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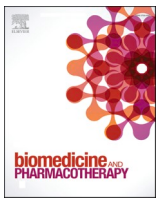

Discovery of tetrazolo-pyridazine-based small molecules as inhibitors of MACC1-driven cancer metastasis

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ABSTRACT

Metastasis is directly linked to poor prognosis of cancer patients and warrants search for effective anti-metastatic drugs. MACC1 is a causal key molecule for metastasis. High MACC1 expression is prognostic for metastasis and poor survival. Here, we developed novel small molecule inhibitors targeting MACC1 expression to impede metastasis formation. We performed a human MACC1 promoter-driven luciferase reporter-based high-throughput screen (HTS; 118.500 compound library) to identify MACC1 transcriptional inhibitors. HTS revealed 1,2,3,4-tetrazolo[1,5-*b*]pyridazine-based compounds as efficient transcriptional inhibitors of MACC1 expression, able to decrease MACC1-induced cancer cell motility in vitro. Structure-activity relationships identified the essential inhibitory core structure. Best candidates were evaluated for metastasis inhibition in xenografted mouse models demonstrating metastasis restriction. ADMET showed high drug-likeness of these new candidates for cancer therapy. The NFκB pathway was identified as one mode of action targeted by these compounds. Taken together, 1,2,3,4-tetrazolo[1,5-*b*]pyridazine-based compounds are effective MACC1 inhibitors and pose promising candidates for anti-metastatic therapies particularly for patients with MACC1-overexpressing cancers, that are at high risk to develop metastases. Although further preclinical and clinical development is necessary, these compounds represent important building blocks for an individualized anti-metastatic therapy for solid cancers.

1. Introduction

The search for novel and effective small-molecule inhibitors is crucial for improving cancer therapy. Since metastasis is the major clinical challenge in cancer therapy, novel compounds able to interfere with metastasis formation are of great interest.

Colorectal cancer (CRC) is one of the leading causes of cancer-associated death worldwide [1]. Metastasis of CRC is directly linked to poor patient survival, accounts for about 90% of all CRC-related deaths and is a key barrier for successful treatment of CRC patients. Identification of causative key drivers for metastasis is the basis for efficient drug intervention targeting these drivers.

Our lab newly discovered the gene MACC1 (Metastasis Associated in Colon Cancer [1,2]. MACC1 induces proliferation, migration and invasion of cancer cells in vitro and drives tumor progression and metastasis in vivo [3–5]. MACC1 prognosticates development of metastasis and CRC patient survival. Tumor progression and metastasis formation of more than 20 solid cancer entities are associated with high MACC1

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expression levels in tumor tissue and patient blood (e.g. solid cancers, digestive system, hepatocellular cancer, CRC, gastric, breast and gynecological cancers; 6–9). Further, MACC1 is a predictive marker for therapy response, e.g. for treatment with 5-fluorouracil and platinum compounds, and is indicative for chemoresistance [10–14]. Taken together, MACC1 plays a causal role in inducing tumor progression and metastasis, and is an independent prognostic and predictive biomarker for CRC and many other cancer entities.

Since MACC1 represents a valuable target for effective intervention of metastasis, we used siRNA/shRNA or knockout approaches. We demonstrated that lowering MACC1 expression is associated with reduced cell motility and metastasis in mouse models [2,3,5]. Further, repositioned small molecules such as rottlerin and statins acting on MACC1 transcription were also efficient for metastasis intervention [15, 16].

Here, we focus on novel compounds acting on MACC1 transcription with the goal of further improving the therapeutic outcome. By high-throughput screening (HTS) we tested more than 118,500 compounds (EMBL, Heidelberg, Germany). We identified a novel, 1,2,3,4- tetrazolo [1,5-*b*]pyridazine-based compound and analogues thereof acting as potent MACC1 transcriptional inhibitors. These compounds were functionally probed for intervening in MACC1-driven cell migration and proliferation in cell culture, and most importantly for restriction of MACC1-induced metastasis formation in mouse models. The 1,2,3,4-tetrazolo[1,5-*b*]pyridazine represents a promising lead structure. Related chemical structures provided a first indication that such building blocks could exert biological activity in vitro and also in vivo. However, at the given time point this had not yet been proven in the context of MACC1 [17–20]. However, using such small molecules holds promise for finding novel, active anti-metastatic compounds.

2. Material and methods

All analyses described were performed independently at least 3 times (biological replicates with several technical replicates per experiment). Detailed description for the following experimental procedures can be found in the Supplementary Information: cell culture, generation of MACC1-promoter reporter cell lines, high throughput screening (HTS), Steady-Glo® Luciferase assay, MTT cell viability assay, quantitative real-time reverse transcription PCR (qRT-PCR), detection of human satellite DNA in mouse liver, gene expression analysis after TNF-α stimulation, Western blot analysis, transwell migration assay (Boyden chamber assay), wound healing assay, immunohistochemistry, prediction of NFκB transcription factor binding sites of the MACC1 promoter, ADME/TOX methods, in vivo tolerability for MACC1 inhibitors, in vivo pharmacokinetics study, in vivo testing of inhibitors for metastasis, and statistical analysis.

3. Results

3.1. Identification of novel MACC1 transcription inhibitors

We performed HTS with more than 118.500 compounds (Supplementary Table 1). We employed HCT116 CRC cells expressing the human MACC1 promoter-driven luciferase reporter gene (HCT116-MACC1p-Luc, Fig. 1A, Fig. 1B). After screening for compound-mediated reporter expression inhibition and counter screening for cytotoxicity and exclusion of luciferase enzyme activity inhibitors, 66 compounds were selected for the validation screen with a compound concentration of 30 μM. Six compounds (no. 23, 25, 26, 41, 49, 52) were excluded due to high cytotoxicity. The remaining 60 compounds were tested for MACC1 transcriptional inhibition by qRT-PCR (Fig. 1C). Compound no. 22, N-(2-ethyl-6-methylphenyl)− 5-(tetrazolo[1,5-*b*]pyridazine-6-ylthio)− 1,3,4-thiadiazol-2-amine, was identified as most effective for MACC1 inhibition. Compound 22 has a linear structure, which is rarely reported in a biological context. Compound 22 and its analogues consist of a tetrazolo[1,5-*b*]pyridazine essential core structure that is linked to a thiadiazole via a thioether bridge. Distal to the thioether, the thiadiazole ring is decorated with different substituted anilines or amines.

