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Untersuchungen zur anti-tumorigenen Wirkung von pflanzlichen Substanzen - Möglichkeiten der therapeutischen Intervention und Chemoprävention bei malignen Neoplasien

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1 Zusammenfassung

Malignome stehen weltweit nach Herzkreislauferkrankungen und Infektionskrankheiten an dritter Stelle der Todesursachen. Das Mammakarzinom ist der häufigste letale Tumor der Frau. Neben den etablierten operativen, medikamentösen und radiologischen Therapieansätzen stehen auch zunehmend komplementärmedizinische Therapiemöglichkeiten im Fokus der Tumortherapie. Insbesondere Pflanzenstoffe wie Curcumin oder Pflanzenstoff-Derivate wie Artesunate, eignen sich hierfür aufgrund ihrer optimalen Wirkungs-/Nebenwirkungsverhältnisse. Die molekularen Wirkmechanismen von Curcumin und Artesunate sind bis jetzt noch nicht gänzlich entschlüsselt. Ohne genaues Verständnis der Wirkmechanismen sind sie nicht in der klinischen Tumor-Therapie einsetzbar.

Zur Klärung weiterer zellulärer Regulationspunkte soll in der vorliegenden Arbeit die Wirkung von Curcumin auf Ebene der miRNAs untersucht werden.

Wir konnten zeigen, dass Curcumin das miRNA-Expressionsmuster von metastasierenden Mammakarzinom-Zellen moduliert. Via *Micro-Array*-Untersuchungen filterten wir miR181b heraus, die mehr als 3-fach überexprimiert war und deren mögliche Zielgene eine große Schnittmenge mit den bereits bekannten Zielmolekülen der Curcumin-Wirkung haben. Wir haben die miR181b-Expression durch *knock in*- und *knock out*-Experimente beeinflusst und zudem zur Validierung des Kausalzusammenhangs zwischen miR181b-Expression und der Curcumin-Wirkung Doppelmodulationsexperimente durchgeführt. Hierbei gelang es uns zu zeigen, dass die proinflammatorischen Zytokine CXCL1 und -2, sowie die mit der Apoptose assoziierten Gene BCL-2 und SURVIVIN nach miR181b-Überexpression inhibiert werden. Ebenso konnte eine Inhibierung der Expression von MMP-1 und -3 nachgewiesen werden, welche mit der Invasionsfähigkeit von Tumorzellen korreliert.

Untersuchungen der Malignom-korrelierten Zellfunktionen ergaben, dass miR181b sowohl die Proliferation als auch die Invasivität der Tumorzellen beeinflusst.

Die *in vitro* gefundenen Ergebnisse ließen sich auch in einem murinen hämatogenen Metastasenmodell - *in vivo* - mit dem Nachweis einer geringeren Metastasenbildung unter miR181b-Überexpression bestätigen. Die Induktion von miR181b und Repression von CXCL1 nach Curcumin-Behandlung primärer Mammakarzinom-Zellen (*ex vivo*) macht eine Übertragung der Ergebnisse auf die klinische Ebene möglich. Diese Daten präsentieren miR181b als wichtigen Mediator der Wirkung von Curcumin auf molekularer Ebene und unterstreichen erneut das chemopräventive Potenzial dieser Substanz. Des weiteren könnte miR181b auch als Prädiktor für die Wirkung von Curcumin fungieren, um eine präzisere Differenzierung zwischen *Respondern* und *Non-Respondern* zu ermöglichen.

Genauso wichtig wie die Entschlüsselung des Wirkmechanismus einer Substanz ist die Klärung einer Resistenz-Entstehung. Als weitere potenzielle, chemopräventive Substanz haben wir Artesunate bzgl. ihrer anti-tumorigenen Eigenschaften untersucht. Hierbei konnten wir feststellen, dass die metastasierende Mammakarzinom-Zelllinie MDA-MB-231 im Laufe der Behandlung eine Resistenz gegenüber Artesunate entwickelte. Diese manifestierte sich in einer anfänglichen, später jedoch ausbleibenden Hemmung der Tumor-assoziierten Transkriptionsfaktoren AP-1 und NFκB. Die steigende Präsenz von AP-1 und NFκB führt zu einer zunehmenden Resistenz gegenüber Apoptose, was auch in einer vermehrten Expression von BCL-2 und einer Reduktion von BAX resultiert. Die beiden mit dem programmierten Zelltod assoziierten Gene zeigten einen in Richtung Apoptose-Resistenz verschobenen Quotienten (BAX/BCL-2). Des Weiteren konnten wir zeigen, dass es mit Erreichen der Resistenz zu einer deutlichen Überexpression von MMP-1 kommt, deren Relevanz bzgl. der Tumorprogression bereits bekannt ist.

Nur durch das Verstehen einer Resistenzentwicklung lassen sich zuverlässige Aussagen über mögliche *Responder* und *Non-Responder* machen. Je detaillierter das Wissen über die molekularen Wirkmechanismen von Naturstoffen ist, um so zielgerichteter können Substanzen wie Curcumin und Artesunate in die therapeutische Praxis integriert werden.

1.1 Summary

In causes of death, cancer ranks third worldwide, after cardiovascular and infectious diseases. Breast cancer is the leading cause of cancer-related mortality in women. Next to established surgical, drug and radiological forms of therapy, more and more complementary and alternative approaches are brought to attention for cancer therapy. In this context phytopharmaceuticals such as Curcumin, or derivates of phytopharmaceuticals such as Artesunate, play an important role as they show an excellent effect to side effect ratio. To this day, the underlying molecular mechanisms of Curcumin and Artesunate are not yet fully understood. Without knowing the detailed molecular mode of action, both substances cannot be applied in clinical tumor therapy.

In order to further clarify the mechanisms of Curcumin, the here presented thesis should specify Curcumin's mode of action regarding microRNAs. Thus we could show Curcumin modulates the miRNA expression pattern of breast cancer cells. Using Microarray expression profiling we found miR181b, which was elevated 3-fold and whose putative targets build a large overlap with the already known molecular targets of Curcumin.

We modulated miR181b expression by *knock-in* and *knock-out* experiments and performed sophisticated double modulation experiments to validate the causality between miR181b expression and curcumin effect. In particular, we show that miR181b overexpression leads to inhibition of proinflammatory cytokines CXCL1 and -2 and apoptoses associated genes BCL-2 and SURVIVIN. In the same way we showed a inhibited expression of invasiveness correlated MMP-1 and -3. Investigation of tumor associated cell functions results in an interaction between miR181b and tumor cell proliferation and invasivness. The findings *in vitro* could be confirmed *in vivo* in a murine model of heamatogenous metastasis, by detection of a lower number of metastases while miR181b overexpression. The induction of miR181b and the resulting repression of CXCL1 following Curcumin treatment of primary breast cancer cells (*ex vivo*) provides transferability to the clinical situation.

These data present miR181b as an important mediator of the effect of Curcumin on molecular level and points out the chemopreventive potential of the substance. Furthermore miR181b seems to be well suited as a predictor of Curcumin efficacy, to get a better differentiation between responder and non-responder.

As important as to know the way of action of a drug, it is equally important to understand the mechanisms that lead to resistance. As a further potentially chemopreventive substance we analyzed Artesunate concerning its anti-tumorigenic properties. In this case we observed an occurring resistance during the process of treatment of metastatic breast cancer cells (MDA-MB-231) with artesunate.

This is shown by an inhibition of tumor associated transcription factors AP-1 and NFκB in the beginning of the experiment, till in the end the effect stays away.

The rising amount of AP-1 and NFκB leads to a progressive resistance against apoptosis, reflected also in an increased expression of BCL-2 and a decreased level of BAX. Both genes show a shift of their ratio (BAX/BCL-2) towards apoptosis resistance. Additionally, with progressing resistance, metastatic breast cancer cells (MDA-MB-231) produce higher amounts of MMP-1, well known to be relevant in tumor progression and metastasis.

Only the understanding of a developing resistance allows a safer discrimination between responder and non-responder. The more detailed the knowledge of the molecular effects of natural compounds, the better substances like Curcumin or Artesunate can be integrated into new therapeutic schemes.

2 Das Mammakarzinom

Nach Schätzungen der WHO (*World Health Organization*) beträgt die Inzidenz von Malignomen bei Frauen im Jahr 2012 ca. 6,6 Millionen weltweit. Den größten Anteil davon (ca. 25 %) stellen maligne Neoplasien der Brust (ca. 1,6 Millionen), gefolgt von Tumoren des Dickdarms (ca. 0,61 Millionen) und der Lunge (ca. 0,58 Millionen). Mit 190.000 Todesfällen pro Jahr ist das Mammakarzinom in den Industrieländern die zweithäufigste Todesursache unter den malignen Tumoren, nach dem Lungenkarzinom (Globocan.iarc, 2014).

Die Risikofaktoren für die Entstehung eines Mammakarzinoms lassen sich in nicht-modifizierbare (Alter, Mammakarzinom assoziierte Mutationen, Familienanamnese, frühere Mammakarzinom-Erkrankungen, Brustdichte, Strahlenexposition und Anzahl der Menstruationszyklen) und modifizierbare Faktoren (Stilldauer, BMI, Diabetes mellitus Typ II, Nahrungszusammensetzung, Vitamin-D-Mangel, Hormontherapie, Genussdrogen, Schlafmangel, Nacht-/Schichtarbeit, reduzierte körperliche Aktivität, chemische Noxen während der fetalen und frühkindlichen Entwicklung) unterteilen (Ago-online, 2014).

Bei einem gesicherten Karzinom stellt die operative Therapie weiterhin den Goldstandard dar. Sie kann sowohl in eine brusterhaltende (BET), als auch in eine radikale Therapie mit Sentinellymphknoten-Entfernung und/oder Axilladissektion unterteilt werden. Im Anschluß an eine BET wird üblicherweise eine adjuvante Radiotherapie durchgeführt. Zur primär systemischen Therapie zählt einerseits die klassische Chemotherapie, anderseits der Einsatz von Antikörpern wie z.B. Trastuzumab oder endokrine Therapiesubstanzen wie z.B. Tamoxifen. Auch der primär systemischen Therapie kann eine Radiotherapie nachgeschaltet werden.

Durch verbesserte Therapiemöglichkeiten konnte 2010 in Deutschland ein 5-Jahres-Überleben von 87 % verzeichnet werden. In wie weit sich die Etablierung von Screening-Untersuchungen auf die Überlebenszeiten auswirken, wird erst in den kommenden Jahren beurteilt werden können (krebsdaten.de, 2010).

Problematisch im Rahmen der klassischen Tumortherapie sind häufig auftretende Nebenwirkungen der Chemotherapie sowie die Entstehung von Resistenzen gegen die zielgerichtete Therapie mit z.B. Antikörpern. Patientinnen mit Mammakarzinomen in Frühstadien erleiden in ca. 30 % der Fälle ein Rezidiv (Gonzalez-Angulo et al., 2007). Verbesserte Therapiemodalitäten ermöglichen eine Behandlung bereits fortgeschrittener Tumorstadien. Durch ein Vermeiden von Resistenzen soll die Überlebenszeit verlängert werden. Erste Studien zur Resistenzentstehung zeigen, dass bei einem Hormonrezeptor-positiven, Her2-negativen (*human epidermal growth factor receptor 2* - negativen*)* Mammakarzinom die Resistenzentwicklung gegenüber der endokrinen Therapie durch die Ergänzung eines mTOR-Inhibitors (*mechanistic Target Of Rapamycin*-Inhibitor*)* um fast 12 Monate herausgezögert werden kann (Beck et al., 2014).

Insbesondere zur Vermeidung der teils schwerwiegenden Nebenwirkungen der Chemotherapie werden Substanzen benötigt, die sich durch eine gute Verträglichkeit auszeichnen. Vor allem pflanzliche Stoffe, wie das natürlich vorkommende Polyphenol Curcumin, weisen ein exzellentes Wirkungs-/Nebenwirkungsverhältnis auf. Zudem haben Naturstoffe den großen Vorteil, an vielen unterschiedlichen Regulationspunkten anzusetzen, was die Entstehung einer Resistenz deutlich verlangsamt.

3 *small noncoding RNAs* - miRNAs

3.1 Biogenese und Regulationsmechanismen

miRNAs (microRNAs) sind kurze, 18-23 nt (*nucleotide*) lange, nicht kodierende, hochkonservierte RNA-Sequenzen. Sie beeinflussen die Regulierung der Gen-Expression auf posttranskriptioneller Ebene (He et al., 2004). Die Entdeckung gelang Lee et al. im Jahr 1993. Aktuell sind auf miRBase 1881 humane miRNA-Vorläufer und 2588 reife miRNA-Sequenzen beschrieben (miRBase Stand 09/2016).

miRNAs werden meist durch die RNA-Polymerase II transkribiert (Lee et al., 2004) und durchlaufen einige Prozessierungsschritte, bis sie aktiv ins Zytoplasma sezerniert werden (Yi et al., 2003). Dort bilden sie mit einem Argonaut-Protein den *RNA-induced silencing complex* (RISC). Die miRNA bindet meist im Bereich der 3'-UTR (*3'-untranslated region*) an der Ziel-mRNA (Lujambio et al., 2012). Eine perfekte oder fast perfekte Basenpaarung initiiert die RNAi (*RNA interference*), mit folgender Degradierung der mRNA (Rhoades et al., 2002; Esquela-Kerscher et al., 2006). Häufig liegt jedoch eine nicht-perfekte Bindung der miRNA an deren Ziel-mRNA vor, wodurch die Translation unterdrückt und das entsprechende Protein nicht synthetisiert wird. In diesem Fall kommt es zu einer Reduktion der Proteinlevel bei nahezu unveränderter mRNA-Expression (Bagga et al., 2005; Lim et al., 2005). Aktuelle Studien machen deutlich, dass miRNAs auch im Bereich der 5'-UTR (5'-*untranslated region)* oder dem ORF (*open reading frame*) (Moretti et al., 2010; Qin et al., 2010) binden und die Expression der Ziel-mRNA induzieren können (van Diest et al., 2004; Garzon et al., 2010).

Um alle Zielgene einer einzelnen miRNA, sowie deren teils mehrfache Bindungsstellen an einer mRNA (Cai et al., 2009) vorherzusagen, werden sog. *Target-Prediction-Tools* für *in silico*-Analysen verwendet. Hierzu zählen Datenbanken wie EMBL-EBI Microcosm-Targets, www.microrna.org, www.targetscan.org, www.mirbase.org und uvm. (Vlachos et al., 2013). Auf Ebene der miRNAs reicht in der Regel schon eine geringe Änderung der Expression, um aufgrund der *Target*-Multiplizität einen relevanten Effekt auf nachfolgende Prozesse zu verursachen. Da jede miRNA viele Zielgene reguliert und jedes Zielgen von vielen unterschiedlichen miRNAs reguliert wird, ist ersichtlich wie komplex und konzertiert die Aufgabe der miRNAs ist.

In Tumorzellen kommt es generell zu einer verminderten miRNA-Expression. Je geringer eine Zelle differenziert ist, umso niedriger ist die miRNA-Expression (Lujambio et al., 2012). Hierfür kann die Hemmung der miRNA-Transkription durch onkogene Transkriptionsfaktoren und eine Veränderungen der Biogenese der miRNAs verantwortlich gemacht werden (Chang et al., 2008; Thomson et al., 2006).

miRNAs können sowohl Onkogen, als auch Tumorsuppressor, oder beides gleichzeitig sein (Fabbri et al., 2007; Gebeshuber et al., 2009). Sie beeinflussen nicht nur die Tumorentstehung, sondern wirken sich auch auf die Tumor-Progression inklusive der Metastasierung und den Tumor-Metabolismus aus (Ma et al., 2007; Godlewski et al., 2010; Zhang et al., 2012).

MiRNAs weisen im Vergleich zur mRNA, eine deutlich größere Stabilität gegenüber RNAsen und Umwelteinflüssen, wie z.B. der Temperatur auf (Xi et al., 2007). Auch extrazellulär lassen sich miRNAs z.B. im Serum von Patienten nachweisen und stellen damit einen bequem messbaren Biomarker zur Therapiekontrolle bei Tumorerkrankungen dar (Mitchell et al., 2008). Ebenso können insbesondere *circulating*-miRNAs wichtige Prädiktoren zur frühen Erkennung von Tumoren wie zum Beispiel dem hepatozellulären Karzinom sein (Yin et al., 2015).

3.2 miRNAs beim Mammakarzinom

Da miRNAs in Karzinomen unterschiedlich exprimiert werden (Lu et al., 2005), lassen sich individuelle Expressionsprofile erstellen. Somit wird eine präzisere Klassifizierung und eine spezifischere Therapie und Prognose möglich (Yanaihara et al., 2006; Calin et al., 2005). Iorio et al. beschrieben 2005 erstmals die miRNA-Dysregulation beim Mammakarzinom nach Anwendung eines miRNA-*Microarray*.
MiRNAs haben Einfluss auf den Zellzyklus und wirken sich auf die Proliferationsfähigkeit von Zellen aus. Yu et al. (2008) zeigten, dass miR-17-5p/miR-20a Cyclin-D durch direktes Binden am 3'-UTR in MCF7-Zellen (*Michigan Cancer Foundation-7*) moduliert. Auch der E2/ERα/Sp1-Signaltransduktionsweg, welcher den Zellzyklus durch Aktivierung von Cyclin-D1 beim Mammakarzinom beeinflusst, wird durch miRNAs reguliert. miR-206 ist in Östrogenrezeptor-positiven Tumoren hochreguliert und hemmt die Östrogenrezeptor-Translation durch direktes Binden am 3'-UTR der ERα-mRNA (Adams et al., 2007).
Der Begriff der epithelialen-mesenchymalen-Transition ist eng mit der Metastasierung verknüpft. Diese wird unter anderem durch Regulation des Cadherin-1-Gen durch miR-9 vermittelt und äußert sich in einer Modulation der Invasionsfähigkeit und Zellbeweglichkeit (Vincent-Salomon et al., 2003; Tryndyak et al., 2010; Ma et al., 2010).
Matrix-Metalloproteinasen (MMPs) sind primär für den Abbau der extrazellulären Matrix und der Basalmembran verantwortlich und lockern Zellverbindungen. Unter physiologischen Bedingungen existiert eine feine Balance zwischen MMPs und deren natürlichen Inhibitoren, den TIMPs (*Tissue Inhibitor of Matrix Metalloproteinase).* Dieses Gleichgewicht ist in Tumorzellen oft gestört. Es konnte gezeigt werden, dass der physiologische MMP-3-Inhibitor (TIMP-3) in einigen Mammakarzinom-Zellen durch direkte Bindung von miRNA-21 herabreguliert wird. Dies hat eine erhöhte Invasionsfähigkeit zur Folge (Gabriely et al., 2008; Song et al., 2010). Ferner beeinflussen miRNAs spezifisch die Angiogenese. Beim metastasierenden Mammakarzinom kommt es nicht zur Repression von VEGF-A (*Vascular Endothelial Growth Factor-A*) durch miR-126. Durch ein stark verringertes miRNA-126-Level wird eine Aktivierung des VEGF/PI3K/AKT-Signaltransduktionsweges beobachtet (Zhu et al., 2011; Nikolic et al., 2010).

Betrachtet man die ausgeprägte Dysregulation der miRNA-Expressionsprofile maligner Tumoren, so ist den miRNAs, dem aktuellen Stand der Forschung zufolge, nicht nur eine maßgebliche Rolle bei der Entstehung und Progression von Mammakarzinomen, sondern von Tumoren im Allgemeinen zuzuschreiben.

4 Chemoprävention

Die heutige Zeit wird durch das Streben nach einer bewussteren Ernährung, einem gesünderen Lebensstil, sowie dem Ziel der Prophylaxe von Krankheiten gekennzeichnet. Detailliertes Wissen über die Wirkung von Nahrungsbestandteilen, Nahrungsergänzungsmitteln und Naturstoffen im Allgemeinen führt zunehmend zur Verlagerung des Therapieansatzes in eine Phase vor der Entstehung einer Krankheit. Man ist bereits lange auf der Suche nach Substanzen, deren Substitution die Bildung von malignen Tumoren verhindert oder verzögert.
Nach Manifestation einer Tumorerkrankung sind die Heilungschancen meist limitiert und der Gewinn an Lebenszeit durch etablierte anti-neoplastische Therapien oft gering. Zudem ist die verbleibende Lebenszeit und -qualität häufig stark beeinträchtigt durch Nebenwirkungen. Daher liegt es Nahe nach Substanzen zu forschen, durch die das Entstehen von Tumoren verhindert oder zumindest verzögert werden kann. Gerade die Zeit, die vor der Tumorentstehung gewonnen wird, ist Zeit von höchster Lebensqualität. Insbesondere Naturstoffe ohne nennenswerte Nebenwirkungen sind im Rahmen der primären Chemoprävention hierfür von großem Interesse. Der Ansatzpunkt ist, die Anfangsphase der Tumorentstehung zu blockieren (Landis-Piwowar et al., 2014). Zielgruppe ist die gesamte Bevölkerung, mit Hauptaugenmerk auf Risikogruppen.
Kam es bereits zur Entstehung eines Malignoms, dient die sekundäre Chemoprävention dazu, das Fortschreiten der Erkrankung, bzw. den Übergang einer Karzinomvorstufe in einen malignen Tumor zu verlangsamen oder aufzuhalten. Diese chemopräventiven Substanzen werden oft als „*suppressive agents*“ beschrieben, beispielhaft ist Curcumin zu nennen.
Im Rahmen der tertiären Chemoprävention soll durch den Einsatz von Curcumin die Entstehung eines Zweittumors nach Heilung des Primarius verhindert werden (Lippman, 1998). Elementar ist jedoch auch die Verhinderung bzw. Verringerung der Metastasierung bei vorliegender maligner Neoplasie. Dass dies möglich ist, haben präklinische Studien aus unserer Arbeitsgruppe bereits gezeigt. Das natürlich vorkommende Polyphenol Curcumin beugt der Bildung von Metastasen beim Mammakarzinom vor (Bachmeier et al., 2007).

4.1 Curcumin

Vogel und Pelletier entdeckten Curcumin (Diferruloylmethan, 1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) Anfang des 19. Jahrhunderts (1815). Curcumin ist als Gemisch der drei Curcuminoide Curcumin, Demethoxycurcumin und Bisdimethoxycurcumin (Zustand: Ketoenol-Äquilibrium (Grynkiewicz et al., 2012)) definiert. Die biologisch höchste Aktivität, vor allem bezogen auf die Regulierung von NFκB (*Nuclear Factor kappa-light-chain-enhancer of activated B cells*), wird dem reinen Curcumin zugeschrieben. Die meisten Studien wurden jedoch mit der Mischung der drei Curcuminoide durchgeführt (Sandur et al., 2007).
Die *in vitro* gezeigten Effekte begründen sich auf Interaktionen an vielen Punkten der Signaltransduktion. So wurden anti-inflammatorische und anti-oxidative, pro-apoptotische, antiproliferative, chemo-präventive und -therapeutische sowie wundheilende Eigenschaften beschrieben (Gupta et al., 2012). Interessant ist die Tatsache, dass Curcumin in allen Stadien der Karzinomentwicklung eingreift, wodurch es zu einer Modulation der Entstehung und Progression eines malignen Tumors kommt (Kawamori et al., 1999).

Klinische Studien zeigten, dass Curcumin gut bekömmlich und nahezu nebenwirkungsfrei ist, sodass es auch von der FDA als unbedenklich beschrieben wurde (*U.S.*

Food and Drug Administration, 2013). In Phase-I-Studien waren bei Patienten mit metastasiertem Mammakarzinom tägliche Dosen bis 6 g und in Phase-II-Studien beim Pankreaskarzinom bis 8 g nebenwirkungsarm (Bayet-Robert et al., 2010; Dhillon et al., 2008). Ab Dosen von 3,6 g/d ist Curcumin im Plasma/Gewebe detektierbar (Sharma et al., 2007). In toxikologischen Studien mit Ratten waren auch bei einer Dosis von 5 g/kg-Körpergewicht keine toxischen Effekte nachweisbar (Wahlstrom et al., 1978).

Antineoplastische Effekte von Curcumin wurden bereits bei Magen-, Colon-, Cervix- und Mammakarzinomen, Lymphomen, Leukämien, Melanomen und vielen anderen Tumorentitäten beschrieben (Bachmeier et al., 2007; Killian et al., 2012; Agrawal et al., 2010). Um das chemopräventive Potenzial von Curcumin noch genauer untersuchen zu können sind weitere Studien notwendig.
Problematisch und bis dato ungelöst ist auch die Frage, wie die Wirksamkeit von Curcumin kalkulier- und messbar gemacht werden kann. Nur damit wäre eine Differenzierung zwischen Patienten, die auf die Therapie ansprechen und sog. „Therapieversagern" möglich. Um den Einsatz von Curcumin in der Klinik zu etablieren, werden zuverlässige und leicht messbare Marker-Moleküle benötigt. Durch tiefgreifende präklinische Studien zum molekularen Wirkmechanismus von Curcumin könnten solche Marker-Moleküle identifiziert werden.

Bislang zeigten molekulare *in vitro*-Studien, dass Curcumin meist an Signaltransduktionswegen im Bereich Proliferation (Cyclin-D1, c-myc), *Cell-Survival* (BCL2, BCLxL, cFLIP, XIAP, c-IAP1), Tumor-Suppression (p53, p21), Caspasen-Aktivierung (Caspase-8, -3, -9), sowie den *Death*-Rezeptoren (DR4, DR5) und Proteinkinasen (JNK, Akt, AMPK) ansetzt. Somit werden zahlreiche Gene wie EGFR/ERBB1 und ERBB2/HER2, IGF-1R, PPAR-g, SHH, PI3K, AKT, AP-1, EGR1, STATs, NFκB, IL-6, COX2 und MMPs reguliert (Nagaraju et al., 2012; Dhandapani et al., 2007; Bharti et al., 2003; Singh et al., 1995; Kunnumakkara et al., 2009; Yodkeeree et al., 2009).
Weitere durch Curcumin differenziell exprimierte Gene sind NOS, TNF und zahlreiche Chemokine wie CXCL-1 und -2 (Onoda et al., 2000; Zambre et al., 2006; Bachmeier et al., 2008). Ferner beeinflusst Curcumin die für die Biotransformation verantwortlichen Enzyme, wie das Cytochrom P450 (Nagaraju et al., 2012).

Durch die oben beschriebenen Regulationswege wird die Tumorbiologie des Mammakarzinoms durch Curcumin verändert. So kommt es zu einer Induktion des G2/M-Zellzyklus-Arrestes (Venkiteswaran et al., 2007), der Apoptose (Cheah et al., 2009; Rowe et al., 2009) und Paraptose, sowie zu einer Inhibierung der Metastasierung (Bachmeier et al., 2007), Migration (Chiu et al., 2009), Invasion (Boonrao et al., 2010) und Angiogenese (Kunnumakkara et al., 2008).
Trotz einer Vielzahl bekannter, von Curcumin modulierter Gene, ist der zugrunde liegende molekulare Mechanismus der Curcuminwirkung, vor allem auf Ebene der posttranskriptionellen Regulation durch miRNAs bis jetzt nur lückenhaft verstanden. Bis dato fehlte eine objektive Messgröße, die ein Ansprechen auf Curcumin messbar macht. Klinische Studien können aber ohne Messgröße für die Wirksamkeit von Curcumin nicht durchgeführt werden.
Einen möglichen Ansatzpunkt hierfür liefern miRNA-Expressionsprofile, welche tumorspezifisch sind und durch Curcumin moduliert werden können. Aus präklinischen Studien zur Expressionsanalyse von Curcumin-regulierten miRNAs in verschiedenen Tumorentitäten und -stadien kann Wissen darüber gewonnen werden ob und welche miRNAs als Biomarker für klinische Studien nützlich sein könnte.

