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Der konträre Einfluss von p53 auf die extrinsische und intrinsische Apoptoseinduktion in Tumorzellen

Dissertation
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1. Einleitung

1.1 Hintergrund

Nach den Krankheiten des Herzkreislaufsystems sind bösartige Neubildungen die zweithäufigste Todesursache in westlichen Ländern. So starben in Deutschland im Jahr 2011 221 591 Menschen an einer Krebserkrankung[1]. In den letzten 5 Jahrzehnten wurde durch die Optimierung der chirurgischen Tumorresektionstechniken, Einführung von Strahlentherapie, Entwicklung der zytostatischen Chemotherapie und zielgerichteter Therapien das rezidivfreie Überleben von Patienten mit malignen Erkrankungen bedeutend verlängert. Bei fortgeschrittener und metastasierter Tumorerkrankung hat sich die Prognose insgesamt nur minimal verbessert und ist, trotz optimierter Chemotherapie, nicht zufriedenstellend. Auch die neuen zielgerichteten Therapien konnten die Hoffnungen nicht erfüllen und in dieser Patientengruppe noch nicht den endgültigen Durchbruch erzielen. Weitere Herausforderungen der Krebstherapie sind primäre Resistenzen, die Entwicklung von Resistenzen gegen konventionelle Zytostatika und Tumorzellen, die sich in Seneszenz befinden und für ein Rezidiv der Tumorerkrankung verantwortlich sind. Die Kenntnis der Signalwege des Zelltodes und die Wechselwirkung dieses Signalnetzwerks sind für die Optimierung der Kombination konventioneller Zytostatika und für die Entwicklung neuer zielgerichteter Therapien von enormer Bedeutung. Zu beachten hierbei ist, dass zur Optimierung der Therapien nicht nur das Verständnis des Wirkmechanismus der Einzelsubstanz bedeutend ist, sondern vielmehr Kenntnisse über die Vorgänge und Interaktionen bei der Kombination von mehreren Zytostatika erlangt werden müssen.

1.2 Apoptose – programmierter Zelltod

Die Apoptose ist eine Form des programmierten Zelltods und bedeutend für die embryologische Entwicklung von Organen, die Gewebshomöostase und ist letztlich der Mechanismus der physiologischen Kontrolle des Wachstums. Apoptose eliminiert nutzlose, alte und gefährliche Zellen (Kerr JF et al., 1972). Apoptose ist, ebenso wie die Mitose, ein regulierter physiologischer Prozess, der nach einem festen Schema verläuft. Apoptotische Zellen verlieren die Adhäsion zu ihrem Zellverband, runden sich ab und schrumpfen. Die Zellmembran und Kernmembran stülpen sich ein und schnüren einzelne Zellteile voneinander ab („Membrane Blebbing“). Das Chromatin kondensiert und es folgt die Fragmentierung des Zellkerns, die DNA und intrazelluläre Proteine und Strukturen werden in Stücke zerlegt (Wyllie AH et al., 1980). Die so entstandenen apoptotischen Körperchen werden von Makrophagen oder Nachbarzellen phagozytiert. Apoptose ist unschädlich für die

[1] Statistisches Bundesamt https://www.destatis.de/DE/ZahlenFakten/GesellschaftStaat/Gesundheit/Todesursachen/Tabellen/EckdatenTU.html, abgerufen am 08.07.2013 um 19.15Uhr

Umgebung der Zelle und löst keine Entzündungsreaktion aus (Kerr JF et al., 1972 und Hengartner MO, 2000). In der Medizin ist die Apoptose zur Therapie von onkologischen Erkrankungen relevant. Ein bedeutender Regulator der Apoptose ist p53. Die Aktivierung von p53 und die Induktion der Apoptose ist sowohl in der konventionellen Therapie maligner Erkrankungen als auch in den neuen zielgerichteten Therapien ausschlaggebend (Kaufmann SH et al., 2000 und Herr I et al., 2001). Um Krebstherapien zu optimieren, müssen jedoch die Funktion der Regulatoren der Apoptose sowie die Signalwege der Apoptose bekannt sein.

1.3 Signalwege der extrinsischen und intrinsischen Apoptose

Die extrinsische Apoptose wird aktiviert, nachdem ein Todesligand, z.B. TRAIL (**T**umor necrosis factor **R**elated **A**poptosis **I**nducing **L**igand) an den Todesrezeptor der Zelloberfläche bindet und über die Caspasenkaskade oder das Mitochondrium Apoptose induziert (Walczak H et al., 2000). TRAIL ist eine vielversprechende zielgerichtete neue Therapie, da es Apoptose selektiv in malignen Zellen induziert, ohne systemische Toxizität zu zeigen (Wiley SR et al., 1995). Im Gegensatz zu konventionellen Zytostatika, die intrinsisch über das Mitochondrium Apoptose induzieren, induziert TRAIL über die Todesrezeptoren DR4 und DR5 extrinsisch Apoptose (Abb.:1, links). Nachdem TRAIL an den Rezeptor bindet, trimerisiert dieser. Über die Todesdomäne des Rezeptors und eine homophile Bindung wird die FAS-associated-Death-Domain (FADD) rekrutiert (Walczak H et al., 2000). FADD rekrutiert an diesem aktivierten Rezeptor Caspase 8 und 10, somit ist der CD95 death-inducing signaling complex (DISC) gebildet. Caspase 8 aktiviert direkt die Effoktorcaspase 7 und 3 oder führt über die Freisetzung von pro-apoptotischen Proteinen aus dem Mitochondrium zum programmierten Zelltod (Scaffidi C et al., 1998).

Zytostatika induzieren im Wesentlichen intrinsische Apoptose (Abb.:1, rechts) über die Freisetzung von pro-apoptotischen Proteinen aus dem Intermembranraum des Mitochondriums. Dieser Vorgang führt zur Aktivierung von Effektorcaspasen (Saelens X et al., 2004). Zwischen intrinsischer und extrinsischer Apoptose Induktion gibt es allerdings keine strikte Trennung, z.B. aktivieren manche Zytostatika auch Caspase 8 innerhalb der intrinsischen Signalkaskade – eigentlich ein Protein der extrinsischen Kaskade (Saelens X et al., 2004).

Die Induktion der Apoptose ist vielfältig reguliert. BCL-2 und BCL-XL wirken anti-apoptotisch, indem sie die Freisetzung von pro-apoptotischen CytochromC aus dem Intermembranraum des Mitochondriums hemmen. (Braun F et al., 2013). Die Inhibitoren der Apoptose (IAPs) inhibieren die Caspasen-3, -7 und die Procaspase-9 (Deveraux QL et al., 1999). Die IAPs wiederrum werden durch Smac/Diabolo antagonisiert. Smac / DIABLO ist ein pro-apoptotisches mitochondriales Protein, das in das Zytosol als Reaktion auf diverse

apoptotischen Stimuli, einschließlich gebräuchlichen Chemotherapeutika, freigesetzt wird (Martinez-Ruiz G et al., 2008). Ein weiterer Regulator ist Omi/HtrA2, eine Serin-Protease, die ebenfalls Inhibitoren der Apoptose (IAPs) antagonisiert (Yang QH et al., 2003).