To evaluate Compound 22 efficacy in vitro, HCT116-MACC1p-Luc cells were treated with increasing concentrations (5–50 μM) for 24 h. Compound 22 significantly reduced MACC1 promoter-driven luciferase expression (**** = $p < 0.0001$, Fig. 1D), starting at 5 μM, with an inhibiting concentration 50 (IC_{50}) of 7.36 μM in moderate MACC1-expressing HCT116 and an IC_{50} of 6.39 μM in high MACC1-expressing SW620 CRC cells (Fig. 1E).

To further improve efficacy of Compound 22 for reducing MACC1 expression at low toxicity, we tested available 1,2,3,4-tetrazolo[1,5-*b*] pyridazine-based analogues to better define the functional groups and core pharmacophoric structure, being essential for the effective MACC1 expression inhibition.

3.2. Hit expansion study reveals main active core substructure

To analyze the structural requirements for transcriptional MACC1 inhibition based on Compound 22, 75 analogues were selected and analyzed with structure-activity relationship (SAR) for their inhibitory potential over several iterative testing rounds. In vitro qRT-PCR, Western blot, cell viability, cell migration and wound healing assays were used to verify MACC1 expression inhibition and its functional consequences in HCT116 (Fig. 2, Supplementary Fig. 1) and SW620 cells (Supplementary Fig. 2, 3). Compared to the initial structural features of Compound 22, and according to the change of the chemical structure of its analogues, the number of screened compounds was gradually narrowed down.

To investigate the role of the distal phenyl ring and preserving the tetrazolo[1,5-*b*]pyridazine thiadiazol elements, eight analogues of Compound 22 (Analogue 2–9) were selected (Supplementary Fig. 1). These analogues have a similar chemical substructure as Compound 22, maintaining the basic core structure (Supplementary Fig. 1 A). The qRT-PCR in HCT116 and SW620 cells showed that Analogue 5, 7, 8 and 9 inhibited MACC1 expression, while Analogue 2, 3, 4 and 6 did not (Supplementary Fig. 1B, 2, 3). Cell viability testing by MTT assay revealed that the IC_{50} of these eight analogues was different due to the difference in substructures, rather than the core part. MACC1 expression inhibition and reduction in MACC1 protein is exemplified for the highly active Analogue 5, 7 and 8 (Supplementary Fig. 1B, 2, 3). These analogues possess low toxicity with IC_{50} of 10.9 μM, 18.11 μM and 21.34 μM in HCT116 cells (Supplementary Fig. 1B, 2), and comparable IC_{50} values in SW620 cells (Supplementary Fig. 2, 3). In summary, Analogue 7 and 8 had favorable characteristics outperforming Compound 22, carrying a bulky alkyl substituent (i.e. iso-propyl and tert-butyl) instead of the phenyl ring of Compound 22 (Supplementary Table 2, 3).

We next examined the role of the tetrazolo[1,5-*b*]pyridazine moiety by replacing it in Analogue 19, 20, and 22 (Supplementary Fig. 1 C) with a quinoxaline or pyrimidine ring. However, no inhibitory effect on MACC1 expression in HCT116 cells was detected, indicating the crucial role of the bicyclic tetrazolo[1,5-*b*]pyridazine ring system (Fig. 2D).

Next, we focused on the connecting 5-membered thiadiazole ring using nine analogues with either a thioether or an amino linkage: Analogue 10–17 (Fig. 2A). Four analogues containing an oxazole (Analogue 10), thiazole (Analogue 13) or oxadiazole (Analogue 15, Analogue 17) were active even with major changes at phenyl ring region essentially truncating this portion in Analogue 10 and 13. All analogues bearing this substructure and retaining the thioether linkage inhibited MACC1 mRNA expression in HCT116 and SW620 CRC cells shown by qRT-PCR (Fig. 2B; Supplementary Fig. 3), while the other analogues incorporating an amine linkage and other than thiadiazole ring systems had no effect (Fig. 2A). According to the MTT assay, Analogue 10 and 13 had the lowest toxicity in HCT116 cells, with IC_{50} of 22.08 μM and 24.42 μM (Fig. 2B). By comparing both EC_{50} and IC_{50} analyses the