Es ist durchaus denkbar, dass in Zukunft einzelne miRNAs oder eine Gruppe von miRNAs verwendet werden können, um die Wirksamkeit von Curcumin bzw. anderer chemopräventiver Substanzen bei einzelnen Patienten im klinischen Alltag vorauszusagen bzw. zu überprüfen.

4.2 Artesunate

Die bereits in der Malaria-Therapie etablierte Substanz Artesunate, gehört wie Curcumin zur Gruppe möglicher Chemopräventiva phytogenen Ursprungs. Hierbei handelt es sich um ein halbsynthetisches Derivat von Artemisinin, dem Wirkstoff der chinesischen Pflanze Artemisia annua (Price, 2000). Das Schizontozid verursacht durch seine Peroxidstruktur in Anwesenheit von Eisenionen die Bildung von freien Radikalen. Dies führt bei Malaria-Patienten zur Abtötung der Plasmodien. Artesunate stellt die wasserlösliche Form dar.
Voraussetzung zur Anwendung als Chemopräventivum sind möglichst geringe Nebenwirkungen auf gesunde Gewebe während der Therapie. Vergleicht man die erreichten Plasma-Spiegel von Artesunate in standardisierter Dosis während der Malaria-Therapie mit den Spiegeln, die im Rahmen der Untersuchungen auf ihr chemopräventives Potenzial verwendet wurden, erscheint es wahrscheinlich, eine Dosis zu erreichen, die inhibierend auf die Progression der Tumorzellen wirkt (Efferth et al., 2003).
Der vermutete antitumorigene Wirkmechanismus von Artemisinin/Artesunate ähnelt dem antiparasitären Effekt. Man geht davon aus, dass nach Aktivierung durch reduziertes Eisen freie Radikale entstehen, die zur Zerstörung der Lysosomen und zur Bildung weiterer ROS (*Radical oxygen species*) führen. Somit resultieren DNA-Doppelstrangbrüche und es kommt zu einer Aktivierung der Caspasen-Kaskade und letztendlich zum Zelltod (Crespo-Ortiz et al., 2012).

5 Eigene Untersuchungen

5.1 Zielsetzung

5.1.1 Wirkung von Curcumin auf die Expression von miRNAs

Ziel der vorliegenden Arbeit war es, die bereits vorhandenen Erkenntnisse über die antitumorigenen Effekte von Curcumin, durch Untersuchung der zugrunde liegenden Mechanismen auf miRNA-Ebene, zu vertiefen.

1. Erstellung eines miRNA-Expressions-Profils aller unter Curcumin-Behandlung differenziell exprimierten miRNAs mittels *Array*-Technologie.
2. Bioinformatische Zielgen-Analyse aller unter Curcumin differenziell exprimierten miRNAs und *Matching* der Resultate mit den bereits bekannten, durch Curcumin differenziell exprimierten Genen (mRNAs).
3. Transiente und stabile Überexpression von miR181b in MDA-MB-231 Mammakarzinom-Zellen.
4. Untersuchung der Expressionslevel Metastasen-korrelierter Gene nach Modulation der miR181b-Expression via *knock-in*- und *knock-out*-Experimente auf Ebene der mRNA und Proteine.
5. Untersuchung funktioneller Aspekte des Einflusses von miR181b auf die Tumorentstehung und Metastasierung.
6. Validierung des Einflusses von miR181b auf das Metastasierungspotenzial im Tiermodell.
7. Translation der Ergebnisse auf die klinische Situation durch Untersuchung des Einflusses von Curcumin auf die miR181b- und CXCL1-Expression in Mammakarzinom-Zellen aus primären Tumoren.

5.1.2 Wirkung von Artesunate in einem Zellmodell des Mammakarzinoms

Auf der Suche nach einem weiteren Chemopräventivum sollten die antitumorigenen Eigenschaften von Artesunate beim Mammakarzinom präzisiert werden. Im Verlauf erfolgte die Untersuchung der zugrunde liegenden molekularen Mechanismen der Resistenzentwicklung.

1. Studien zur antitumorigenen Wirkung von Artesunate beim Mammakarzinom.
2. Untersuchung der molekularen Mechanismen der Resistenzentstehung gegenüber Artesunate.
3. Studien zur Resistenzentwicklung gegenüber Artesunate beim Mammakarzinom im Tiermodell.

5.2 Zellmodelle

Die aggressive und schnell proliferierende Zelllinie MDA-MB-231 dient als Modell eines stark malignen, multiresistenten Karzinoms. Sie entstammt dem Pleura-Punktat einer 51-jährigen Europäerin und ist einem Adenokarzinom der Brustdrüse zuzuordnen. Neben dem *epidermal growth factor* (EGF) exprimiert sie den *transforming growth factor alpha* (TGF alpha) und ist mit ihrem negativem Hormonrezeptorstatus, ein optimales Beispiel des Hormonrezeptor-negativen, vielfach resistenten Mammakarzinoms (*Non-Responder*). In Kultur wächst die rasch proliferierende Zelllinie in adhärenter Formation (lgcstandards MDA-MB-231).
Um im Vergleich eine gegenüber den meisten Therapiesubstanzen sensible Zelllinie zu untersuchen, kam die nicht invasive Mammakarzinom-Zelllinie MDA-MB-468 zur Anwendung. Sie wurde aus dem Pleura-Punktat einer 51-jährigen farbigen Patientin isoliert und exprimiert die gleichen Wachstumsfaktoren. Die Generationszeit ist geringer als die der MDA-MB-231-Zellen einzustufen. In Kultur zeigt sich ein adhärentes Wachstum (lgcstandards MDA-MB-468).

Des Weiteren wurde auch ein *ex vivo* Modell etabliert, um die Aussagekraft insbesondere bzgl. der miR181b-Expression und deren Zielgene CXCL1 und -2 zu stärken. Dazu wurden aus primären Mammakarzinom-OP-Resektaten Zellen isoliert und in Kultur genommen. Die Zellen wurden darauf mit Curcumin behandelt und anschließend molekularbiologisch analog der Zelllinien untersucht.

5.3 Wirkmechanismus von Curcumin auf miRNA-Ebene / Identifikation von Biomarkern – (Publikation A)

5.3.1 Effekt von Curcumin auf die Expression von miRNAs

Um die Anwendung einer Substanz im klinischen Alltag zu etablieren, ist es wichtig deren Wirkweise zu kennen und die zugrunde liegenden molekularen Mechanismen zu verstehen. Lassen sich durch *in vitro* Studien die Schlüsselmoleküle und zellbiologischen Prozesse einer Therapie definieren, so können Prädiktoren (Biomarker) für die Wirkung/Wirksamkeit der Substanz identifiziert werden. Als besonders geeignete Prädiktoren für Naturstoffe wie Curcumin könnten miRNAs dienen. Grund hierfür ist die Tatsache, dass ähnlich zur vielschichtigen und fein regulierten Wirkung von Curcumin, miRNAs bereits durch eine minimale Änderung der Expression einen vielfältigen Effekt auf biochemische Prozesse vermitteln.

In einem ersten Schritt untersuchten wir den Einfluss von Curcumin auf die Expression von miRNAs beim Mammakarzinom. Aus Vorarbeiten war bereits ein *Array*-Datensatz aller unter Curcumin-Behandlung differenziell exprimierten miRNAs in der malignen Mammakarzinom-Zelllinie MDA-MB-231 vorhanden.

Im Vergleich zur *carrier*-behandelten Kontrolle konnten wir 13 verschiedene miRNAs identifizieren die mindestens 2-fach über- (10 miRNAs) oder unterexprimiert (3 miRNAs) sind. Für alle weiteren Untersuchungen habe ich mich mit der humanen miRNA miR181b beschäftigt, welche gemeinsam mit der nahezu identischen miR181d (gleiche *seed-region* und damit übereinstimmende *target-genes)* mehr als dreifach überexprimiert war.

Sogenannte *Targetprediction-Tools* (bioinformatische Analysealgorhythmen wie PicTar, microRNA.org, TargetScan) ermöglichen es, alle voraussichtlichen Zielgene, die theoretisch (*in silico*) von miR181b/d reguliert werden, zu identifizieren.

In Folge haben wir einen in Vorarbeiten von uns generierten Genexpressionsdatensatz, aller unter Curcumin-Einfluss differenziell exprimierten Gene (Bachmeier et al., 2008), den Resultaten der bioinformatischen Analyse gegenüber gestellt und beide Datensätze miteinander abgeglichen.

Aufgrund der enormen Fülle an *Targets* konzentrierte ich mich zunächst auf eine Auswahl von Genen, die gleichermaßen im *in silico*-Datensatz, als auch im vorbekannten *Microarray*-Genexpressionsdatensatz zu finden waren.

5.3.2 Wirkung von Curcumin via miR181b auf die proinflammatorischen Zytokine CXCL1 und -2

Ob ein kausaler Zusammenhang zwischen der miR181b-Überexpression und dem durch Curcumin verursachten *knock-down* der CXCL1 und -2 (*Chemokine (C-X-C Motif) Ligand 1/-2*) Expression besteht, sollte zunächst durch eine isolierte transiente Induktion der miR181b-Expression in Mammakarzinom-Zellen untersucht werden. Wir zeigten, dass die transiente Überexpression von miR181b zu einer statistisch signifikanten Reduktion der CXCL1 und -2 Expression sowohl auf Transkripte-, als auch kongruent auf Protein-Ebene führt. Mittels *Luciferase-Assay* konnten wir bestätigen, dass miR181b direkt an der *in silico* vorhergesagten Bindungsstelle im 3'-UTR der mRNA beider Zytokine bindet.

Im weiteren erfolgten komplexe Doppelmodulationen mit gleichzeitiger miR181b Überexpression (transient oder stabil) oder miR181b *knock-down* durch spezifische *Hairpin*-Inhibitoren und gleichzeitige Curcumin-Behandlung. Hier konnte ich nachweisen, dass die unter Curcumin beobachtete Hemmung der CXCL1 und -2 Expression, durch den gleichzeitigen Einsatz von miR181b spezifischen Hairpin-Inhibitoren aufgehoben wurde. Parallel kam es zu ansteigenden Expressionslevel von CXCL1 und -2 unter alleiniger miR181b Antagonisierung. Somit war es mir möglich, *in vitro* den Nachweis zu erbringen, dass miR181b ursächlich für die Wirkung von Curcumin auf die differenzielle Expression der Zytokine CXCL1 und -2 ist. Damit kommt miR181b eine maßgebliche Rolle an der anti-tumorigenen Wirkung von Curcumin zu (siehe schematische Abbildung der Curcuminwirkung via miR181b in Abb.1).

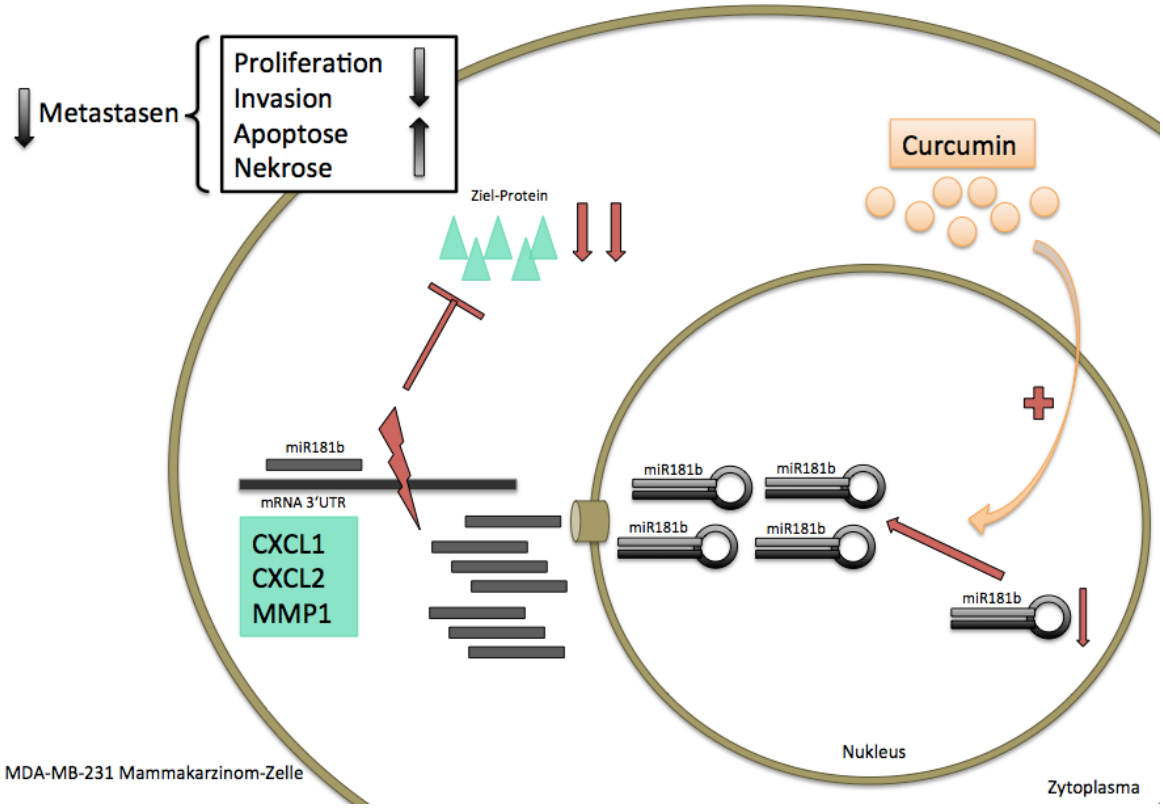

Abb. 1 (vorherige Seite): Abbildung einer MDA-MB-231 Mammakarzinom-Zelle (schematisch). Wie in den meisten Tumorzellen zeigt sich das physiologische miRNA-Expressionsmuster dysreguliert. Unter Behandlung mit Curcumin kommt es zu einer statistisch signifikanten Erhöhung der miR181b Expression. Diese wird zuletzt als reife miRNA aktiv via Exportin 5 in das Zytoplasma sezerniert und kann hier durch Bindung an den spezifischen miR181b-Sequenzen im Bereich der 3'-UTR der Zielgene (z. B. CXCL1/CXCL2/MMP-1) eine Hemmung der Translation bzw. Degradierung der mRNA hervorrufen. Durch Reduktion der Synthese der proinflammtorischen Zytokine CXCL1 und -2 und der matrixdegradierenden Metalloproteinase 1 kommt es durch Verminderung der Proliferations- und Invasionsfähigkeit sowie durch Verstärkung der Apoptose- und Nekrose-Rate der Tumorzellen in Summe zu einer Reduktion des Metastasierungspotenzials.

5.3.3 Die Wirkung von miR181b auf die Tumorprogression

Wichtige mit der Progression eines Tumors verbundene Charakteristika, sind Zellproliferation, Invasivität, sowie Apoptoserate eines Tumors. Daher war es unser Ziel zu zeigen, dass miR181b an der durch Curcumin verminderten Tumorzell-Proliferation und -Invasion und der gesteigerten Apoptose-Rate beteiligt ist.

Die Proliferationsfähigkeit der Tumorzellen untersuchten wir mit Hilfe eines funktionalen *Proliferation-Assays* (BrdU-Assay, Roche). Nach transienter miR181b-Überexpression mittels miR181b-*Mimics* konnte ich eine deutliche Proliferationshemmung von ca. 25% in den MDA-MB-231-Zellen, gegenüber den entsprechenden Kontroll-Zellen nachweisen. Interessanterweise war es möglich den bereits bekannten Effekt der Proliferationsinhibierung unter Curcumin (Bachmeier et al., 2010), bei gleichzeitiger Anwendung des spezifischen miR181b-Inhibitors nahezu vollkommen aufzuheben. Dieses Phänomen zeigt eindeutig eine kausale Beteiligung von miR181b an der anti-proliferativen Wirkung von Curcumin.

Ein weiteres Kriterium, das unmittelbar die Tumorprogression beeinflusst, ist die Fähigkeit von Tumorzellen die Apoptoserate zu reduzieren. Nun soll der Einfluss von miR181b auf die proapoptotischen Eigenschaften von Curcumin untersucht werden.

Mittels *Apoptose-Assay* (Cell Death ELISA, Roche) konnte ich bestätigen, dass eine miR181b-Überexpression zu einer statistisch signifikanten Induktion der Apoptose-Rate führt. Eine transiente miR181b-Überexpression resultiert in einer Verdoppelung der Apoptose-Rate im Vergleich zur Kontrolle. Ergänzend hierzu stieg auch die Nekrose-Rate um bis auf das Fünffache an.

Hierbei könnten potenziell BCL-2 (*B-cell lymphoma 2)* und SURVIVIN (anti-apoptotisch), die ich zuvor im Rahmen der bioinformatischen Analyse als vermeintliche miR181b-Zielgene ausgemacht hatte, verantwortlich sein. *In vitro* konnte ich dann tatsächlich nach miR181b-Überexpression eine statistisch hochsignifikante Reduktion (BCL-2 um ca. 60 %, SURVIVIN um ca. 50 %) gegenüber der mit einem Kontrolloligonukelotid transfizierten Vergleichszelllinie nachweisen.

Eine hohe Invasivität bedeutet eine raschere Progredienz der Erkrankung und damit eine höhere Malignität des Tumors. Aus diesem Grund muss zur Beurteilung der Tumorprogression auch die Invasionfähigkeit herangezogen werden. Für die entsprechende experimentelle Untersuchung wurde ein *Invasion-Assay* (Boyden Chamber Matrigel Assay*,* Chemicon) durchgeführt. Hierbei war zu beobachten, dass die Invasivität nach miR181b-Überexpression im Vergleich zur unbehandelten Kontrolle um ca. 50 % reduziert war. Die aktive Invasion der Tumorzellen ist als Resultat des proteolytischen Abbaus der extrazellulären Matrixschicht (ECMatrix^{TM}) zu sehen.

Für das erhöhte invasive Potenzial der Tumorzellen sind sehr wahrscheinlich die bereits *in silico,* als potenzielle Zielgene von miR181b nachgewiesenen Matrix-Metalloproteinasen-1 und -3 (MMP-1 und -3) mitverantwortlich zu machen. Beide sind eng mit der Degradierung der extrazellulären Matrix verknüpft. Quantitative RT-PCR-Analysen der zwei MMPs ergaben beim Vergleich von stabil miR181b über-

exprimierenden MDA-MB-231-Zellen mit Kontroll-Zellen, eine 93 %ige (MMP-1) und 75 %ige (MMP-3) Erniedrigung der Expressionslevel. Auch auf Proteinebene war via *Western Blot,* unter identischen Untersuchungsbedingungen, eine statistisch signifikante Reduktion der MMP-1 und -3-Konzentrationen zu finden (siehe schematische Abbildung der Curcuminwirkung via miR181b in Abb.1).
Zusammenfassend können wir nach den durchgeführten Studien feststellen, dass ein Großteil der Curcuminwirkung auf die Mammakarzinom-Zelllinie MDA-MB-231 über miR181b vermittelt wird. Insbesondere auf Ebene funktionaler Zellprozesse wie der Proliferation konnte ein ähnlicher Effekt wie unter Curcumin, auch unter alleiniger miR181b-Überexpression beobachtet werden. Die Aufhebung der Curcuminwirkung durch die Hinzugabe eines spezifischen miR181b Inhibitors spricht klar für den kausalen Zusammenhang.

5.3.4 Hemmung der Metastasierungsfähigkeit in vivo

Frühere Untersuchungen unserer Arbeitsgruppe ergaben eine statistisch signifikant verminderte Bildung von Metastasen unter Curcuminbehandlung (Bachmeier et al., 2007). Im Folgenden sollte nun geklärt werden, ob dies über miR181b vermittelt wird. Für die *in vivo* Studien kam ein in unserer Arbeitsgruppe bereits etabliertes Tiermodell zum Einsatz. Es wurde die Bildung hämatogener Metastasen in weiblichen, immundefizienten, fünf Wochen alten, CD-1 Foxn1nu Mäusen (Charles River Laboratories, Calco, Como, Italien) analysiert.
Diesen Mäusen wurden entweder stabil miR181b-überexprimierende Zellen (MDA-MB-231$^{miR181b+}$), oder entsprechende Kontrollzellen (MDA-MB-231^{MOCK}) intrakardial injiziert. Je nach Art der injizierten Zelle wurden die Mäuse in zwei gleich großen Studiengruppen geteilt. Nach 5 Wochen erfolgte die Tötung und pathologische Aufarbeitung aller Mäuse. Gehirn, Humerus, Femur und Wirbelsäule der Mäuse waren frei von Metastasen. Die intra- und peripulmonal gefundenen Metastasen wurden von einem Pathologen histologisch untersucht und statistisch ausgewertet.
Wir konnten zeigen, dass in der Studiengruppe der Mäuse denen miR181b-überexprimierende MDA-MB-231$^{miR181b+}$-Zellen injiziert wurden, im Vergleich zur Kontrollgruppe statistisch signifikant weniger Metastasen gebildet wurden. Gemittelt bildeten die Mäuse nach Injektion von MDA-MB-231^{MOCK}-Zellen ca. 15 pulmonale Metastasen pro Tier, während die MDA-MB-231$^{miR181b+}$-Gruppe im Schnitt ca. 6 Metastasen bildete. Die detaillierte Aufarbeitung der Tumoren beider Populationen ergab eine identische Dimension, Morphologie sowie Histologie. Der menschliche Tumorursprung wurde durch Detektion des humanen p53-Proteins bestätigt. Die Vitalität der Tumorzellen konnte anhand der Zahl der proliferierenden Ki-67 positiven Zellen gemessen werden.
Zur Untermauerung dieses Ergebnisses untersuchten wir Gene, deren Expression mit der Entwicklung von Lungenmetastasen beim Mammakarzinom korrelieren (Minn et al., 2005). Hier konnten wir nachweisen, dass die Expression von SPARC (*Secreted Protein Acidic and Rich in Cysteine*), CXCR4 (C-X-C *chemokine receptor type* 4), COX2 (Cyclooxygenase-2), ANGPTL4 (*Angiopoietin-like* 4), EFEMP1 (EGF-*containing fibulin-like extracellular matrix protein* 1), IL6 (Interleukin-6) und EGR1 (*Early growth response protein* 1) 72 h nach Behandlung mit miR181b-Oligonukleotiden, statistisch signifikant gegenüber den mit Kontroll-Oligonukleotiden transfizierten MDA-MB-231-Zellen reduziert ist. Diese Gene konnten von Ramaswamy et al. (2003) beim Vergleich der Genexpressionsprofile metastasierter Tumoren versus Primärtumoren, als für die Metastasierung typisch herausgearbeitet werden. Es war sogar möglich ANGPTL4, EFEMP1, SPARC unmittelbar dem

Expressionsprofil des metastasiertem Mammakarzinom zu zuordnen (Albini et al., 2008).
Ergänzend analysierten wir in 424 Fällen von Mammakarzinomen die Korrelation der Expression dieser Gene mit dem Rezidiv-freien Überleben. Wir fanden hier eine statistisch signifikante Korrelation einer niedrigeren Expression von SPARC, COX2 und ANGPTL4 mit Rezidiv-freiem Überleben, während bei den anderen Genen kein statistisch signifikanter Effekt zu beobachten war.

5.3.5 Einfluss von Curcumin auf primäre Mammakarzinom-Zellen

Zur Übertragung der bis dato mit Hilfe von Zelllinien gewonnen Ergebnisse (*in vitro* und *in vivo*) auf die klinische Situation, haben wir Untersuchungen an primären, aus Mammakarzinomen isolierten Tumorzellen (*ex vivo*) durchgeführt, um die Wirkung von Curcumin auf die miR181b-Expression und die daraus resultierende Expression von CXCL1 zu evaluieren. Darüberhinaus galt es auch zu prüfen, ob miR181b generell in primären Mammakarzinomen exprimiert wird und somit als Biomarker in Frage kommen würde. Die Analyse der primären Mammakarzinom-Zellen bzgl. ihrer miR181b-Expression unter Curcumin-Behandlung ergab eine deutliche Überexpression, gegenüber der *carrier*-behandelten Kontrolle, von ca. 50 %. Betrachtet man die resultierende CXCL1-Expression, so konnten wir mittels ELISA eine 3,5-fache Reduktion der Protein-Level gegenüber der *carrier*-behandelten Kontrolle nachweisen. Unsere Studien haben bis zu diesem Punkt gezeigt, dass unsere Strategie des Screenings und der Suche nach molekularen Mechanismen und Markern für die Wirksamkeit und Wirkung von Curcumin erfolgreich war. miR181b ist als Prädiktor für die Curcumin-Wirksamkeit sowie für deren weitere klinische Validierung denkbar. Somit gelang es uns, mit dem oben beschriebenen Ansatz, die Ergebnisse der *in vitro*- und *in vivo*-Studien auf die klinische Situation zu übertragen.

5.4 Entwicklung von Resistenzen unter der chemopräventiven Substanz Artesunate (Publikation B)

Auf der Suche nach weiteren Substanzen mit chemopräventivem Potential wurde die in der Malaria-Behandlung etablierte Substanz Artesunate untersucht. Diverse Studien zeigten, dass Artesunate bei über 70 unterschiedlichen Karzinom-Zelllinien zytotoxische Effekte verursacht (Efferth et al., 2001). Daher war es naheliegend diese nebenwirkungsarme Substanz bzgl. ihrer antineoplastischen Wirkung beim Mammakarzinom zu untersuchen. Zu diesem Zweck haben wir zwei Zelllinien unterschiedlicher Tumorigenität verwendet, zum einen die hochmaligne Metastasen-bildende, *triple*-negative Zelllinie MDA-MB-231, zum anderen die weniger maligne, nicht invasive Zelllinie MDA-MB-468.
Hierbei bestand mein Anteil der Arbeit zur Co-Autorenschaft der beschriebenen Veröffentlichung, in Zellkultur-Arbeiten, *in vitro* mRNA-/Protein-Analysen mittels quantitativer RT-PCR, respektive *Westerblots* sowie dem Cell Death ELISA für die Apoptose-Analysen.