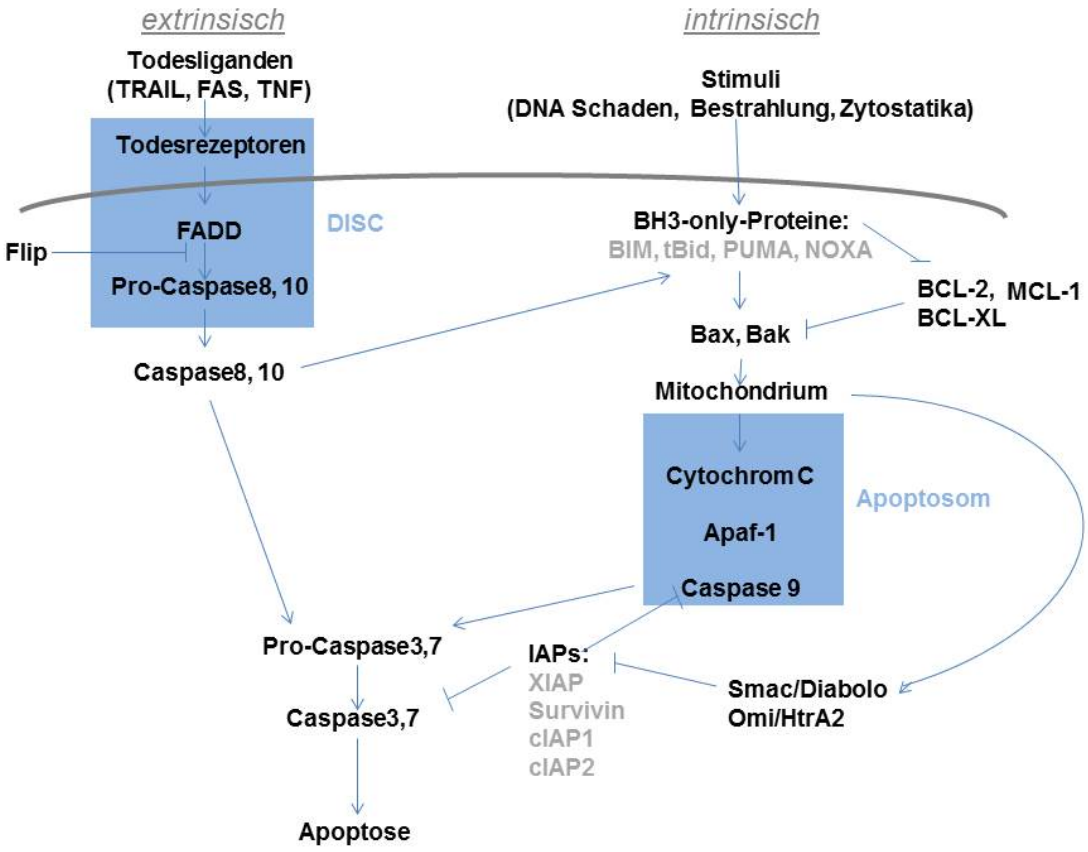

Abbildung 1: Schematisches Diagramm der Apoptose Signalwege
Die linke Seite zeigt den extrinsischen Apoptose Signalweg, die rechte Seite die intrinsische Apoptose Signalkaskade. Die beiden Signalwege treffen sich an der Stelle der Caspasenkaskade. DISC und Apoptosom sind in blau hervorgehoben.

modifiziert nach: Guangwu Xu, Yufang Shi: Apoptosis signaling pathways and lymphocyte homeostasis. Cell Research (2007) 17:759-771.

1.4 Die Vielfältigkeit von p53 – dem Wächter des Genoms

p53 wurde 1979 entdeckt (Lane DP et al., 1979 und Linzer DI et al., 1979) und ist das am besten untersuchte Apoptoseprotein. p53 ist vor allem bekannt als Tumorsuppressor, in dieser Funktion induziert p53 Zellzyklusarrest oder Apoptose in prämalignen Zellen. Der sogenannte Wächter des Genoms schützt den Körper vor der Entstehung maligner Erkrankungen. p53 kontrolliert die Transkription von verschiedenen pro-apoptotischen Proteinen (Abb.:2), so zum Beispiel PUMA, Caspase 8, BAX, NOXA und DR5. Die Aktivierung von p53 ist auf transkriptionaler Ebene, aber auch durch posttranskriptionelle

Modifikationen wie Phosphorylierung gesteuert. Erst in letzter Zeit tritt die diverse Bedeutung von p53 mehr in den Mittelpunkt der Forschung. p53 reguliert eine Vielzahl von Funktionen der Zelle (Abb.:3), darunter Apoptose, Zellzyklusarrest, zelluläre Seneszens, DNA Reparatur, Angiogenese und Migration. Zudem hat p53 auch Einfluss auf den Metabolismus der Zelle, Autophagie, Stammzellerneuerung, Embryogenese und auf Vorgänge in der Fertilität und Immunität des Organismus (Hollstein M et al., 2010 und Vousden KH et al., 2009).

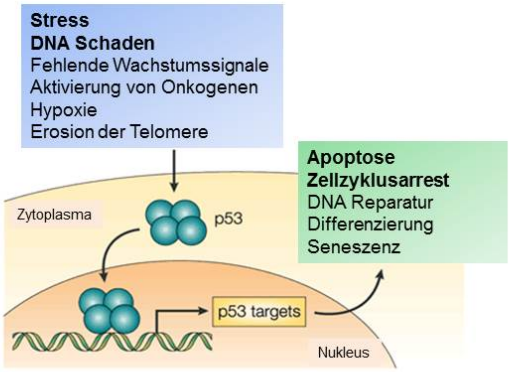

Abbildung 2: p53 als Transkriptionsfaktor
In Abwesenheit von Stressfaktoren ist die Konzentration von p53 in der Zelle sehr gering. In der Folge von DNA Schäden wird p53 durch Phosphorylierung stabilisiert und akkumuliert. p53 bindet als Tetramer und induziert die Transkription von verschiedenen p53 Zielgenen. So reguliert p53 den Zellzyklus, die Apoptose, DNA Reparaturmechanismen, Differenzierung und Seneszenz.
modifiziert nach: Patrick Chène Nature 2003 Reviews Cancer 3: 102-109.

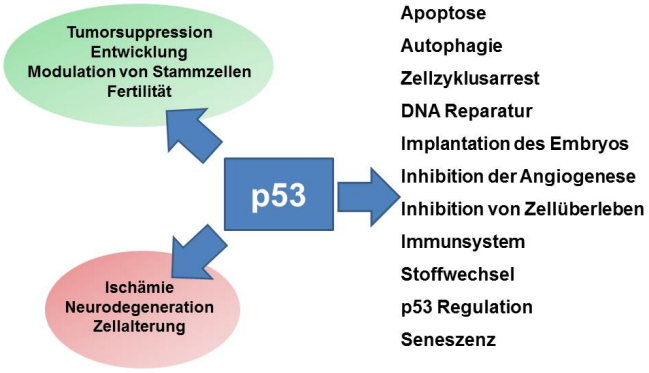

Abbildung 3: Die vielfältigen Funktionen von p53
p53 ist neben der bekannten Rolle als Wächter des Genoms in viele weiter physiologische Prozesse der Zelle involviert.
modifiziert nach: Vousden KH, Prives C. Blinded by the Light: The Growing Complexity of p53. Cell. 2009 May 1;137(3):413-31

1.5 Stabilisierung von p53 als Ansatzpunkt neuer Therapiemöglichkeiten

Bis vor wenigen Jahren war die wissenschaftliche Lehrmeinung, dass p53 in Wildtypkonformation immer pro-apoptotisch wirkt, während mutiertes p53 immer als ein Faktor der Entstehung maligner Zellen galt. Erst Studien der letzten Jahre zeigen die Komplexität der Apoptoseregulation durch p53. So kann p53 den Zelltod auch verhindern (Vousden KH et al., 2009, Jänicke RU et al., 2008, DeBerardinis et al., 2008). Aus den Erkenntnissen der Forschung über die pro-apoptotische Funktion von p53 hat sich die Stabilisierung von p53 als Ansatzpunkt neuer Therapiemöglichkeiten entwickelt, dabei sollten neue Erkenntnisse über die anti-apoptotische Wirkung von p53 berücksichtigt werden.