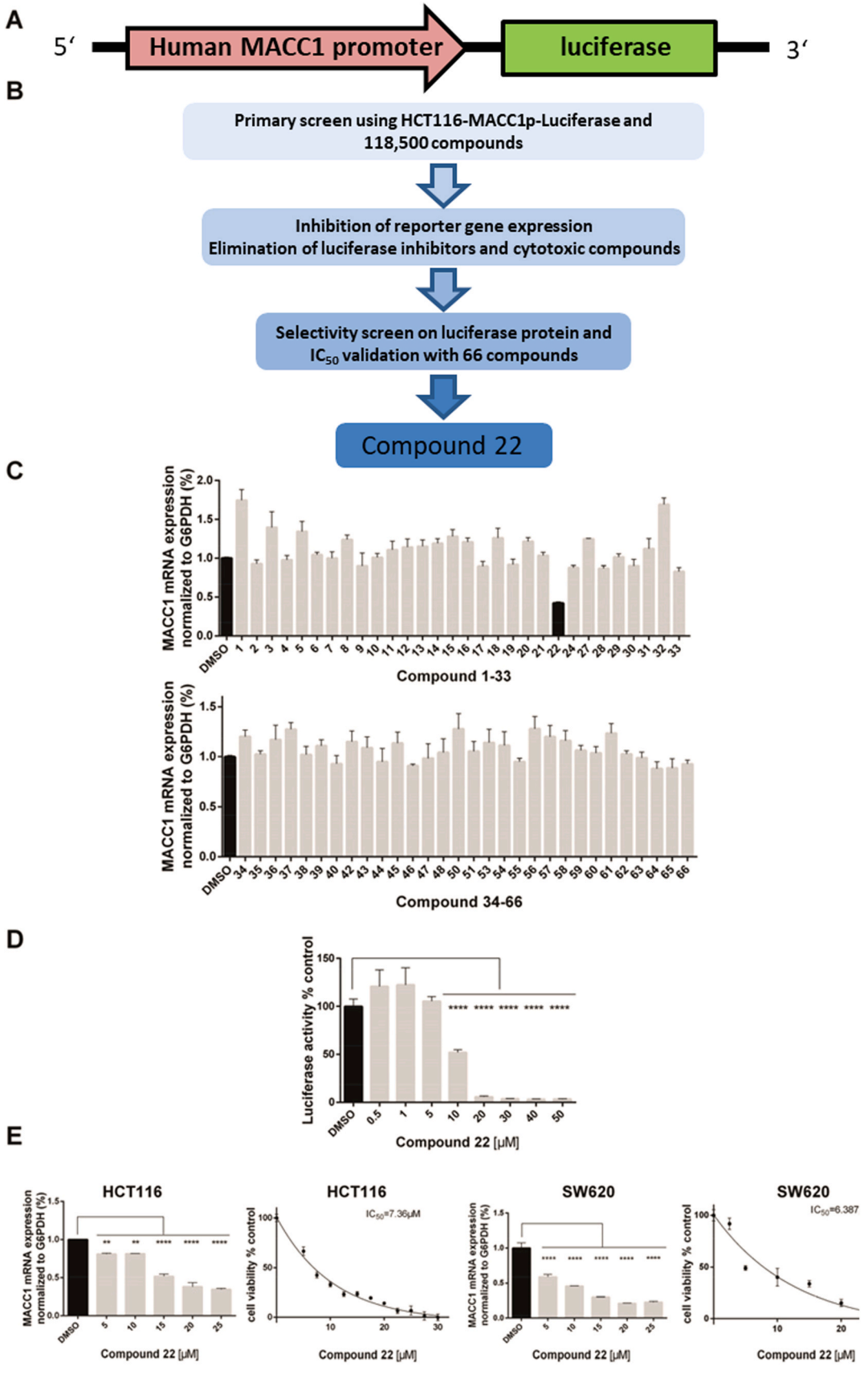

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Fig. 1.. Identification of novel small molecules for efficient MACC1 transcription inhibition. (**A**) Scheme of luciferase reporter gene construct. Luciferase is driven by the human MACC1 promoter (MACC1p). This construct was stably transfected into HCT116 cells, generating HCT116-MACC1p-Luc cells used for HTS. (**B**) Transcription inhibitors of MACC1 were identified from the compound library through HTS and underwent a stepwise selection/exclusion process, resulting in the lead Compound 22. (**C**) 60 pre-selected compounds were tested for their ability to down-regulate intrinsic MACC1 expression in HCT116 cells in vitro of which Compound 22 was most effective. (**D**) Then, HCT116-MACC1p-Luc cells were treated with increasing concentrations of Compound 22 for 24 h to evaluate best concentration for MACC1 expression inhibition in the reporter assay. (**E**) Compound 22 inhibits endogenous MACC1 expression (left panels) in HCT116 (moderate MACC1 expression) and SW620 (high MACC1 expression) cells (right panels). MACC1 mRNA levels were normalized to G6PDH mRNA expression and respective DMSO treated controls (black bar, DMSO). Results for mRNA represent mean values ± Standard error of the mean (SEM) of three independent experiments. Significant results were determined by one-way ANOVA and multiple comparison was done by Dunnett's post tests (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

strongest MACC1 expression inhibition and reduction in MACC1 protein was measured for Analogue 10 and 13 compared to other active analogues (Fig. 2B; Supplementary Fig. 3; Supplementary Table 2, 3). Therefore, we further evaluated the impact of these two analogues in functional in vitro assays.

When comparing the chemical structure of these analogues with Compound 22, it is apparent that all analogues that inhibit MACC1 expression carry the 6-(methylsulfanyl) [1–4] tetrazolo[1,5-*b*]pyridazine element and a thioether linked 5-membered heterocycle (red marked, Fig. 2A). Loss of effectivity towards MACC1 gene expression was observed by replacing a thioether linkage to an amine linker (inactive analogues, Fig. 2A, Supplementary Table 3) which was detrimental for MACC1 inhibition. This substructure marked in red may be the minimal pharmacophoric substructure for effective inhibition of MACC1 expression. To test this hypothesis, we investigated additional very similar analogues bearing only gradual changes (Fig. 2C, D; Supplementary Table 2, 3).

Thirteen analogues have the same 6-(methylsulfanyl) [1–4] tetrazolo [1,5-*b*]pyridazine substructure as Compound 22. Forty-three molecules had a modification at the aforementioned substructure (e.g. Analogue 56). This heterocycle lacks one nitrogen at the 5-membered ring of the parent tetrazolopyridazine, but carries a bulky trifluoromethyl group. Analogue [21] and with the substructure of 6-(methylsulfanyl) [1–4] tetrazolo[1,5-*b*]pyridazine (marked in red) did inhibit MACC1 expression, while Analogue 27 and 28 did not, shown by qRT-PCR. Only 11 of the 43 analogues had an inhibitory MACC1 expression effect (Supplementary Table 2). This indicates the highly discriminative nature of the interaction in this region, meaning that a few single atom changes or altered substituting groups completely abolish the inhibitory ability on MACC1 expression (Supplementary Table 3).

Since MACC1 is causal for metastasis formation of various solid cancers, we tested if the identified primary structure and the Analogues 10 and 13 are able to inhibit MACC1 gene expression in cell lines of other cancer entities. HupT3 (pancreatic adenocarcinoma), OE33 (esophageal adenocarcinoma) and SKBR3 (breast adenocarcinoma) cells with different MACC1 mRNA expression levels were used (Supplementary Fig. 4). Compound 22 and the two most promising analogues 10 and 13 are able to inhibit MACC1 expression in a dose-dependent manner (Supplementary Fig. 4; Supplementary Table 4). Interestingly, the effect is stronger with higher endogenous MACC1 expression (HupT3 >OE33 >SKBR3). This generalizes the MACC1-inhibitory effects of Compound 22 and of the two analogues. Taken together, these gradual changes in the molecule indicate where potential further modifications of the molecule might be advantageous for compound optimization.

3.3. Cancer cell motility inhibition

We identified two highly active analogues, Analogue 10 and 13, inhibiting MACC1 gene expression at tolerable cytotoxicity (Supplementary Table 2). Since MACC1 is a key driver for increased migration, we investigated the effect of Compound 22 and the two Analogues 10 and 13 on MACC1-mediated cell migration in the Boyden chamber assay. HCT116 cells treated with different compound concentrations (all below the respective IC_{50}) for 24 h showed significantly reduced cell migration compared to solvent-treated control cells in concentration-dependent manner (Fig. 3A).

We further analyzed the effect of these compounds on directed migration in a wound healing assay (Fig. 3B). Lower effective concentrations than the IC_{50} were selected. The inhibitory effect reached its maximum when compound treatment was performed for 24–36 h (Fig. 3C, Supplementary Fig. 5). After 36 h, the wound in the solvent-treated control group was completely closed, while wounds were still open in the compound-treated groups. Thus, both migration and wound healing assays, confirmed that these highly active analogues not only inhibit MACC1 expression, but also efficiently reduce MACC1-mediated functions such as cell motility.