5.4.1 Beobachtung einer Resistenzentwicklung

Zur Bestimmung der notwendigen Konzentration von Artesunate sowie der optimalen Behandlungszeitpunkte, erfolgten in einem ersten Schritt Studien zu Zellvitalität und Proliferationsfähigkeit. Hierfür kam der *MTT-Assay* (Thermo Fisher Scientific Inc.) zur Anwendung.
Anfänglich zeigte sich wie erwartet, bei beiden Mammakarzinom-Zelllinien der zytotoxische Effekt von Artesunate, statistisch signifikant, konzentrationsabhängig bei allen Bedingungen, mit bis zu 55 % (MDA-MB-231) respektive 45 % (MDA-MB-468) Reduktion der Zellvitalität bei 50 µM Artesunate. Zu späteren Zeitpunkten (>24 h) ließ sich dieser Effekt in den metastasierenden MDA-MB-231-Mammakarzinom-Zellen nicht mehr nachweisen. Es kam nicht nur zu einem Ausbleiben der Vitalitäts-hemmung, sondern sogar zu einem Anstieg der Vitalität nach Vorbehandlung der Zellen (20 µM Artesunate über 24 h), gemessen an deren metabolischer Aktivität. Die weniger tumorigenen MDA-MB-468-Zellen sprachen jedoch nach identischer Vorbehandlung mit Artesunate, im Vergleich zu den „aggressiveren" MDA-MB-231-Zellen weiterhin auf die Behandlung an. So war bei einer maximalen Artesunate-Konzentration von 50 µM eine Reduktion der Zellvitalität um 55 % gegenüber der unbehandelten Kontrolle zu beobachten. Dieses Phänomen werteten wir als ersten Hinweis für eine erworbene Resistenz der MDA-MB-231-Zellen gegenüber Artesunate.

Neben der Vitalität stellt auch die Zu- bzw. Abnahme der Apoptose-Rate eine wichtige Messgröße zur Beurteilung der Wirkung einer Substanz dar. Hierfür wurde der *Cell Death ELISA* (Roche Applied Biosystems) verwendet. Nach Behandlung mit 20 µM bzw. 50 µM Artesunate reagierten beide Zelllinien mit einer statistisch signifikanten Verdopplung der Apoptose-Rate. Behandelte man jedoch die Zelllinien über 24 h mit 20 µM Artesunate vor, konnte bei den MDA-MB-231-Zellen keine Apoptose-Induktion erzielt werden. Die weniger malignen MDA-MB-468-Zellen zeigten jedoch unabhängig von der Vorbehandlung einen konzentrations-abhängigen Anstieg der Apoptose-Rate.

In einem dritten, unabhängigen Experiment, untersuchten wir mittels *Flow*-Zytometrie Apoptose und Nekrose nach Artesunate-Behandlung (20 µM). Nach Ernte der Zellen wurden diese mit FITC (Fluorescein Isothiocyanat)-konjugiertem Annexin V und Propidiumiodid inkubiert. Auch hier konnten wir beobachten, dass MDA-MB-468-Zellen

mit und ohne Vorbehandlung, sowohl in frühe als auch in späte Apoptose-Stadien übergingen. Konträr hierzu zeigten MDA-MB-231-Zellen nach Vorbehandlung keine vermehrte Apoptose.
In Summe der oben beschriebenen, drei unabhängigen Untersuchungen, konnten wir zeigen, dass Artesunate beim Mammakarzinom entgegen der Erwartung, kein wirksames Chemotherapeutikum darstellt und stattdessen die hochmaligne Mammakarzinom-Zelllinie (MDA-MB-231) eine Resistenz gegenüber Artesunate entwickelt.

5.4.2 *Untersuchung der molekularen Mechanismen der Resistenzentwicklung unter Artesunte*

Neben dem Verständnis der antitumorigenen Wirkung chemopräventiver Substanzen wie Artesunate, ist auch die Entschlüsselung einer Resistenzentwicklung (im Hinblick auf Therapie-*Non-Responder*) von großer Bedeutung. Bis dato sind die molekularen Mechanismen, die zu spezifischen Resistenzen, aber auch zur MDR (*Multi-Drug-Resistance*) führen, nicht bis ins Detail verstanden und sollen im Folgenden weiter untersucht werden.

5.4.2.1 *Transkriptionsfaktoren NFκB und AP-1*

Die Transkriptionsfaktoren NFκB und AP-1 sind eng mit Proliferation, Adhäsion, Angiogenese und Inflammation verbundenen. NFκB wurde bereits als wichtiger Faktor im Bezug auf die Resistenzentwicklung gegen Chemotherapeutika durch Inhibierung der Apoptose beschrieben (Arlt et al., 2002). Kommt es zur nukleären Aktivierung von NFκB durch z.B. DNA-Schäden, werden pro-apoptotische Gene herunter- und mit Zell-Überleben korrelierte Gene hochreguliert. Dadurch kann es zur Reduktion der Apoptose und zur Entstehung einer Apoptose-Resistenz kommen.
Neben NFκB wurde auch für AP-1 eine Rolle bei der Entstehung von Resistenzen gegenüber Chemotherapeutika beschrieben (Efferth et al., 2002). Durch vermehrte Expression von AP-1 oder dessen Untereinheiten c-jun und c-fos, werden die Homo- und Heterodimere (aus c-jun und c-fos) in den Zellkern transloziert und beeinflussen dort Apoptose-regulierende Gene. Es ist bereits bekannt, dass AP-1 die mit Resistenzen verbundenen Proteine P-Glykoprotein/MDR1 und Glutathion-S-Transferase-pi hochreguliert. Durch Glutathion-S-Transferasen werden zytotoxische Substanzen an Glutathion gebunden und aus der Zelle geschleust. Auch die zelluläre Antwort von Tumorzellen auf die Behandlung mit Artesunate, wird im Hinblick auf Resistenzen mit der Expression von Glutathion-S-Transferasen verbunden (Efferth et al., 2005).
Wir haben daher die nukleäre Aktivität dieser beiden mit der Resistenzentwicklung verbundenen Transkriptionsfaktoren nach Artesunate-Behandlung mittels EMSA (*electrophoretic mobility shift assay*) untersucht. Mit der Messung der nukleären Aktivität kann direkt die Reaktion eines Transkriptionsfaktors auf einen Stimulus und damit die Initiierung der zellulären Antwort abgebildet werden. Die Artesunate-Behandlung (20 µM) von MDA-MB-231-Zellen zeigte eine statistisch signifikante nukleäre Aktivierung von NFκB und AP-1, bzw. deren Untereinheiten p65 und c-jun. Durch die Artesunate-Behandlung und die verursachten DNA-Doppelstrang-Brüche (Li et al., 2008) dissoziiert NFκB von IκB (Inhibitor of κB). Hierauf translociert NFκB in den Nukleus und führt zu einer gesteigerten nukleäreren Aktivität und damit letztendlich zu einer vermehrten Expression von *Cell-Suvival*-korrelierten Genen wie z.B. BCL-2.
Quantitative Messungen der mRNA-Expression der NFκB-Untereinheit p65 und der AP-1-Untereinheit c-jun (mittels qRT-PCR) ergaben, dass es bei MDA-MB-231-Zellen nach Artesunate-Behandlung zunächst zu erhöhten p65 und c-jun-

Expressions-Level kam. Nach Artesunate-Vorbehandlung konnte keine Änderung der p65- und c-jun-Expression, insbesondere keine Reduktion der Expression (und damit Ansprechen auf Artesunate und Erhöhung der Apoptose), mehr nachgewiesen werden. Somit scheint sich in den MDA-MB-231-Zellen eine Resistenz gegen Artensunate entwickelt zu haben.
Die MDA-MB-468-Zellen zeigten eine von Vorbehandlung und Behandlung unabhängige Reduktion der p65-Expression, was vereinbar mit der konstant erhöhten Apoptose-Rate ist. Ein identisches Ergebnis ergab die Untersuchung der c-jun-Expression. So konnten wir den ersten Schritt zu Klärung der erworbenen Resistenz gegenüber Artesunate auf molekularer Ebene erbringen.

5.4.2.2 *Apoptose-korrelierte Proteine BAX und BCL-2*
Die Familie der BCL-2-Proteine ist eng mit der Regulation der Apoptose verbunden. Mitglieder sind unter anderen das pro-apoptotische BAX (BCL2 *associated X protein*) und das anti-apoptotische BCL-2. Bekannterweise korreliert insbesondere deren Quotient mit der Apoptoserate, so dass die Gen-Expression beider Mitglieder der BCL-2-Familie nach Artesunate-Behandlung/Vorbehandlung von Interesse war. Unter Berücksichtigung der bestehenden Ergebnisse, im Hinblick auf die erworbene Resistenz, vermuteten wir, dass es nach Artesunate-Vorbehandlung zur Reduktion der BAX-Expression (pro-apoptotisch) und zu einer Induktion der BCL-2-Expression (anti-apoptotisch) kommen müsste. In Folge dessen sollte sich auch der BAX/BCL-2-Quotient zu Gunsten eines höheren Zellüberlebens erniedrigen.
Die Expression beider Gene wurde mittels quantitativer RT-PCR-Analyse der mRNA-Produkte von BAX und BCL-2 nach Behandlung bzw. Vorbehandlung mit 20 µM Artesunate gemessen.
MDA-MB-231-Zellen wiesen bereits unmittelbar (2 h) nach Artesunate-Behandlung eine signifikante Induktion von BCL-2 (anti-apoptotisch) auf (siehe Abb. 2, Punkt „1“). Im Verlauf konnte die BCL-2-Expression der Zellen nach Vorbehandlung aufgrund der entstandenen Resistenz nicht mehr induziert werden. Parallel hierzu kam es nach Erst-Behandlung mit Artesunate zu einer statistisch signifikanten Reduktion von BAX (pro-apoptotisch). Auch hier konnte nach Vorbehandlung mit Artesunate keine weitere Repression mehr erzielt werden, so dass die BAX-Expression aufgrund der Resistenzentwicklung stabil blieb.
Insgesamt ist zu vermuten, dass die Induktion des anti-apoptotischen BCL-2 und die Reduktion des pro-apoptotischen BAX nach Artesunate-Behandlung zur Entwicklung der Resistenz führt. Betrachtet man den Quotienten von BAX und BCL-2, ist ein zu Beginn der Behandlung hoher Wert zu erkennen (entsprechend einer erhöhten Apoptose-Rate, wie auch zuvor im Cell-Death-ELISA nachgewiesen), welcher jedoch im Verlauf des Experiments zunehmend abnimmt (Entwicklung der Resistenz und Abnahme der Apoptose) und sich schließlich nach Artesunate-Vorbehandlung auf niedrigem Niveau einpendelt.

Betrachtet man die BAX/BCL-2-Expression der MDA-MB-468-Zellen unter Artesunate, zeigt sich entsprechend der vorbeschriebenen Wirksamkeit und ausbleibenden Resistenz, sowohl nach Behandlung, als auch nach Vorbehandlung eine signifikante Inhibierung von BCL-2, sowie eine beinahe linear verlaufende Induktion von BAX. Die Untersuchung der MDA-MB-468-Zellen ergab unabhängig ob mit oder ohne Vorbehandlung mit Artesunate einen klaren, zeitabhängigen Anstieg des BAX/BCL-2-Quotienten. Somit konnten wir zeigen, dass in dieser weniger malignen Zelllinie keine Artesunate-Resistenz auftrat und die Zellen durchwegs sensibel auf die Substanz reagierten.

Sowohl NFκB als auch AP-1 sind als Regulatoren einiger Mitglieder der BCL-2-Familie bekannt (Sevilla et al., 2001). In wie weit die Induktion von NFκB mit der Regulation von BAX und BCL-2 kausal verbunden ist, haben wir mittels Promotoranalysen validiert. Hier zeigte sich, dass beide Proteine die selben Transkriptionsfaktor-Bindungsstellen (ELK4, ELF5, SPIB, NFκB, ZNF354C, SP1, ELK1, MZF1_1-4, MZF 5-13, Bapx1) aufwiesen, wobei BAX eine und BCL-2 zwei mutmaßliche Bindungsstellen für NFκB enthielt.
Die Entwicklung einer Resistenz der MDA-MB-231 gegenüber Artesunate, kann bei gleichen Transkriptionsfaktor-Bindungsstellen von BAX und BCL-2 nicht alleine mit der unterschiedlichen Anzahl an NFκB-Bindungsstellen begründet werden. Als weiterer Regulationsmechanismus ist eine Modulation über p53 wahrscheinlich. Es ist bekannt, dass BAX über p53 reguliert wird (Miyashita et al. 1995). Die Aktivierung von p53 erfolgt wahrscheinlich über die durch Artesunte verursachten Doppelstrangbrüche in der DNA (siehe auch Abb. 2, Punkt „1“) (Powell et al., 2014; Berdelle et al., 2011). Da die Zelllinie MDA-MB-231, wie etwa die Hälfte aller malignen Tumorzellen, eine Mutation im p53-Gen aufweist (Powell et al., 2014), ist eine deutlich geringere Transkriptions-Rate und damit eine reduzierte Aktivität am BAX-Promotor zu erwarten. In Folge dessen ergibt sich auch über diesen Weg der Regulation ein erniedrigter BAX-Spiegel, womit sich das Gewicht in Richtung Anti-Apotose verlagert und damit die Resistenz-Entwicklung möglich wird.

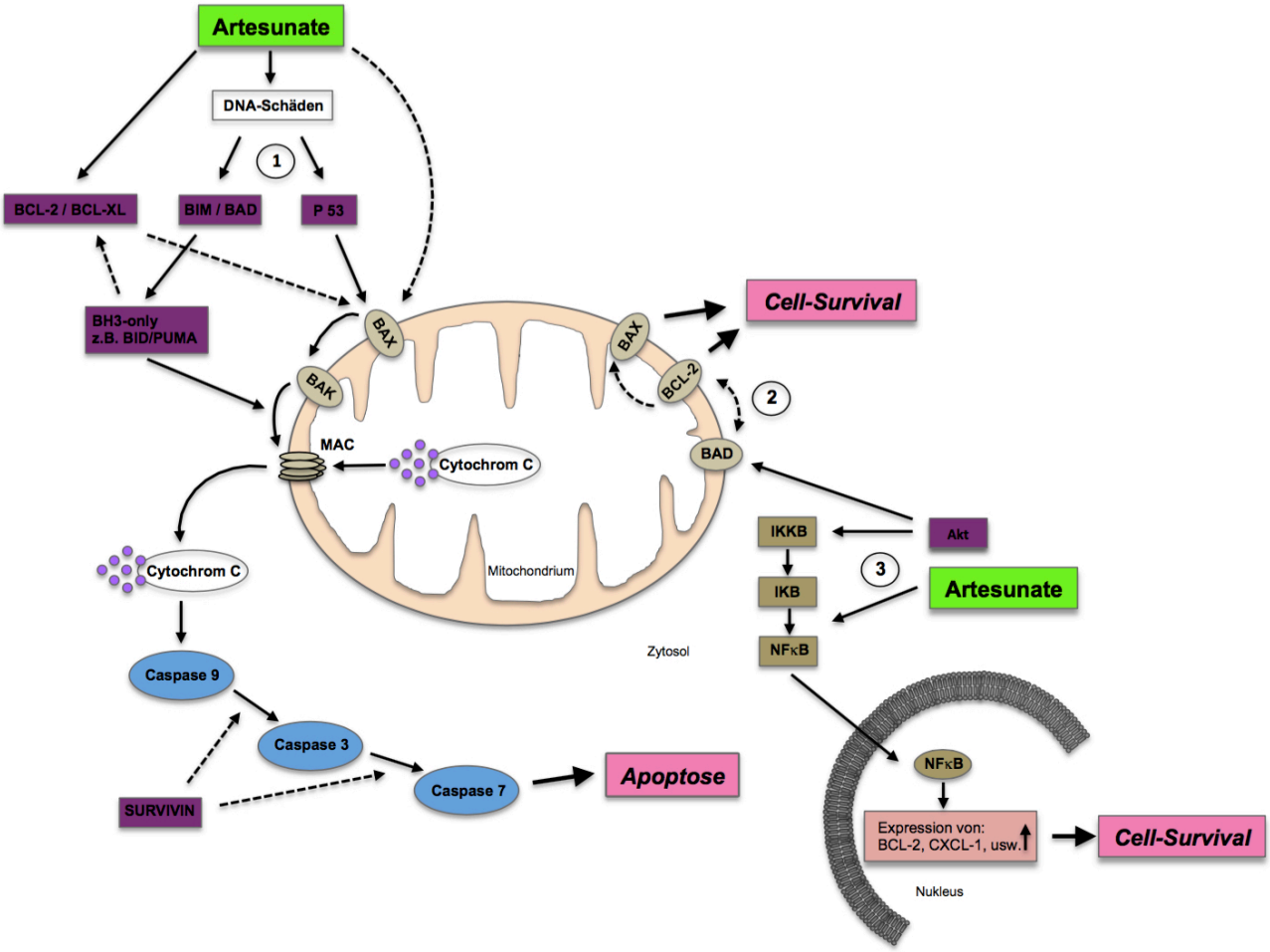

Abb. 2 (vorherige Seite): Auszug des Apoptose-Signaltransduktionsweges unter Artesunate-Behandlung, schematisch dargestellt. Durchgezogene Pfeile → Induktion/Verstärkung; gestrichelte Pfeile → Hemmung/ Verringerung. Artesunate-Behandlung führt wie durch „1" gekennzeichnet mutmaßlich zu DNA-Schäden/-Doppelstrangbrüchen wodurch über die Faktoren BIM/BAD (bcl-2 *interacting mediator of cell death*/ Bcl2-*associated agonist of cell death*), sowie p53 die Bildung des BAX/BAK-Komplexes (BAX/Bcl-2 *homologous antagonist killer*) und damit die Entstehung des MAC (*membran attac complex*) gefördert wird. Via Cyctochrom-C-Ausschüttung aus den Mitochondrien kommt es über die Caspasen-Kaskade letztendlich zur Apoptose-Induktion. Die Induktion der BCL-2-Expression mit resultierender Hemmung von BAX, sowie die Reduktion von BAX selbst mit folgender Hemmung der MAC-Bildung sind vermutlich verantwortlich für die Resistenz-Entwicklung.
Punkt „2" stellt den Schnittpunkt von BCL-2 und BAX mit dem Akt-*Pathway* dar. Nach Aktivierung von Akt kommt es zur Phosphorylierung von BAD, wodurch dieses von BCL-2 dissoziiert und eine Hetero-dimerisierung von BCL-2 mit BAX ermöglicht. Das Resultat ist eine Blockade von BAX und damit ein vermehrtes Zell-Überleben.
Die Wirkung von Artesunate auf NFκB ist unter „3" dargestellt. Durch Artesunate erfolgt die Aktivierung von NFκB, mit dessen Translokation in den Zellkern und die daraufhin vermehrte Expression von mit der Resistenz-Entwicklung-korellierten Genen wie z.B. BCL-2 (welches darauf wieder in Punkt „1" angreifen kann).

5.4.2.3 *MMP-1*

Da invasive und multiresistente Karzinomzellen bekannterweise vermehrt MMP-1 produzieren (Yang et al., 2003; Minn et al., 2005), haben wir den Effekt von Artesunate (20 µM) auf die MMP-1-Expression von MDA-MB-231-Zellen untersucht. Mittels RT-PCR (mRNA), Westernblot (Proteine) und Zymographie (enzymatische Aktivität) konnten wir durchgehend, bis zu einer Behandlungsdauer von 6 h, eine Reduktion der jeweiligen Level der MMP-1-Produkte, im Vergleich zur unbehandelten Kontrolle nachweisen. Hingegen kam es im Zeitverlauf nach 7-24 h zu einer klaren Induktion, sowohl der MMP-1-mRNA, der MMP-1-Proteinlevel, als auch der enzymatischen Aktivität von MMP-1. Somit konnte auch hier ein klarer Hinweis auf entstehende Resistenz unter Artesunate gefunden werden (Bachmeier et al., 2011).

5.4.3 *Untersuchung der Artesunate-Resistenz in vivo*

Um den bereits *in vitro* beobachteten Effekt der erworbenen Resistenz der MDA-MB-231-Mammakarzinom-Zellen gegenüber Artesunate auch *in vivo* zu validieren, kam ein Mausmodell zum Einsatz.
24 weiblichen Nacktmäusen (Ncr:nu/nu) wurden jeweils 10^7 MDA-MB-231-Zellen in physiologischer Kochsalzlösung suspendiert, subkutan injiziert. Danach wurden die Mäuse in 4 Gruppen á 6 Tiere geteilt. Je nach Gruppe wurde entweder Artesunate (200 oder 400 mg/kg), Trägerlösung (10 % Tween 80 in physiologischer Kochsalzlösung) oder Doxorubicin (8 mg/kg) an fünf aufeinander folgenden Tagen intraperitoneal injiziert. Zweimal wöchentlich erfolgte die Erfassung der gebildeten Tumore und die Messung der Raumforderung mit einem Kaliper (Volumen-berechnung → $V = (\text{Länge} \times \text{Breite}^2)/2$). Mit Erreichen eines Tumordurchmessers von 5-6 mm begann die Behandlung. Hierbei zeigte sich unter Artesunate erst bei einer Dosis von 400 mg/kg eine marginale Inhibierung des Tumorwachstums (insbesondere bis zum fünften Tag nach Behandlungsbeginn), die Dosis 200 mg/kg erbrachte keinen nachweisbaren Effekt. In der Kontrollgruppe (Doxorubicin-Behandlung) ließ sich eine klare Inhibierung des Tumorwachstums nachweisen. Dies lässt erkennen, dass es im Gegensatz zur Kontrolle nach repetitiver Artesunate-Behandlung zu einer Resistenz gegenüber Artesunate kommt. Eine Verlangsamung des Tumorwachstums, wie unter Doxorubicin, ließ sich nicht beobachten.

Letztendlich konnten wir mit unseren Artesunate-Studien sowohl *in vitro* als auch i*n vivo* zeigen, dass es in der hochmalignen metastasenbildenden, *triple*-negativen Mammakarzinom-Zelllinie MDA-MB-231 nach Artesunate-Vorbehandlung zu einer erworbenen Resistenz kommt. Die beobachtete Resistenz gegen Apoptose wird vordergründig über eine anfängliche Induktion von NFκB und AP-1 ausgelöst. Im Zeitverlauf ist kein weiterer Effekt auf NFκB und AP-1 zu beobachten, die Zellen sind nun gegen Artesunate resistent. NFκB scheint hierbei hauptsächlich über die Proteine BAX und BCL-2 die Apoptose-Reduktion zu regulieren. Wir konnten hier einen deutlich reduzierten und damit hin zur Apoptose-Resistenz verschobenen BAX/BCL-2-Quotienten beobachten. Die eng mit Metastasierung und Medikamenten-Resistenzen verbundene MMP-1, welche von multiresistenten Zellen häufig über-exprimiert wird, konnte in MDA-MB-231-Zellen nach Artesunate-Vorbehandlung in deutlich erhöhter Expression nachgewiesen werden, womit der Status einer multiresistenten Zelllinie unterstrichen werden kann.

6 Ausblick

Naturstoffe wie das pflanzliche Polyphenol Curcumin spielen bereits seit Jahrhunderten eine große Rolle in der empirischen Therapie von Wunden, Schmerzzuständen, Entzündungsprozessen und vielem mehr. Eine ähnliche Rolle nehmen Derivate pflanzlicher Substanzen wie Artesunate ein.

Vielfach wurde gezeigt, dass nicht alle natürlichen Substanzen einen Benefit in der Behandlung von Tumoren versprechen. Während Curcumin beispielsweise in einigen, jedoch nicht in allen Tumorentitäten antineoplastisch wirksam ist (Bachmeier B. E. et al., 2007; Killian P. H. et al., 2012), zeigte sich unter Artesunate sogar die Entstehung einer Resistenz. Im Hinblick auf diese teils komplett unterschiedlichen Effekte, ist es von besonderer Bedeutung Biomarker zu identifizieren, mit deren Hilfe diagnostisch zwischen Wirksamkeit und Resistenz, bzw. zwischen *Respondern* und *Non-Respondern* unterschieden werden kann.

Die ubiquitär vorliegenden miRNAs sind als Biomarker gut geeignet, da die kleinen, gegen Umwelteinflüsse relativ robusten RNA-Sequenzen, nicht nur in soliden Geweben, sondern auch in leicht zugänglichem Patientenmaterial, wie Vollblut, Serum oder Plasma nachweisbar sind (Mitchell et al., 2008; Volinia et al., 2006; Schwarzenbach, 2011). Somit könnte die Wirksamkeit eines Chemopräventivums oder Tumortherapeutikums schnell und zuverlässig gemessen werden.

Neben der Funktion als Biomarker gewinnen miRNAs auch als eigentliches Therapeutikum bzw. Therapieziel an Bedeutung. Erste klinische Studien über miRNAs und deren spezifische Inhibitoren sind bereits angelaufen. Als exemplarisch für eine klinische Anwendung kann miR121 herausgegriffen werden, deren Inhibierung bei chronischen Hepatits-C-Infektionen vielversprechend scheint. Nach Studien an Primaten (Lanford et al., 2010) zeigte sich beim Menschen im Rahmen von Phase IIa-Studien, unter miR121-Repression eine starke Reduktion der Viruslast (Hydbring et al., 2013).

Die Ergebnisse der in dieser Arbeit beschriebenen Studien unterstreichen die Rolle pflanzlicher Substanzen in der modernen Tumortherapie. Sie verdeutlichen aber auch den dringenden Bedarf einer eingehenden Untersuchung des vielschichtigen Wirkmechanismus, sowie des Findens von Indikatoren zur Messung der Wirksamkeit. Nur so können erworbene Resistenzen, wie sie unter Artesunate beobachtet wurden, optimalerweise bereits im Vorfeld einer Behandlung detektiert und alternative Behandlungswege beschritten werden. Besonderes Augenmerk sollte hierbei auch auf die große Gruppe der miRNAs gelegt werden, deren Verantwortlichkeit für zahlreiche Funktionen von Naturstoffen wie Curcumin zu erwarten ist. Ähnlich wie bei Naturstoffen im Allgemeinen, die meist nur zu geringen Veränderungen jedoch an vielen unterschiedlichen Regulationspunktion führen, kommt es bereits bei einer geringen Expressionsänderung der miRNAs an multiplen Zielgenen zu gravierenden Auswirkungen. Somit steht die Vielfalt des Einflusses von miRNAs kongruent zur „*Target*-Multiplizität“ von Naturstoffen.

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miR181b is induced by the chemopreventive polyphenol curcumin and inhibits breast cancer metastasis via down-regulation of the inflammatory cytokines CXCL1 and -2

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ABSTRACT

Chronic inflammation is a major risk factor for the development and metastatic progression of cancer. We have previously reported that the chemopreventive polyphenol Curcumin inhibits the expression of the proinflammatory cytokines CXCL1 and -2 leading to diminished formation of breast and prostate cancer metastases. In the present study, we have analyzed the effects of Curcumin on miRNA expression and its correlation to the anti-tumorigenic properties of this natural occurring polyphenol.

