Novel functions of MDMX and innovative therapeutic strategies for melanoma

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CHAPTER 6

General discussion

Oncogenic functions of MDMX in uveal melanoma

Malignant cells often highly express MDMX and/or MDM2 as means to dampen p53 activity [1]. MDM2 is an E3 ubiquitin ligase whose activity results in ubiquitin-dependent p53 degradation, while MDMX shields the transactivation domain of p53. However, the oncogenic functions of MDMX are not limited to the inhibition of p53 activity [2, 3]. Chapter 2 reports which genes are transcriptionally controlled by MDMX and to what degree this regulation is p53-dependent. The data presented indicate that MDMX regulates cell cycle progression, at least partially via the p53-p21-DREAM (DP, RB-like, E2F4 and MubB) axis, and apoptosis via p53 and FOXO's. It has been generally accepted for some time now that p53 promotes the expression of p21 and thereby induces transcriptional repression by the DREAM complex [4, 5]. However, to what level this particular indirect p53 activity could be inhibited by MDMX had not yet been studied. The observation that MDMX depletion, which only minimally effects p21 expression, is capable of inducing this transcriptional repression has never been reported before. Although possibly not very surprising, since MDMX is known to inhibit p53 transcriptional activity [6, 7], it further strengthens the proposition that MDMX could serve as alternative therapeutic target, since its inhibition provokes a similar downstream effect as inhibition of MDM2. While having a comparable therapeutic potential as MDM2 inhibition, MDMX is less commonly expressed and not always essential for cell survival in the adult tissue [8-14]. Therefore, it could well be that targeting MDMX has less adverse effects in patients compared to inhibition of MDM2, potentially making it the preferred way of reactivating p53.

In chapter 2 we established that the downregulated cell cycle controlling genes upon MDMX knockdown could indeed be regulated via the p53-p21-DREAM axis. However, the transcriptional repression is, at least partly, p53-independent, suggesting the existence of another transcription factor under the control of MDMX. Not only repressed, but also some of the upregulated genes show clear p53 independency. Although 38% of the upregulated genes were identified as direct p53 target genes the results demonstrate that MXD4 (also named MAD4) and PIK3IP1 expression is controlled by MDMX in a p53-independent manner. The major DNA binding consensus site identified in 65% of the up-regulated genes is the Forkhead-Boxes DNA binding site. Indeed, FOXO1 levels are slightly increased upon MDMX depletion in a p53-independent manner. Inhibition of FOXO1 activity could explain the p53-independent function of MDMX. Mechanistically it remains unsolved as yet how MDMX inhibits FOXO1. It is known that MDM2 is capable of directly binding and ubiquitinating FOXO1, 3 and 4 [15, 16]. Preliminary data show that reduced MDMX expression results in increased nuclear localization of FOXO1 (data not shown). If the inhibition of FOXO1 is the result of a direct interaction with MDMX, the development of specific compounds antago-

nizing this interaction could lead to new therapeutic option for tumors overexpressing MDMX.

Interestingly, MXD4 affects p53 activity potentially suggesting a novel back-up mechanism (see Figure 1). This back-up mechanism would include that upon MDMX depletion MXD4 is upregulated, mediated by FOXO1, and competes with p53 for SIN3A. By forming a complex with SIN3A, which is known to be involved in transcription repression, MXD4 could be part of a potential pathway leading to the p53-independent gene repression mentioned earlier. Interestingly, SIN3A might not only potentially enable p53-independent repression, it can also form a transcription repressive complex which not only includes E2F4 but also p27 [17, 18]. The latter protein was previously described as being stabilized upon MDMX depletion, independently of p53 [2]. Based on these earlier studies and the data described in chapter 2 a larger picture is emerging in which MDMX is controlling a large repressive complex via the regulation of SIN3A and E2F4 and inhibition of MXD4 transcription. Furthermore, releasing p53 from SIN3A allows MDM2 to bind p53 and to target p53 for ubiquitination-mediated degradation [19]. This pathway would imply a p53-independent back-up mechanism

