMSO treated controls (black bar, DMSO) and detected by RT-qPCR. Statistical analysis was conducted using one-way ANOVA and multiple comparison was done by Dunett’s post tests. (\* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001).

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**Supplementary Figure 6:** Second tumor in vivo model using SW620 CRC cells. A) Immunohistochemistry pictures of livers of untreated (DMSO, left) and Compound 22 treated mice (50 mg/kg, right) stained with anti-human CK19 antibody (brown staining) at 10 fold and 20 fold (inner picture) resolution. Scale bars indicate 100 µM. B) Human satellite expression in the livers of untreated (DMSO) and Compound 22 (50 mg/kg) treated mice. C) Relative MACC1 expression in the liver of untreated (DMSO) and Compound 22 (50 mg/kg) treated mice. Expression levels were normalized to respective DMSO treated controls (black bar, DMSO). Statistical analysis was conducted using unpaired student’s t-test (\* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001)

**Supplementary Table 1:** Small molecule screening data

|  |  |  |
| --- | --- | --- |
| **Category** | **Parameter** | **Description** |
|
| Assay | Type of assay | Cell-based in vitro screening |
| Target | MACC1 promoter |
| Primary measurement | MACC1 promoter driven firefly luciferase |
| Key reagents | Britelite plus Reporter gene assay system ATPlite ATP detection assay system (for cytotox measurement)  Both: Perkin Elmer |
| Assay protocol | Cell-based assay performed as described in the manuals of the reagents |
| Additional comments |  |
| Library | Library size | 118850 compounds |
| Library composition | Diversity Library of Chemical Biology Core Facility at EMBL (Heidelberg) |
| Source | Drug-like molecules selected from big vendor collections (e.g. Enamine, ChemDiv, ChemBridge, Asinex, Wuxi) |
| Additional comments |  |
| Screen | Format | 384 well plates |
| Concentration(s) tested | High-Throughput Screen with compounds at a single concentration of 10 µM |
| Plate controls | Gentian violet [20 µM] as positive control (100% inhibition) |
| Reagent/ compound dispensing system | FlexDrop IV Exi, Perkin Elmer |
| Detection instrument and software | Instrument: EnVision Xcite, Perkin Elmer  Software: EnVision Manager  1.13.3009.1401 |
| Assay validation/QC | Average Z-prime (370 plates): 0.64 |
| Correction factors | - |
| Normalization | Normalized to positive (Gentian violet, 100% inhibition) controls |
| Additional comments | Screen carried out by the Chemical Biology Core Facility at EMBL, Heidelberg |
| Post-HTS  analysis | Hit criteria | Reduction of MACC1 promoter driven firefly luciferase activity, no firefly luciferase inhibition in a cell-free assay, low cytotoxicity (IC50 > EC50) |
| Hit rate | Inhibition > 90%: 0.08% (97 cmpds)  Inhibition > 80%: 0.13% (160 cmpds)  Inhibition 70%: 0.18% (216 cmpds)  Inhibition 58% (3x SD): 0.27% (332 cmpds) |
| Additional assay(s) | Counterscreen using luciferase protein, cytotox assay |
| Confirmation of hit purity and structure | Hit compounds were re-ordered and compound was quality checked via UPLC-measurement |
| Addtitional comments |  |

**Supplementary Table 2:** Chemical structure and summary of key characteristics of compound activities of all active Compound 22 analogues

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Compound** | **Structure** | **Cell line** | **Maximal inhibition**  [**%**] | **At concentration** [µM] | **Active concentration range** [µM] | **EC50 (activity)** [µM] | **IC50 (Tox/MTT)** [µM] |
| normalized | normalized |
| Analogue 5 |  | HCT116 | 80 | 30 | 5-30 | 12.96 | 10.9 |
| SW620 | 53.5 | 20 | 1-20 | 2.302 | 23.61 |
| Analogue 9 |  | HCT116 | 64 | 50 | 30-50 | 29.64 | 17.52 |
| SW620 | 66 | 40 | 10-50 | 12.16 | 22.29 |
| Analogue 10 | | HCT116 | 56 | 50 | 1-50 | 4.42 | 22.08 |
| SW620 | 78 | 40 | 1-50 | 2.6 | 36.5 |
| Analogue 13 | | HCT116 | 70 | 50 | 0.5-50 | 9.44 | 24.42 |
| SW620 | 63 | 50 | 1-50 | 8.73 | 31.1 |
| Analogue 21 |  | HCT116 | 91 | 30 | 1-50 | 6.78 | 7.49 |
| SW620 | 72 | 30 | 1-30 | 9.96 | 12.14 |
| Analogue 23 |  | HCT116 | 88 | 30 | 10-50 | 10.82 | 1.025 |
| SW620 | 75 | 30 | 1-30 | 0.88 | 3.021 |
| Analogue 24 |  | HCT116 | 90 | 50 | 10-50 | 11.49 | 14.68 |
| SW620 | 69 | 30 | 10-30 | 10.53 | 17.63 |
| Analogue 25 |  | HCT116 | 68 | 50 | 30-50 | 17.64 | 14.3 |
| SW620 | 65 | 50 | 1-50 | 8.4 | 12.36 |
| Analogue 26 |  | HCT116 | 87 | 30 | 10-30 | 7.089 | 7.697 |
| SW620 | 79 | 30 | 1-30 | 5.987 | 14.73 |