Using microarray miRNA expression analyses, we show here that Curcumin modulates the expression of a series of miRNAs, including miR181b, in metastatic breast cancer cells. Interestingly, we found that miR181b down-modulates CXCL1 and -2 through a direct binding to their 3′-UTR. Overexpression or inhibition of miR181b in metastatic breast cancer cells has a significant impact on CXCL1 and -2 and is required for the effect of Curcumin on these two cytokines. miR181b also mediates the effects of Curcumin on inhibition of proliferation and invasion as well as induction of apoptosis. Importantly, overexpression of miR181b in metastatic breast cancer cells inhibits metastasis formation *in vivo* in immunodeficient mice. Finally, we demonstrated that Curcumin up-regulates miR181b and down-regulates CXCL1 and -2 in cells isolated from several primary human breast cancers.

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Taken together, these data show that Curcumin provides a simple bridge to bring metastamir modulation into the clinic, placing it in a primary and tertiary preventive, as well as a therapeutic, setting.

1. Introduction

Breast cancer is the most common cancer in women worldwide and the second leading cause of cancer-related mortality in women. It is estimated that about 20% of all patients with invasive breast cancer develop metastatic disease, which is essentially incurable (Alvarez). In these cases the aims of therapy include palliation of symptoms, delay of disease progression, and prolongation of overall survival time without negatively impacting quality of life (Irvin et al.). To develop successful anti-dissemination approaches, also in the context of cancer chemoprevention, we need to understand the molecular mechanisms of how tumors metastasize. Over the last decade several genes that regulate metastasis or act as molecular markers of metastatic disease have been discovered. These include metastasis genes (Nguyen et al., 2009) and metastasis repressor genes (Shoushtari et al., 2011) as well as multi-gene or multi-protein "signatures" often developed using microarray approaches (Pfeffer, 2013). In addition, a newly discovered class of small (19–25 nucleotides) non-coding RNAs, the microRNAs (miRs or miRNAs) have been linked to several human diseases, in particular cancer (Kong et al., 2012; Lujambio and Lowe, 2012). miRNAs post-transcriptionally regulate protein expression: each miRNA can control the expression of several proteins through binding to the mRNA 3′ UTR, thus resulting in mRNA degradation or inhibition of mRNA translation (Krol et al.). miRNAs involved in cancer can act as either tumor promoters ("oncomirs") or tumor suppressors (tumor suppressor miRs) (Kong et al., 2012; Lujambio and Lowe, 2012). Several miRNAs have been associated to metastasis ("metastamirs") with both prometastatic and antimetastatic effects (Hurst et al., 2009; White et al., 2011).

Translation of the information concerning metastasis associated genes and gene expression regulators into the clinic has been difficult (Kong et al., 2012; Shoushtari et al., 2011) since tumor cell dissemination and the formation of metastases begins long before the primary tumor is diagnosed and surgically removed. Studies on metastasis-associated genes have largely focused on the cancer cells themselves, rather than genes that modify the microenvironment. The regulation of the microenvironment and the key role this plays both in permitting tumor development (Bissell and Hines, 2011; de Visser et al., 2006; DeNardo et al., 2010), regulating progression (Albini et al., 2012; Bierie and Moses, 2006; Mantovani and Sica, 2010; Noonan et al., 2008), and even in preparing the "soil" for metastatic dissemination (Joyce and Pollard, 2009; Psaila and Lyden, 2009) is now coming to light.

One approach to reducing the morbidity and mortality of cancer is through prevention, in particular chemoprevention (Sporn). The concept of chemoprevention has been recently extended to the microenvironment (Albini and Sporn, 2007; Albini et al., 2012), potentially preventing tumor insurgence, progression and metastasis. The largest group of potential chemoprevention molecules are the phytochemicals and their derivatives (Noonan et al., 2011), which include molecules with known clinical chemoprevention potential such as aspirin and metformin (Albini et al., 2012). Major targets include inflammation, a key element in tumor insurgence (de Visser et al., 2006; DeNardo et al., 2010), progression (Mantovani and Sica, 2010) and metastasis (Joyce and Pollard, 2009; Psaila and Lyden, 2009), as well as angiogenesis (Albini and Sporn, 2007; Albini et al., 2012), a process driven by inflammation (Albini et al., 2005; Noonan et al., 2008).

One of the most promising phytochemicals is Curcumin, a compound with pleiotropic activities targeting both tumor cells and the tumor microenvironment (Gupta et al., 2010; Noonan et al., 2011; Reuter et al., 2011). We have recently demonstrated that Curcumin inhibits metastatic dissemination in breast and prostate cancer models (Bachmeier et al., 2007; Killian et al., 2012) and we have identified Curcumin-induced modulation of the proinflammatory cytokines CXCL1 and -2 as being critical to this anti-metastatic effect (Bachmeier et al., 2008). Curcumin interrupts the feed-back loop between NFκB, the chemokines CXCL1 and -2 and the pro-metastatic players whose transcription is normally induced by the two chemokines (Bachmeier et al., 2008; Killian et al., 2012). Curcumin also acts as a phytoestrogen in estrogen receptor α positive breast cancer cells (Bachmeier et al., 2010b). Recently, Curcumin has been found to modulate miRNA expression in retinoblastoma cells (Sreenivasan et al.) and other cancer cells leading to abrogation of tumor growth (Neelakandan et al.) yet nothing is known on the effect of Curcumin modulated miRNAs on the interaction of the tumor cell with the microenvironment and metastasis.

Using microarray miRNA expression analyses, we show here that Curcumin induces miR181b expression in breast cancer cells that down-regulates the expression of the pro-metastatic cytokines CXCL1 and -2 repressing the metastatic potential. Our data show that Curcumin provides a simple bridge to bring metastamir modulation into the clinic, placing it in a primary and tertiary preventive, as well as a therapeutic, setting.

2. Materials and methods

2.1. Cells and culture conditions

Human metastatic breast cancer cells MDA-MB-231 (ATCC) (Zhang et al., 1991) were selected because of their sustained CXCL1 and -2 expression that is not observed in other

Table 1 – Patient data.

Patient	Age	Grade	Estrogen receptor positive	Progesterone receptor positive	Erb-B2 status	p53 status	Ki67 status	TNM	Histotype
1	64	3	100%	98%	neg	3%	28%	T2 N0 (1s)	Infiltrating ductal carcinoma, poorly differentiated, angioinvasive
2	54	2	100%	2% (weak)	neg	neg	9%	T2 N3	Infiltrating lobular carcinoma, discreetly differentiated, no signs of angioinvasion or neuroinvasion.
3	73	2	100%	70%	neg	15%	15%	T2 N0 (SN)	Infiltrating lobular carcinoma, discreetly differentiated, no signs of angioinvasion or neuroinvasion.

commonly used breast cancer cell lines. The cells were grown as previously described (Killian et al., 2012).

2.2. Isolation of cells from primary breast cancers

Breast cancer tissue samples were collected following surgical resection of tumors larger than 2 cm in diameter from patients of the Ospedale di Circolo, Fondazione Macchi, Varese-Italy, following informed consent in an ethics committee approved protocol. Samples were stored in phosphate buffered saline (LONZA, Basel, Switzerland) with 1% Pen/Strep (Sigma–Aldrich, St. Louis, MO, USA) at 4 °C for less than 18 h prior to processing. The patient characteristics are given in Table 1. Samples were processed as previously described (Bruno et al.). Briefly red blood cell aggregates and excess of adipose tissue were removed, tissues were mechanically minced by scissors and enzymatically digested with a cocktail containing DNAse (100 μg/mL, Roche, Mannheim, Germany), Collagenase IV (1 mg/ml, Sigma–Aldrich) and hyaluronidase (200 U/mL, Sigma–Aldrich) in RPMI 1640 (LONZA) supplemented with Pen/Strep for 2 h at 37 °C. Tissue fragments were processed in a tissue disassociator (GentleMACS dissassociator, Miltenyi, Bologna, Italy) and the suspension was filtered twice through 50 μm pore size cell strainers (Becton Dickinson, BD – San Jose, California, USA). The total single cell suspension was washed twice by centrifugation in PBS at 500 × g for 5 min at room temperature to remove residual enzymes and debris. Cell viability was determined by trypan Blue (LONZA) staining and the vital primary breast cancer cells were seeded into six-well plates in RPMI 1640 supplemented with FBS (Euroclone, UK), 1% Pen/Strep and allowed to attach at 37 °C, 5% CO_2 for 24 h. Cells were treated with 25 μM Curcumin or carrier alone for 24 h. Supernatants were collected and immediately stored at −80 °C for CXCL1 analyses. Cells were lysed with Quiazol (Qiagen, Hilden, Germany) and stored at −80 °C for subsequent miRNA analyses. Each experiment was performed in triplicate.

2.3. Curcumin treatment of cells

Curcumin, 95% pure, was purchased from Fluka (Buchs, Switzerland) and dissolved in 0.5 M NaOH. For the use in cell culture a 2.5 mM solution in sterile PBS was prepared. Curcumin was applied at an end concentration of 25 μM for all assays. For controls the carrier (0.5 M NaOH) was diluted in PBS according to the Curcumin working solutions.

2.4. Total and low molecular weight (LMW) RNA isolation

Isolation of separate fractions of LMW RNA and total RNA (>200 nt) from MDA-MB-231 cells was performed using QIAzol and MiRNeasy kit (Qiagen, Hilden, Germany) following the manufacturers' protocol.

2.5. Microarray miRNA expression analyses

miRCURY LNA probes (miRBase 9.2.) were spotted onto Ultra GAPS II slides (Corning) by using the OmniGrid Accent spotter (GeneMachines) and crosslinked by UV. The probe sets included spike-in control probes, negative capture probes and the capture probes that hybridize to small nuclear RNAs. All hybridizations were made using a reference standard for the control channel prepared from an equimolar pool of short RNAs, extracted from large scale cultures of 20 different cell lines of ecotdermal, mesenchymal and endodermal origin. For labeling of the miRNAs, we used the miRCURY™ LNA microRNA Array Power labeling kit (Exiqon) according to the recommendations of the manufacturer. Hybridization at 56 °C for 16 h in the HS-400 Pro system (Tecan) was followed by washing and nitrogen drying according to the manufacturer's protocol (Exiqon). For image acquisition the Axon Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA) and dedicated software was used. Quality control was performed using Axon and R/BioConductor (Gentleman et al., 2004) software. Only spotted arrays with good quality were used for normalization and data analysis. Normalization was performed on data from individual arrays (intra-slide normalization) using an internal positive control, whereas normalization of different arrays (inter-slide normalization) was based on data from a set of arrays using the quantitative standard reported. All analyses were performed in quadruplicate.

2.6. In silico target gene identification – three public miRNA databases were used to identify putative target genes

TargetScanHuman (http://www.targetscan.org/) Release 6.2
Microrna.org (http://www.microrna.org/microrna/home.do) Release August 2010
MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets(v5/) Release Version 5

2.7. Real-time RT-PCR

mRNA or miRNA was reverse transcribed according to standard protocols using oligo-dT primers and the first strand synthesis kit (GE Healthcare) or the miScript Reverse Transcription Kit together with the miScript Primer Assay (both Qiagen, Hilden, Gemany) respectively. Expression analysis of mRNA was performed using the Light Cycler technology according to standard protocols. Human RPII and HPRT were used as endogenous controls. All primers were designed using primer3 software (Rozen and Skaletsky, 2000) (see primer list, Supplementary Table 1). Expression of mature miRNAs was determined using miRNA specific quantitative real-time PCR using a specific miR181b primer and RNUB6_2 for normalization (MiScript Reverse Transcription Kit and Primer Assay, Qiagen, Hilden, Germany) by Light Cycler technology according to the recommendations of the manufacturer.

2.8. Luciferase assay

The human CXCL1 and CXCL2 3′UTRs (607 bp and 654 bp from the STOP codon respectively) were both PCR amplified from human genomic DNA by using the primers "A" and "B" or primers "E" and "F" respectively. These two primer pairs contain each *KpnI* and *NheI* recognition sites at the 5′ end. The DNA fragments corresponding to the CXCL1 or the CXCL2 3′UTRs were cloned downstream of the luciferase gene in the pGL3-cont PLK + vector (Fontana et al., 2007). From these two wildtype constructs, the mutant derivatives were generated by inverse PCR with primers "C" and "D" or "G" and "H", respectively. HeLa cells were cotransfected with 0.4 g of wildtype or mutant constructs and 50 ng of *Renilla* luciferase vector (pRL-TK Promega), together with 160 nM 2′-O-Methyl oligonucleotides (anti-181 and anti-control; Dharmacon, Lafayette, CO), with Lipofectamine 2000 (Invitrogen). Cells were harvested and assayed with Dual Luciferase Assay (Promega) 48 or 72 h after transfection by using Microlite TLX1 (Dynatech Laboratoires, Chantilly, CA). Three independent experiments were done in triplicate. All sequences all listed in Supplementary Table 1.

2.9. Oligonucleotides and transfection experiments

MDA-MB-231 cells were transfected with mature miR181b (miRIDIAN microRNA mimics), a specific miR181b inhibitor (miRIDIAN microRNA hairpin inhibitor) or the respective scrambled negative controls (miRIDIAN microRNA mimic negative control and hairpin inhibitor negative control) obtained from Thermo Scientific (Dharmacon, Lafayette, USA). In silencing experiments 30 nM or 50 nM specific small interfering oligonucleotids against CXCL1 and CXCL2 were used (Silencer Pre-designed siRNA, Ambion, USA).

2.10. Generation of a stable miR181b overexpressing MDA-MB-231 cell clone

Double stranded DNA fragments were obtained by annealing the oligonucleotides miR181b-s and miR181b-as (Supplementary Table 1, synthesized by TibMolBiol, Berlin, Germany). This 71 base pair DNA fragment was cloned by restriction site ligation into the NheI and KpnI sites of the pcDNA6.2-GW/EmGFP^{mod}-miR vector (Fontana et al., 2008) resulting in a miR181b overexpression vector (MDA-MB-231$^{miR181b+}$). 25.000/well MDA-MB-231 cells were seeded into a 6-well plate and after 24 h transfected with 6 μg MDA-MB-231$^{miR181b+}$ or the corresponding empty vector (MDA-MB-231^{MOCK}) vector DNA respectively by lipofection (Lipofectamine 2000, Invitrogen). Stable clones were selected with 1 mg/ml blasticidin S (Invitrogen). After 6 week of selection with 8 μg/ml blasticidin cells with highest fluorescence were further selected by FACS. Stable clones were maintained in growth medium containing 6 μg/ml blasticidin.

2.11. Preparation of conditioned media

Cell culture supernatants were collected and centrifuged 15 min at 4000 × g.

2.12. Determination of protein concentration

Protein concentrations were determined by the BCA protein assay (Pierce, Oud-Bejierland, Netherlands) with bovine serum albumin as standard.

2.13. Western Blots

Blots were performed as previously described by us in detail (Bachmeier et al., 2008). Conditioned media were analyzed using antibodies against CXCL1, CXCL2 (both Dianova, Germany), MMP-1 and MMP-3 (both Sigma, Germany). In order to verify that equal amounts of total protein of conditioned media (β-actin staining not possible) were loaded to each lane of the gels, we visualized the protein bands blotted onto the nitrocellulose membranes (after the gel run) by Ponceau staining (see Figures 1B, 2B, and 3B, lanes indicated as "loading control"). Semiquantitative evaluation of the bands was performed by densitometric analysis with the ImageJ software provided by the NIH (http://rsb.info.nih.gov/ij/).

2.14. CXCL1 ELISA

CXCL1 was quantified in conditioned media of Curcumin treated and non-treated cells isolated from primary breast cancers using the Quantikine Human CXCL1/GROα Immunoassay (R&D Systems, Minneapolis, USA) according to the recommendations of the manufacturer.

2.15. Apoptosis assay

Apoptotic cell death was determined by an enzyme-linked immunoassay (Cell Death Detection ELISA ^{PLUS}, Roche) to detect fragmented DNA and histones. MDA-MB-231 cells were seeded on 24-well plates 25 h after transfection with the miR181b mimic or the corresponding control oligos respectively in triplicates. After another 24 h cells were washed with PBS lysed and processed following the instructions of the manufacturer.

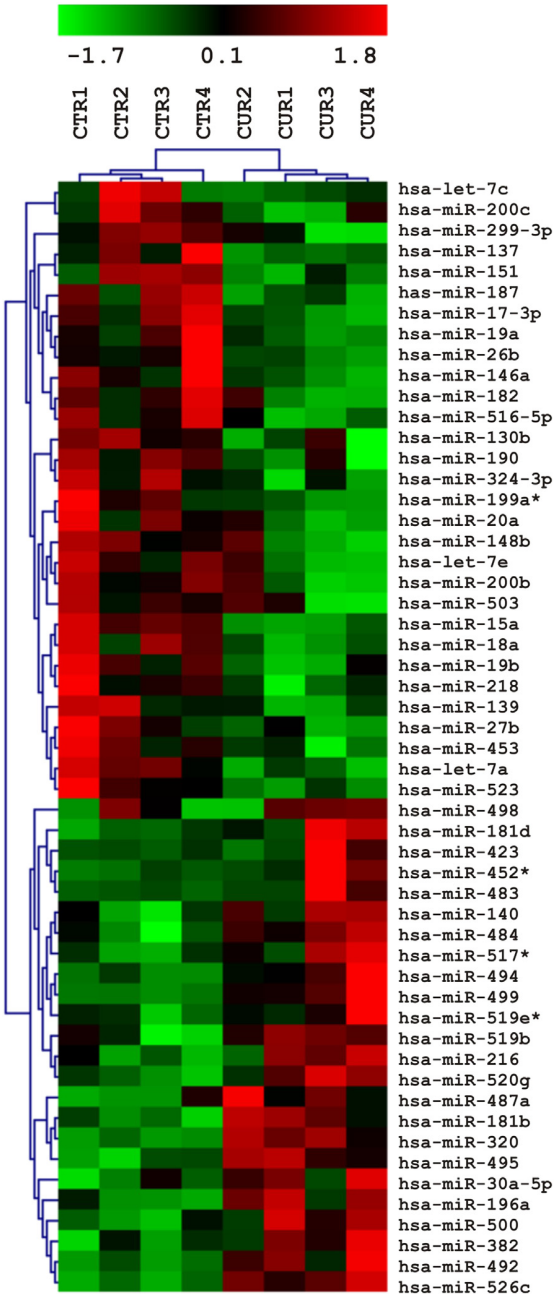

Figure 1 – *Curcumin modulates miRNA expression in human breast cancer cells*. Microarray miRNA expression analysis. Four samples each of Curcumin treated (6 h) MDA-MB-231 cells and carrier-treated controls were analyzed by hierarchical clustering applying euclidean distance and average linkage. Expression levels are indicated by a color code, green = expression below, red = expression above mean levels of expression, black = mean level.

2.16. *Proliferation assay*

We used the BrdU Cell Proliferation ELISA (Roche, Germany) according to the recommendations of the manufacturer. Briefly, two thousand cells were seeded in a 96-well plate and left overnight to attach. After several time periods, according to the experiment (e.g. 24 h, 48 h), BrdU was added and incubated for 2 h. Afterwards cells were fixed, stained and colorimetric analysis was performed with an ELISA plate reader (MPP 3408T, Mikrotek Laborsysteme GmbH, Germany).

2.17. *Invasion assay*

A fluorescent quantitative cell invasion assay in a 96-well format (QCM™96-Well Cell Invasion Assay, Chemicon, USA) was used to examine the ability of MDA-MB-231^{mock} and MDA-MB-231^{miR1871b} cells to penetrate the extracellular matrix (ECM). The assay was performed according to the recommendations of the manufacturer. Briefly, cells in a density of 1×10^6/ml were re-suspended in serum-free medium and added to the upper chambers while the lower chambers were filled with human serum (PAA, Germany) that served as a chemo-attractant. Cells were then incubated for 6 h at 37 °C. After removal of cells on the upper surface of the membrane, cells on the lower surface of the membrane were lysed and stained with CyQuant DR Dye and fluorescence was read in a plate reader (safire^2, Tecan, Switzerland). Data was expressed as the percentage of invasive cells as compared with the control. All experiments were performed in triplicates and results were expressed as mean ± SEM of three independent experiments.

2.18. *Hematogenous metastases in immunodeficient mice*

Animal studies and research protocols were reviewed and approved by the institutional ethics committee of the IRCCS San Martino-IST and were conducted in accordance with the national current regulations and guidelines for the care and use of laboratory animals (D.L. 27/01/1992, n. 116). Five-week-old CD-1 Foxn1nu female mice (Charles River Laboratories, Calco, Como, Italy) were maintained under specific pathogen-free conditions and given sterile food and water ad libitum. Mice were anaesthetized and tumor cells inoculated intracardially. Health and survival rate was observed until their euthanasia. MDA-MB-231$^{miR181b+}$ and MDA-MB-231^{MOCK} were collected by trypsinization, washed and resuspended in PBS. 5×10^5 MDA-MB-231$^{miR181b+}$ or MDA-MB-231^{MOCK} cells were injected into the left cardiac ventricle of 15 mice. All mice in both groups were fed with standard diet (Mucedola, Italy). On day 35, mice were humanely sacrificed, and necropsied and analyzed for the formation of metastases as previously described by us in detail (Bachmeier et al., 2007; Killian et al., 2012).

2.19. *Data analysis*

Statistical significance was assessed by comparing mean (±SD) values, which were normalized to the control group with Student's *t*-test for independent groups. One-way ANOVA was used to test for statistical significance ($p < 0.05$), and when significance was determined, Bonferroni's Multiple Comparison Test was performed *post hoc*, as indicated in the figure legends. Statistical analysis was performed using the Prism software (GraphPad, San Diego, CA, USA).

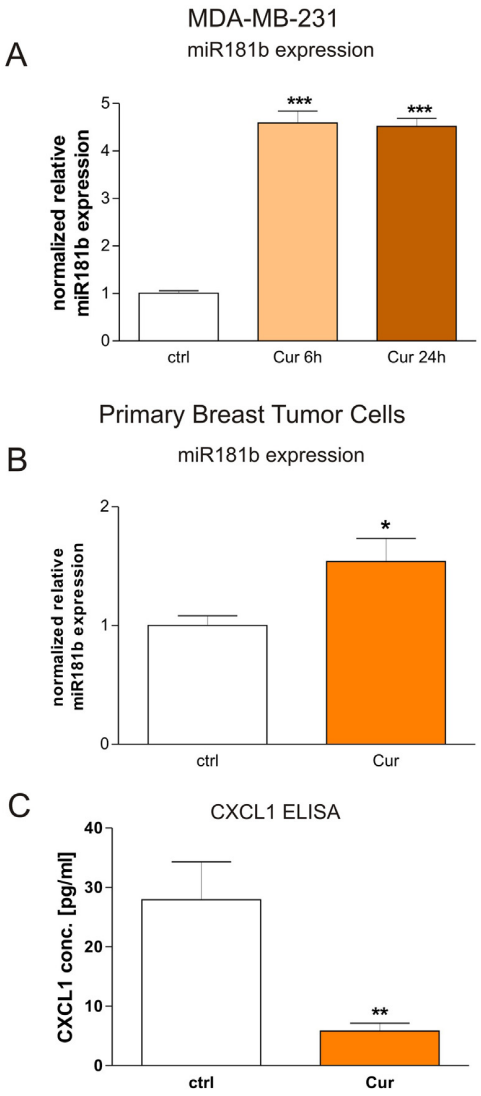

Figure 2 – *Curcumin modulates miR181b expression in human breast cancer cells*. A: Quantitative RT-PCR reveals that human metastatic breast cancer cells (MDA-MB-231) treated with Curcumin for 6 h ("Cur") express four fold higher amounts of miRNA181b in respect to carrier-treated cells ("ctrl"). ***$P < 0.001$, student's *t*-test. B: miR181b expression was analyzed in breast cancer cells from human primary tumor samples, cultured *in vitro* and treated for 24 h with Curcumin with respect to carrier-treated control cells from the same origin. For each case, we observed up-regulation of miR181b after Curcumin treatment (data not shown) and by pooling the results from all patient data we obtained a mean miR181b up-regulation rate of 50%, which was statistically significant (*$p < 0.05$; student's *t*-test). Mean + SD from 3 different patients are shown. C: CXCL1 protein expression secreted from breast cancer cells isolated from primary human tumors that were treated with Curcumin *in vitro* was analyzed by ELISA. CXCL1 concentrations were statistically significantly down-regulated about 3.5-fold in Curcumin treated tumor cells ($p < 0.01$; student's *t*-test). Mean + SD from 3 different patients are shown.**

2.20. *Analysis of genes affecting BC progression*

Association of the expression of miR181b regulated genes with disease free survival (DFS) of basal-like human breast cancers was analyzed using the Breast Mark collection of breast cancer datasets and analysis tools (Madden et al.). The Hazard ratio was generated using Cox regression and a log rank test was used to assign significance.

3. Results

3.1. *Curcumin modulates miRNA expression in human breast cancer cells*

We performed Microarray analysis to evaluate if treatment of MDA-MB-231 human breast cancer cells with Curcumin leads to a modulation of miRNA expression. Of 351 miRNAs detected on the microarray, we found 58 miRNAs to be statistically significantly (*$P < 0.05$) differentially expressed in Curcumin treated cells as compared with controls (Figure 1). Among these 58 miRNAs, we selected by bioinformatics analysis those miRNAs with at least a 2.5-fold increase or decrease in response to Curcumin treatment and found 10 miRNAs up-regulated and 3 miRNAs down-regulated (Supplementary Table 2).

By using different bioinformatic programs (see Material and Methods) we searched for putative target genes of the selected miRNAs. Interestingly, we found CXCL1 and CXCL2 as putative target genes of miR181b. We previously demonstrated that treatment of MDA-MB-231 cells with Curcumin leads to a down-modulation of CXCL1 and CXCL2, which in turn mediates the anti-metastatic effect of Curcumin (Bachmeier et al., 2008). Therefore, we hypothesized that Curcumin may regulate CXCL1 and CXCL2 expression through miR181b.

Subsequently we validated the microarray data by measuring levels of miR181b by qRT-PCR. Consistently, Curcumin treatment (6 h) causes a four-fold up-modulation of miR181b in MDA-MB-231 cells (Figure 2A) corresponding very well with the microarray data.

We searched in depth for all possible targets of miR181 (see Supplementary Table 3 pages 1–3). In brief, since miR181a, b, c and d are identical in their seed regions, they all have the same target genes. We also performed a thorough data bank search to obtain a complete picture of all putative miRNAs binding to the 3′-UTR of CXCL1 and -2 (see Supplementary Table 3 pages 4–6). Interpretation of our *in silico* data showed that the pro-inflammatory cytokines CXCL1 and -2 are highly probable targets of miR181b and miR181d (see Supplementary Table 3, page 6).