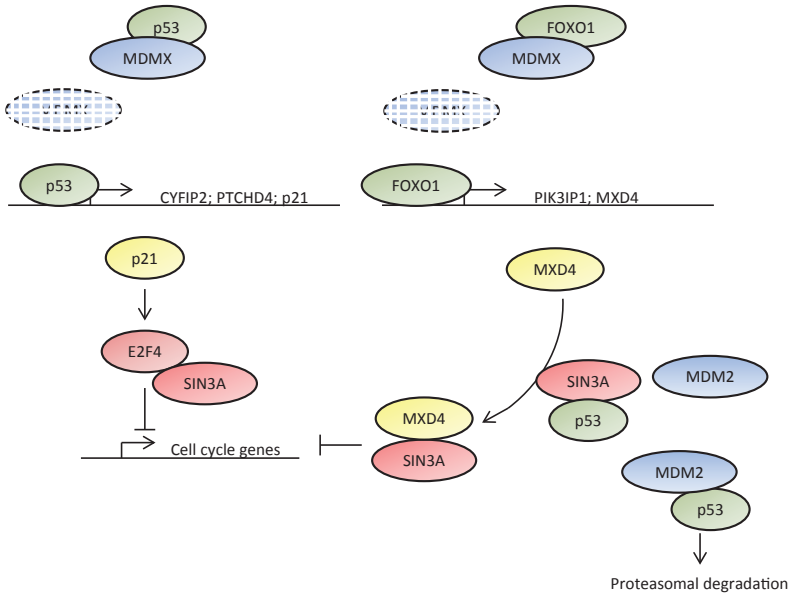

Figure 1. Working model of MDMX's oncogenic functions. In this model MDMX inhibits both p53 and FOXO1 which prevents target gene transcription. Upon MDMX depletion both transcription factors bind DNA and promote transcription resulting in, among other effects, the repression on cell cycle regulatory genes. Simultaneously, FOXO mediated upregulation of MXD4 transcription could prime p53 for MDM2 mediated degradation by competing with p53 for SIN3A binding.

in which a cell with high levels of MDMX rewires towards more MDM2 mediated p53 inhibition once MDMX is depleted. Not only does this SIN3A/MXD4 mediated 'back-up' mechanism clarify the p53-independent transcription repression upon MDMX depletion, it could also provide new therapeutic targets. Depletion of MXD4 resulted in p53 stabilization, possibly due to an increased SIN3A binding attenuating MDM2 binding to p53. MXD4 depletion resulted in a slight, p53-dependent, growth inhibition and it synergized with p53 activating and stabilizing drugs, showing that to fully unleash p53 and further exploit current p53 activating strategies a way to target MXD4 should be elucidated.

In conclusion, the data presented in chapter 2 show that p53-independent oncogenic functions of MDMX could be partially explained by the p53-independent effects on the transcriptome. In addition to a better understanding of the oncogenic functions of MDMX in melanoma, a possible new route to potentiate current anti-cancer strategies in various malignancies was uncovered.

MDMX as enhancer of current therapeutic interventions for metastasised uveal melanoma

The feasibility and clinical advantages of p53 reactivation by MDM2 inhibition have been established [20]. Unfortunately, clinical studies have shown strong adverse, on target, effects in patients upon MDM2 inhibition [21]. Although both MDM2 and MDMX are essential for restraining p53 during embryonic development [22-26], in adult cells and tissue MDM2 loss is always lethal whereas MDMX loss can be compatible with life [8-13]. The lack of general expression of MDMX compared to MDM2 would indicate potentially less adverse effects when targeting an MDMX expressing cancer. The therapeutic potential of MDMX targeting has been established by our lab and others for various cancer types, partly independent of p53 status [2, 3, 27, 28]. It has become evident and generally accepted that using a monotherapy on a cancer will in general not result in a durative and curative response [29]. Therefore, combinations of drugs are tried in more studies in order to hit a cancer cell from multiple angles at the same time, making acquired resistance less likely to occur. Interestingly, in melanoma concurrent targeting of MDMX and BRAF has proven to be an effective combination [3].

In uveal melanoma an activating mutation in GNAQ or GNA11 is the main driver towards malignancy. Constitutive active GNAQ/GNA11 activates a number of signalling pathways, including the proliferation stimulating MAPK pathway. An essential node in this cascade is the activation of protein kinase c (PKC) isoforms, which has been

recognized as a valuable therapeutic target for uveal melanoma, e.g. by means of the PKC inhibitor Sotrastaurin/AEB071 [30].