Dieser Therapieansatz entwickelte sich auch aus der Idee, dass Mutationen in den Signalwegen von p53 zur Tumorentstehung führen. In der Mehrzahl der Krebspatienten ist p53 entweder selbst mutiert oder Gene, die Signalproteine des p53 Netzwerkes kodieren, sind mutiert (Stiewe 2007 und Vousden KH et al., 2007). Bei zellulären Stress oder DNA Schaden induziert p53 entweder

Apoptose oder führt zu einem Arrest im Zellzyklus, um der Zelle Zeit für DNA Reparaturmechanismen zu geben. Die Wiederherstellung von funktionellem p53 oder die Aktivierung von p53 ist ein vielversprechender Ansatz der neuen zielgerichteten Therapien (Rozan LM et al., 2007 und Petitjean A, et al. 2007). Die Steigerung des p53 Expressionslevels durch direkte und indirekte Stabilisierung von p53 sowie die selektive Modulation von mutierten p53 hat sich in Studien als vielversprechend gezeigt (Mandinova A et al., 2011 und Cheok CF et al., 2011). Doch wurde in anderen Studien beschrieben, dass die Aktivierung von p53 in der Tumortherapie hinderlich sein kann (Jänicke RU et al., 2008). Um zielgerichtete Therapien zu entwickeln, ist es wichtig, die vielfältigen Funktionen von p53 genau zu untersuchen, denn die Induktion der Apoptose ist nur eine von vielen Funktionen von p53.

2. Ziele dieser Arbeit

Die genaue Kenntnis der Signalwege der Apoptose ermöglicht es, Zytostatika sinnvoller als bisher zu kombinieren und neue zielgerichtete Medikamente einzusetzen. Ein zentraler Regulator der Apoptose ist p53.

In der folgenden Arbeit wurden für einzelne experimentelle Settings folgende Situationen untersucht:

I.) die Bedeutung des basalen p53 Status von Tumorzellen auf die extrinsische und intrinsische Apoptose

II.) die Folgen des durch p53 Aktivierung induzierten Zellzyklusarrests auf die Apoptoserate

III.) der Effekt der Aktivierung von p53 im Rahmen der Therapie mit Zytostatika-Kombinationen

Da p53 ein zentraler Regulator ist und vielfältige und auf den ersten Blick teils widersprüchliche Funktionen ausübt, ist die genaue Kenntnis von p53 und der Interaktion von p53 im Apoptose-Netzwerk eine Möglichkeit für weitere Therapieverbesserungen, die letztlich ein verbessertes Überleben der Patienten mit malignen Erkrankungen ermöglichen.

3. Ergebnisse eigener wissenschaftlicher Arbeiten

3.1 Arbeiten zur kumulativen Promotionsleistung

3.1.1 Die Bedeutung des alterierten basalen p53 Status auf die extrinsische und intrinsische Apoptoseinduktion

Wachter F, Grunert M, Blaj C, Weinstock DM, Jeremias I, Ehrhardt H: **Impact of the p53 status of tumor cells on extrinsic and intrinsic apoptosis signaling**. Cell Commun Signal. 2013 Apr 17;11(1):27.

Die bisherige Forschung fokussierte sich vor allem auf die positiven Effekte der p53 Aktivierung, nach Präinkubation mit Zytostatika oder anderer direkter p53 Stimuli, auf die Wirksamkeit einer zweiten Substanz, wie z.B. TRAIL (Zhang L et al., 2000, Ehrhardt H et al., 2011, und Rozan LM et al., 2007).

In der Arbeit „Impact of the p53 status of tumor cells on extrinsic and intrinsic apoptosis signaling“ wurde die Bedeutung des alterierten konstitutiven p53 Status auf die extrinsische und intrinsische Apoptoseinduktion untersucht. Dazu wurden verschiedene molekulare Herangehensweisen gewählt, die bereits bei Untersuchungen über die Bedeutung von mutierten p53 für die Tumorentstehung verwendet wurden (Olive KP et al., 2004 und Liu G et al., 2000), nämlich

I.) die Expression von p53 in wildtyp oder mutierter Konformation in Tumorzellen mit Hilfe der lentiviralen Überexpression durch den pCDH Vektor. Folgende fünf Mutationen wurden mittels Mutagenese eingefügt: p53 V143A zeigt eine reduzierte Stabilität des Proteins, p53 R248W und R273H haben einen Defekt in der DNA Bindung zur Folge und p53 R175H und p53 R249S sind strukturell instabil (Grafik 2 und Zusatzgrafik1: Grafik S2, Publikation1)

II.) die Herunterregulierung (knockdown) von p53 durch RNA Interferenz

III.) die Verwendung von Tumorzellpaaren mit p53 Expression und mit dem somatischen knock-out von p53.

Die basale Expression von p53 inhibiert die extrinsische Apoptose Induktion durch TRAIL

Um die Bedeutung von p53 auf die extrinsische Apoptose Induktion durch TRAIL zu untersuchen verwendeten wir HCT-116 Zellen, die p53 in Wildtypkonfiguration exprimieren und Zellen mit somatischen knock-out von p53 (diese wurden von Bert Vogelstein, Johns Hopkins University bezogen). Um die Daten zu validieren, wurde zusätzlich ein Rettungsexperiment durchgeführt und p53 in Wildtypkonfiguration in HCT-116 p53-/- Zellen re-exprimiert. Die Apoptose Induktion durch TRAIL war höher in den HCT-116 Zellen, die kein p53 exprimierten (Grafik 1A, Publikation 1). Die gesteigerte Apoptose Sensitivität war nicht verursacht durch Proteine des TRAIL Apoptose Signalweges (Grafik 1C, Publikation1). Auch auf Rezeptorebene konnte keine Änderung der Expression der Todesrezeptoren DR4

und DR5 festgestellt werden (Grafik 1B, Publikation 1). Auch die Verteilung im Zellzyklus und das spontane Wachstum wurde nicht durch p53 beeinflusst (Grafik 1D und E, Publikation 1). Erstaunlicherweise konnte somit gezeigt werden, dass basal exprimiertes p53 die Apoptose Induktion durch TRAIL inhibiert.

Der Einfluss von häufigen p53 Mutationen auf die extrinsische und intrinsische Apoptoseinduktion

Zunächst untersuchten wir den Einfluss von mutiertem p53 auf die intrinsische Apoptoseinduktion, wie sie unter anderem durch Zytostatika eingeleitet wird. Wie bereits publiziert erhöhte die Expression von p53 Wildtyp die, durch Doxorubicin und 5-Fluorouracil induzierte (intrinsische) Apoptose in HCT-116 Zellen; hingegen zeigte die Expression von mutierten p53 keine Apoptose-Sensitivierung (Grafik 2A, Publikation1). Nach Stimulation der HCT-116 Zellen mit TRAIL wiesen jedoch alle p53 Mutanten eine ähnliche Reduktion der Apoptoseinduktion wie der p53 Wildtyp auf (Grafik 2B, Publikation 1). In SHEP Zellen konnte der gleiche Phänotyp gezeigt werden.

Zusammenfassend konnten bereits publizierte Daten der intrinsischen Apoptose bestätigt werden und erstmals gezeigt werden, dass extrinsische Apoptose unabhängig von der p53 Konformation abläuft.