3.4. ADMET (absorption, distribution, metabolism, excretion and toxicity) studies confirm drug-like behavior of novel compounds

As important parameter, ADMET properties of the compounds were assessed (Fig. 4A). The displayed predictions of the OSIRIS property explorer are in accordance with the following assays. Compared to Compound 22 the analogues displayed improved solubility. The drug-score, summarizing the predictions made, was 0.28 for Compound 22 and improved for Analogue 10 with 0.43 and Analogue 13 with 0.76.

The physical and chemical properties were calculated using the chemical editor MarvinSketch (ChemAxon, Budapest, Hungary). All three molecules fulfill the Lipinski rules of five, indicating their drug-likeness (Supplementary Table 5). Increased solubility was shown for the analogues vs. Compound 22. Regarding plasma stability and plasma protein binding (Supplementary Table 6), all three tested molecules were stable for at least 1 h with more than 93% still remaining after incubation in human plasma. Compound 22 showed a plasma protein binding of 59.7% compared to the analogues with only 39–45%. No redox activity was detected for the three molecules. The molecules displayed a high permeability with little efflux determined by PAMPA and CaCo-2 Assay (Supplementary Table 7). No efflux was measured by MDR1-MDCKII assay (Supplementary Table 7).

Assays on CYP (Cytochrome P450) inhibition in human liver microsomes and toxicity (Supplementary Table 8) showed no major CYP inhibition. IC_{50} values for more than half of the tested CYP enzymes were above 50 μM concentration. Compound 22 displayed an IC_{50} of 5.94 μM against human PBMCs (peripheral blood mononuclear cells) in a luminescent cell viability assay after 72 h incubation which was increased to above 30 μM for the analogues. This IC_{50} shift was also seen in plateable primary murine and human hepatocytes (Supplementary Table 8). All compounds showed no hERG (human ether-a-go-go-related gene) potassium channel binding in a Fluorescence Polarization assay up to 30 μM, indicating that these compounds may not present potential cardiotoxic hERG channel liabilities.

In summary, Compound 22 and the analogues display a drug-like behavior. The analogues show improved properties compared to the primary hit structure.

3.5. In vivo metastasis reduction by novel MACC1 inhibitors

First, we determined in vivo tolerability of daily oral application of Compound 22 at escalating doses of 25–200 mg/kg in mice (Fig. 4B). We

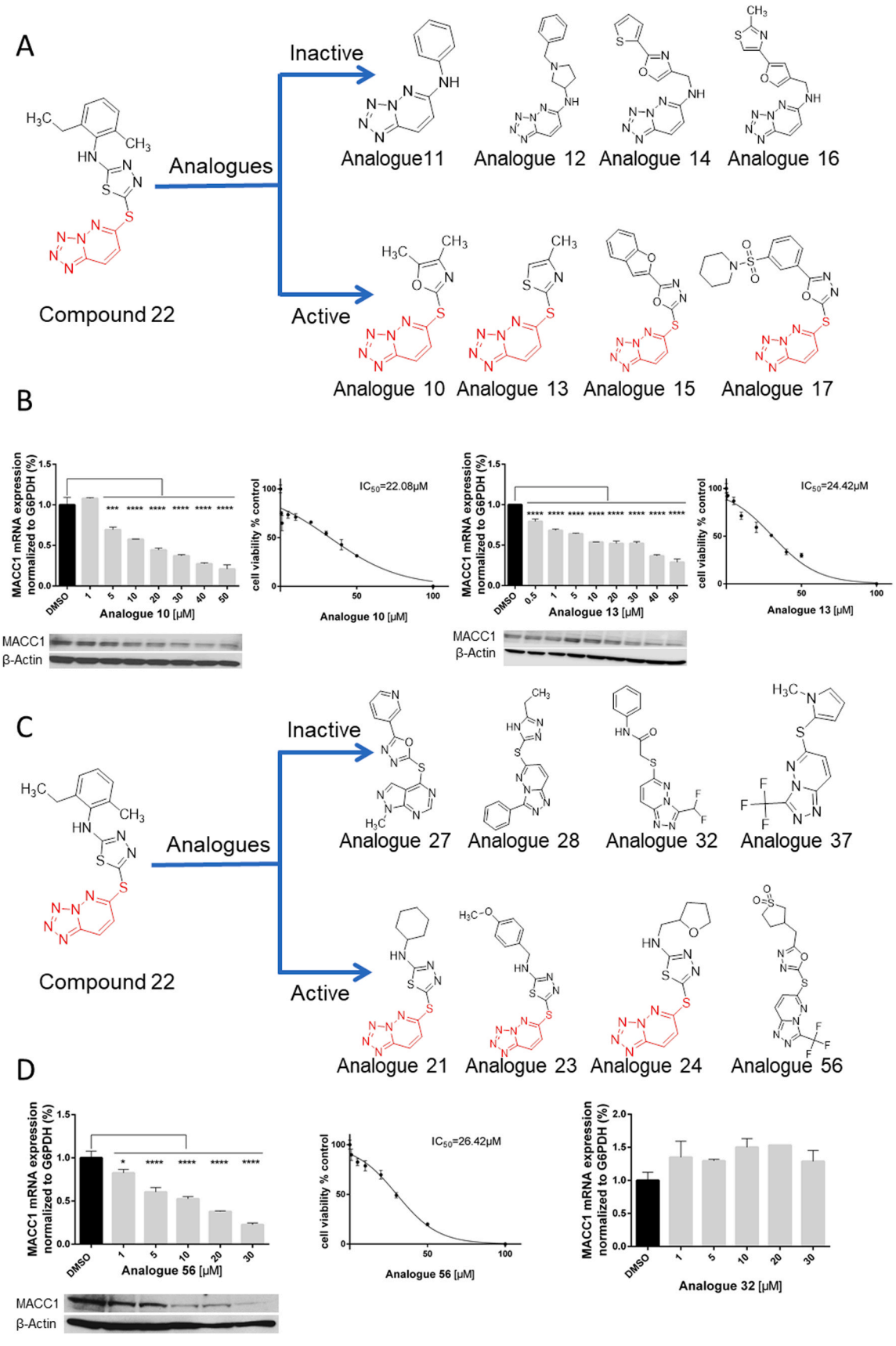

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Fig. 2.. The effect of Compound 22 analogues on MACC1 expression, cell viability in association with functional substructure screening. (**A**) All analogues with the 6-(methylsulfanyl) [1–4] tetrazolo[1,5-*b*]pyridazine element and a thioether linked 5-membered heterocycle (red marked) reduced (**B**) MACC1 expression at mRNA and protein level. (**C, D**) Replacement of 6-(methylsulfanyl) [1–4] tetrazolo[1,5-*b*]pyridazine (marked in red), but also of thioether linkage to an amine linker led to loss of activity, shown by qRT-PCR and Western blot of MACC1 expression (exemplified by the active Analogue 56 versus inactive Analogue 32). 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. For Western blot analysis, 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 triplicates. Significant results were determined by one-way ANOVA and multiple comparison was done by Dunnett's post tests (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