In order to verify that Curcumin is able to regulate miR181b in primary human breast cancers, we isolated breast cancer cells from 3 human primary tumor samples, all of which were ER+ (Table 1). The tumor cells were treated for 24 h *in vitro* with Curcumin or carrier as controls, then analyzed for miR181b expression (Figure 2B). In each case, we observed up-regulation of miR181b after Curcumin treatment that indicated a statistically significant miR181b up-regulation of 50%

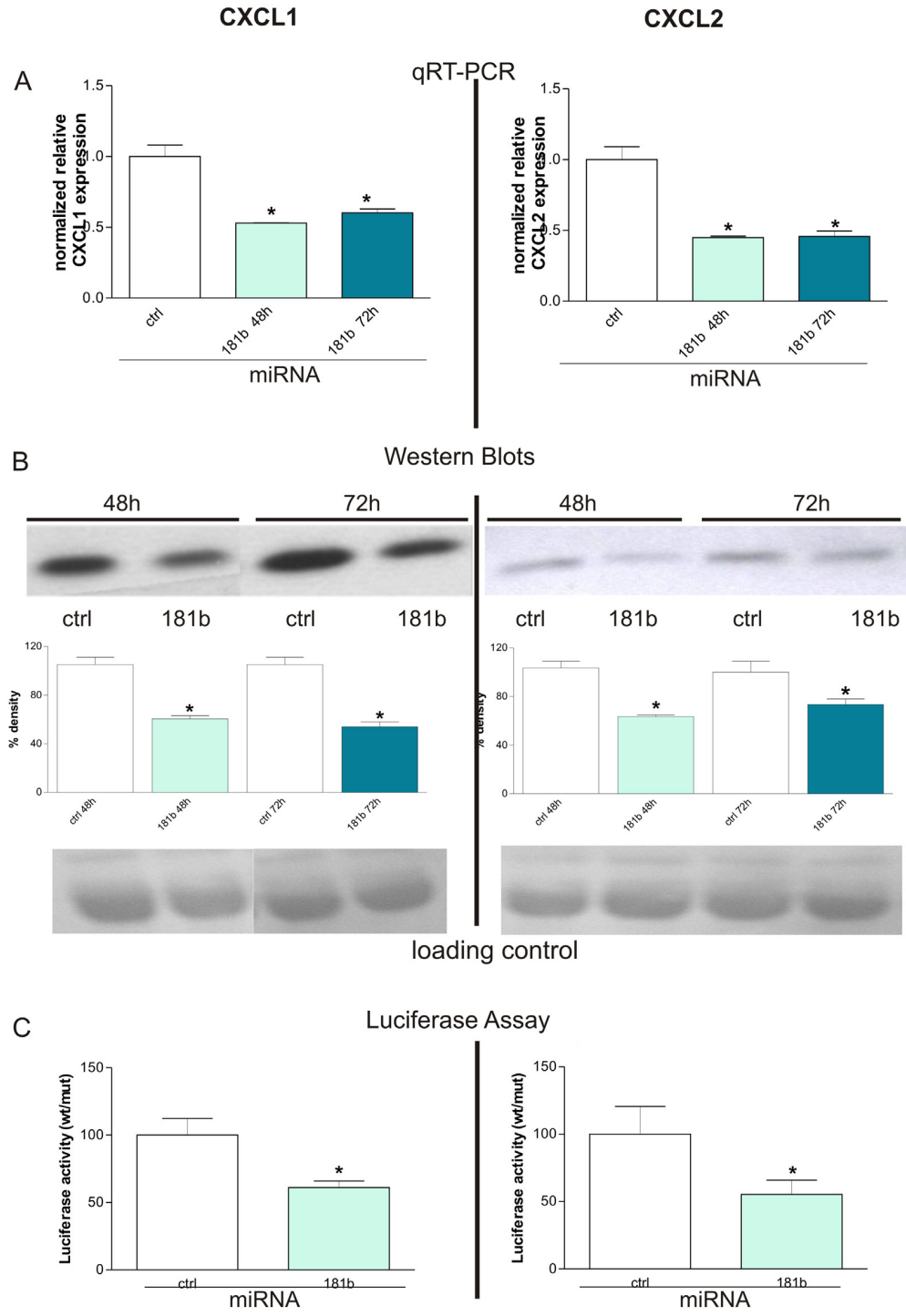

Figure 3 – *miR181b regulates CXCL1 expression through a direct binding to its 3′ UTR*. A: Transient overexpression of miR181b in MDA-MB-231 breast cancer cells leads to a ~50% down-regulation of CXCL1 (left side) and CXCL2 (right side) transcripts as evidenced by qRT-PCR (*$P < 0.05$, student's *t*-test). Mean + SD from 3 independent experiments normalized to the respective controls are shown. B: On the corresponding protein level the same effect could be seen as indicated by Western Blot (upper panel) analysis 48 h and 72 h after transient introduction of miR181b into the tumor cells. Densitometric analysis of the bands (middle panel) reveals that CXCL1 (left side) and CXCL2 (right side) protein syntheses were statistically significantly (*$P < 0.05$, student's *t*-test) impaired by about 50% 48 h after transfection. Ponceau staining was used as loading control to evaluate that equal amounts of proteins were applied (lower panel). Mean + SD from 3 independent experiments are shown. C: Luciferase assays demonstrated that regulation of CXCL1 and -2 occurs through direct binding of miR181b to their corresponding 3′UTRs. Co-transfection of pGL3-cont-CXCL1 UTR-wt or pGL3-cont-CXCL2 UTR-wt together with miR-181b, but not with a scrambled miR181b oligonucleotide, caused a decrease of the luciferase activity in He–La cells. Conversely, miR181b oligonucleotide did not inhibit luciferase activity of pGL3-cont-CXCL1 UTR-mut or pGL3-cont-CXCL2 UTR-mut. Mean + SD from 3 independent experiments are shown.

(Figure 2B). This suggests that Curcumin regulation of miR181b is present in both ER- (the MDA-MB-231 model) and in ER+ (primary samples) breast cancers.

3.2. *The metastasis-related cytokine CXCL1 is down-regulated by curcumin in primary breast cancer cells*

We have previously published that curcumin regulates CXCL1 expression in breast cancer cell lines (Bachmeier et al., 2008), we therefore examined whether Curcumin was able to mediate CXCL1 protein down-regulation in primary human tumor *in vitro*. ELISA results demonstrated that CXCL1 concentrations were statistically significantly down-regulated about 3.5-fold in Curcumin treated primary tumor cells with respect to controls (Figure 2C). These data obtained on the same samples show a potential relation between Curcumin effects on miR181b and CXCL1 modulation.

3.3. *miR181b regulates CXCL1 and -2 expression through a direct binding to its 3′ UTR*

We then hypothesized that CXCL1 and CXCL2 expression is regulated by miR181b. In order to test this hypothesis, we transiently transfected MDA-MB-231 cells with miR181b or a control miRNA. Transfection of MDA-MB-231 cells with miR181b oligos led to a down-modulation of CXCL1 and -2 in breast cancer cells similar to that obtained by treatment of the cells with Curcumin, as published by us previously (Bachmeier et al., 2008). This effect is not observed in cells transfected with unspecific miRNAs. Furthermore qRT-PCR and Western blot analysis showed very consistently that over-expression of miR181b significantly down-modulated expression of CXCL1 and CXCL2 about 50% at 48 and 72 h post-transfection on the transcript (Figure 3A) as well as at the protein (Figure 3B) level.

To demonstrate that this regulation occurs through a direct binding of miR181b to the CXCL1 and CXCL2 3′ UTR, we cloned a portion of the CXCL1 or CXCL2 3′ UTR containing the miR181b putative binding sites into the pGL3-Control vector downstream the luciferase gene, generating the pGL3-cont-CXCL1 UTR-wt and pGL3-cont-CXCL2 UTR-wt constructs. As a control, we cloned a region of CXCL1 or CXCL2 3′ UTR containing a mutated miR181b recognition site (pGL3-cont-CXCL1 UTR-mut and pGL3-cont-CXCL2 UTR-mut). Co-transfection of pGL3-cont-CXCL1 UTR-wt or pGL3-cont-CXCL2 UTR-wt together with miR181b, but not with a scrambled miR181b oligonucleotide, caused a decrease of the luciferase activity in He–La cells (Figure 3C). Conversely, miR181b oligonucleotide did not inhibit luciferase activity of pGL3-cont-CXCL1 UTR-mut or pGL3-cont-CXCL2 UTR-mut, demonstrating that mutation of the miR181b binding site in the CXCL1 or CXCL2 3′ UTR abolished the ability of miR181b to regulate their expression (Figure 3C).

3.4. *miR181b mediates Curcumin-related down-modulation of CXCL1 and CXCL2*

In order to investigate the role of miR181b in the regulation of CXCL1 and CXCL2 expression by Curcumin, MDA-MB-231 cells were treated with Curcumin in the presence or absence of a miR181b inhibitor (miRIDIAN microRNA hairpin inhibitor, Dharmacon, Lafayette, USA (see also material and methods). Curcumin down-modulates CXCL1 (Figure 4 left panel) and CXCL2 (Figure 4 right panel) at both transcript (Figure 4A) and protein (Figure 4B) levels. However, in the presence of the miR181b inhibitor this down-modulation was abolished. Transfection of MDA-MB-231 cells with a hairpin inhibitor directed against mir181b induces CXCL1 (Figure 4 left panel) and CXCL2 (Figure 4 right panel) expression when compared to cells transfected with an unspecific scrambled hairpin inhibitor control. These results clearly indicate that Curcumin inhibits CXCL1 and CXCL2 through the induction of miR181b. Interestingly, in the presence of the miR181b inhibitor, Curcumin-mediated down-regulation of CXCL1 was completely restored (left panel) thus indicating that Curcumin regulates CXCL1 exclusively through miR181b. Conversely, CXCL2 expression was only partially restored in cells treated with Curcumin plus the miR181b inhibitor (right panel), suggesting that additional regulation mechanisms may exist. The data obtained from analysis on protein level (Western Blots) and transcript level (qRT-PCR) are in strong agreement, supporting the hypothesis that miR181b mediates the down-modulation of CXCL1 and -2 by Curcumin.

3.5. *Curcumin inhibits proliferation via miR181b*

We wished to know whether miR181b triggers the anti-proliferative effect of Curcumin (Bachmeier et al., 2010a), and therefore modulated miR181b expression using small hairpin inhibitors directed against miR181b along with the corresponding controls in the presence of Curcumin, in double modulation experiments. Curcumin inhibited MDA-MB-231 cell proliferation (Figure 5A), however, Curcumin treatment together with the specific miR181b hairpin-inhibitor abolished the anti-proliferative effect, returning cell proliferation to control-levels. The effect of miR181b expression on proliferation was highly statistically significant. Thus inhibition of miR181b abrogates the ability of Curcumin to inhibit proliferation in MDA-MB-231 cells, strongly indicating that miR181b is responsible for Curcumin's anti-proliferative effects.

3.6. *miR181b induces apoptosis and necrosis in breast cancer cells*

Since Curcumin induces tumor cell apoptosis (Bachmeier et al., 2010a), a function critical for suppression of tumor formation and metastasis, we examined if miR181b is likewise able to induce apoptosis. Functional apoptosis/necrosis assays revealed that miR181b over-expression led to enhanced apoptosis (Figure 5B) as well as necrosis (Figure 4C) in a highly statistically significant manner. Over-expression of miR181b for 24 h in MDA-MB-231 cells doubled the apoptosis rate as compared to wildtype MDA-MB-231 cells or to MDA-MB-231 cells transfected with an appropriate control oligo (Figure 5B). Likewise, necrosis rate was increased approximately 5 fold in miR181b over-expressing MDA-MB-231 cells as compared to wildtype cells and over 2.5 fold as compared to MDA-MB-231 cells expressing an appropriate control oligo (Figure 5C).

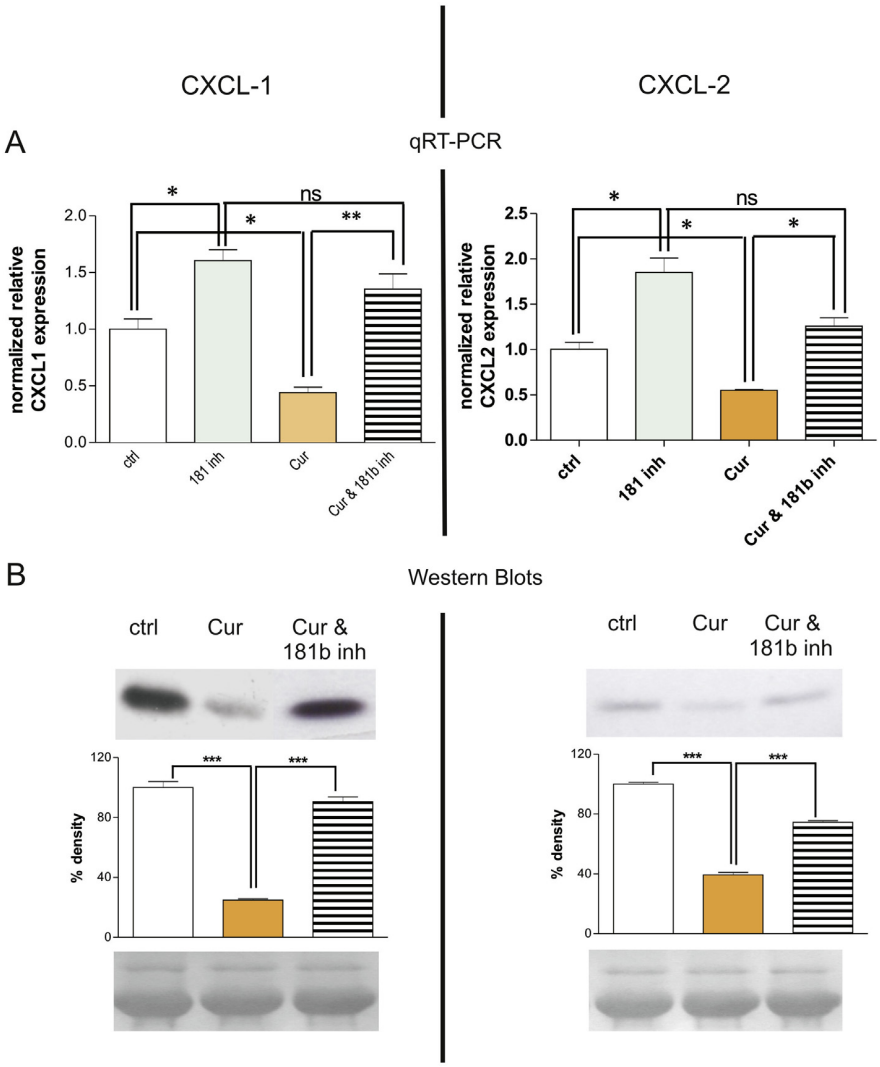

Figure 4 – *miR181b mediates Curcumin-related down-modulation of CXCL1 and CXCL2*. A: qRT-PCR results reveal that treatment of MDA-MB-231 cells with Curcumin for 24 h leads to inhibition of CXCL1 expression (left side, "Cur") that can be reverted to original expression levels ("ctrl") by concomitant application of a miR181b hairpin inhibitor ("Cur &181b inh"). Conversely, CXCL2 expression (right side) was only partially restored in cells treated with Curcumin plus the miR181b hairpin inhibitor, indicating that additional regulation mechanisms may exist. Inhibition of mir181b by specific small hairpin inhibitors leads to induction of CXCL1 and -2 expression compared to control cells transfected with scrambled unspecific hairpin inhibitors (*$P < 0.05$; **$P < 0.001$; ANOVA with Bonferroni's post-test). Mean + SD from 3 independent experiments are shown. "ns": not significant. B: The corresponding analysis on protein level (Western Blots, upper panel) confirm the data obtained from qRT-PCR, strengthening the evidence that miR181b mediates down-modulation of CXCL1 (left side) and CXCL2 (right side) by Curcumin. Densitometric analysis of the bands (middle panel) reveals that differences in expression levels of CXCL1 and -2 after Curcumin treatment were statistically significant (*$P < 0.001$; ANOVA with Bonferroni's post-test). Loading controls (bottom panel) indicate that equal amounts of total protein were subjected to each lane of the gel. Mean + SD from 3 independent experiments are shown.**

Biostatistic analysis of apoptosis related miR181 target genes revealed that BCL2 was among the putative candidates (see Supplementary Table 3, page 4). Using qRT-PCR, we found a statistically significant down-regulation of the apoptosis related factors BCL2 and survivin/BIRC5 in MDA-MB-231 over-expressing miR181b, 72 h after transfection with a double stranded miR181b oligo as compared to the appropriate controls (Figure 5D).

3.7. *miR181b inhibits expression of MMPs in metastatic breast cancer cells*

We evaluated the correlation between miR181b and the expression of extracellular matrix degrading proteases (MMPs – matrix metalloproteinases), as expression of MMPs is a prerequisite for tumor growth, invasion and metastasis. In an initial step we found that MMPs are present among the *in silico*

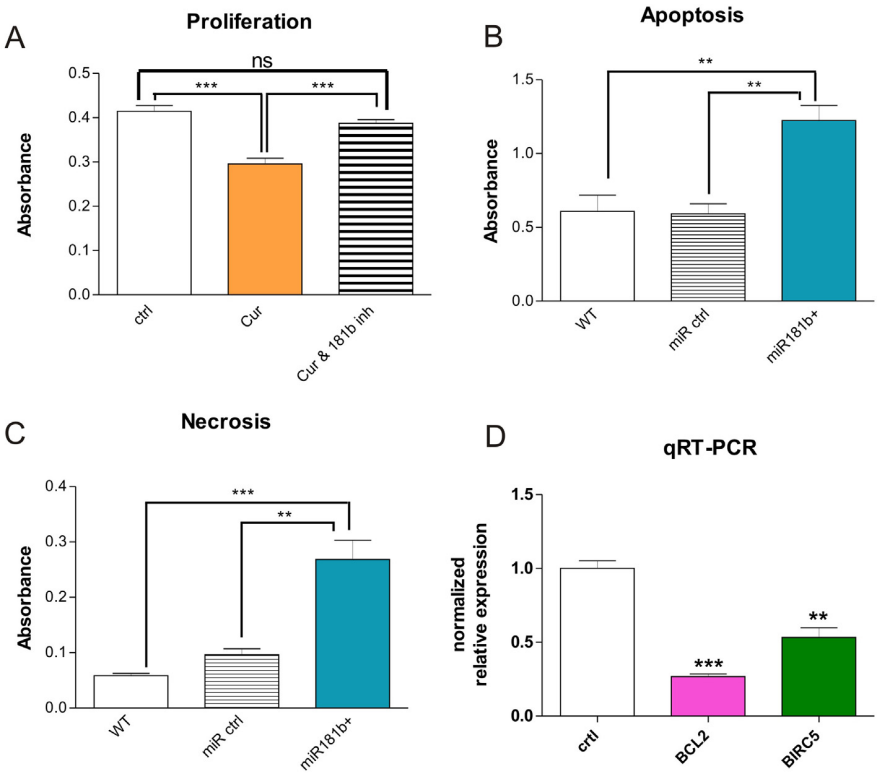

Figure 5 – *Involvement of miR181b in tumor cell proliferation, apoptosis and invasion*. A: Curcumin inhibits proliferation via miR181b. Treatment of MDA-MB-231 breast cancer cells for 24 h with Curcumin ("Cur") inhibits cell proliferation. However, treatment with Curcumin together with a specific miR181b hairpin-inhibitor ("Cur & 181b inh") abolishes the anti-proliferative effect of Curcumin, returning cell proliferation to control-levels. The effect of miR181b expression on proliferation was statistically highly significant as evidenced by student's *t*-test (*$P < 0.001$). "ns": not significant. B, C: miR181b overexpression induces apoptosis and necrosis in breast cancer cells. Functional apoptosis/necrosis assays revealed that miR181b over-expression led to enhanced apoptosis (left panel) as well as necrosis (middle panel). 24 h after transfection with miR181b oligos apoptosis rate in MDA-MB-231 cells was doubled as compared to wildtype MDA-MB-231 cells or to MDA-MB-231 cells transfected with an appropriate control oligo (left panel). Likewise, necrosis rate was increased approximately 5 fold in miR181b over-expressing MDA-MB-231 cells as compared to wildtype cells and over 2.5 fold as compared to MDA-MB-231 cells expressing an appropriate control oligo (**$P < 0.01$; ***$P < 0.001$; ANOVA with Bonferroni's post test). Mean + SD from 3 independent experiments are shown. D: Using quantitative RT-PCR, expression of the apoptosis related factors BCL2 and survivin/BIRC5 in MDA-MB-231 over-expressing miR181b showed a statistically significant down-regulation achieved 72 h after transfection with a double stranded miR181b oligo as compared to the appropriate controls (right panel) (**$P < 0.01$ and ***$P < 0.001$; student's *t*-test). Mean + SD (SEM?) from 3 independent experiments are shown.**

targets of miR181 (Supplementary Table 3, page 5). Furthermore, our qRT-PCR data revealed that MMP-1 and -3 transcripts were down-regulated by 93% and 75% respectively, in MDA-MB-231 cells stably transfected with a miR181b over-expression vector when compared to cells stably transfected with the appropriate control vector (Figure 6A). Western blots (Figure 6B) of conditioned media from MDA-MB-231 cells stably over-expressing miR181b have a lower MMP-1 and -3 protein expression than appropriate control cells. Quantification of the bands by densitometry indicated that the differences in expression levels were approximately 15% and 34% for MMP-1 and MMP-3 (respectively) and were statistically significant.

3.8. *miR181b impairs the invasive capacity of breast cancer cells*

Given the significant effect on MMP expression, we investigated the influence of miR181b expression on invasive capacity using the chemoinvasion assay (Albini et al.). As the invasion chambers are covered with a reconstituted basement membrane (Matrigel) active invasion as result of proteolytic digestion of the matrix is required for the tumor cells to pass the barrier. Invasion of MDA-MB-231 breast cancer cells stably over-expressing miR181b was significantly reduced by about 50% after an incubation period of only 6 h (Figure 6C). This establishes a functional consequence of decreased MMP production.

3.9. *Breast cancer cells over-expressing miR181b have a lower capacity to metastasize* in vivo

We previously reported that Curcumin prevents the formation of hematogenous breast cancer metastases in immunodeficient mice in a highly significant manner (Bachmeier et al., 2007). We therefore examined whether miR181b is responsible for the diminished formation of lung metastases by creating a

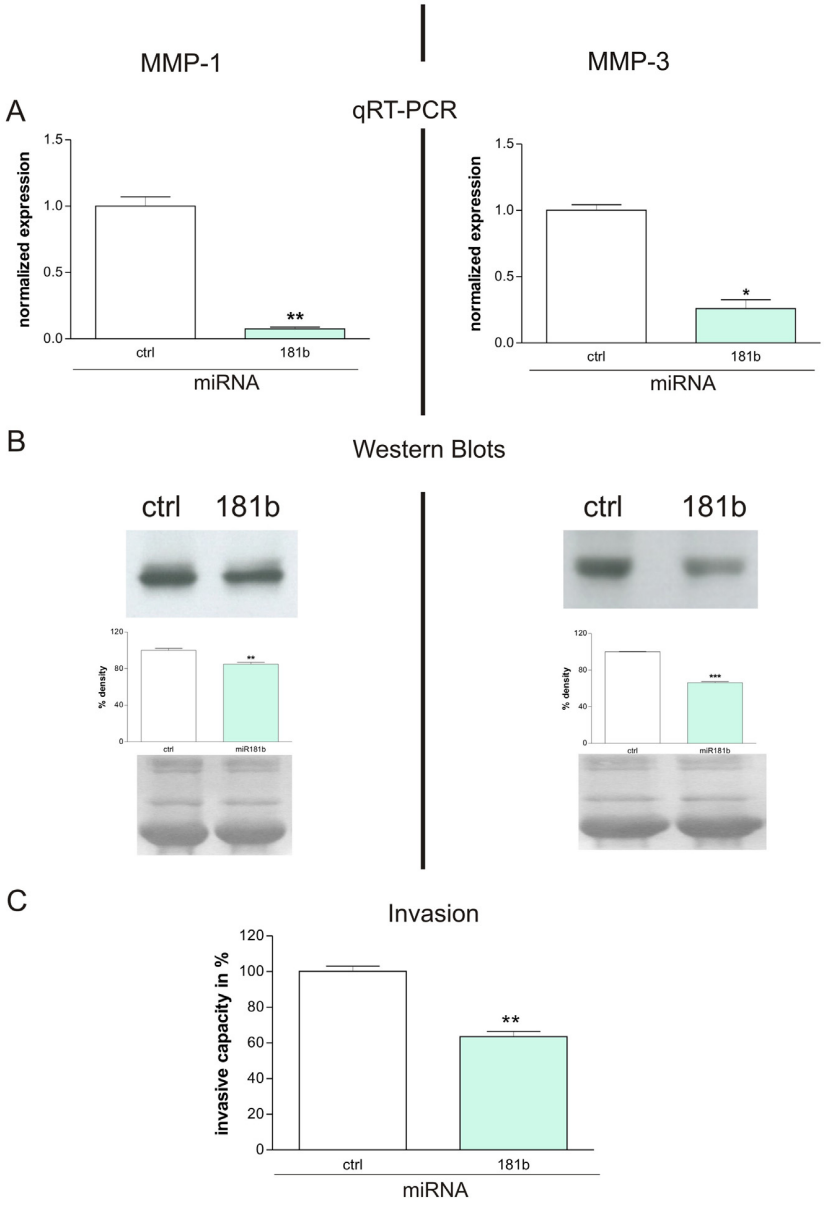

Figure 6 – *miR181b impairs the expression of MMP-1 and MMP-3*. A: qRT-PCR shows that MMP-1 (left side) and MMP-3 (right side) transcripts were downregulated 93% ($P = 0.0059$; student's *t*-test) and 75% (*$P = 0.0109$; student's *t*-test) respectively in MDA-MB-231 cells stably transfected with a miR181b overexpression vector (lanes indicated with 181b) when compared to cells stably transfected with an appropriate control vector (lanes indicated with ctrl). Mean + SD from 3 independent experiments are shown. B: Western blots (upper panel) of conditioned media from MDA-MB-231 cells stably transfected with a miR181b overexpression vector (lanes indicated with 181b), reveal a downregulation of MMP-1 and -3 protein when compared to cells stably transfected with an appropriate control vector (lanes indicated with ctrl). This effect was quantified by subsequent densitometry (middle panel) showing that the differences in expression levels were about 15% (**$P = 0.0068$; student's *t*-test) for MMP-1 and about 34% (***$P < 0.0001$; student's *t*-test) for MMP-3. Equal amounts of total protein were loaded to each lane of the gels and to verify this, we visualized the protein bands blotted onto the nitrocellulose membranes (after the gel run) by Ponceau staining (lower panel). Mean + SD from 3 independent experiments are shown. C: miR181b over-expression impairs the invasive capacity of breast cancer cells. Invasion of MDA-MB-231 breast cancer cells stably over-expressing miR181b through a reconstituted basement membrane (Matrigel) was significantly reduced (**$P < 0.01$; student's *t*-test) by about 50% after an incubation period of 6 h. Mean + SD from 3 independent experiments are shown.**

stably miR181b over-expressing cell clone from MDA-MB-231 cells (MDA-MB-231^{miR1871b}) along with the corresponding MOCK control cell clone (MDA-MB-231^{mock}) (see material and methods). As an *in vivo* model we used mice injected with either the miR181b over-expressing or the MOCK cell clones and divided them into two study groups accordingly. At the end of the experimental time period, brains, humeri, femurae and vertebral columns of the mice were free of metastases. Tumor cells/cell aggregates were found in the intrapulmonary and the peripulmonary compartment, however since