Die Heterogenität von p53 in der extrinsischen Apoptose

Um den beschrieben Sachverhalt weiter zu erforschen, wurden die Apoptoseinduktion durch TRAIL in n=12 Tumorzelllinienpaaren untersucht. Ein Partner exprimierte p53, in der anderen Partnerzelllinie wurde p53 durch RNA Interferenz herunterreguliert oder es handelte sich um einen somatischen knock-out. Dabei zeigte sich bei n=5 Tumorzelllinien, dass die Expression von p53 die Apoptoseinduktion durch TRAIL verminderte, bei n=5 hatte das Expressionslevel von p53 keinen Einfluss auf die Apoptose durch TRAIL und nur bei n=2 war die Apoptoseinduktion durch TRAIL in den Zellen, die p53 exprimieren, höher als in den Kontrollzellen (Grafik 3A, Grafik 3B und Additional file 1: Grafik S3, Publikation 1). Die verminderte Apoptoseinduktion durch TRAIL in Anwesenheit von p53 wurde in soliden, hämatopoetischen und mesenchymalen Tumoren gezeigt. Zusätzlich wurde dieser Phänotyp sowohl in Zellen mit p53 wildtyp Konfiguration beobachtet, als auch in Zellen mit mutiertem p53 (Grafik 3C, Publikation1). Der Expressionslevel von p53 variierte in den verschiedenen Zelllinien und zeigte keinen Einfluss auf die Apoptosesensitivität durch TRAIL (Grafik 3D, Publikation 1).

Um die klinische Relevanz der Apoptoseinduktion durch TRAIL in Abhängigkeit von p53 zu zeigen, führten wir Untersuchungen an Patientenproben durch. Dazu verwendeten wir die Zellen von Patienten mit akuter leukämischer Leukämie (ALL), die in Nod-Scid-Gamma-

Mäusen passagiert worden waren. Durch die Verwendung der Patientenzellen wird das Anhäufen von in-vitro Spontanmutationen reduziert, wie es in etablierten Zelllinien unvermeidlich ist (Peller S et al., 2003, Sandberg R et al., 2005, Greshock et al., 2007 und Shultz LD et al., 2007). Das Level der p53 Expression wurde, durch eine kürzlich in meinem Labor entwickelte Methode zur Herunterregulierung von Proteinen mittels RNA Interferenz in Patientenproben, variiert (Ehrhardt H et al., 2011, Ehrhardt H/Höfig I et al., 2012 und Höfig I et al., 2012). In den Patientenproben konnten ähnliche Ergebnisse wie in den Zelllinien dargestellt werden (Grafik 4B, Publikation 1). Allerdings unterschied sich das basale Expressionsniveau von p53 weniger als in den Zelllinien (Grafik 4D, Publikation 1). Zusammenfassend bestätigten die Experimente mit den Tumorzellen der Patientenproben die Ergebnisse der Zelllinien.

Der Einfluss von p53 auf die extrinsische Apoptoseinduktion zeigte sich als komplex, und ist nicht direkt mit klassischen Kategorien wie Therapiesensitivität, Ätiologie des Tumors oder p53 Mutationen der Zelle erklärbar. Der Einfluss von p53 auf die Apoptoseinduktion durch TRAIL ist von der jeweiligen Tumorzelle abhängig.

Bisherige Forschung untersuchte lediglich den Einfluss der p53 Aktivierung auf die extrinsische Apoptose, in unserer Arbeit konnte erstmals gezeigt werden, dass die basale Expression von p53 Einfluss auf die Apoptoseinduktion durch TRAIL hat. Diese Daten bedeuten, dass die Restoration von p53 nicht immer von Vorteil ist. Zukünftige Therapien sollten die Komplexität von p53 und die Diversität der Tumorzellen berücksichtigen.

Eigener Anteil der Arbeit:

Im Wesentlichen wurden alle Experimente von mir durchgeführt, dabei wurde ich von Cristina Blaj, Michaela Grunert und Harald Ehrhardt unterstützt. Cristina Blaj hat im Rahmen ihrer Diplomarbeit einige Stimulationsexperimente durchgeführt und eine der p53 Mutanten hergestellt. Michaela Grunert hat mich in den molekularbiologischen Arbeitstechniken angeleitet. David Weinstock hat die Sequenzierung der Patientenproben durchgeführt. Irmela Jeremias hat die Entwicklung des Projekts mit wissenschaftlichen Beiträgen unterstützt. Unter der Supervision von Harald Ehrhardt habe ich die Versuche geplant und durchgeführt sowie die Daten ausgewertet, interpretiert und die Abbildungen erstellt.

3.1.2 Erhöhung der Apoptoseinduktion durch TRAIL über p53 Aktivierung induzierten Zellzyklusarrest

Ehrhardt H*, **Wachter F***, Grunert M, Jermias I: **Cell cycle-arrested tumor cells exhibit increased sensitivity towards TRAIL-induced apoptosis**. Cell Death Dis. 2013 Jun 6;4:e661.

***beide Autoren haben mit dem gleichen Anteil beigetragen**

Ruhende Tumorzellen stellen wegen ihrer schlechten Behandelbarkeit eine große Herausforderung für die Therapie maligner Erkrankungen dar. Diese seneszenten Tumorzellen sind verantwortlich für einen späteren Rückfall (Li Y et al., 2012, Vermeulen L et al., 2012, Mordant P et al., 2012 und Müller V et al., 2010).

In der Arbeit „Cell cycle-arrested tumor cells exhibit increased sensitivity towards TRAIL-induced apoptosis" zeigten wir, dass TRAIL induzierte Apoptose verstärkt wurde, wenn Doxorubicin über p53 Aktivierung Zellzyklusarrest induzierte. Um zu beweisen, dass dabei der Zellzyklusarrest selbst verantwortlich war, wurden folgende Experimente durchgeführt: Der Arrest des Zellzyklus in G0, G1 oder G2 wurde in Tumorzelllinien und in Patientenzellen, die in Mäusen passagiert worden waren („Xenograftzellen"), induziert. Dazu wurden Zytostatika, phasenspezifische Inhibitoren oder die Herunterregulierung von CyclinB und E mittels RNA Interferenz verwendet. Der biochemisch oder molekular induzierte Zellzyklusarrest erhöhte an jedem Punkt des Zellzyklus die Apoptose durch TRAIL. Sehr bedeutend für die klinische Translation ist, dass TRAIL auch in drei pädiatrischen Patientenproben mit B-Vorläufer-Leukämie und mit akuter lymphoblastischer T-Zellleukämie, die in Mäusen passagiert worden waren, nach molekularen Zellzyklusarrest vermehrt Apoptose induzierte.

Wir schließen aus diesen Daten, dass TRAIL ein potentiell geeignetes Therapeutikum gegen ruhende Tumorzellen darstellt und weiter entwickelt werden sollte. Im Rahmen der TRAIL induzierten Apoptose sind die Aktivierung von p53 und der damit folgende Zellzyklusarrest von Vorteil für die Behandlung der Patienten.

Eigener Anteil der Arbeit:

Alle wesentlichen Experimente wurden von mir persönlich durchgeführt, Harald Ehrhardt führte die Transfektionen der Xenograftzellen durch. Im Rahmen der Revision wurde ich von meinen Koautoren unterstützt. Michaela Grunert leitete mich bei den molekularbiologischen Techniken an, so dass ich diese dann selbst durchführen konnte. Unter Supervision von Harald Ehrhardt führte ich die Versuchsplanung durch sowie die Interpretation und Auswertung der Daten. Irmela Jeremias beteiligte sich durch wissenschaftliche Diskussionen.