did not observe any toxicity or side effects, only reversible body weight losses ranging between 3% and 5% (Fig. 4B) [22]. We selected the dose of 50 mg/kg to determine tolerability of Analogue 10 and 13 in vivo (Fig. 4C). Compared to vehicle (solvent)-treated animals there was no decrease of the body weight. Therefore, a dose of 50 mg/kg was applied in the forthcoming therapeutic animal experiments. Then the pharmacokinetics were explored by administering a single dose of 50 mg/kg applied orally (Fig. 4L). Applying Compound 22 via the oral route led to a fast increase of the Compound 22 plasma levels. The $t_{1/2}$ was calculated to be 9,83 h.

In a pilot study, we used Compound 22 to determine the anti-metastatic activity in SCID-beige mice, which received HCT116-CMVp-Luc CRC cells intrasplenically to develop liver metastasis. Tumor growth and metastasis formation was monitored by BLI for 21 days (Fig. 4D, E). BLI from the lateral view (primary tumor in spleen) and the ventral view (liver metastases) showed a Compound 22-mediated inhibitory effect on tumor growth (Fig. 4D) and liver metastasis formation (Fig. 4E). Based on this, all three compounds (Compound 22, Analogue 10 and 13) were tested and BLI monitoring of tumor growth and liver metastasis formation was performed (Fig. 4F, G). BLI images of whole mice and isolated spleens and livers taken at day 21 showed an impact on tumor growth by compound treatments (Fig. 4F) and more importantly, a clear reduction in liver metastases formation (Fig. 4G).

We next analyzed the presence of human satellite DNA in the livers of control vs. compound-treated mice as a molecular parameter for liver metastases (Fig. 4H). All Compound 22-, Analogue 10- and 13-treated groups showed significant reductions in human satellite DNA, which was reduced by 55%, 75% and 70%, respectively, vs. control animals. Further, all compound-treated animals showed significantly reduced MACC1 mRNA expression in livers and spleens (Fig. 4I-J). This was supported by human CK19-specific immunohistochemistry, to detect HCT116 metastases in mouse liver (Fig. 4K). Number and size of metastases was clearly reduced, with best results for Analogue 10 and 13, vs. controls. This confirms that all three compounds act as transcriptional inhibitors of MACC1 thereby inhibiting MACC1-induced metastasis formation in vivo. This strongly supports that 1,2,3,4-tetrazolo[1,5-*b*]pyridazine–based compounds are of value as anti-metastatic compounds, targeting the causal driver of metastasis MACC1. This finding was supported by another in vivo study employing a different cell model (SW620 CRC cells, Supplementary Figure 6). Metastasis formation, human satellite DNA and MACC1 expression in the liver of Compound 22 (50 mg/kg, daily application) treated mice was significantly reduced. In another study it was explored whether a dose escalation of Compound 22 leads to a stronger reduction of metastasis formation. It can be seen in the supplementary figure 5 that the concentration of 100 mg/kg did not outperform 50 mg/kg concentration used in the pilot experiment regarding bioluminescent liver signal and MACC1 expression reduction in the primary tumor and liver metastasis.

3.6. Mode of action of Compound 22

By literature search of tetrazolo-pyridazine-containing small molecules, the compound Ro106–9920 was identified as a possible analogue of Compound 22. It was described as a NFκB inhibitor, irreversibly inhibiting IκBα ubiquitination. In human PBMCs and in rat the NFκB-dependent cytokine expression was blocked [23]. Further, we recently showed that TNF-α increases MACC1 gene expression in HCT116 CRC cells [24]. Based on this, we hypothesized that Compound 22 might target the NFκB pathway, leading to a reduced MACC1 gene expression. First, we predicted p65 and p50 transcription factor binding sites in the MACC1 promoter using the online tool PROMO 3.0 (23, Fig. 5A). Interestingly, we showed that 10 μM of Compound 22 blocks the TNF-α-induced increase of the MACC1 gene expression (Fig. 5B) compared to the DMSO control.

4. Discussion

There is still a strong need for effective treatments of cancer, as it poses the second leading cause of death worldwide, and CRC has the second highest mortality rate [25,26]. Although surgery, chemotherapy and radiotherapy are efficacious for early-stage CRC, the treatment outcome becomes limited for advanced disease [27,28]. Thus, intervention in cancer metastasis is essential to improve patient outcome. Search for novel compounds inhibiting metastasis is of growing importance to efficiently treat cancer. MACC1 represents an appropriate target, as it is a causal driver of metastasis, is found to be overexpressed in a variety of solid cancers and is responsible for tumor progression and metastasis formation [5,29].

The search for selective and effective inhibitors is strongly supported by large compound libraries that serve as the basis for HTS [16,30], which was successfully used for finding small molecule compounds for many different diseases including cancer and acting by different modes of action [31–36]. The advantage of using library screens is the direct, unbiased access to the particular compound of interest, to continue with extended in vitro and in vivo studies. HTS frequently employ gene-specific promoters in reporter assays to identify selective transcriptional inhibitors of a particular gene. In earlier studies, we used such HTS approaches to identify repositioned small molecule transcriptional inhibitors of metastasis genes, S100A4 and also of MACC1 [15,37,38].

In this study, we pinpointed 1,2,3,4-tetrazolo[1,5-*b*]pyridazine-based compounds as hit compounds to more selectively target MACC1 expression. Interestingly, such compounds and analogues thereof have already been associated with biological activity [17–20], however were never in focus for anti-metastatic therapy.