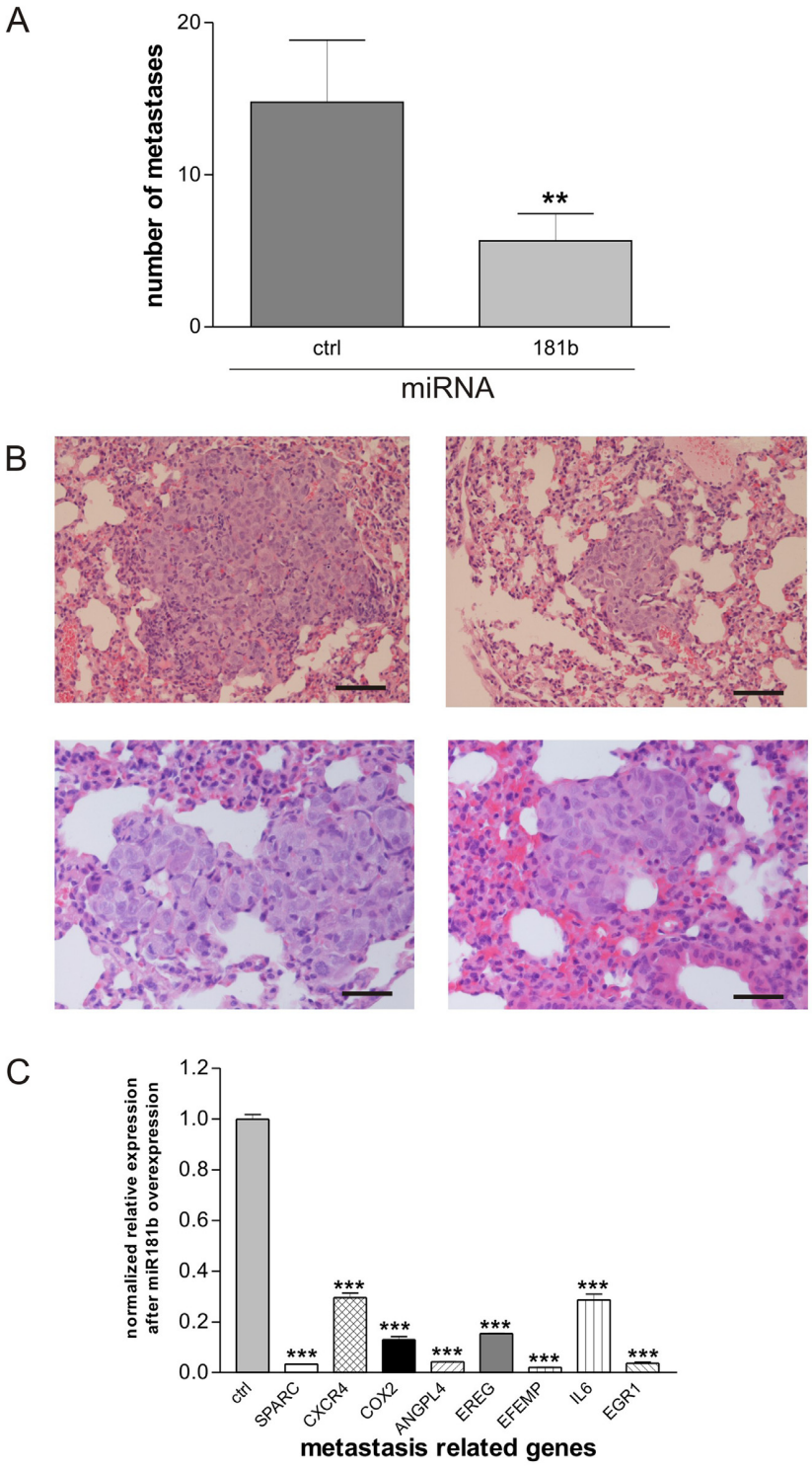

Figure 7 – *Breast cancer cells over-expressing miR181b have a lower capacity to metastasize in vivo*. Breast cancer cells over-expressing miR181b had a statistically significant (Mann–Whitney test, $p = 0.0070$) reduction in their capacity to metastasize into the lung of the animals (A). While the average number of metastasis in mice belonging to the control group was approximately 15 per animal, animals carrying MDA-MB-231^{miR1871b} cells developed an average of only 6 metastases per animal. The tumor cell morphology showed characteristic atypia, significant expression of human p53 protein was observed only in human tumor cells. Vitality of the tumor cells was confirmed by a high number of proliferating Ki-67 positive cells thus excluding tumor cell dormancy (B). Bars in the right lower corners of all photos are equivalent to 50 μm. Expression analysis (qRT-PCR) of a series of metastasis-related genes in MDA-MB-231 cells transiently over-expressing miR181b in comparison to corresponding MDA-MB-231 control cells showed that expression of the metastasis-related genes SPARC, CXCR4, COX2, ANGPL4, EFEMP, IL-6, and EGR1 to be statistically highly significantly down-regulated (C) 72 h after transfection (***$p < 0.001$; student's *t*-test). Mean + SD from 3 independent experiments are shown.

peripulmonary metastases are more likely to be derived from direct dissemination during the intercardiac injection, we limited our analysis to intrapulmonary metastases of hematogenous origin.

Breast cancer cells over-expressing miR181b had a highly statistically significant reduction (Mann–Whitney test, $p = 0.0070$) in lung metastasis (Figure 7A). The average number of metastasis in mice belonging to the control group was approximately 15 per animal. Animals inoculated with MDA-MB-231^{miR181b} cells developed an average of only 6 metastases per animal. Tumors of animals in both study groups were similar in dimension, morphology and histology. The tumor cell morphology showed characteristic atypia, significant expression of human p53 protein was observed only in human tumor cells. Vitality of the tumor cells was confirmed by a high number of proliferating Ki-67 positive cells thus excluding tumor cell dormancy (Figure 7B). The application of antagomirs in this type of experimental setting of hematogenous metastasis was not possible, due to the undesired toxicity of antagomirs in systemic application (data not shown).

To identify the molecular players involved in the diminished metastatic capacity of breast cancer cells over-expressing miR181b, we analyzed the expression of a series of metastasis-related genes in MDA-MB-231 cells transiently over-expressing miR181b through transfection with double stranded miRNA oligos in comparison to MDA-MB-231 cells transfected with the corresponding control oligos. We chose genes whose functional involvement has been shown through the analysis of highly metastatic tumor cells with pulmonary tropism (Minn et al., 2005). In this context we found expression of the metastasis-related genes SPARC (osteonectin), CXCR4, PTGS2 (prostaglandin-endoperoxide-synthase 2, COX2), ANGPTL4 (angiopoetin-like 4), EFEMP1 (EGF containing fibulin like extracellular matrix protein 1), IL-6, and EGR1 to be statistically highly significantly down-regulated 72 h after transfection (Figure 7C). These genes are comprised in the general metastasis signature developed by Ramaswamy et al. (2003) and SPARC, EFEMP1 and ANGPTL4 are also present in a signature of extracellular matrix genes that predict breast cancer metastasis (Albini et al., 2008). In addition, we analyzed the association of the expression of these genes with disease free survival in 424 cases of basal like human breast cancers (with 124 events). Low expression of SPARC ($p = 0.002$), PTGS2 ($p = 0.013$) and ANGPTL4 ($p = 0.016$) was significantly associated with disease free survival, IL6 ($p = 0.054$) showed a similar trend whereas EFEMP1, CXCR4, IL6 and EGR were not significantly associated with DFS of basal like breast cancer (see Supplementary Figure 1).

4. Discussion

In the metastatic process tumor cells are not the only participants; tumor-associated cells like macrophages, vascular and lymphatic endothelial cells or fibroblasts that reside in the tumor microenvironment of the host are clearly involved (Peinado et al., 2008). Inflammation is considered as a major factor for tumor progression (Balkwill and Mantovani, 2001) and recruitment of host cells from the microenvironment mediated by chemokines and their receptors play a critical role in this context. The two pro-inflammatory cytokines CXCL1 and -2 have recently been found to be linked to breast (Bachmeier et al., 2008; Minn et al., 2005) and prostate (Killian et al., 2012) cancer metastasis to the lung involving NFκB and CXCR2 (Bachmeier et al., 2008; Killian et al., 2012; Minn et al., 2005). Very recently CXCL1 and its cognate receptor CXCR2 have been shown to facilitate homing of mammary cancer cells (Halpern et al.) and CXCL1 has been found to be up-regulated in lymphatic endothelial cells from lymph node metastases suggesting a role in lymphangiogenesis (Xu et al.). Chemokine production is common for but not limited to inflammatory breast cancer and characterizes a specific subset of triple negative breast cancer for which the MDA-MB-231 cells are a suitable model.

Curcumin is a plant-derived compound that is particularly suited for prevention of tumor progression in inflammatory cancer because it is known to act on the central activator of inflammation, NFκB (Bachmeier et al., 2007, 2010a, 2008; Killian et al., 2012). In previous studies we have shown that Curcumin prevents formation of breast and prostate metastasis by targeting CXCL1 and -2 (Bachmeier et al., 2008; Killian et al., 2012). In this study we focused on the involvement of small non-coding RNAs (miRNAs) in the anti-metastatic effect of Curcumin. We addressed this problem in particular as little is yet known about the effect of this natural polyphenol on miRNA expression. Curcumin has been shown to modulate the expression of a variety of miRNAs in cancer cells *in vitro* of different origin (Gandhy et al.; Sreenivasan et al.; Sun et al., 2008; Yang et al., 2010). This in turn has a functional impact on proliferation, migration and apoptosis of the tumor cells (Sreenivasan et al.; Subramaniam et al.). However, these reports do not address how Curcumin regulates miRNA expression in the context of metastasis.

Our microarray and bioinformatics analyses identified miR181b to be modulated by Curcumin and linked to CXCL1 and -2. We validated the causal link between miR181b and CXCL1 and -2 expression by promoter binding assays and knock-in/knock-out experiments in a cell model of human metastatic breast cancer and found that miR181b mediates Curcumin-related down-modulation of the inflammatory cytokines CXCL1 and CXCL2, which are both tightly related to metastases (Bachmeier et al., 2008; Killian et al., 2012).

Curcumin is a known inhibitor of NFκB activation that most likely acts through the inhibition of degradation of the Inhibitor of Kappa Light Polypeptide Gene Enhancer In B-Cells, IκB. We have previously shown that Curcumin reduces NFκB activation and translocation in breast (Bachmeier et al., 2007, 2008) and prostate cancer cells (Killian et al., 2012) thereby affecting the expression of pro-metastatic genes, among which CXCL1 and −2. The Curcumin mediated induction of miR181b described here has similar effects. Figure 4 shows that the addition of an inhibitor of miR181b to Curcumin treated MDA-MB-231 cells only partially restores the CXCL1 and -2 expression levels of untreated cells. This is particularly evident for CXCL2, and at least in this case, Curcumin shows effects that cannot entirely be explained by miR181b induction, most likely due to inhibition of NFκB. On the other hand, miR181b on its own has been described as an inhibitor of NFκB therefore the fact that Curcumin induces

miR181b is expected to result in a strong inhibition of NFκB activation (Olarerin-George et al.; Sun et al.).

Our results also demonstrate that miR181b inhibits expression of the matrix degrading enzymes MMPs (matrix metalloproteinases) which leads to reduced invasion. Given the presence of miR181b target sequences in the transcripts of MMP1 and -3 their downregulation in miR181b over-expressing cells is likely to be direct. However, indirect, CXCL1 mediated effects might contribute through miR181b dependent disruption of the positive feedback loop between CXCL1 and NFκB (Bachmeier et al., 2008; Killian et al., 2012) that also affects MMP expression.

Additionally we show that the anti-proliferative effect of Curcumin on breast cancer cells is mediated via miR181b and that apoptosis and necrosis – both processes dysregulated in metastatic tumor cells – are induced in metastatic breast cancer cells over-expressing miR181b.

We found that miR181b over-expression in metastatic breast cancer cells down-regulates expression of genes belonging to the breast cancer lung metastases signature (Minn et al., 2005) and miR181b over-expression in breast cancer cells inhibits metastases formation *in vivo*. So far there are only a few reports regarding the role of miR181 expression in tumor progression and it seems that miR181b has both oncomir and tumor suppressor functions depending on tumor type and context with inflammation (Bisso et al.; Seoudi et al.; Taylor et al.).

Finally we verified the clinical relevance of our results by showing identical responses to Curcumin treatment in tumor cells from primary breast cancers. We show here that Curcumin impacts on miRNA expression in primary tumors inducing miR181b expression, which translates into a down-regulation of the pro-inflammatory cytokine CXCL1 and subsequent loss of metastatic potential. The data presented here together with our previously published data (Bachmeier et al., 2007, 2008) can be easily translated into the clinic, in particular because Curcumin can be easily administered orally and dosages up to 8 g per day have been shown to be safe (Dhillon et al., 2008). For example breast cancer patients that are either at high risk for, in treatment for or in remission from breast cancer, could receive a combination of Curcumin prevention with standard therapeutic approaches in controlled clinical trials eventually stratifying patients for ABCA1 mediated resistance to Curcumin (Bachmeier et al., 2009).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.01.005.

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Development of Resistance towards Artesunate in MDA-MB-231 Human Breast Cancer Cells

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Abstract

Breast cancer is the most common cancer and the second leading cause of cancer death in industrialized countries. Systemic treatment of breast cancer is effective at the beginning of therapy. However, after a variable period of time, progression occurs due to therapy resistance. Artesunate, clinically used as anti-malarial agent, has recently revealed remarkable anti-tumor activity offering a role as novel candidate for cancer chemotherapy. We analyzed the anti-tumor effects of artesunate in metastasizing breast carcinoma *in vitro* and *in vivo*. Unlike as expected, artesunate induced resistance in highly metastatic human breast cancer cells MDA-MB-231. Likewise acquired resistance led to abolishment of apoptosis and cytotoxicity in pre-treated MDA-MB-231 cells. In contrast, artesunate was more cytotoxic towards the less tumorigenic MDA-MB-468 cells without showing resistance. Unraveling the underlying molecular mechanisms, we found that resistance was induced due to activation of the tumor progression related transcription factors NFκB and AP-1. Thereby transcription, expression and activity of the matrix-degrading enzyme MMP-1, whose function is correlated with increased invasion and metastasis, was up-regulated upon acquisition of resistance. Additionally, activation of the apoptosis-related factor NFκB lead to increased expression of ant-apoptotic *bcl2* and reduced expression of pro-apoptotic *bax*. Application of artesunate *in vivo* in a model of xenografted breast cancer showed, that tumors growth was not efficiently abolished as compared to the control drug doxorubicin. Taken together our *in vitro* and *in vivo* results correlate well showing for the first time that artesunate induces resistance in highly metastatic breast tumors.

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Introduction

Breast cancer belongs to the most fatal cancer types in industrialized countries [1]. While treatment options have considerably improved over the past decades, cure from the disease is still not a reality for all women suffering from breast cancer. Among the reasons for this situation are the development of drug resistance and severe side effects of chemotherapy, which still are unresolved problems in clinical oncology. Therefore, the search for novel anticancer compounds with improved features is mandatory.

A couple of years ago, we focused our efforts on artesunate [2,3]. This is a semi-synthetic derivative of artemisinin, the active principle of *Artemisia annua* L. Artemisinin and its derivatives are valuable drugs treating multidrug-resistant *Plasmodium falciparum* and *P. vivax* infections. In addition to their efficacy in malaria treatment, they are cytotoxic towards cancer cells and multidrug-resistant tumor cells. More than 70 cell lines from different tumor types have been reported to be inhibited by artesunate and its related compound artemisinin [2,4]. Over-expressing ATP-binding cassette-type drug transporters (MDR1/P-gp, MRP1, BCRP) do not reveal cross-resistance to artesunate [4]. We have also shown that normal cells are minimally or not affected by artesunate [5]. In addition, there are several reports by us and others that artesunate and artemisinin inhibit tumor growth in xenograft tumors *in vivo* [6,7,8,9]. Case reports on the activity of this drug class in tumor patients [10] and a clinical study on 120 non-small cell lung cancer prove the anticancer activity of artesunate [11].

Despite the far-reaching lack of resistance in malaria and cancer, the first reports appeared concerning development of resistance in *Plasmodia* [12,13,14] implying that resistance to artesunate may also occur in cancer cells. To address the question of development of artesunate resistance in cancer cells, we have chosen breast cancer as suitable tumor type. The response rates of breast cancer towards standard chemotherapy show that this entity belongs to the tumor types, where women can benefit from cytotoxic treatment. Therefore, further improving treatment strategies in breast cancer might be more promising than in other tumor types poorly responding the chemotherapy. For this reason, we used MDA-MB-231 breast cancer cells. This cell line reveals several features of an aggressive phenotype such as invasiveness and formation of metastasis *in vivo* and insensitivity to anticancer drugs.

In the present investigation, we demonstrated that a resistance phenotype could be induced in MDA-MB-231 cells. Up-regulation of the transcription factors NFκB and Ap-1 associated with

increased expression of ant-apoptotic *bcl-2* and reduced expression of pro-apoptotic *bax* can be discussed as underlying mechanism of action. These results obtained *in vitro* correspond with the weak activity of artesunate in MDA-MB-231 xenograft tumors *in vivo*.

Materials and Methods

Cell Culture Conditions

We obtained the estrogen receptor negative cell lines MDA-MB-231 and MDA-MB-468 [15,16] (referred to as 231 and 468 cells) from ATCC_LGC (Wesel, Germany). MDA-MB-231 cells injected into the mammary fat pad of nude mice result in the formation of tumors and distant metastases in lungs, brain, and lymph nodes of most mice [17], whereas MDA-MB-468 are less tumorigenic and do not form metastasis *in vivo*.

Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in MEM (Eagle's) with Earle's salts supplemented with 5% heat inactivated fetal calf serum, 1% L-glutamine solution (200 mM), 1% sodium pyruvate solution (100 mM), non-essential amino acids and vitamins. All cell culture material was obtained from PAA (Cölbe, Germany). Medium was changed every two days.

Treatment of Cells

Artesunate was obtained from Saokim Ltd (Hanoi, Vietnam). The drug was dissolved in sterile DMSO (SIGMA-Aldrich; Taufkirchen, Germany) as a 1 mM stock solution and stored at −20°C. For the use in cell culture sterile dilutions in culture media were prepared. The maximal dilution of DMSO in the cell cultures did not exceed 1:100. Therefore, toxic effects by DMSO can be excluded.

Preparation of Conditioned Media

Cell culture supernatants of artesunate and carrier-treated-treated MDA-MB-231 cells were collected and centrifuged 15 min at 4000×g. The supernatants were used for Western Blots and zymography analyses.

Determination of Protein Concentration

Protein concentrations were determined by the BCA protein assay (Pierce, Oud-Bejierland, Netherlands) with bovine serum albumin as standard.

Preparation of RNA and cDNA synthesis

Total RNAs were isolated from cells treated with artesunate for several time periods using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Thereafter, oligo dT primed cDNAs were synthesized using the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Irvine, CA) following the manufacturer's instructions.

Quantitative RT-PCR

Expression analysis of a variety of genes was performed by quantitative real-time RT-PCR. All primers for the genes tested were designed using primer3 software [18] with a Tm optimum of approximately 60°C and a product length of 100–150 nt (see primer list, Table S1). Real time PCR was performed on an I-Cycler (Biorad Hercules, CA) using iQ Supermix (Biorad) supplemented with 10 nM fluorescein (Biorad), 0.1× SYBR-Green I (Sigma-Aldrich), 2.5 µL of cDNA (5× diluted), 3 pmol sense and antisense primers in a final reaction volume of 25 µL. After an initial denaturation step of 3 min during which the well factor was measured, 40 cycles of 15 sec at 95°C followed by 30 sec at 60°C were performed. Fluorescence was measured during the annealing step in each cycle. After amplification melting curves with 80 steps of 15 sec and 0.5°C increase were performed to monitor amplicon identity. Amplification efficiency was assessed for all primer sets utilized in separate reactions, and primers with efficiencies >94% were used. Expression data were normalized on HPRT, GAPDH and on RNA polymerase II (RPII) gene expression data obtained in parallel using the software BestKeeper [19]. Relative expression values with standard errors and statistical comparisons (unpaired two-tailed t-test) were obtained using Qgene software [20].

Western Blots

Conditioned media from artesunate treated (3, 5, 7, 15, or 24 h) and non-treated control cells were analyzed using antibodies against MMP-1 (kind gift from Ralf Lichtinghagen, Medical School, Hannover) as previously described [21]. Enhanced chemiluminescence was used for visualization of the protein bands as recommended by the manufacturer (GE Healthcare, Little Chalfont, U.K.). Semiquantitative evaluation of the bands was performed by densitometric analysis with the ImageJ software provided by the NIH (http://rsb.info.nih.gov/ij/).

MTT assay

The anti-proliferative effects of artesunate on MDA-MB-231 and MDA-MB-468 breast carcinoma cell lines were determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye uptake method as previously described [22]. Pretreatment with artesunate was performed for 24 h by adding 20 µM of the drug to the culture media. Afterwards cells were washed 3 times with PBS and incubation with different concentrations of artesunate (2, 5, 10, 20, or 50 µM) was continued for another 24 h.

Apoptosis Assay

Apoptotic cell death was determined by an enzyme-linked immunoassay (Cell Death Detection ELISA^{PLUS}, Roche) to detect fragmented DNA and histones (mononucleosomes and oligonucleosomes). Human breast cancer cells MDA-MB-231 were seeded on 24-well plates and pretreated with 20 µM artesunate or carrier for 24 h. Afterwards cells were washed with PBS and treated with the carrier alone or different concentrations of artesunate in for another 24 h. Lysates prepared from the cells were processed following the instructions of the manufacturer.

The breast cancer cell lines MDA-MB-231 and MDA-MB-468 were grown in 24-well plates and incubated with Artesunate as described in the result section. The cells were harvested and treated with (FITC)-conjugated annexin V and propidium iodide (Annexin-V-FLUOS Staining kit from Roche Diagnostics (Mannheim, Germany) according to the recommendations of the manufacturer. Ten thousand events were counted for each sample. Data were analyzed using a Flow-Cytometer (Beckmann Coulter XL-MCL, Software: System II).

Electrophoretic Mobility Shift and Supershift Assays

Cells were seeded onto 150 cm^2 culture dishes with 25 mL culture medium and treated with artesunate or the carrier alone for several time periods (2, 4 or 6 h). Nuclear extracts were prepared as previously described [23]. Oligonucleotides corresponding to the consensus sequences (AP-1 site: 5′-GAT CTG TGA CTC AGC GCG AG-3′; NFκB site: 5′-GTT AGT TGA GGG GAC TTT CCC A-GGC-3′) were labeled with [^{32}P]dATP (3000 Ci/mM) and Klenow enzyme and were incubated with 10 µg of nuclear protein in 20 µL of 7 mM Hepes-KOH (pH 7.9), 100 mM KCl, 3.6 mM $MgCl_2$, and 10% glycerol on

ice for 20 min. Poly[d(I-C)] (0.05 mg/mL) was added as an unspecific competitor. The samples were run on a 5% non-denaturing polyacrylamide gel in a buffer containing 25 mM Tris-HCl (pH 8.0), 190 mM glycine, and 1 mM EDTA. Gels were dried and analyzed by autoradiography. In order to prove the specificity of the probes, a 50-fold excess of unlabeled probe was incubated with the binding reaction mixture for 45 min on ice before adding the radiolabeled DNA fragment. Specificity of binding ascertained by Superhift was performed using a specific antibodies against the p65 subunit of NFκB and c-jun (Santa Cruz, USA).

Casein Zymography

Gelatin zymography was essentially performed as previously described in detail [24]. A molecular mass standard (Biorad, Germany) was used in all experiments. These experiments were repeated three times.

Promoter analysis

Promoter sequences were analyzed for the presence of transcription factor binding sites using oPossum (www.cisreg.ca/oPOSSUM; [25]. Sequence 5000 nts upstream and 2000 nts downstream of the transcription start site were considered, the matrix match threshold was set to 85%.

Data analysis

Statistical significance was assessed by comparing mean (±SD) values, which were normalized to the control group with Student's t-test for independent groups. One-way analysis of variance was used to test for statistical significance ($P<0.05$), and when

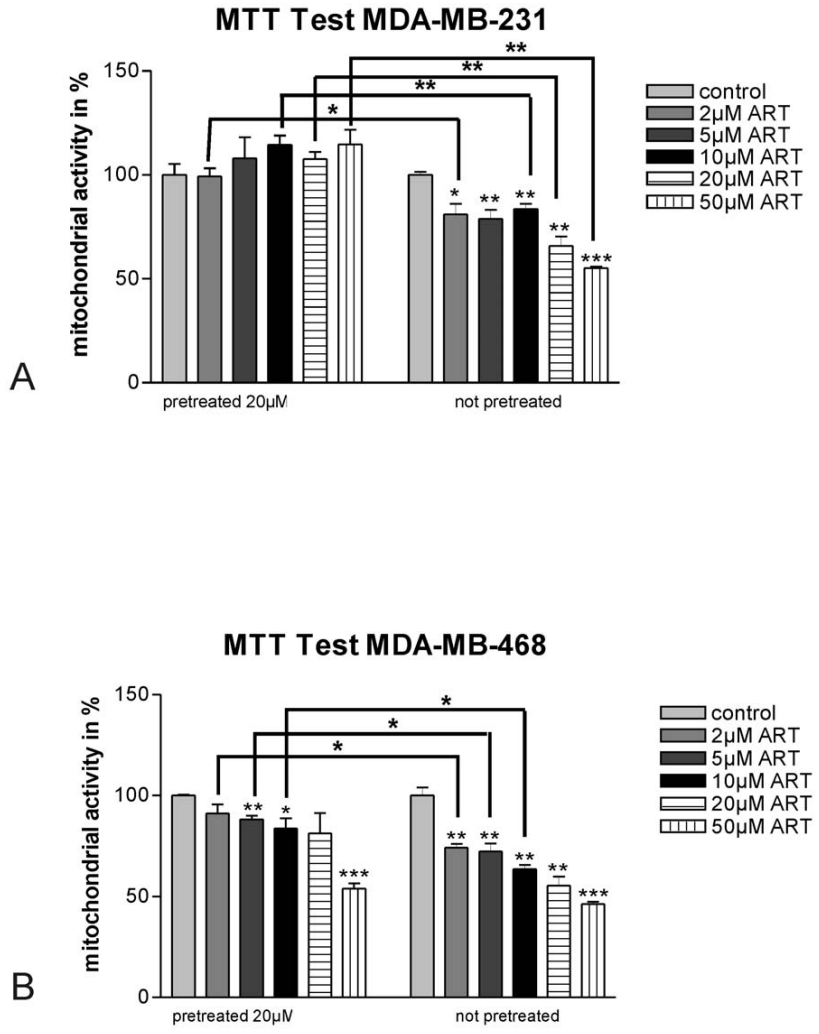


Figure 1. Cell viability. A) Different concentrations of artesunate reduce cell viability measured by MTT assay up to approximately 40% (50 μM) after 24 h in MDA-MB-231 cells (right panel), which was statistically significant (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; one way Anova with Bonferroni's post test). In contrast, MDA-MB-231 cells pretreated with 20 μM artesunate for 24 h acquired resistance and did not longer respond to application of the drug at various concentrations for further 24 h (left panel). B) Human non-metastastc breast cancer cells MDA-MB-468 responded to artesunate treatment at various concentrations with a statistically significant decline in cell viability up to 50% (** $P<0.01$; *** $P<0.001$; one way Anova with Bonferroni's post test) (right panel). After 24 h pretreatment with 20 μM artesunate, MDA-MB-468 cells still responded to the drug with decreased cell viability down to about 55%, which was statistically significant (*** $P<0.001$; one way Anova with Bonferroni's post test).
doi:10.1371/journal.pone.0020550.g001

significance was determined, Bonferroni's multiple comparison test was performed post hoc, as indicated in the figure legends. Statistical analysis was performed using the Prism software (GraphPad, San Diego, CA).