3.1.3 p53 Aktivierung als Inhibitor Vincristin induzierter Apoptose

Ehrhardt H, Schrembs D, Moritz C, **Wachter F**, Haldar S, Graubner U, Nathrath M, Jeremias I: **Optimized anti-tumor effects of anthracyclines plus vinca alkaloids using a novel, mechanism-based application schedule.** Blood. 2011 Sep 16;118(23):6123-31.

Monotherapien sind in der Krebstherapie wenig effektiv, deshalb werden maligne Erkrankungen in der Regel mit einer Kombination mehrerer Zytostatika behandelt (Dy GK et al., 2008, Frei E et al., 1985 und Ramaswamy S et al., 2007). In den Behandlungsplänen maligner hämatopoetischer Erkrankungen werden Vincaalkaloide und Anthrazykline am gleichen Tag verabreicht. Diese Strategie wurde in klinischen Studien implementiert und zeigte eine Überlegenheit gegenüber der Gabe von Vincaalkaloiden alleine, allerdings wurden andere Applikations-Schemata als die simultane Gabe niemals getestet (Frei E et al., 1972 und Frei E et al., 1974). Die gemeinsame Gabe beruht lediglich auf Tierexperimenten der späten 1970er Jahren, die nur an Maus und Rattentumorzellen durchgeführt wurden (Goldin A et al., 1975 und Zeller WJ et al., 1979).
Wir zeigten in der Veröffentlichung „Optimized anti–tumor effects of anthracyclines plus vinca alkaloids using a novel, mechanism-based application schedule“, dass bei gleichzeitiger Applikation Doxorubicin die Apoptoseinduktion durch Vincristin inhibierte. Der antagonistische Effekt war auf 83% der 18 getesteten Zelllinien zu sehen (Grafik1A-E, Publikation3) sowie ebenfalls in einem in vivo Mausmodell (Grafik1F, Publikation3). Außerordentlich relevant für die klinische Anwendung ist, dass auch 34% der frischen ALL Patientenproben reduzierte Apoptose durch Vincristin zeigen, wenn Vincristin gemeinsam mit Doxorubicin verabreicht wurden (Grafik 2, Publikation3), obwohl die Patienten, von denen die Proben genommen wurden, genau diese Kombination in der Therapie ihrer Erkrankung erhielten. Der molekulare Signalweg konnte charakterisiert werden: Während bekannt ist, dass Doxorubicin p53 aktiviert und so Zellzyklusarrest induziert, beobachteten wir erstmals, dass in arretierten Zellen Vincristin die anti-apoptotische Funktion der anti-apoptotischen Mitglieder der Bcl-2-Familie nicht mehr inhibieren kann und somit keine Apoptoseinduktion durch Vincristin erfolgte (Grafik 4, Publikation3). Demzufolge benötigt Vincristin sich teilende Zellen, um die volle Apoptose induzierende Wirkung zu zeigen.

Eigener Anteil der Arbeit
Ich habe im Rahmen dieser Arbeit gezeigt, dass die Apoptose durch Vincristin in Zellen mit herunterregulierten CyclinA, die daraufhin im Zellzyklus inhibiert waren, ebenfalls reduziert ist. Im Rahmen dieses Experimentes wurde auch auf molekularer Ebene der oben beschriebene Phänotyp gezeigt (Abbildung 6D, Publikation 3).

3.2 Zusätzliche Publikationen im Rahmen der Promotionsarbeit

Ehrhardt H, Schrembs D, Pfeiffer S, **Wachter F**, Grunert M, Jeremias I: Activation of the DNA damage response by antitumor therapy counteracts the activity of vinca alkaloids. Anticancer Res. 2013 Dec;33(12):5273-87.

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Ehrhardt H, Höfig I, **Wachter F**, Obexer P, Fulda S, Terziyska N, Jeremias I: NOXA as critical mediator for drug combinations in polychemotherapy. Cell Death Dis. 2012 Jun 21;3:e327.

Ehrhardt H, **Wachter F**, Maurer M, Stahnke K, Jeremias I: Important role of Caspase-8 for chemo-sensitivity of ALL cells. Clin Can Res. 2011 Oct 18;17(24):7605-13.

4. Schlussfolgerung und Ausblick

Mechanistisches Verständnis und individuelle Therapie als Erfolgskonzepte der Krebstherapie

Aus der oben geschilderten Darstellung wird deutlich, dass es noch zusätzlicher Untersuchung in diesem Gebiet bedarf. Viele Funktionen des bereits 1979 entdeckten p53 sind noch unklar, obwohl p53 zu den am besten erforschten Proteinen gehört. p53 hat neben seiner Funktion als Wächter des Genoms noch viele weitere Funktionen im Stoffwechsel, der Immunität und der Fertilität. Doch auch die Aufgaben von p53 in der Kontrolle des Wachstums und der Zellteilung sind noch nicht endgültig geklärt. p53 ist bekannt als Wächter des Genoms, doch seine basale Expression oder seine Akkumulation verursachen kontext- und teils zellspezifisch pro- bzw. anti-apoptotische Wirkungen. Dies konnte in meinen eigenen und verschiedenen weiteren Arbeiten der Arbeitsgruppe klar herausgestellt werden. So inhibierte die basale Expression von p53 die Apoptoseinduktion durch TRAIL in einem Teil der Tumorzellen, während in einem anderen Teil die Apoptose-induzierende Wirkung verstärkt wurde. Die Aktivierung von p53 und der dadurch entstehende Zellzyklusarrest reduzierte die Apoptose durch Vincristin. Andererseits führte die Aktivierung von p53 durch Doxorubicin oder Bestrahlung zum Zellzyklusarrest und damit zur Sensitivierung der Apoptoseinduktion durch TRAIL.

Aktuelle präklinische und klinische Studien (Phase 1-3), die die Aktivierung von p53, sowie dessen Stabilisierung und Modulation untersuchen, sollten weitere Funktionen von p53 in Erwägung ziehen und kritisch hinterfragen, ob p53 Restitutio bzw. Aktivierung immer positiv für den Gesamteffekt ist.

Die Schlussfolgerung daraus ist, dass die Auswahl von Medikamenten basierend auf einem mechanistischen Verständnis ein neues und vielversprechendes Konzept für die Entwicklung potenter neuer Chemotherapieprotokolle darstellt.

Allerdings muss bedacht werden, dass das reine Verständnis des Wirkmechanismus eines Medikamentes nicht ausreicht. Im Rahmen der extrinsischen Apoptoseinduktion zeigt sich, dass die Regulation der Apoptose viel komplexer ist und der Erfolg der Therapie von der individuellen Tumorzelle abhängt. Die Erforschung weiterer molekularer Marker ist ein Grundbaustein, um in Zukunft individualisierte Therapien anbieten zu können, welche die individuelle Wirkung von p53 berücksichtigen.

5. Zusammenfassung

Obwohl in den letzten Jahrzehnten in der Therapie maligner Erkrankungen große Fortschritte erzielt wurden, erfordern vor allem weit fortgeschrittene, metastasierte Krebserkrankungen und Rezidive die Entwicklung neuer, innovativer Therapiekonzepte. Ein Großteil der Chemotherapeutika wirkt über die Induktion von Apoptose (programmierter Zelltod). Die genaue Kenntnis der Signalwege der Apoptose ermöglicht es, Zytostatika sinnvoller als bisher zu kombinieren und neue zielgerichtete Therapien zu entwickeln. Ein zentraler Regulator der Apoptose ist p53, der Hauptangriffspunkt der konventionellen Therapien sowie neuer zielgerichteter Therapien. Obwohl p53 zu den am intensivsten erforschten Proteinen gehört, ist die Vielfalt und Heterogenität der Funktion von p53 noch unklar.