Here, we identified a novel 1,2,3,4-tetrazolo[1,5-*b*]pyridazine compound, which is referred to as Compound 22, as transcriptional inhibitor of MACC1 via the luciferase reporter-based HTS [39]. This compound not only reduced MACC1 expression, but also impacted MACC1-driven cell motility in vitro and metastasis formation in vivo. Based on this particular 1,2,3,4-tetrazolo[1,5-*b*]pyridazine structure, we searched for even more effective MACC1 inhibitors and screened a large number of analogues with a similar core structure. We found four highly active analogues verifying the tetrazolo[1,5-*b*]pyridazine moiety as essential to exert its anti-metastatic activity via MACC1 inhibition. Changes in this core chemical substructure highly affect the effectiveness of the compound. This SAR will be important for future compound structure modifications to improve efficacy and applicability [40,41]. Further, we were able to identify the NFκB signaling pathway as potential target of these novel MACC1 transcriptional inhibitors, identifying binding sites of NFκB transcription factors on the MACC1 promoter and showing the complete abolishment of the TNF-α induced increase of MACC1 gene

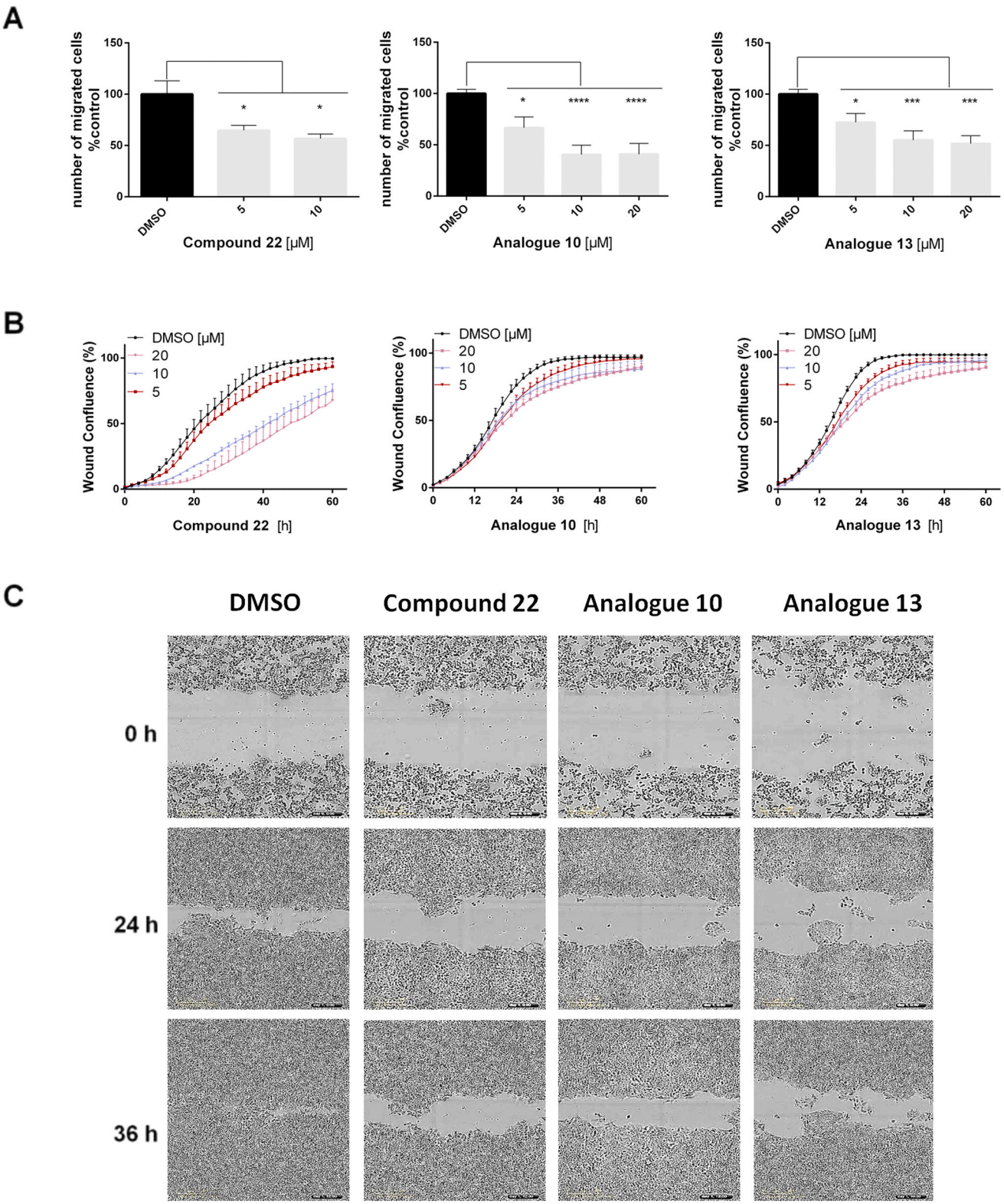

Fig. 3.. Evaluation of inhibitory effect of Compound 22 and of most active Compound 22 analogues (Analogue 10 and 13) on cell migration and wound healing. (A) For in vitro migration, HCT116 cells were treated with different compound concentrations (below respective IC_{50}) for 24 h and migration was measured in the Boyden chamber assay, expressed as % migrated cells versus DMSO-treated control. (**B**) Impact of compound treatments on wound healing assay. Wound closure was measured every 2 h by IncuCyte for 60 h in total. Compound 22 and two highly active analogues decreased the percentage of wound confluence of HCT116 cells over time. (**C**) Microphotographs of wound confluence of HCT116 cells were taken at 0 h, 24 h and 36 h, treated with 10 μM for Compound 22 and with 20 μM for Analogue 10 and 13. Results are shown as mean ± SEM of three independent experiments, performed in triplicates. Control cells were DMSO treated in all assays. Significant results were determined by one-way ANOVA and multiple comparison was done by Dunnett's post tests (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