**Supplementary Table 2** continued

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Compound** | **Structure** | **Cell line** | **Maximal inhibition**  [**%**[ | **At concentration** [µM[ | **Active concentration range** [µM] | **EC50 (activity)** [µM] | **IC50 (Tox/MTT)** [µM] |
| normalized | normalized |
| Analogue 15 |  | HCT116 | 78 | 50 | 50 | 33.99 | 49.31 |
| Analogue 17 |  | HCT116 | 85 | 50 | 30-50 | 26.99 | 12.87 |
| Analogue 30 |  | HCT116 | 35.24 | 30 | 5-30 | N/A | 133.70 |
| Analogue 39 |  | HCT116 | 76.89 | 30 | 1-30 | 3.278 | 19.88 |
| Analogue 41 |  | HCT116 | 54.17 | 30 | 20-30 | 12.45 | 21.16 |
| Analogue 46 |  | HCT116 | 62.16 | 30 | 10 | 7.217 | 5.95 |
| Analogue 47 |  | HCT116 | 53.28 | 30 | 20-30 | 8.483 | 16.93 |
| Analogue 51 |  | HCT116 | 87.41 | 20 | 5-30 | 4.973 | 10.66 |

**Supplementary Table 2** continued

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Compound** | **Structure** | **Cell line** | **Maximal inhibition**  [**%**] | **At concentration** [µM] | **Active concentration range** [µM] | **EC50 (activity)** [µM] | **IC50 (Tox/MTT)** [µM] | |
| normalized | | normalized |
| Analogue 56 |  | HCT116 | 77.33 | 30 | 1-30 | 4.558 | | 26.42 |
| Analogue 62 |  | HCT116 | 75.89 | 30 | 5-30 | 6.1 | | 22.33 |
| Analogue 65 |  | HCT116 | 83.08 | 30 | 10-30 | 8.369 | | 15.42 |
| Analogue 66 |  | HCT116 | 86.46 | 30 | 1-30 | 2.042 | | 18.91 |
| Analogue 71 |  | HCT116 | 78.03 | 30 | 1-30 | 3.203 | | 20.70 |
| Analogue 73 |  | HCT116 | 83.32 | 20 | 1-30 | 1.207 | | 4.83 |
| Analogue 74 |  | HCT116 | 87.79 | 30 | 1-30 | 1.149 | | 10.18 |
| Analogue 75 |  | HCT116 | 86.72 | 30 | 1-30 | 1.013 | | 12.57 |

**Supplementary 3:** Chemical structure of all inactive Compound 22 analogues.

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound** | **Structure** | **Compound** | **Structure** |
| Analogue 2 |  | Analogue 18 |  |
| Analogue 3 |  | Analogue 19 |  |
| Analogue 4 |  | Analogue 20 |  |
| Analogue 6 |  | Analogue 22 |  |
| Analogue 11 |  | Analogue 27 |  |
| Analogue 12 |  | Analogue 28 |  |
| Analogue 14 |  | Analogue 29 |  |
| Analogue 16 |  | Analogue 31 |  |

**Supplementary Table 3** continued

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound** | **Structure** | **Compound** | **Structure** |
| Analogue 32 |  | Analogue 42 |  |
| Analogue 33 |  | Analogue 43 |  |
| Analogue 34 |  | Analogue 44 |  |
| Analogue 35 |  | Analogue 45 |  |
| Analogue 36 |  | Analogue 48 |  |
| Analogue 37 |  | Analogue 49 |  |
| Analogue 38 |  | Analogue 50 |  |
| Analogue 40 |  | Analogue 52 |  |

**Supplementary Table 3** continued

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound** | **Structure** | **Compound** | **Structure** |
| Analogue 53 |  | Analogue 63 |  |
| Analogue 54 |  | Analogue 64 |  |
| Analogue 55 |  | Analogue 67 |  |
| Analogue 57 |  | Analogue 68 |  |
| Analogue 58 |  | Analogue 69 |  |
| Analogue 59 |  | Analogue 70 |  |
| Analogue 60 |  | Analogue 72 |  |
| Analogue 61 |  | Analogue 76 |  |