Wir untersuchten die Bedeutung von p53 auf die intrinsische und extrinsische Apoptose (Wachter F et al., 2013). Dazu wurden verschiedene molekularbiologische Ansätze gewählt und sowohl Tumorzelllinien, als auch primäre Tumorzellen von Kindern mit akuter Leukämie untersucht. Apoptose wird intrinsisch über die Freisetzung von pro-apoptotischen Proteinen aus dem Intermembranraum des Mitochondriums ausgelöst, z.B. durch Zytostatika wie Doxorubicin, oder extrinsisch über eine Aktivierung der Todesrezeptoren z.B. durch TRAIL, den **T**umor necrosis factor **R**elated **A**poptosis **I**nducing **L**igand. In Einklang mit der Literatur führte die Expression von wildtyp p53 zu vermehrtem intrinsischen Zelltod, wohingegen verschiedene Mutationen die Apoptoseinduktion reduzierten. Unsere Daten zeigen, dass die Bedeutung von p53 für die extrinsische Apoptoseinduktion dagegen viel komplexer ist: p53 führte entweder zu erhöhter, unveränderter oder reduzierter Induktion des Zelltods; dabei zeigte interessanterweise p53 in wildtyp und p53 in mutierter Konfiguration den gleichen Effekt.

Als nächsten Schritt untersuchten wir den Einfluss des durch aktiviertes p53 induzierten Zellzyklusarrests auf die Apoptoseinduktion durch TRAIL (Ehrhardt/Wachter et al., 2013). Der Zellzyklus wurde in Tumorzelllinien und in Patientenproben durch Zytostatika, phasenspezifische Inhibitoren oder RNA-Interferenz gegen ZyklinB und E in G0, G1 oder G2 induziert. Zellzyklusarrest erhöhte bei Arrest in jedem Checkpoint des Zellzyklus die Apoptoserate durch TRAIL. Wurde der biochemisch induzierte Zellzyklusarrest im umgekehrten Ansatz durch Coffein aufgehoben, war die Apoptoserate durch TRAIL wieder reduziert. Die extrinsische Apoptose durch TRAIL ist in arretierten Tumorzellen erhöht. Das ist vor allem wichtig zur Therapie der „Minimal Residual Disease“ und zur Prävention von Rezidiven, die durch ruhende Tumorzellen ausgelöst werden.

Zusätzlich untersuchten wir den Einfluss der p53 Aktivierung auf die Apoptoserate im Rahmen von Polychemotherapie (Ehrhardt, Schrembs, Moritz, Wachter et al., 2011). Monotherapien sind in der Krebstherapie wenig effektiv, deshalb werden maligne Erkrankungen in der Regel mit einer Kombination mehrerer Zytostatika behandelt. In den Behandlungsplänen maligner hämatopoetischer Erkrankungen werden Vincaalkaloide und Anthrazykline am selben Tag

verabreicht. Erstmals zeigten wir, dass bei gleichzeitiger Applikation Doxorubicin die Apoptoseinduktion durch Vincristin inhibierte. Während bekannt ist, dass Doxorubicin p53 aktiviert und so Zellzyklusarrest induziert, beobachteten wir erstmals, dass in arretierten Zellen Vincristin die anti-apoptotische Funktion der anti-apoptotischen Mitglieder der Bcl-2-Familie nicht mehr inhibieren kann und somit keine Apoptoseinduktion durch Vincristin erfolgte.

Zusammenfassend haben wir die Komplexität der Wirkung von p53 in der extrinsischen und intrinsischen Apoptose gezeigt. Der Einfluss des p53 Status auf die extrinsische Apoptoseinduktion sollte vor allem im Kontext von neuen p53 wiederherstellenden Therapiekonzepten bedacht werden. p53 kann pro- und anti-apoptotisch wirken, abhängig von der individuellen Tumorzelle, dem applizierten Medikament und Signalinteraktionen. Da die Wirkung von p53 in seinem komplexen Netzwerk für ein bestimmtes Therapiesetting derzeit kaum abschätzbar ist, stellt der Ansatz der individualisierten Therapie nach in vitro Testung für den Patienten ein sinnvolles Therapiekonzept dar. Weitere Untersuchungen werden benötigt, um das therapeutische Potenzial dieser erlangten Erkenntnisse komplett zu verstehen.

6. Summary

In the past view decades the therapy of malignant diseases has improved significantly. However especially metastatic diseases and relapses still require the development of new therapeutic modalities. Most chemotherapeutic agents induce apoptosis. A better understanding of apoptosis signaling is needed in order to combine cytostatic drugs in a more efficient way and to develop new targeted therapies. Conventional cytotoxic drugs and new therapies target p53, a key regulator of apoptosis. p53 is one of the most intensively studied target proteins but the heterogeneity and diversity of the function of p53 remains unclear.

We investigated the importance of p53 in the intrinsic and extrinsic apoptosis signaling, via a variety of molecular biological approaches (Wachter et al., 2013). Apoptosis is induced intrinsically by the release of pro-apoptotic proteins from the inter-membrane space of the mitochondrion (eg. doxorubicin) or via activation of death receptors on the surface of the cell (eg. TRAIL, **T**umor necrosis factor **R**elated **A**poptosis **I**nducing **L**igand). As described in the literature, wildtype p53 leaded to an increase in intrinsic cell death, whereas different p53 mutations reduced apoptosis. In our work we discovered that the influence of p53 status on extrinsic cell death induction was much more multifaceted. The presence of p53 in tumor cell lines and xenograft tumor cells resulted in augmented, unaffected or decreased cell death. With great interest we discovered that the replacement of wildtype p53 by mutant p53 did not disturb the extrinsic apoptosis inducing capacity.

As a next step we studied the influence of cell cycle arrest induced by p53 activation on TRAIL apoptosis sensitivity (Ehrhardt/Wachter et al., 2013). Cell cycle arrest was induced in tumor cell lines and patient samples in G0, G1 or G2 with cytostatic drugs, phase-specific inhibitors or RNA interference against cyclinB and E. Molecular or biochemical arrest at any point of the cell cycle augmented the rate of apoptosis by TRAIL. Consequently, when cell cycle arrest was deactivated by adding caffeine, the antitumor activity of TRAIL was decreased. The extrinsic apoptosis by TRAIL was increased in senescent tumor cells. This is especially important for the therapy of minimal residual disease and for the prevention of relapse caused by resting tumor cells.

Additionally, we studied the impact of p53 activation on the rate of apoptosis induced by combinations of cytostatic drugs (Ehrhardt, Schrembs, Moritz, Wachter et al., 2011). A single drug is not very effective in cancer therapy therefore malignant diseases are usually treated with a combination of cytostatic agents. Co-administration of anthracyclines and vinca alkaloids on the same day is a standard of care for hematopoietic malignancies. If both drugs are applied simultaneously, doxorubicin inhibits apoptosis induction of vincristine. While it is well understood that doxorubicin activates p53 and induces cell-cycle arrest, we observed that cell-cycle arrest disabled inactivation of anti-apoptotic Bcl-2-family members by

vincristine. Therefore, it follows that vincristine was unable to activate downstream apoptosis signaling. Cycling cells are required for vincristine to kill cancer cells efficiently.

In summary we have shown the heterogeneity of p53 in extrinsic and intrinsic apoptosis signaling. The impact of the p53 status of tumor cells on extrinsic apoptosis signaling should be considered, especially in the context of therapeutic approaches that aim to restore p53 function to enable cell death. p53 can act pro- and anti-apoptotic, depending on the individual patient tumor cell and on drug and signal interactions. Finally, this suggests that patients benefit from an individualized therapeutic regimen. Further investigation is required in order to better understand the therapeutic potential of these complex interactions. .