Animal Experiments

Animals: For the animal experiments, female nude mice (Ncr:nu/nu), ages 4 to 6 weeks and weighing 20–24 g, were used as described earlier [26]. All animal experiments were performed according to the German Animal Protection Law and by approval of the local responsible authorities (Approval Number: GV247/98; Ethics Committee of the *Landesamt für Gesundheit und Soziales*, Berlin, Germany).

Tumor Inoculation: Each mouse received 10^7 tumor cells subcutaneously without anesthesia. The injected cells were diluted in isotonic salt solution and subcutaneously injected into the mice. The diameter of the tumors was measured twice weekly using a caliper-like mechanical instrument and the tumor volume (V) was calculated according to the empirical equation $V = (\text{length} \times \text{width}^2)/2$. The median volumes of each group were normalized to the initial tumor volume resulting in the relative tumor volume.

Treatment modalities: Each six MDA-MD-231-transplanted animals received artesunate (200 and 400 mg/kg, respectively) or vehicle (10% Tween80 in saline) intraperitoneally (i.p.) at five consecutive days. Treatment started when tumors were palpable (about 5–6 mm diameter). An additional group of xenografted animals were treated with doxorubicin (i.v., 8 mg/kg) once a week for two weeks.

Results

Acquired resistance in terms of cell viability

As a first step, the effect of artesunate on the viability of the human breast cancer cell lines MDA-MB-231 and MDA-MB-468 was analyzed by MTT assays. Artesunate induced resistance in the highly metastatic MDA-MB-231 cells (Fig. 1A) and to a lesser extent also in non-metastatic MDA-MB-468 cells (Fig. 1B).

In comparison to carrier-treated cells, cell viability of MDA-MB-231 cells treated for 24 h with different concentrations of artesunate (2, 5, 10, 20 and 50 μM) declined in response to the dose applied to about 55% (50 μM) with high statistical significance: $P<0.05$ for 2 μM; $P<0.01$ for 5, 10 and 20 μM; $P<0.001$ for 50 μM (Fig. 1A, right panel).

When MDA-MB-231 cells were pretreated for 24 h with 20 μM artesunate, the cells did not respond to any further treatments showing that the cells acquired drug resistance (Fig. 1A, left panel).

MDA-MB-468 cells responded to application of artesunate and cell viability dropped in a dose-dependent manner to about 45% with statistical significance: $P<0.01$ for 2, 5, 10 and 20 μM; $P<0.001$ for 50 μM (Fig. 1B, right panel). In comparison to MDA-MB-231 cells, pretreatment of MDA-MB-468 cells with 20 μM artesunate for 24 h induced resistance to a lesser extent with a constant decline in cell viability in a dose-dependent manner to further application of the drug with statistical significance: $P<0.05$ for 10 μM, $P<0.01$ for 5 μM and $P<0.001$ for 50 μM (Fig. 1B, left panel). In contrast to the carrier-treated controls, cell viability dropped to further 55%.

Acquired resistance in terms of apoptosis

Apoptosis measured by means of increased nucleosomes in cell lysates was induced in MDA-MB-231 and MDA-MB-468 cells in a dose-dependent manner after 24 h treatment (Fig. 2 (■)). Thereby, the apoptosis rate was increased about 2-fold by 20 μM and about 3-fold by 50 μM artesunate. Pretreatment of MDA-MB-231 and MDA-MB-468 cells with 20 μM ART for 24 h induced resistance to apoptosis in the cells and likewise the number of nucleosomes in lysates of the pretreated cells did not increase upon further treatment with various doses of artesunate (Fig. 2 (▲)).

In order to confirm our results obtained by measuring the increase of nucleosomes and to clearly distinguish between early and late apotosis, we performed flow cytometry analysis of apoptosis and necrosis in non-resistant MDA-MB-468 and resistant MDA-MB-231 cells (Fig. 3). MDA-MB-468 cells treated with ART for 24 h, showed clear evidence of early and late apotosis, no matter whether they were pretreated for 24 h with ART. In contrast, ART treatment induced early and late apotosis only in non-pretreated MDA-MB-231 cells, whereas pretreated MDA-MB-231 cells did not further undergo early and late apotosis.

Activation of NFκB and AP-1 (EMSA) by artesunate

In order to unravel the molecular mechanism of acquired resistance in MDA-MB-231 cells, we analyzed the effect of artesunate on the transcription factors NFκB and AP-1. In case of nuclear activation of these factors, apoptosis is down-regulated due to over-expression of cell survival related genes and repression of pro-apoptotic genes.

Nuclear extracts from artesunate-treated and carrier-treated cells were subjected to electrophoretic mobility shift assays (EMSA), where the binding of transcription factors is revealed by the retarded electrophoretic migration of radioactively labeled oligonucleotides of the specific binding sequence. Binding of the transcription factors NFκB and AP-1, which are both associated with resistance to standard anti-cancer drugs, was monitored (Fig. 4A and B). Artesunate treatment for 2, 4, and 6 h of MDA-MB-231 cells (lanes 2, 3, and 4, respectively) showed a clear induction in the specific bands for the NFκB transcription factor subunit p65 (Fig. 4A) and the AP-1 subunit *c-jun* (Fig. 4B) in comparison to carrier-treated cells (lanes 1). The specificity of the binding was assessed by addition of a 50-fold excess of cold oligonucleotides that abolished the band shifts observed (lanes 5). Furthermore, the bands disappeared upon addition of appropriate

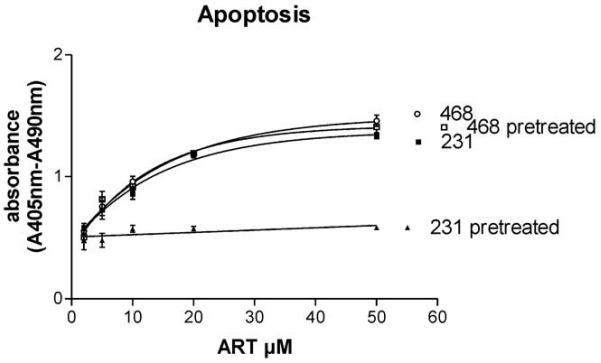


Figure 2. Detection of nucleosomes in cytoplasmic fractions (Cell Death ELISA, Roche Applied Biosystems) of MDA-MB-231 and MDA-MB-468 cells treated for 24 h with various concentrations of artesunate in comparison to the cells pre-treated for 24 h with 25 μM artesunate. While MDA-MB-468 cells undergo apoptosis upon artesunate treatment no matter if cells are pretreated for 24 h with artesunate (indicated with □) or not (indicated with ○), MDA-MB-231 cells acquired resistance to artesunate treatment and do not undergo apoptosis (indicated with ■ or ▲ respectively) when pretreated for 24 h with artesunate.
doi:10.1371/journal.pone.0020550.g002

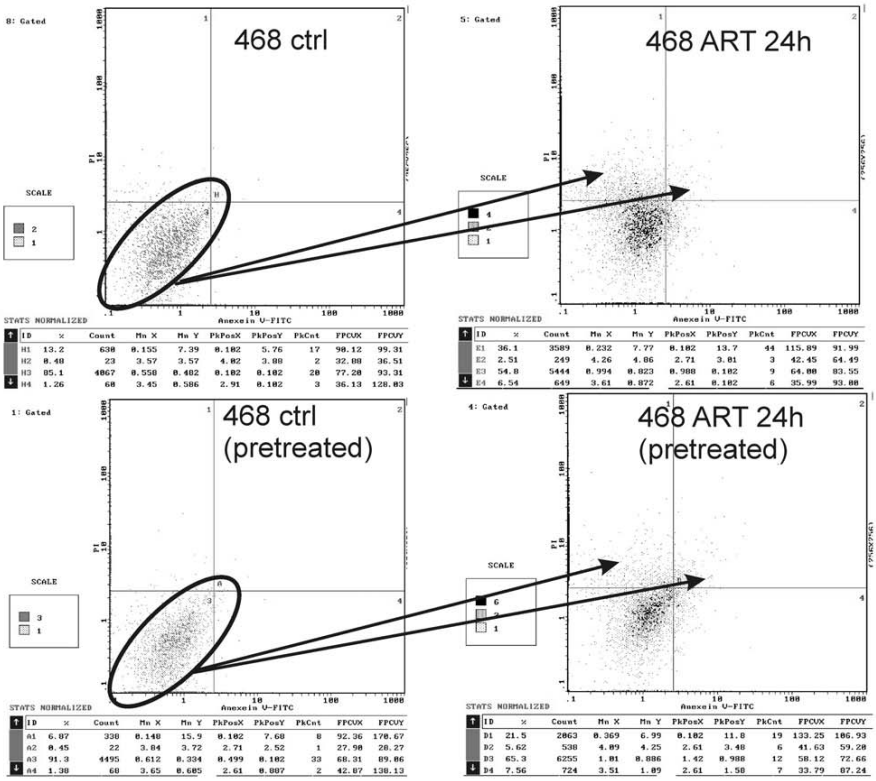

468 ctrl
468 ART 24h
468 ctrl (pretreated)
468 ART 24h (pretreated)
231 ctrl
231 ART 24h
231 ctrl (pretreated)
231 ART 24h (pretreated)
Annexin V-FITC
PI
SCALE
STATS NORMALIZED

Figure 3. Flow cytometry analysis of early and late apoptosis in non-resistant MDA-MB-468 and resistant MDA-MB-231 cells. Evidence of early apoptosis in terms of translocation of phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane, where it becomes accessible to annexin V staining, can be seen in quadrants 4 and 2. Cells reaching a late apoptotic propidium iodide positive state can be seen in quadrant 1. MDA-MB-468 cells treated with ART for 24 h, showed clear evidence of early and late apoptosis, no matter if they were pretreated for 24 h with ART. The two arrows indicate the shift from the lower left quarter to the upper right and upper left quarters. In contrast ART treatment induced early and late apoptosis only in non pretreated MDA-MB-231 cells, whereas pretreated MDA-MB-231 cells did not further undergo early or late apoptosis as illustrated by the two circles and the arrow in the lower panel of the figure.
doi:10.1371/journal.pone.0020550.g003

specific antibodies (supershift) against p65 or c-jun respectively. The interference of the antibodies with the binding of the proteins (transcription factors) to the labelled probes resulted in the formation of very faint or rather diffuse double bands (lanes 6–9). Addition of an unrelated mutant oligonucleotide had no effect on NFκB binding (data not shown). Experiments were repeated at least three times.

Expression of the NFκB subunit p65 (Fig. 4C–F) and the AP-1 subunit *c-jun* (Fig. 4G–K) were analyzed by quantitative RT-PCR. mRNA expression of p65 was induced in MDA-MB-231 cells upon treatment throughout 24 h, whereby the peak of induction was reached after 18 h (* $P<0.05$; one way Anova with Bonferroni's post test). After pre-treatment of MDA-MB-231 cells with ART for 24 h, no statistically significant changes in p65 expression could be observed. In non-resistant MDA-MB-468 cells p65 expression is down-regulated statistically significantly (* $P<0.05$; one way Anova with Bonferroni's post test) after 18 h and 24 h upon ART treatment no matter whether the cells were pretreated with ART or not. The effect of acquired resistance as seen for p65 expression could also be found concerning the expression of c-jun. While c-jun was statistically significantly (** $P<0.01$; one way Anova with Bonferroni's post test) up-regulated in MDA-MB-231 cells after 18 h and 24 h, no significant change could be observed for cells pretreated 24 h with ART. Again MDA-MB-468 cells responded always to ART treatment with down-regulation of c-jun expression, which was statistically significant at least after 24 h no matter if the cells were pretreated with ART or not.

Our results show that human metastatic breast cancer cells MDA-MB-231 acquired resistance to ART treatment and thereby expression of p65 and c-jun do not alter upon further treatment with the substance.

Acquired resistance becomes also clear by comparing the percentage of cells undergoing early and late apotosis upon further ART treatment in pretreated or non pretreated breast cancer cells (Table 1). While MDA-MB-468 cells have a significant increase in early (6.18%) and late apoptosis (14.63%), MDA-MB-231 cells showed only a minimal increase in early apoptosis (2.29%) and no increase in late apoptosis 24 h after additional ART treatment.

Bcl-2 and bax are involved in the development of resistance

As recent evidence accumulates that the ratio between *bcl-2* and *bax* is of special interest for the induction of apoptosis in cancer cells, we examined the expression of these two members of the *bcl-2*-family, in order to evaluate their role in the development of resistance against artesunate.

Treatment of the human metastatic breast cancer cell line MDA-MB-231 with 20 μM artesunate, led to induction of *bcl-2* expression already after 2 h (** $P<0.01$). Up-regulation of this anti-apoptotic factor reached a level of about two fold after 18 h with statistical significance of * $P<0.05$ in comparison to carrier-treated control cells (Fig. 5A). Pretreatment with ART rendered MDA-MB-231 cells resistant to this compound and hence bcl-2 expression could not be induced in these cells (Fig. 5B). In contrast, pretreatment with ART did not lead to resistance in MDA-MB-468 cells. Consequently, bcl-2 expression was diminished in these cells already after 2 h with further decline up to 24 h, no matter whether cells were pretreated (Fig. 5C) or not (Fig. 5D).

Expression of the pro-apoptotic factor *bax* was repressed in MDA-MB-231 cells upon artesunate treatment. This anti-apoptotic effect became statistically significant after 6 h with ongoing decline up to 24 h with a significance of *** $P<0.001$ in comparison to carrier-treated cells (Fig. 5E). As already seen for bcl-2 expression, pretreatment with ART rendered MDA-MB-231 cells resistant and, therefore, bax expression could not be inhibited by ART in these cells (Fig. 5F). On the other hand, expression of bax in MDA-MB-468, which did not acquire resistance against ART, could be induced by the compound, already after 2 h with further significant increase up to 24 h, no matter whether cells were pretreated (Fig. 5H) or not (Fig. 5G).

Apoptosis results from the balance of pro- and anti-apoptotic members of the bcl-2 family. Therefore, we calculated the ratio of the pro-apoptotic bax and the anti-apoptotic bcl-2 as indicator for induction or repression of apoptosis by artesunate. High ratios indicate cellular proficiency to induce apoptosis, while low ratios may occur in more apoptosis-resistant cells. As shown in Figure 5 (I), the bax/bcl-2 ratio was high in non-pretreated MDA-MB-231 cells and decreased over time, indicating that the cells acquired resistance to artesunate-induced apoptosis. In contrast, the bax/bcl-2 ratio was low in pretreated cells and did not increase up to 18 h and only a little bit after 24 h. this may indicate that the cells remained apoptosis-resistant towards artesunate. The strongly increasing bax/bcl-2 ratios in both pretreated and non-pretreated MDA-MB-468 cells indicate that artesunate pretreatment did not result in apoptosis-resistance towards artesunate.

The correlation between the induction of the transcription factors NFκB and the regulation of the apoptotis-related genes *bcl-2* and *bax* was confirmed by promoter analysis. Both genes share all of the transcription binding factor sites identified by high stringency analysis of the promoter region from −5000 to +2000 (ELK4,ELF5, SPIB, NFκB, ZNF354C, SP1, ELK1, MZF1_1–4, MZF 5–13, Bapx1) indicating a common regulation. *bax* had a *bona fide* NFκB in position −363 (sequence: GGGCCTGCCC) and *bcl-2* had two of binding sites in position −641 and +139 (sequences: GGCAATTTAC), relative to the transcription start site (Table 2).

Effect of artesunate on expression and activity of MMP-1 in resistant breast cancer cells

We analyzed the amount of the metastasis-related protease MMP-1 secreted into the cell supernatants of artesunate treated human breast cancer cells MDA-MB-231 by Western blots with specific antibodies against MMP-1 (Fig. 6A). Thereby, accumulation of newly released MMP-1 secreted into fresh serum-free medium added at the beginning of the time course was monitored in the presence (+) or in the absence (−) of 20 μM artesunate. We found that MMP-1 protein was released into the supernatants of MDA-MB-231 cell cultures. For shorter time periods (3 and 5 h, left upper panel), the amount of MMP-1 protein secreted into the

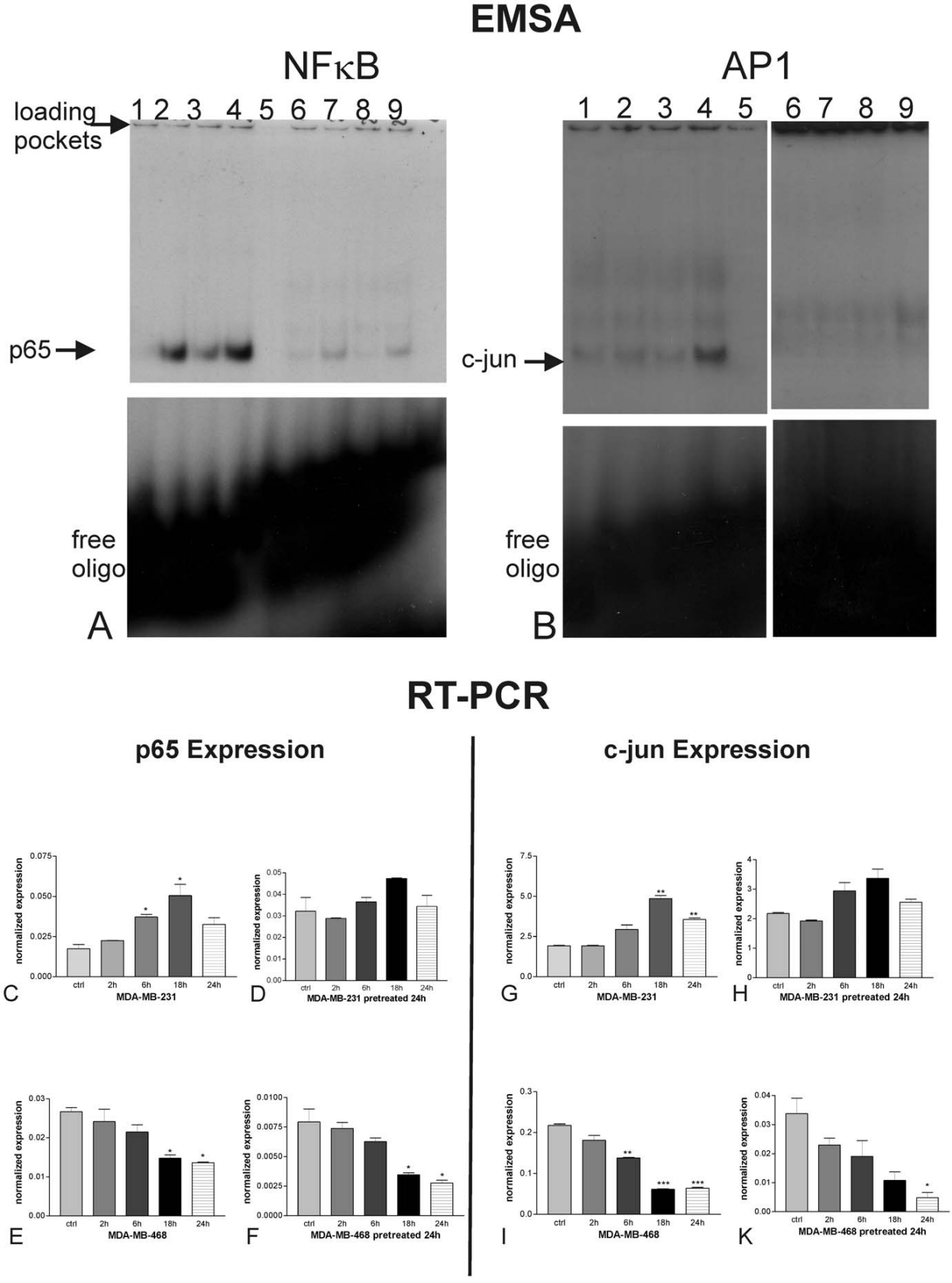

EMSA
NFκB
AP1
loading pockets
1 2 3 4 5 6 7 8 9
p65
c-jun
free oligo
A
B
RT-PCR
p65 Expression
c-jun Expression
normalized expression
ctrl
2h
6h
18h
24h
MDA-MB-231
MDA-MB-231 pretreated 24h
MDA-MB-468
MDA-MB-468 pretreated 24h
C
D
E
F
G
H
I
K

Figure 4. Effects of artesunate on NFκB (A) and AP-1 (B). Binding of ^{32}P-labeled NFκB and AP-1 specific oligonucleotides to the cognate transcription factor present in nuclear extracts of MDA-MB-231 cells was monitored by EMSA. Artesunate treatment of MDA-MB-231 cells for different time intervals (lane 2, 2 h; lane 3, 4 h; lane 4, 6 h) led to a induced binding of NFκB (A) and AP-1 (B) to its response element as compared to carrier-treated cells (lane 1). The specificity of the binding was assessed for all three oligos by addition of a 50× molar excess of cold oligonucleotides (lanes 5). Furthermore, addition of appropriate specific antibodies(supershift) against p65 or c-jun respectively resulted in the formation of very faint or rather diffuse double bands (lanes 6–9). Experiments were repeated at least three times. Fig. 4 C-K illustrates the expression of p65 and c-jun in MDA-MB-231 and -468 cells. mRNA expression of p65 was induced in MDA-MB-231 cells upon artesunate treatment only in non-pretreated cells (C), while p65 expression did not alter upon artesunate treatment in pre-treated cells (D). MDA-MB-468 cells p65 expression was down-regulated statistically significantly after 18 h and 24 h upon artesunate treatment, no matter whether the cells were pretreated with artesunate (F) or not (E). The effect of acquired resistance as seen for p65 expression could also be found concerning the expression of c-jun. While c-jun was statistically significantly up-regulated in MDA-MB-231 cells after 18 h and 24 h, no significant change could be observed for cells pretreated 24 h with artesunate. (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; one way Anova with Bonferroni's post test). Experiments were performed in triplicates.
doi:10.1371/journal.pone.0020550.g004

media of treated cells was lower compared to those of non-treated cells. After 7 h of artesunate treatment, the effect switched and supernatants of treated cells had elevated MMP-1 concentrations in comparison to non-treated breast cancer cells. Up-regulation of MMP-1 secretion was observed for up to 24 h artesunate treatment (7, 15 or 24 h, upper right panel). All experiments have been performed in triplicates. According to determination of protein concentrations by Pierce assay equal amounts of secreted protein were subjected to SDS-PAGE, and as loading control, the amount of protein blotted onto the membranes was visualized with Ponceau red before blocking (data not shown). Densitometric analysis of the bands (Fig. 6A, lower panels) revealed that the down-regulation of MMP-1 protein secretion in response to artesunate treatment, seen for early time points (3 and 5 h; Fig. 6A, lower left panel), was about 60–65%. Up-regulation of MMP-1 secretion was about 1.6-fold after 7 h, 3 fold after 15 h and 2.3-fold after 24 h of artesunate treatment (Fig. 6A, lower right panel). The statistical power for all observed regulation events was $P<0.001$ throughout the whole experiments (one way Anova with Bonferroni's post test).

In order to visualize the enzymatic activities present in cell culture supernatants, we performed zymography analyses on casein-containing gels (Fig. 6B). As MMP-1 is able to degrade casein, electrophoretically separated proteases, present in cell culture supernatants, were detected as translucent bands on the Coomassie brillant blue stained substrate background.

MDA-MB-231 breast cancer cells responded to the artesunate treatment (lanes labeled with "+") with a transient (3 and 5 h, left panel) reduction of MMP-1 activity released into fresh serum-free medium in comparison to carrier-treated cells (lanes labeled with "−"). In contrast, cells treated for longer periods (15 h and 24 h) with artesunate (+) showed a clear induction of MMP-1 as depicted here in Fig. 6A, right panel. Interestingly, treatment with artesunate for 7 h did not result in any differences in MMP-1 secretion into the culture medium of MDA-MB-231 cells when compared to carrier-treated cells (−). MMP-1 was identified according to its migration behavior as compared to a molecular mass standard (lanes labeled with M).

In order to monitor the effect of artesunate on the level of MMP-1 transcription, we performed quantitative Real Time RT-PCR (Fig. 6C) and normalized expression values on those obtained for the housekeeping genes RPII and HPRT. In accordance with the results from Western blots and zymography, shown in the sections before, artesunate treatment of MDA-MB-231 cells resulted in slightly diminished levels of MMP-1 mRNA after 2 and 6 h. However, after 18 h treatment the reduction of MMP-1 mRNA expression by artesunate faded out and treatment with 20 μM of the drug for longer time periods led to statistically significant inductions of MMP-1 of 1.6- and 1.9-fold after 24 and 31 h respectively. The levels of statistical significance were $P<0.01$ at 24 h and $P<0.05$ at 31 h (student's t-test).

Activity of artesunate towards MDA-MB-231 xenograft tumors in nude mice

The anti-tumor activity of artesunate was examined *in vivo* in MDA-MB-231 xenografted tumors in nude mice. The results are shown in Fig. 7. While treatment with 200 mg/kg artesunate did not result in inhibition of tumor growth as compared to vehicle-treated tumors, a marginal inhibition was observed with 400 mg/kg artesunate. By contrast, a considerable inhibition of tumor growth was reached using a control drug, doxorubicin. We did not observe any signs of toxicity of artesunate (loss of weight etc.).