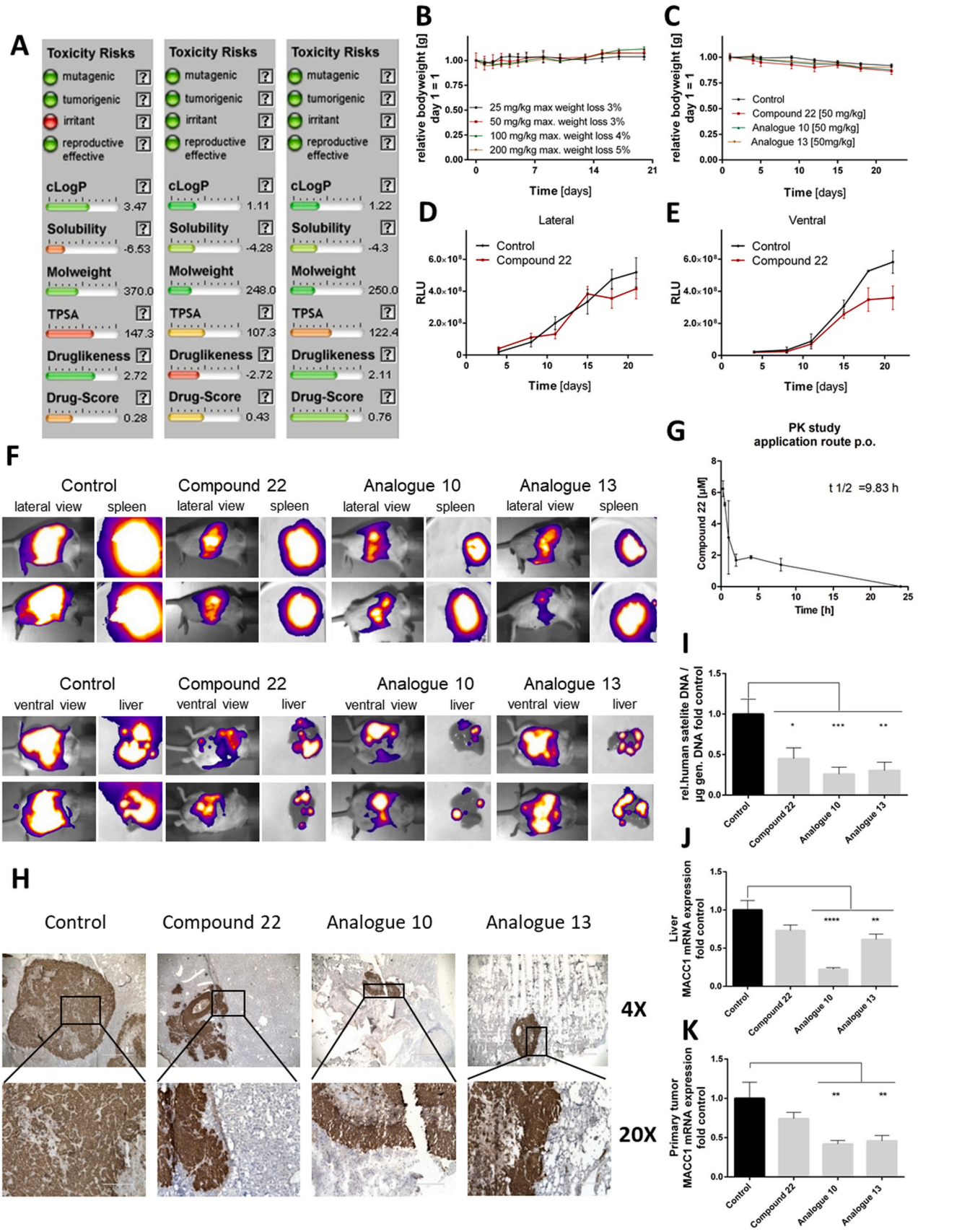

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Fig. 4.. The effect of MACC1 transcriptional inhibitors Compound 22, Analogue 10 and 13 on tumor growth and liver metastasis of HCT116 cells in vivo. (**A**) Toxicity risks of Compound 22 and analogues predicted by OSIRIS property explorer. In the top panel, prediction of mutagenic, tumorigenic, irritant effects or effects on the reproductive system are shown. Green color indicates drug-conform behavior and red undesired effects, all based on known effects of substructures of the main compound structure. In the lower panel, values predicted for the cLogP (partition coefficient), solubility, molecular weight, TPSA (the polar surface area), drug-likeness (calculated using a substructure fragment database with associated drug-likeness scores) and drug-score (combination of all predicted values). Green color indicates drug-conform behavior, orange/yellow indicate less ideal behavior and red undesired behavior. (**B**) Tolerability of Compound 22 (escalating dose 25, 50, 100 and 200 mg/kg) and of (**C**) analogues (fixed dose of 50 mg/kg) was determined in female SCID-beige mice. During treatment body weight was measured. (**D, E**) First study to determine proof-of-concept of Compound 22 treatment for tumor growth and metastasis inhibition. SCID-beige mice were intrasplenically transplanted with HCT116-CMVp-Luc cells and were orally treated each day with MACC1 transcriptional inhibitor Compound 22. BLI intensity was quantified for Compound 22 treatment (lateral images for tumor growth, ventral images for liver metastases) via ImageJ and averaged per group and day. (**F**) SCID-beige mice were intrasplenically transplanted with HCT116-CMVp-Luc cells and orally treated each day with three MACC1 transcriptional inhibitors: Compound 22, Analogue 10 and Analogue 13. BLI was determined laterally (for tumor growth, top panel) and ventrally (for liver metastases bottom panel). (**G**) PK study to determine terminal half-life of Compound 22. After sacrifice, BLI of spleens and livers was determined. (**H**) Detection of metastases of HCT116 cells by immunohistochemical staining with anti-human CK19 antibody (brown staining) in control and in compound-treated mice at low (4-fold) and high (20-fold) resolution. Quantification of human satellite DNA in mouse livers was quantified by qPCR, of human MACC1 mRNA levels in mouse livers (**I,J**) and the spleens (**K**) using qRT-PCR. Data represent mean ± SEM. Significant results were determined by one-way ANOVA and multiple comparison was done by Dunnett's post tests (n = 10 animals/group, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