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8. Abbildungsverzeichnis

Abbildung 1:
modifiziert nach: Guangwu Xu, Yufang Shi. Apoptosis signaling pathways and lymphocyte homeostasis. Cell Research (2007) 17:759-771.

Abbildung 2:
modifiziert nach: Patrick Chène Nature 2003 Reviews Cancer 3: 102-109.

Abbildung 3:
modifiziert nach: Vousden KH, Prives C. Blinded by the Light: The Growing Complexity of p53. Cell. 2009 May 1;137(3):413-31.

9. Sonderdrucke der Publikationen 1-3 im Rahmen der Promotionsarbeit

Publikation 1:

Wachter F, Grunert M, Blaj C, Weinstock DM, Jeremias I, Ehrhardt H: Impact of the p53 status of tumor cells on extrinsic and intrinsic apoptosis signaling. Cell Commun Signal. 2013 Apr 17;11(1):27. (Impaktfaktor 2012: 5,09)

Publikation 2:

Ehrhardt H*, **Wachter F***, Grunert M, Jeremias I: Cell cycle-arrested tumor cells exhibit increased sensitivity towards TRAIL-induced apoptosis. Cell Death Dis. 2013 Jun 6;4:e661. (Impaktfaktor 2012: 6,04)

*** beide Autoren haben mit dem gleichen Anteil beigetragen**

Publikation 3:

Ehrhardt H, Schrembs D, Moritz C, **Wachter F**, Haldar S, Graubner U, Nathrath M, Jeremias I: Optimized anti-tumor effects of anthracyclines plus vinca alkaloids using a novel, mechanism-based application schedule. Blood. 2011 Sep 16;118(23):6123-31. (Impaktfaktor 2011: 9,89)

CELL COMMUNICATION & SIGNALING

RESEARCH Open Access

Impact of the p53 status of tumor cells on extrinsic and intrinsic apoptosis signaling

Franziska Wachter[1], Michaela Grunert[1], Cristina Blaj[1], David M Weinstock[2], Irmela Jeremias[1,3] and Harald Ehrhardt[1,4*]

Abstract

Background: The p53 protein is the best studied target in human cancer. For decades, p53 has been believed to act mainly as a tumor suppressor and by transcriptional regulation. Only recently, the complex and diverse function of p53 has attracted more attention. Using several molecular approaches, we studied the impact of different p53 variants on extrinsic and intrinsic apoptosis signaling.

Results: We reproduced the previously published results within intrinsic apoptosis induction: while wild-type p53 promoted cell death, different p53 mutations reduced apoptosis sensitivity. The prediction of the impact of the p53 status on the extrinsic cell death induction was much more complex. The presence of p53 in tumor cell lines and primary xenograft tumor cells resulted in either augmented, unchanged or reduced cell death. The substitution of wild-type p53 by mutant p53 did not affect the extrinsic apoptosis inducing capacity.

Conclusions: In summary, we have identified a non-expected impact of p53 on extrinsic cell death induction. We suggest that the impact of the p53 status of tumor cells on extrinsic apoptosis signaling should be studied in detail especially in the context of therapeutic approaches that aim to restore p53 function to facilitate cell death via the extrinsic apoptosis pathway.

Keywords: p53, Mutant p53, Extrinsic, Intrinsic, TRAIL, Doxorubicin, Apoptosis

Background

The known functions of p53 are becoming increasingly complex and involvement of p53 in transcriptional control impacts many cellular functions including cell death control, cell cycle arrest, cellular senescence, DNA repair, angiogenesis, cell migration and other fundamental physiologic cellular activities including cell metabolism, autophagy, stem cell renewal, embryogenesis, innate immunity and fertility [1]. The prediction of the precise action of p53 on a specific cell and within a special context remains the focus of research activities and the impact on transcriptional control and the cell cycle is strictly context-specific [2-7]. p53 is classically viewed for its transcriptional control of pro-apoptotic proteins like DR5, Caspase-8, Bax, PUMA or NOXA, and its accumulation and activation is regulated by transcription and by a panel of post-transcriptional modifications like phosphorylation, subcellular localization and interaction with negative regulators [1].

Targeted therapies that specifically modulate a specific step of cell death induction in tumor cells represent one of the main goals of ongoing preclinical and clinical studies to improve cancer therapy. For p53, the selective modulation of mutant p53 and the variation of p53 expression levels by direct or indirect stabilization represent the most promising approaches within current studies [8,9]. In contrast to the many ongoing studies using mostly biochemical modulations, the specific impact of p53 gene alterations was investigated in a limited number of studies with respect to the general impact on intrinsic cell death induction [5,7,10-12]. Within the research activities of the extrinsic cell death induction by death inducing ligands like TRAIL, the focus was exclusively drawn to the promising approaches of p53

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activation by the addition of or pre-incubation with chemotherapeutic drugs or targeted stimuli [3,4,13].

In the study presented here, the specific impact of the altered p53 status on extrinsic and intrinsic apoptosis induction was studied in detail. We used different molecular approaches as has been done i.e. for tumorigenesis in different p53 genetic backgrounds before [14,15].

Results

Restoration of p53 functionality and activation of p53 to improve the efficacy of tumor therapy have attracted much attention within many different tumor entities and have demonstrated superior cell death induction for many combinations [3,13,16]. In contrast, the presence or activation of p53 within certain therapeutic settings was shown to be of disadvantage for the therapy efficacy in vitro and in vivo [2,17]. Here we aimed to study the impact of the p53 status itself on extrinsic and intrinsic apoptosis sensitivity.

Generation of constructs to modulate the p53 status

To study the impact of p53 on extrinsic and intrinsic apoptosis induction after defined stimuli, we used three different approaches: I) expression of p53 in wild-type or mutant conformation in tumor cells. II) knockdown of p53 by RNA interference. III) the use of pairs of tumor cells with baseline p53 expression and with the somatic knock-out of p53. For the first experimental setting, we generated p-CDH constructs either containing p53 in wild-type conformation or with specific point mutations for lentiviral transduction. The FKBP destabilization domain tagged to overexpressed p53 conducts the proteasomal degradation. Transient expression of the transgene was achieved after adding SHIELD-1.

Constructs were expressed in different tumor cells either with somatic knockout of p53 (Figures 1 and 2), loss of p53 (Additional file 1: Figure S3C) or with expression of wild-type p53 (Additional file 1: Figure S2). Five different mutant forms of p53 were introduced by mutagenesis which are known to be associated with reduced protein stability (V143A), defect of DNA binding (R248W, R273H) or structural instability (R175H, R249S) (Figure 2 and Additional file 1: Figure S2) [16]. For the second approach, the previously described technique of RNA interference against p53 using a lentiviral system was used [2-4] to generate an efficient knockdown of p53 (Figure 3A and Additional file 1: Figure S3). For the last approach, access to pairs of tumor cells either expressing p53 or with somatic knock-out of p53 was obtained (Figures 1, 2 and 3A, Additional file 1: Figure S3B).

Impact of p53 on extrinsic apoptosis sensitivity in HCT116 cells

First we used HCT116 cells either expressing p53 in wild-type conformation or with somatic knock-out of p53. Additionally p53 in wild-type conformation was re-introduced in the p53-/- cells. To our surprise, cell death induction by the death inducing ligand TRAIL was augmented in the absence of p53 (Figure 1A). The increased apoptosis sensitivity was not attributable to any regulation of apoptosis signaling proteins involved in TRAIL signaling. On the receptor level, the p53 status did not impact DR4 and DR5 expression levels (Figure 1B). In an alternative approach, cells were transfected with the TRAIL death receptors DR4 and DR5, and overexpression did not impact apoptosis induction by TRAIL (Additional file 1: Figure S1). Downstream of the TRAIL death receptors, the p53 status did not change the expression levels of the further members of the DISC and of the most important members of the Bcl-2 and the IAP-family (Figure 1C). Beyond transcriptional control, neither the cell cycle distribution nor the spontaneous growth was affected by the p53 status (Figure 1D and E).