In chapter 3 it is described that combined p53 activation and PKC inhibition result in synergistic growth inhibition of uveal melanoma cells. In our studies the dual MDM2/X inhibitor Nutlin-3 was used, whereas the MDM2 inhibitor CGM097 was used in another study describing the beneficial effects of combining p53 reactivation with PCK inhibition [31]. Interestingly, in the latter study no synergistic effects upon concurrent PKC inhibition and p53 reactivation could be demonstrated *in vitro*, in contrast to our results, suggesting that full release of p53 from both MDM2 and MDMX is essential for synergism. Depletion of MDMX, like the pharmacological activation of p53 by MDM2 inhibitors, attenuates the proliferation and survival of UM cells, which is further enhanced by a combination with PKC inhibition. Thus, MDMX inhibition in combination with existing therapeutic interventions for uveal melanoma could serve as a promising therapeutic intervention, stressing the need for the development of specific MDMX inhibitors, which thus far has been proven very difficult.

Replacing pan-PCK inhibition for PKCδ targeting

Activated protein kinase C (PKC) isoforms is a common feature of UM and has shown potential as therapeutic intervention for UM patients [30]. Unfortunately, pan-PKC inhibition as single treatment appears to have only limited clinical benefit and elicits adverse effects in patients [32]. Combining PKC inhibition with activation of p53, which is rarely mutated in UM, by MDM2 inhibitors has shown promising results in pre-clinical studies. Therefore, alternative approaches were investigated to achieve similar anti-cancer effects, but with potentially less adverse effects. Since the PKC family consists of 10 different isoforms it can be hypothesized that targeting a single PKC isoform would have less adverse effects compared to a pan-PKC inhibitor. It has been demonstrated that, despite the great structural homology between the different PKC isoforms, especially within a certain subclass, they appear to have separate and non-redundant functions. Furthermore, it has been observed that all PKC isoforms tested (α, β, θ, ε and δ) are essential for uveal melanoma cell viability [30, 33], emphasising that specific targeting of a single PKC isoform could yield an effective treatment. PKCε and PKCδ were shown to be responsible for the activation of RASGRP3, a guanine nucleotide exchange factor promoting the GTP loading of RAS. So, activation of RASGRP3 serves as a RAS activator driving the RAS/MEK/ERK pathway in uveal melanoma [34]. In chapter 3 it is confirmed that uveal melanoma cell viability depends on PKCδ expression and, therefore, could be regarded a potential drug target, especially interesting since PKCδ does not seem to be required for development and normal cell proliferation [35, 36]. As mentioned above, it is unlikely that the specific targeting

of a single signalling molecule will result in a curative response it was investigated whether PKCδ depletion would also enhance the effect of MDM2/MDMX inhibition. Like pan-PKC inhibition, the specific depletion of PKCδ resulted in synergistic growth inhibition of UM cells in combination with p53 reactivation.

In conclusion, the data presented in chapter 3 show that the synergistic effects of p53-activation by MDM2/MDMX inhibition and broad spectrum PKC inhibition on survival of UM cells can largely be achieved by the presumably less toxic combination of depletion of MDMX and targeting a specific PKC isoform, PKCδ. Although PKCδ appears to be a promising therapeutic target until recently no small molecule compound specifically targeting this kinase had been described. The functional non-redundancy between the various isoforms suggests the opportunity for developing a selective inhibitor. Recently, the development of a selective PKCδ inhibitor (B106) has been described [35], but our experiments with this inhibitor indicate that the growth inhibitory effect is not dependent on PKC activity in uveal melanoma cells (data not shown). The approach to develop this inhibitor was to design the structure of B106 on the reported Rottlerin's capability to selectively bind PKCδ compared to PKCα and to combine this with parts of the kinase inhibitor Staurosporin. However, it has now been widely accepted that Rottlerin does actually not bind PKCs and Staurosporin is considered to be one of the most a-selective kinase inhibitors commercially available. An alternative approach to selectively target PKCδ, or members of the novel-PKC family, could very well consist of modifying a known pan-PKC inhibitor such as Sotrastaurin or GF109203X. These selective pan-PKC inhibitors have a strong structural overlap with the a-selective Staurosporin with regard to the 'head' domain of these compounds, suggesting that the selectivity of the compounds has to be acquired from the 'tail' residues. A valid approach for the development of a selective PKCδ inhibitor would be to chemically modify the tail region of either GFX or Sotrastaurin and determine the specificity using *in vitro* kinase assays.