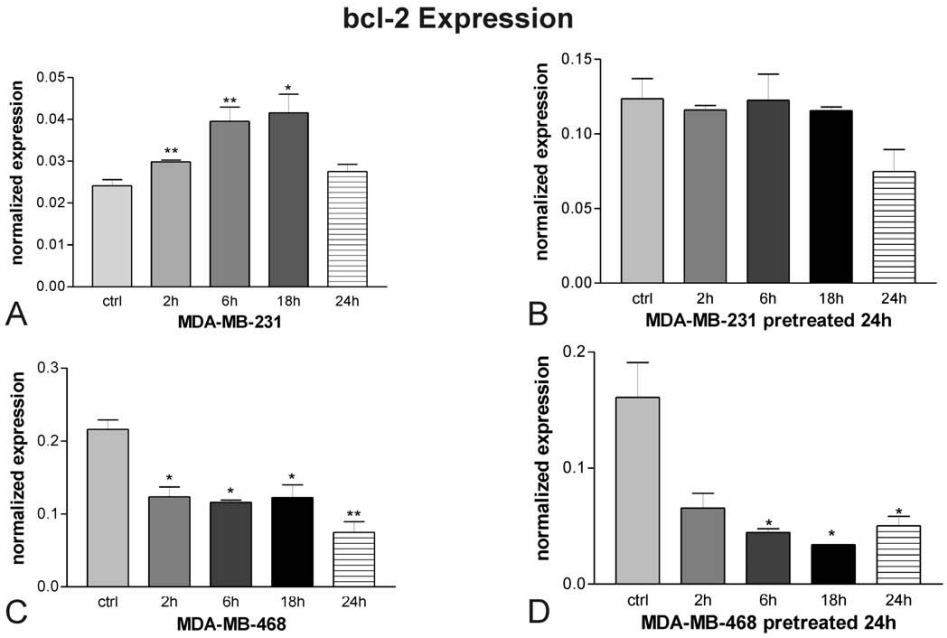
Discussion

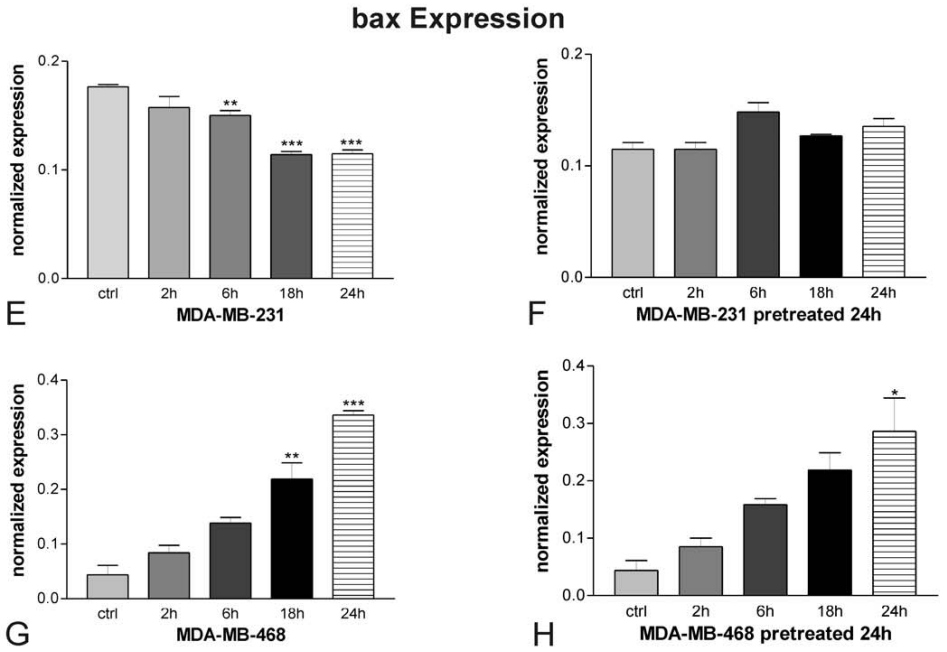
In the present investigation, we showed that sub-lethal doses of artesunate resulted in the development of resistance towards this drug. While inherent differences of cancer cell lines in response towards artesunate and modulation of inherent resistance by ferrous iron were previously described [4,27], the present study is to the best of our knowledge the first to show an acquired resistance phenomenon towards artesunate in cancer cells. The

Table 1. Percentage of cells undergoing apoptosis or necrosis.

	MDA-MB-468				MDA-MB-231			
			ART pretreatment				ART pretreatment	
	ctrl	24 h ART	ctrl	24 h ART	ctrl	24 h ART	ctrl	24 h ART
Early apoptosis	1.26	6.54	1.38	7.56	1.65	5.41	3.58	5.87
Late apoptosis	13.2	36.1	6.87	21.5	6.58	25.1	11.9	11.7

Early and late apoptosis measured by annexin V and propidium iodide staining in human breast cancer cells revealed that the metastatic MDA-MB-231 cells pretreated for 24 h with artesunate, became resistant to further treatment. While MDA-MB-468 cells showed significantly increased early (6.18%) and late apoptosis (14.63%), MDA-MB-231 cells showed only a minimal increase in early apoptosis (2.29%) and no increase in late apoptosis 24 h after additional artesunate treatment.
doi:10.1371/journal.pone.0020550.t001


bcl-2 Expression
normalized expression
ctrl
2h
6h
18h
24h
A
MDA-MB-231
B
MDA-MB-231 pretreated 24h
C
MDA-MB-468
D
MDA-MB-468 pretreated 24h


bax Expression
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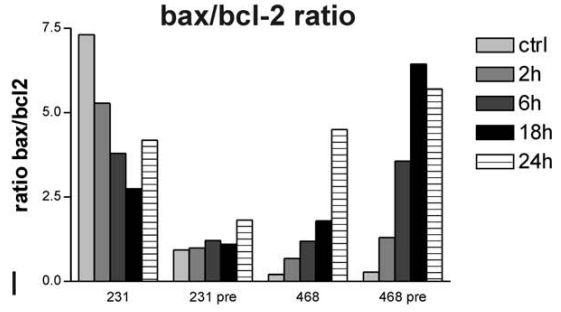
bax/bcl-2 ratio
ratio bax/bcl2
ctrl
2h
6h
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24h
231
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468
468 pre
I

Figure 5. Expression of apoptosis-related genes as determined by RT-PCR. (A–D) Treatment with 20 μM leads to induction of *bcl-2* expression in MDA-MB-231 cells after 2 h (** $P<0.01$) and reaches a level of about two fold after 18 h with statistical significance of * $P<0.05$ in comparison to carrier-treated control cells (A). Pretreatment with artesunate rendered MDA-MB-231 cells resistant to this compound and, hence, bcl-2 expression could not be induced in these cells (B). In contrast, pretreatment with artesunate did not lead to resistance in MDA-MB-468 cells. Consequently, bcl-2 expression was statistically significantly diminished in these cells already after 6 h with further decline up to 24 h, no matter if cells were pretreated (C) or not (D). (E–H) Expression of the pro-apoptotic factor *bax* was repressed in MDA-MB-231 cells upon treatment between 6 and 24 h(E). As already seen for bcl-2 expression, pretreatment with artesunate rendered MDA-MB-231 cells resistant and, therefore, bax expression could not be inhibited by artesunate in these cells (F). On the other hand, expression of bax in MDA-MB-468, which did not acquire resistance against artesunate, could be induced by the compound, already after 2 h with further significant increase up to 24 h, no matter whether cells were pretreated or not. Experiments were performed in triplicates (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; one way Anova with Bonferroni's post test). (I) Since apoptosis results from the balance of pro- and anti-apoptotic members of the bcl-2 family, we calculated the expression ratios of the pro-apoptotic bax and the anti-apoptotic bcl-2 as indicator for induction or repression of apoptosis by artesunate. High ratios indicate cellular proficiency to induce apoptosis, while low ratios may occur in more apoptosis-resistant cells.
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induced resistance observed *in vitro* corresponds to the unresponsiveness at lower artesunate doses (200 mg/kg) and only minor responsiveness after treatment with high artesunate doses (400 mg/kg) after repeated injection of the compound to nude mice as shown here.

Recently, it was reported that artemisinin treatment can confer resistance to other anti-cancer drugs such as doxorubicin [28]. Resistance towards artesunate-type drugs occurs only infrequently in malaria parasites, but has been reported both in patient samples and under *in vitro* conditions [12,13,14]. The role of several plasmodial proteins, including multidrug resistance transporters (pfmdr1), sarcoendoplasmatic Ca^{2+}-dependent ATPase (SERCA), translationally controlled tumor protein (TCTP) and others have been discussed.

Here, we present evidence that MDA-MB-231 human breast cancer cells reveal reduced sensitivity after repeated artesunate treatment. Likewise diminished cytotoxicity was seen in pretreated highly metastatic MDA-MB-231 cells in contrast to less tumorigenic non-metastatic MDA-MB-468 breast cancer cells. This observation implicates that the drug could be beneficially applied as therapy in less advanced breast cancer. Additionally apoptosis was down-regulated in metastatic breast cancer cells pretreated with the drug for 24 h.

Analysis of molecular mechanisms underlying the acquired resistance against ART in the breast cancer cells showed that this was due to activation of the transcription factors, NFκB and AP-1. NFκB represents a central player for many cellular processes such as proliferation, adhesion, angiogenesis, inflammation and others. It mediated apoptosis resistance towards various stimuli, including anti-cancer agents and is activated by DNA damage [29].

NF-κB is a resistance factor for established anticancer drugs by inhibiting apoptosis [30,31]. Recently, we have shown that artesunate induces double stand breaks [32]. In non-activated cells, NFκB is complexed with IκB in the cytosol. Upon stimulation by appropriate stimuli, the complex is dissociated and NFκB translocates into the nucleus, where it binds to specific promoters of target genes, *e.g.* survival-related genes, and stimulates their transcriptional activation.

The AP-1 complex consists of c-Fos, FosB, Fra-1, or Fra-2, each of which can dimerize with c-Jun, JunB, or JunD. This complex binds to specific binding motifs in the promoter of target genes regulating, apoptosis, proliferation, or differentiation. A role for AP-1 for resistance to anti-cancer drugs has also been proposed [33]. In a previous investigation, we found that AP-1 (together with Sp1) was activated in host cells upon infection with human cytomegalovirus and that a single artesunate treatment suppressed AP-1 activation and production of virus-specific proteins in host cells [34]. AP-1 acts as transcription factor for anticancer drug resistance genes such as P-glycoprotein/*MDR1* or glutathione S-transferase-pi. Glutathione S-transferases detoxify harmful xenobiotic molecules by binding them to glutathione. Then, glutathione-drug conjugates are transported out of cells. The expression of glutathione S-transferases was also associated with cellular response of tumor cells to artesunate [35,36].

Here, we observed that repeated treatment of highly metastatic breast cancer cells induced AP-1 activation leading to artesunate resistance. Whether a similar phenomenon can also be found in anti-viral therapy with artesunate is unknown yet.

Similar to AP-1, we found an activation of NFκB associated with development of artesunate resistance in MDA-MB-231

Table 2. Analysis of NFκB binding sites in the apoptosis genes.

Promoter Analysis

Gene	ENSEMBL ID	Chr.	Transcription factor binding site	Position relative to transcription start	Orientation	Z-Score
BAX	ENSG00000087088	19	GGGCCTGCCC	354	−1	7.01e+00
BCL2	ENSG00000171791	18	GGGACTTCCA	3745	−1	9.80e+00
			GGGACTTCCA	2966	−1	9.80e+00
			GGCAATTTAC	650	1	7.04e+00
			GGCAATTTAC	−130	1	7.04e+00

The promoter regions of BAX and BCL2 from −5000 to +2000 relative to the transcription start site were analyzed for the occurrence of NFκB binding sites using the Opossum web-service. The identity of the sequence used for analysis (ENSEMBL ID), the chromosome number (Chr.), the actual transcription factor binding site, the position relative to the transcription start site and the orientation are indicated. The Z-score uses the normal approximation to the binomial distribution to compare the rate of occurrence of a TFBS in the target set of genes to the expected rate estimated from the pre-computed background set. The likelihood that the number of TFBS nucleotides detected for the included target genes was significant as compared with the number of TFBS nucleotides detected for the background set. Z-score was expressed in units of magnitude of the standard deviation.
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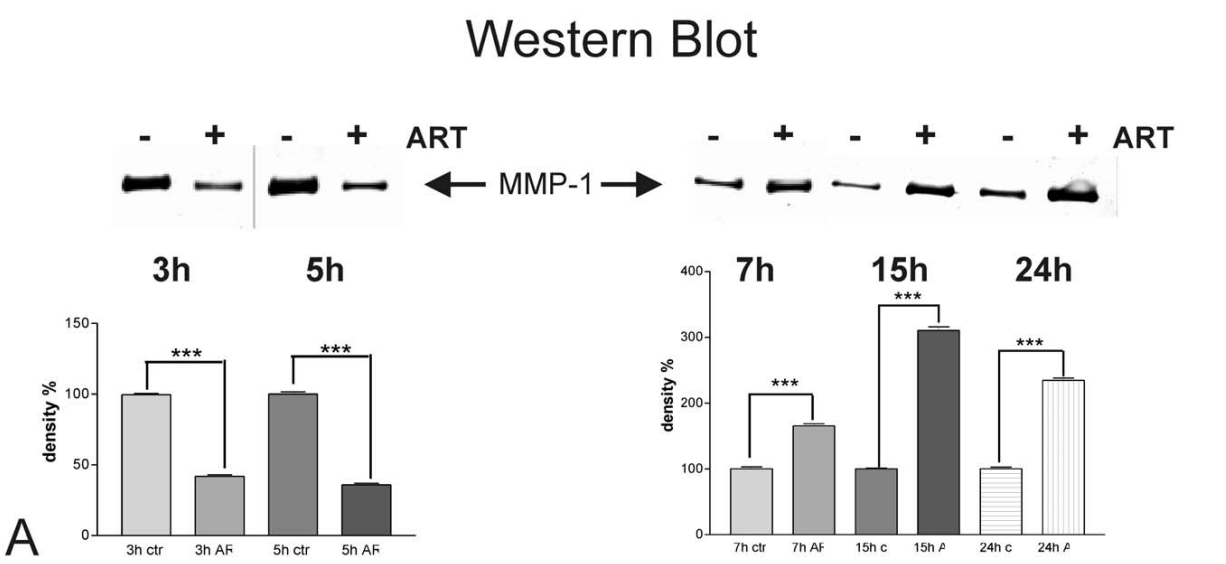
Western Blot
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MMP-1
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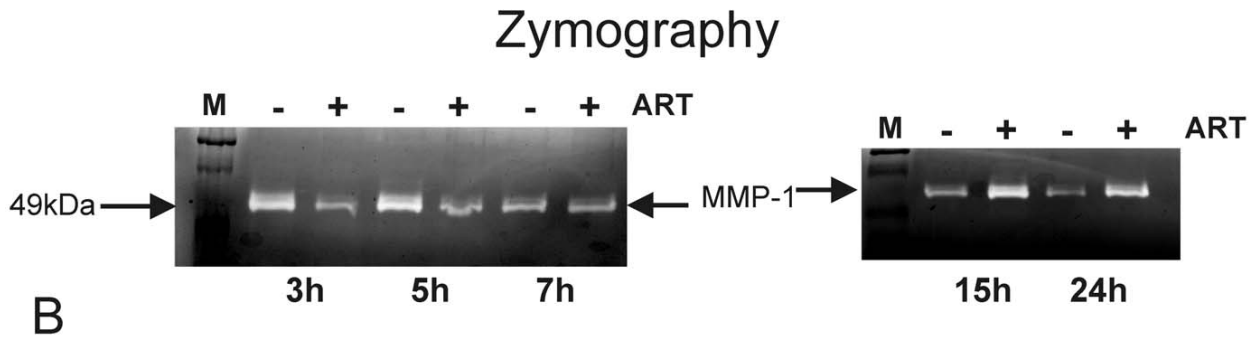
Zymography
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49kDa
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3h
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15h
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B

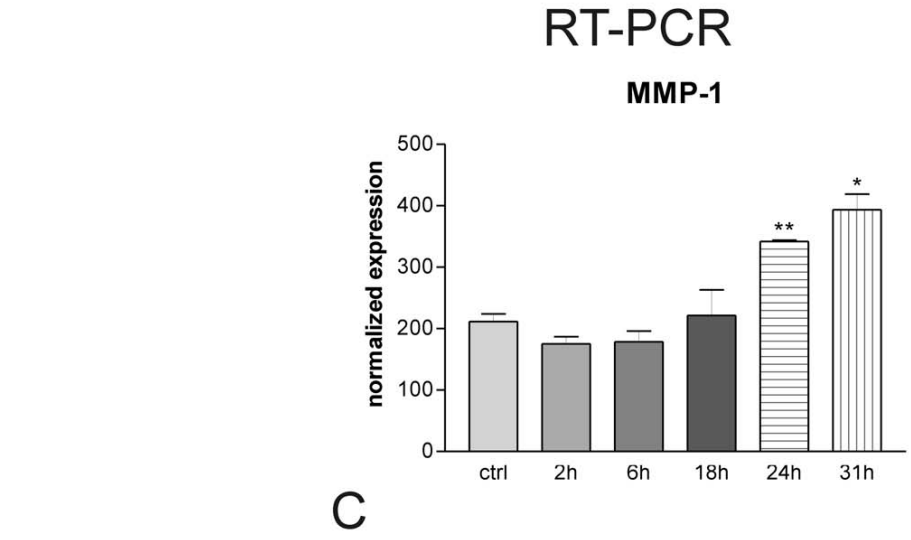
RT-PCR
MMP-1
normalized expression
ctrl
2h
6h
18h
24h
31h
C

Figure 6. Effect of artesunate on MMP-1 expression and activity. A) Western Blots of conditioned media from MDA-MB-231 cells treated with 20 μM artesunate for several time periods (lanes indicated with "+") in comparison to carrier-treated control cells (lanes indicated with "−") using specific antibodies against MMP-1 reveal that artesunate inhibited MMP-1 secretion when applied for shorter time periods (3 h, 5 h, left panel), while it induced MMP-1 when applied for longer time periods (7, 15 and 24 h, right panel). According to densitometrical analysis of the bands (lower panels), down-regulation of MMP-1 protein secretion in response to artesunate treatment, seen for early time points (3 and 5 h, lower left panel), was about 60–65% and up-regulation was about 1.6-fold after 7 h, 3-fold after 15 h and 2.3-fold after 24 h of artesunate treatment (lower right panel). *** $P<0.001$ (one way Anova with Bonferroni's post test). B) Casein Zymography (analysis of MMP-1 activity) was in accord with the results obtained from Western Blot analysis and showed a reduction of proteolytic activitiy present in conditioned media from MDA-MB-231 cells treated for 3 and 5 h with 20 μM artesunate (ART) (lanes indicated with "+") as compared to those of carrier-treated cells (lanes indicated with "-"). In contrast, cells treated for longer periods (15 and 24 h) with artesunate (lanes indicated with "+") showed a clear induction of MMP-1 as depicted here in Fig. 5A, right panel. Interestingly, treatment with artesunate for 7 h did not result in any differences in MMP-1 secretion into the culture medium of MDA-MB-231 cells when compared to carrier-treated cells (lanes indicated with "−"). MMP-1 was identified according to its migration behavior as compared to a molecular mass standard (lanes indicated with M). C) Quantification of MMP-1 mRNA expression by real time RT-PCR of MDA-MB-231 breast cancer cells treated with 20 μM artesunate for several time periods revealed a statistically significant induction of MMP-1 only after 24 up to 31 h treatment. Shorter treatment periods did result in a slight reduction of MMP-1 transcripts. Experiments were performed in triplicate; error bars indicate SD; * $P<0.05$; ** $P<0.01$ (student's t-test).
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cancer cells. In immune cells, artesunate-type drugs have been described to inhibit NFκB activation causing immunomodulatory effects [37,38,39]. On the other hand, NFκB activation has been reported as potential mechanism of gemcitabine resistance in MCF-7 and MDA-MB-231 breast cancer cells [40]. These results are comparable to our here presented data on artesunate resistance in MDA-MB-231 cells.

Both, NFκB and AP-1 are known to regulate apoptosis-regulating *bcl-2* family members [41]. Dimerization of *bax* with other pro-apoptotic *bcl-2* family members leads to the formation of mitochondrial membrane pores enabling cytochrome C release into the cytosol and onset of cell death. Anti-apoptotic *bcl-2* family members inhibit pore formation and cytochrome C release by binding to *bax*.

The identification of multiple *bcl-2* homologues many of which form homo- or heterodimers suggests that these molecules function at least in part through protein-protein interactions. *bax* heterodimerizes with *bcl-2* and homodimerizes with itself. When *bax* is over-expressed in cells, apoptotic death in response to a death signal is accelerated. When *bcl-2* is over-expressed, it heterodimerized with *bax* and death is repressed [42]. Thus, the ratio of *bcl-2* to *bax* is important in determining susceptibility to apoptosis.

Therefore, we analyzed the expression of the pro-apoptotic and anti-apoptotic genes after treatment with artesunate. The expectation was that expression levels of pro-apoptotic genes will decrease and of anti-apoptotic genes will increase during development of resistance. *Bcl-2* expression increased upon challenge with artesunate. This indicates that *bcl-2* may have a

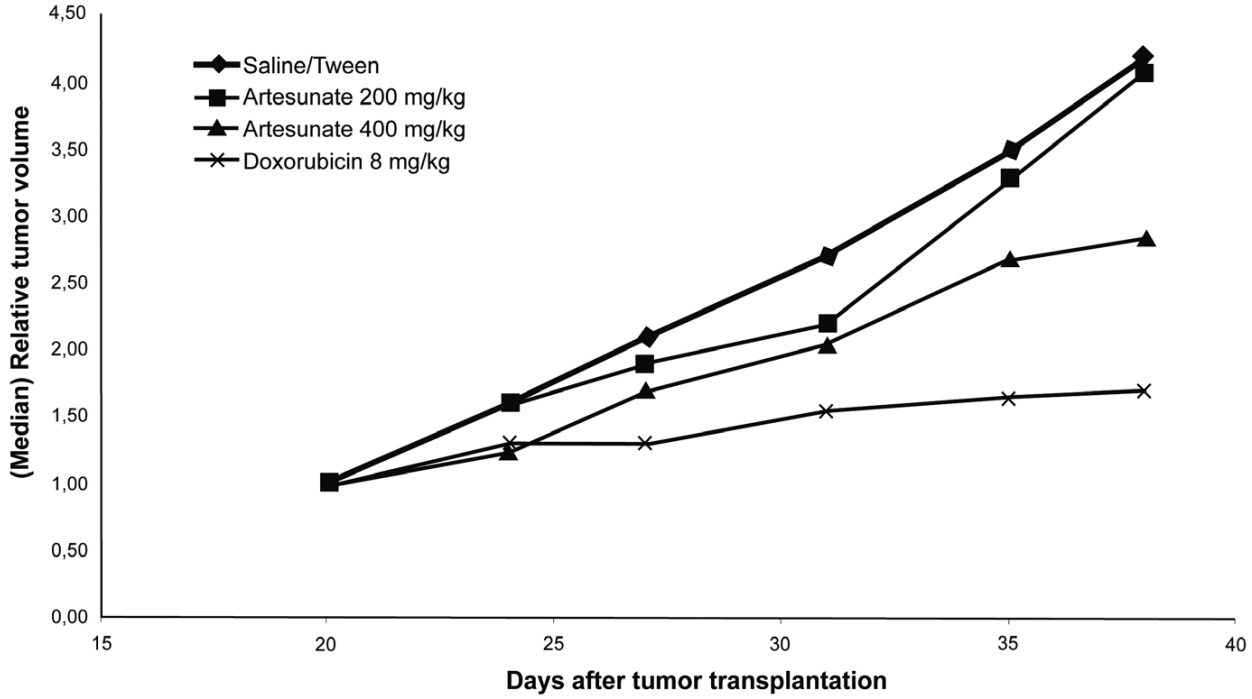

Figure 7. Activity of artesunate towards MDA-MB-231 xenograft tumors in nude mice. Xenografted mice were either treated i.p. with vehicle (10% Tween 80 in saline) or with 200 and 400 mg/kg artesunate, respectively, at five consecutive days. In addition, a control group was treated with doxorubicin 8 mg/kg i.v.) twice in a weekly distance. The relative tumor volume over time is shown of each six animals per treatment group.
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specific function for induction of artesunate resistance. Among the pro-apoptotic genes, *bax* expression decreased after artesunate treatment suggesting that down-regulation of *bax* participated in development of artesunate resistance. The stable expression of bax at 18 and 24 h may be explained by a rheostat of anti-and pro-apoptotic bcl-2 family members, of which bax is only one pro-apoptotic factor. It cannot be excluded that other pro-apoptotic members are also down-regulated supporting the function of bax. Functional analysis of apoptosis by detection of nucleosomes in cytoplasmic fractions of MDA-MB-231 cells treated with various concentrations of artesunate in comparison to MDA-MB-231 cells pre-treated with artesunate revealed that artesunate induced apoptosis only in cells that have not been pre-treated. These results strongly suggest that MDA-MB-231 cells acquire resistance against cell death. *Bcl-2* is up-regulated and *bax* is down-regulated upon treatment with artesunate, though transiently. However, both share similar TF-binding sites, especially sites for NFκB. The differential regulation can therefore not simply be explained by enhanced NFκB activity following ART treatment. *bax* is also under control of p53 which might be induced by artesunate induced double strand breaks. MDA-MB 231 cells have a mutation in exon 5 of the p53 gene (www-p53.iarc.fr) leading to an almost non functional transcriptional activity conserving, however, some activity on the *bax* promoter [43]. It is conceivable that NFκB has an immediate survival effect mediated by *bcl-2* with a potential feedback via *bax*, with the latter being dependent on other factors such as p53. Moreover, *bcl-2* has two bona fide binding sites and *bax* only one; kinetics or the strength of induction might therefore vary between the two genes. It should however be taken into account that NFκB affects other signaling routes (*e.g.* JNK phosphorylation) as well to regulate drug-induced apoptosis [44].

The human metastatic breast cancer cells MDA-MB-231 are a well-known model for studying tumor aggressiveness, invasion and metastasis. Matrix metalloproteinases (MMP), especially MMP-1 that is able to degrade fibrillar native collagen type I, are involved in the metastatic process, and also in the inhibition of apoptosis and drug resistance [45,46]. Likewise it has been shown that multidrug resistant cell lines produced more MMP-1, -2 and -9 compared to their analogous non resistant cells [47].

Therefore, we also analyzed the effect of artesunate on MMP expression and observed that for short treatment periods artesunate transiently down-regulates MMP-1 on the levels of expression, secretion and enzymatic activity in metastatic MDA-MB-231. At later time points, when the cells have already acquired resistance, MMP-1 expression, secretion and activity are induced upon artesunate treatment. The induction of MMP-1 is well in line with the activation of the tumor progression-associated transcription factors NFκB and AP-1, which are induced upon artesunate treatment as evidenced here by EMSA analyses. It has been reported that both NFκB and AP-1 binding sites are present in the promoter region of MMP-1 [48,49,50] and enhanced production of MMP-1 is associated with a more aggressive tumor growth, a higher metastatic potential, and poor clinical outcome of malignant tumors [51,52,53].

In conclusion, treatment of MDA-MB-231 cells with artesunate results in development of resistance, which was associated by NFκB and AP-1-mediated apoptosis resistance with up-regulation of the anti-apoptotic *bcl-2* and down-regulations of the pro-apoptotic *bax* genes. The development of resistance towards artesunate may have important implications for the application of this drug in cancer chemotherapy.

Supporting Information

Table S1 Primer Sequences.
(DOC)

Author Contributions

Conceived and designed the experiments: BB UP IF TE. Performed the experiments: BB IF PHK EK UP. Analyzed the data: BB IF UP TE. Contributed reagents/materials/analysis tools: BB IF UP TE. Wrote the paper: BB TE.

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