**Supplementary Table 4:** Chemical structure and summary of key characteristics of compound activities of Compound 22 and Analogues 10 and 13 in cell lines of different cancer entities.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Compound** | **Structure** | **Cell line** | **Maximal inhibition**  [**%**] | **At concentration** [µM] | **Active concentration range** [µM] | **EC50 (activity)** [µM] | **IC50 (Tox/MTT)** [µM] |
| normalized | normalized |
| Compound 22 |  | HupT3 | 71 | 20 | 5-50 | 3.38 | 14.01 |
| OE33 | 81 | 50 | 1-50 | 8.27 | 13.63 |
| SKBR3 | 66 | 50 | 5-50 | 9.04 | 13.63 |
| Analogue 10 |  | HupT3 | 51 | 50 | 5-50 | 8.49 | 32.38 |
| OE33 | 52 | 50 | 15-50 | 10.04 | 28.21 |
| SKBR3 | 31 | 50 | 15-50 | 10.43 | 19.20 |
| Analogue 13 |  | HupT3 | 60 | 50 | 5-50 | 17.61 | 27.66 |
| OE33 | 53 | 50 | 15-50 | 19.21 | 27.46 |
| SKBR3 | 38 | 20 | 10-50 | 8.7 | 22.96 |

**Supplementary Table 5:** Physical and Chemical properties of Compound 22, Analogue 10 and Analogue 13.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Compound | MW | logD | logP | H-Bond donors | H-Bond acceptors | | PSA | pKb1 | pKb2 | Purity | Sol Rank | Solu-bility | Stability |
| Compound 22 | 370.5 | 4.86 | 4.87 | 1 | 7 | 93.8 | | -0.97 | -2.14 | 97.2 | 191.4 | 1.89  ± 1.69 | 92.3±5.4 101.6±2.7 87.6±2.2 |
| Analogue  10 | 248.3 | 1.77 | 1.77 | 0 | 5 | 82.0 | | -0.47 | -2.12 | 100 | 454.0 | 100.5  ± 5.93 | 92.9±5.0  101±4.5  93.4±1.6 |
| Analogue  13 | 250.3 | 2.28 | 2.28 | 0 | 5 | 68.9 | | 1.05 | -2.1 | 100 | 477.3 | 594.5  ± 58.53 | 103.4±6.1 94.3±3.5 96.7±3.4 |

logD [pH 7.4]; H-Bond: Hydrogen Bond; SolRank: kinetic solubility [rel. conc µM]; solubility: Shake flask solubility in PBS pH7.4 [µg/ml] ± [σ]; stability: Chemical stability [%Remain at 24 h @pH1/7.4/9] ± [σ]

Properties calculated using ChemAxon, MarvinSketch chemical editor: logD, logP, H-Bond donor and acceptor, pKb1, pKb2

**Supplementary Table 6:** Human plasma stability, protein binding and metabolic stability of Compound 22 and Analogues 10 and 13.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Compound | Plasma stability [% remain @1h] ± [σ] | Plasma protein binding [% bound @6h] | Clint Phase I  [µl / min / mg] | Phase II glucoronidation  [% remain @1h] | Redox-Assay  [AU] |
| human | human | HLM | HLM |
| Compound 22 | 95.5 ± 1.53 | 59.7 | 53.7 | 57.2\* ± 2.4 | No redox activity detected |
| Analogue 10 | 102.9 ± 1.14 | 45.3 | 9.4 | 102.4 ± 2.3 | No redox activity detected |
| Analogue 13 | 93.5 ± 0.79 | 39.4 | 19.7 | 94.3 ± 11.7 | No redox activity detected |

\*Non UGT mediated (w/o UDPGA 56% remain); HLM: human liver microsomes

**Supplementary Table 7:** Permeability of Compound 22 and Analogues 10 and 13. PAMPA = parallel artificial membrane permeability assay.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Compound | PAMPA  pH7.4  [% Flux] | CaCo-2 Assay (bidirectional) | | MDR1-MDCKII (bidirectional) | |
| efflux ratio | Papp A->B  [10^6 cm/s] | efflux ratio | Papp A->B  [10^6 cm/s] |
| Compound 22 | 34.6 | NA\* | NA\* | 0.7 | 27.1 |
| Analogue 10 | 67.5 | 0.8 | 12.8 | 0.7 | 67.9 |
| Analogue 13 | 41.2 | 0.9 | 10.7 | 0.7 | 44.0 |

NA: too low recovery Papp A->B

**Supplementary Table 8:** In silico/ in vitro Toxicity of Compound 22 and Analogues 10 and 13.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Compound | CYP inhibition | | | | | Cyto  toxicity | Cardio  toxicity | Hepato  toxicity | | |
| IC50 CYP1A2 [µM] | IC50  CYP2C9 [µM] | IC50 CYP2C19 [µM] | IC50  CPY2D6 [µM] | IC50  CYP3A4 [µM] | IC50  PBMC-CTG [µM] | IC50  hERG binding FP assay [µM] | IC50  HepG2 +  primary  Hepatocytes  CTG [µM] | | |
|  |  |  |  |  |  |  |  | human | mouse | |
| Compound 22 | >50 | 38.25 | ~10 | >50 | 33.34 | 5.94 | >30 | 21.6 | | 15.2 |
| Analogue 10 | >50 | >50 | ~25 | >50 | >50 | >30 | >30 | >30 | | >30 |
| Analogue 13 | 39.09 | >50 | ~25 | >50 | >50 | >30 | >30 | >30 | | >30 |