Deviating from the hypes

Finding curative therapeutic intervention has been the focus of many decades of cancer research. A few success stories, such as Imatinib (targeting the BCR/ABL fusion gene) and Rituximab (targeting CD20), catalysed the targeted therapies hype. Although great results were expected based on these successes, the median increase in survival was 2.1-2.5 months based on 71 drugs approved to treat cancer by the FDA between 2002 and 2014 [37]. According to the ASCO guidelines, regarding improve in quality of life and overall survival, only 42% of these drugs had a meaningful clinical impact. It should be noted that many of the 71 approved drugs contain an overlapping mode of action [37]. When a certain company has developed an approved and

profitable therapy, even if only with a modest improvement in overall survival, other pharmacological companies will develop compounds with a similar mode of action to ensure a piece of the market and the profit. Thereby, they are pushing drug prices to incredible height without really improving the quality of the therapy and patient survival. This is particularly illustrated by the 50 molecules which entered clinical trials for targeting vascular endothelial growth factor (VEGF) as a means of targeting angiogenesis, or the 25 molecules in clinical trials for the targeting of mitosis in solid tumours, the latter with an average response rate of just 1% [38]. These duplication efforts are even further illustrated by the observation that 9 big pharmaceutical companies have a 74% overlap in their molecules with regard to the expected mode of action [37].

This focus on targeted therapies, with only limited clinical benefit, has almost blinded funding agencies, academia and companies. It is nowadays accepted that targeted therapies tend to work efficiently for 'single cause' diseases, but not really for more complex malignancies such as cancer due to tumor heterogeneity [39]. The most recent big breakthrough in cancer treatment is the development of immune checkpoint targeting drugs, discussed in the introduction of this thesis. These developments are currently changing the whole cancer research field, like targeted therapies did a few decades ago. Although promising in some cancer type, stratification of patients appears to be crucial for the success of a treatment, like for targeted therapies [40]. It appears that immunotherapies are most efficient in tumours with a high mutational load. Cutaneous melanomas have a high mutation load in contrast to uveal melanomas. Indeed, uveal melanoma patients did not benefit from immunotherapy in the form of Ipilimumab [41, 42]. It appears that with regard to metastasized uveal melanoma a therapeutic intervention should not come from current immunotherapy nor targeted therapy strategies. Therefore, the remainder of this discussion will be on the use of therapeutics not fitting within targeted- nor immunotherapy, but which are already in clinical trials, to reduce the time from bench to bedside.

EZH2 and HDAC as therapeutic intervention

In uveal melanoma BAP1 expression is frequently lost due to mutation and loss of the second allele [43-45]. In UM 80-90% of the metastases show loss of BAP1 expression resulting in strong predictive power for BAP1 mutations for the occurrence of metastasis [45, 46]. Interestingly, upregulation of Enhancer of Zeste (EZH) 2 expression in mesothelioma cells upon BAP1 loss has been reported [47]. EZH2 is an essential component of the polycomb repressive complex 2 (PRC2), which mediates the tri-methylation of histone H3 at lysine 27 [48, 49]. EZH2 controls the expression of numerous genes by promoting the repressive tri-methylation of histone H3 thereby

reducing transcription [50]. An EZH2 dependency was found in myeloid cells for BAP1 knockout induced transformation and *in vivo* it was demonstrated for BAP1-negative mesothelioma that EZH2 inhibition is an effective treatment for BAP-1 negative cancers [47]. A clinical trial was initiated for patients with BAP1-negative mesothelioma using the EZH2-inhibitor Tazemetostat (identifier: NCT02860286). In a later report, EZH2 inhibition did not appear to affect both BAP-1 positive and negative UM cell proliferation. However, in chapter 4 it is demonstrated that UM cells are responsive to long term EZH2 inhibition.