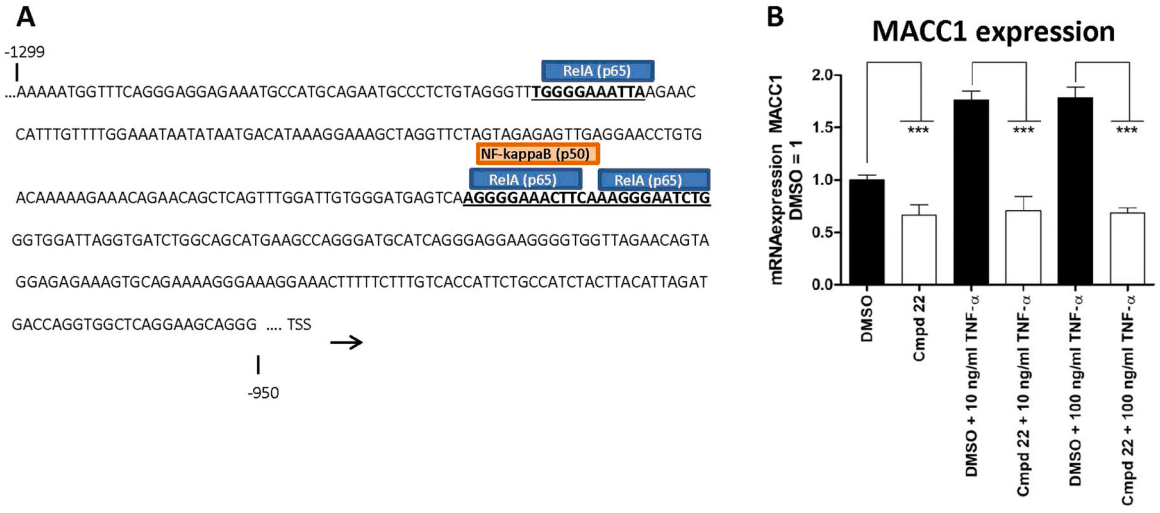

Fig. 5.. NFκB pathway in the context of compound 22-mediated MACC1 expression inhibition. (**A**) Prediction of NFκB transcription factor binding sites in the human MACC1 promoter calculated by PROMO 3.0. RelA (p65) binding was predicted in the following sequence: TGGGGAAATTA, AGGGGAAACTT and AAGGGAATCTG. NFκB (p50) binding was predicted in the following sequence: GGGGAACTTCA. (**B**) The effect of Compound 22 on MACC1 gene expression during TNF-α stimulation. MACC1 gene expression after 24 h incubation with Compound 22 with or without TNF-α stimulation (10 or 100 ng/ml) and DMSO control in HCT116 cells. mRNA levels were normalized to DMSO control and represent means +SEM of three independent experiments. Significant results were determined by one-way ANOVA and multiple comparison was done by Dunnett`s post tests (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.001$, **** = $p < 0.0001$).

expression by Compound 22.

This study represents a promising initial stage of compound development. Thus, this study has some limitations, for example lacking additional mouse models such as PDX, a more developed drug derivative and a more in-depth analysis of the mode of action. However, the newly discovered small molecule transcriptional MACC1 inhibitors create the basis for further developments to improve effectiveness at reduced toxicity, enhanced solubility for better applicability via optimization of the compound structure [42–45]. More in-depth research on the molecular mechanism and pharmacokinetics of the tetrazolo[1,5-*b*]pyridazine-based compounds is needed to optimize the therapeutic efficacy [46], especially, when this drug should be implemented for a

Table 1
Chemical structure and summary of key characteristics of Compound 22 and best analogues.

Compound	Structure	Cell line	Maximal Inhibition [%]	At concentration [μM]	Active concentration range [μM]	EC50 (activity) [μM] normalized	IC50 (Tox/MTT) [μM] normalized
Compound 22	fx1	HCT116	85.4	30	5–30	11.31	7.36
		SW620	78	25	5–25	7.359	6.38
Analogue 7	fx2	HCT116	77	50	10–50	12.3	18.11
		SW620	69	50	5–50	10.99	21.49
Analogue 8	fx3	HCT116	90	50	0.5–50	6.79	21.34
		SW620	82	50	0.5–50	6.6	17.87
Analogue 10	fx4	HCT116	56	50	1–50	4.42	22.08
		SW620	78	40	1–50	2.6	36.5
Analogue 13	fx5	HCT116	70	50	0.5–50	9.44	24.42
		SW620	63	50	1–50	8.73	31.1

first-in-human trial.

Taken together, we identified small molecule compounds for selective transcriptional inhibition of MACC1 (Table 1). The search revealed 1,2,3,4-tetrazolo[1,5-*b*]pyridazine-based compounds as effective inhibitors in vitro and more importantly in vivo. This study showed that compounds with the 1,2,3,4-tetrazolo[1,5-*b*]pyridazine core are promising candidates for anti-metastatic therapies that aim at improving the outcome of cancer patients with MACC1-overexpressing cancers, that especially are at high risk to develop metastases or already suffer from metastatic disease. Such newly identified 1,2,3,4-tetrazolo[1,5-*b*]pyridazine-based compounds represent important building blocks for a more individualized and stratified anti-metastatic therapy not only for CRC but also with cross entity potential. These efficient metastasis inhibitors warrant further preclinical and clinical development to improve anti-metastatic cancer therapy.

In conclusion, metastasis of cancer still represents the major cause of death and correlate with a poor prognosis for patients. Therefore, hitting a target that is causally linked to metastasis formation will contribute to more effective anti-metastatic therapies. We selected MACC1 as a target, as it has been proven to be causal in driving metastasis formation of tumor cells and is prognostic for metastasis and poor outcome. By HTS we identified tetrazolo-pyridazine-based novel small molecule inhibitors, which efficiently target MACC1 expression, leading to reduced tumor cell migration, invasion and metastasis formation in different cancer models. We provide SAR, detailed analyses of drug-likeness and first hints for mode of action for potential clinical development of this anti-metastatic compound. Our data indicate a promising new path for targeted anti-metastatic therapies, aiming at MACC1 as metastatic driver in a personalized medicine setting with high cross-entity potential.

Ethics approval

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.

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CRediT authorship contribution statement

Shixian Yan: Data curation, Investigation and Validation, Writing of the original draft; **Paul Curtis Schöpe:** Data curation, Investigation and Validation, Writing of the original draft; **Joe Lewis:** HTS investigation and Methodology; **Kerstin Putzker:** HTS investigation and Methodology; **Ulrike Uhrig:** HTS investigation and Methodology; **Edgar Specker:** Data curation and Formal analysis; **Jens Peter von Kries:** Data curation, Methodology; **Peter Lindemann:** Data curation and Formal analysis; **Anahid Omran:** Data curation and Formal analysis; **Janice Smith:** Data curation, Investigation and Validation; **Hector E. Sanchez-Ibarra:** Data curation and Formal analysis; **Anke Unger:** ADMET investigation and Methodology; **Mia-Lisa Zischinsky:** ADMET investigation and Methodology; **Bert Klebl:** ADMET investigation, Data curation and Formal analysis; **Wolfgang Walther:** Conceptualization, Data curation, Funding acquisition, Writing of the original draft; **Marc Nazaré:** Conceptualization, Data curation, Funding acquisition, Writing of the original draft; **Dennis Kobelt:** Funding acquisition, Investigation and Validation, Writing of the original draft; **Ulrike Stein:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing of the original draft. All: Reviewing and editing of the final version of the manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ulrike Stein reports financial support was provided by SPARK Berlin. Wolfgang Walther reports financial support was provided by SPARK Berlin. Dennis Kobelt reports financial support was provided by SPARK Berlin. Ulrike Stein reports financial support was provided by German Cancer Consortium. Shixian Yan reports financial support was provided by China Scholarship Council. Paul Schoepe reports financial support was provided by Berlin School of Integrative Oncology. Ulrike Stein has patent #1. Stein U, Kobelt D, Walther W, Shixian Y, Nazaré M, Specker E, Schöpe P, Lindemann P. Identification of novel compounds for targeted therapy of MACC1-driven metastasis. EP 21169702.4, 21.10.2022 issued to none.

Data availability

The data and material used to support the findings of this study are available from the corresponding author on request.

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Consent for publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115698.

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