Nevertheless, it appears that for most cell lines tested the time until onset of growth inhibition was over a week. Furthermore, only 2 of the cell lines tested completely stopped proliferating, meaning that most cell lines tested could be sub-cultured with continuous EZH2 inhibition. These relatively slow and mild effects *in vitro* are far from optimal for translation to a clinical setting, in which a patient with metastasised uveal melanoma generally only has a few months to live. Therefore, it was investigated whether EZH2 inhibition would sensitize UM cells for other compounds, known to be effective in uveal melanoma patients. Concurrent inhibition of EZH2 and HDAC has already been shown to effectively reduced tumor cell survival in various different cancer types [51-53]. Therefore this combination could be considered as an interesting novel strategy, which should be further studied in clinical trials, for multiple malignancies. Importantly, HDAC inhibitors have already been described as a potential therapeutic intervention for uveal melanoma [54, 55]. Indeed it was found that also UM cell lines are sensitized for HDAC inhibition upon EZH2 inhibition due to the induction of cell death. Others have showed that, in KRAS mutated lung cancer cells, EZH2 inhibition sensitized for MEK and PI3K/AKT inhibitor [56]. Furthermore, EZH2 is involved in the transcriptional repression of various DNA repair-related genes, which result in higher sensitivity to chemotherapeutic agents when cells are treated with an EZH2 inhibitor [57-59]. These studies indicate that in addition to the dual inhibition of HDACs and EZH2, other compounds could well synergize with EZH2 inhibition and be a promising therapeutic intervention for metastasised uveal melanoma. Future research has to elucidate which combinations are the most potent and feasible.

Combining HDAC and CDK inhibition as therapeutic strategy

As described previously, overall survival has hardly improved for patients with advanced unresectable cutaneous melanoma or metastatic uveal melanoma in the last decades. This lack of improvement is highlighting the need for novel therapeutic options. In chapter 5 the potential of the combination of another compound with the HDAC inhibitor (Quisinostat) described in chapter 4 was investigated. Both drugs

assessed in this chapter are currently in clinical trials reducing time from bench to bedside.

Encouraging results using histone deacetylase (HDAC) inhibitors indicate a potential therapeutic intervention for uveal and cutaneous melanoma [12–15]. HDAC inhibition often induces of a G1 cell cycle arrest in cancer cells. Although this cell cycle arrest can prevent further outgrowth of a tumor [21], finding drug combinations that synergistically induce cancer cell killing would greatly increase the clinical impact of HDAC inhibitors. For example, apoptosis is induced when both CDKs and HDACs are inhibited in neuroblastoma cell lines [23]. This study aimed at potentiating the effect of HDAC inhibitor Quisinostat by combining the therapy with CDK inhibition using Flavopiridol. Flavopiridol is currently tested in clinical trials, mainly as treatment strategy for acute myeloid leukaemia and lymphoma. Interestingly, stable disease in 7/16 patients with previously untreated metastatic malignant melanoma was induced by Flavopiridol. Unfortunately, according to objective response criteria Flavopiridol failed to achieve significant clinical benefit [28]. Data presented in chapter 5 show that single treatment with Flavopiridol or Quisinostat slows down the growth of UM and CM cells, while concurrent treatment inhibits cell growth synergistically and reduces survival.

Concurrent Flavopiridol and Quisinostat treatment shows a synergistic reduction in melanoma cell survival independent of mutations driving the malignancy. These synergistic effects were also observed in BRAF mutant melanoma cells that had acquired resistance to BRAF inhibition *in vitro*. Induction of apoptosis could at least partly explain the mechanism behind the observed synergism. However, the molecular mechanism underlying this induction of cell death remains undetermined. In a cutaneous melanoma PDX model combined Flavopiridol and Quisinostat treatment induced tumor regression, without enhancing adverse effects. However, the observed combinatory effects did not measure up to the expected results based on the *in vitro* assays. This can most likely be attributed to the lack of clear Flavopiridol activity on a molecular level. Flavopiridol is known to be cleared from the human body in hours [60]. Future research on this drug combination should mainly focus on reaching and maintaining proper CDK inhibition by Flavopiridol in order to reach maximum synergism and thus clinical benefit. When that can be achieved the Flavopiridol/Quisinostat combination could be a promising treatment strategy for metastasized uveal and cutaneous melanoma patients, regardless of earlier received treatments.

Considerations for drug combinatory studies

When two drugs are combined they can influence each other's respective outcome in either a synergistic, additive or antagonistic manner. When synergy between two

drugs occurs this indicates a potential novel therapeutic intervention. However, resolving the underlying mechanism driving the observed synergism could be extremely difficult, especially when the compounds are targeting a number of related molecules, as for instance described in chapter 5. These difficulties arise from the numerous possibilities on how both drugs might influence each other. This complexity is illustrated by a list of basic motives with only 3 theoretical nodes which already results in 21 possible explanations for synergism, let alone when more general pathways or cellular processes are influenced [61].

The other option upon combining drugs is the occurrence of antagonism. Synergism only tends to occur in a minority of drug combinations when systematically tested [62]. There appears to be a bias in both published literature and this thesis towards synergistic drug combinations rather the antagonistic ones based on a PubMed search for 'antagonism' or 'synergism' in combination with cancer'. This search yielded six times more hits for synergism in cancer compared to antagonism in cancer, although according to unbiased screening there should be at least ten times more antagonistic combinations compared to synergistic ones. Although these combinations clearly do not contain a therapeutic benefit, they could reveal potential mechanism of drugs resistance. To mechanistically explain antagonism appears to be easier compared to synergism as there are far less theoretical explanations for the occurrence of antagonism compared to synergism [61]. For example, it has been shown that p53 reactivation in combination with CDK1/2 inhibition results synergistically in growth inhibition of melanoma cells [63]. However, preliminary data from our lab show that the pan-CDK inhibitor Flavopiridol has an antagonistic effect on p53 activation (data not shown). Which could actually make sense since Flavopiridol's main target is the block of transcription by CDK9 inhibition, which would antagonize p53-induced transcription upon activation. So, despite Flavopiridol's lack of synergistic capabilities with p53 the results could help understanding which drugs or CDK inhibitors could provide useful therapeutic enhancers of p53 activation therapies and which will not.

For the studies described in this thesis the 'educated guess' methodology was used to find novel synergistic combinations to inhibit uveal melanoma cell growth. Illustrated by the concurrent targeting of two main hallmarks of uveal melanoma, namely PKC activity and high expression of MDMX/2. Furthermore, knowledge of BAP1 and EZH2 was applied to combine EZH2 and HDAC inhibition, of which the latter had been shown to elicit metastasis regression in uveal melanoma patients. The observed HDAC inhibition induced cell cycle arrest directed us towards the combination with CDK inhibition. Although these combinations work synergistically *in vitro* and hold

true potential it could easily be doubted whether these combinations are the best combinations possible.

An alternative approach consists of performing unbiased synthetic lethal screens to find genes and pathways synergising with a particular drug. The question remains which drugs to select for these approaches. One might select drugs based on their availability in the clinic, proven clinical efficacy, or their ability to induce a growth arrest *in vitro* so the synergism will be clear. However, it could also be considered to select a drug for this screening based on the lack of activity. Especially the lack of strong adverse effects could be considered a pro due to the reduced likeness of enhancing adverse effects in the combination. A prominent downside of this approach could well be that the ideal pathway or molecule identified by these screens cannot -yet- be targeted by a specific small molecule compound. Another drawback could be that no interest exists from the industry to commercialize a certain treatment option.

A last option would be to put all scientific interest aside and, much like the Quisinostat/Flavopiridol combination, focus on drugs already in clinical trials and preferably FDA approved. Also here it boils down to which drugs to investigate. In such a setting it could be wise to test only those compounds the companies are willing to bring forward into clinical trials. Finding a cure for metastasised uveal melanoma following this approach has the great advantage of a relatively short time from bench to bedside. The obvious downside of this approach is that again the combination with strongest effects and with the least adverse side effects could be missed.

Clinical relevance and concluding remarks

When studies, like described in this thesis, are being performed one should always wonder what the true question is that needs to be answered. Whether or not there is a scientific interest in a certain molecule or pathway or just simply the need to find a curative treatment for patients who have none or have run out of treatment options. Regardless of the fact that finding a curative treatment for all metastasised uveal melanoma patients is most likely impossible due to great inter- and intra-tumor variation, the identification of the 'best possible' drug (combination) will require the 'brains' of the educated guess approach, the unbiasedness of the second and unconditional support of pharmacological industries. Concluding that none of the above mentioned approaches is either good or wrong as long as they are fitting with the question needing to be answered.

To date no effective therapy exists for metastasized uveal melanoma. It is therefore that all potential effective or prognosis improving therapeutic strategies need to be

evaluated properly in order to obtain an effective therapy for this deadly malignancy. Findings in this thesis provide several potential new therapeutic interventions mainly based on drugs already in clinical trials in order de reduce time from bench to bedside. However, in all cases of suggested drug combinations additional research needs to be performed, in a (pre-) clinical setting, to show the true potential of these combinations.

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