Different impact of mutant p53 on extrinsic and intrinsic cell death induction

Next we studied the impact of wild-type and mutant p53 on extrinsic and intrinsic cell death induction in the HCT116 cells. As published before, wild-type p53 significantly augmented the cell death induction by doxorubicin, while the five studied p53 mutants did not affect the apoptosis inducing capacity (Figure 2A). Comparable results were obtained when doxorubicin was substituted by 5-fluorouracil, another cytotoxic drug acting by DNA damage and activation of the intrinsic apoptosis signaling cascade (data not shown) [18].

When TRAIL was studied as a classical activator of the extrinsic apoptosis signaling cascade, the impact of mutant p53 was completely different. The five different mutants of p53 reduced the TRAIL sensitivity to a similar extent as p53 in wild-type conformation further arguing against transcriptional control as the central regulatory mechanism of action (Figure 2B) [5,6]. When SHEP cells, which express p53 in wild-type conformation where overexpressed with wild-type p53 or the five different mutant forms of p53, the identical results were obtained as in HCT116 cells. While the sensitivity for doxorubicin or 5-fluorouracil was augmented when wild-type p53 was overexpressed (Additional file 1: Figure S2A and data not shown), wild-type p53 overexpression reduced the sensitivity for extrinsic apoptosis induction. As observed in HCT116 cells, not the p53 status but the overexpression of p53 was associated with reduced TRAIL sensitivity (Additional file 1: Figure S2B). Similar results were obtained, when the extrinsic signaling cascade was stimulated with the death inducing ligand FasL (data not shown).

Taken together, we have identified a distinct regulation of extrinsic and intrinsic cell death by p53. While the previously published results for the intrinsic signaling

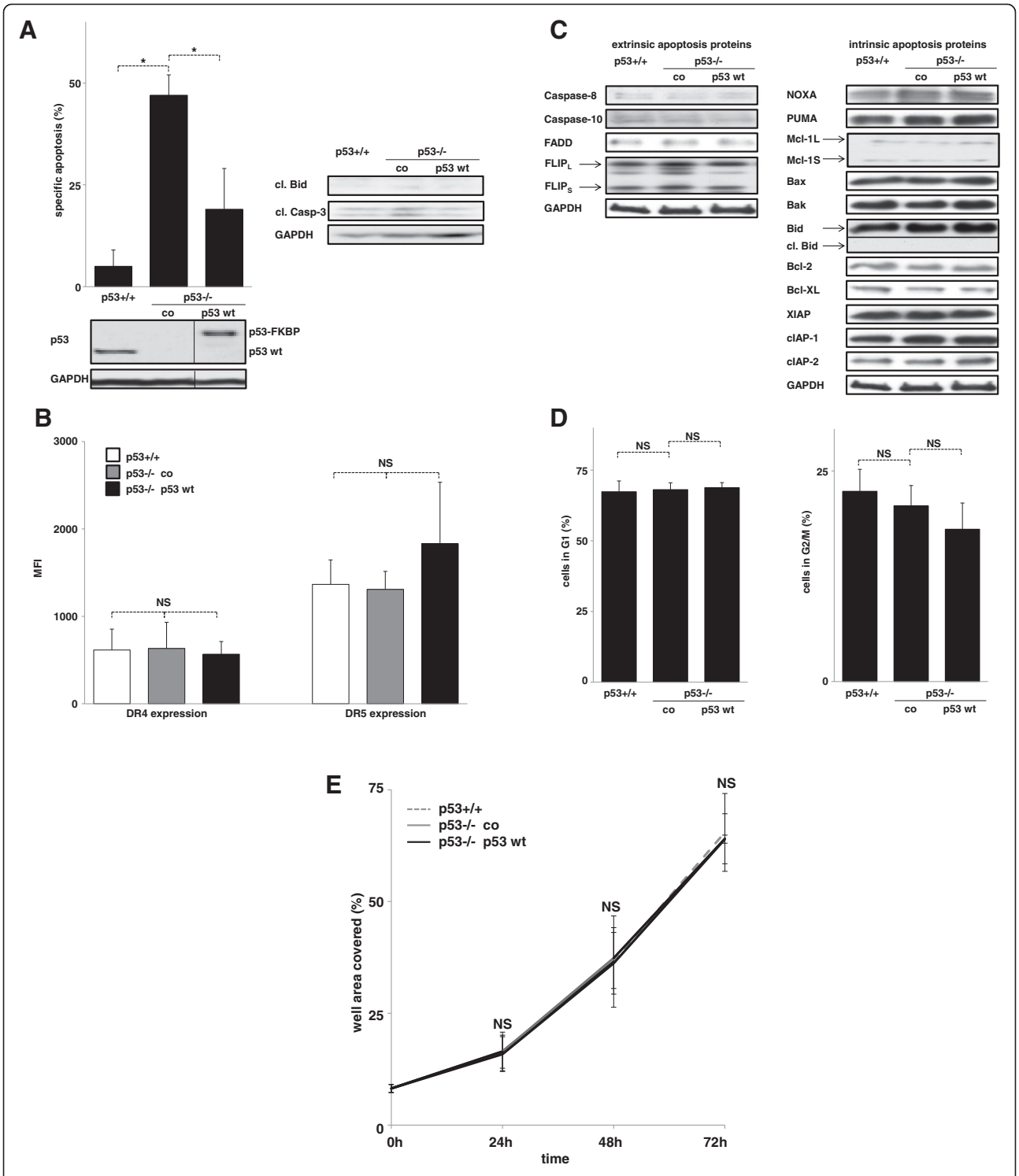

Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 Impact of p53 on extrinsic apoptosis sensitivity in HCT116 cells. A) HCT116 p53+/+, untransfected HCT116 p53-/- cells (co) and HCT116 p53-/- cells transfected with pCDH p53 in wild-type conformation (p53 wt) were stimulated with TRAIL (100 ng/ml) for 48 hours. Measurements of cell death induction (left panel) and cleavage of Bid and Caspase-3 (Casp-3; right panel) are presented. Western blot analysis was performed of total cellular protein. For the ease of reading, the order of samples within the identical blot was rearranged without any further manipulation, indicated by the separating lines. cl = cleaved. **B**, **C**) Cells from Figure 1A were analyzed for TRAIL death receptor (**B**) and apoptosis signaling protein (**C**) expression. DR4 and DR5 expression was determined by FACs surface staining. The MFI (mean fluorescence intensity) was determined in the APC-Cy7 channel of a LSR II flow cytometer as described in Methods. cl. Bid = cleaved Bid. **D**, **E**) Cells from Figure 1A were analyzed for cell cycle distribution using propidium iodide staining (**D**) and for spontaneous growth by automated analyses of the well area covered over time (**E**). Cell death induction of adherent cells was measured by Nicoletti staining. Specific apoptosis was calculated as [(apoptosis of stimulated cells at end point minus apoptosis of unstimulated cells at end point) divided by (100 minus apoptosis of unstimulated cells at end point) times 100]. Statistical analysis was performed using one way RM ANOVA. *p < 0,05, NS = statistically not significant.

cascade were reproduced, the presence of p53 was associated with reduced extrinsic cell death induction in HCT116 and SHEP cells. Unexpectedly and in contrast to intrinsic apoptosis signaling, wild-type and mutant p53 had the identical impact on extrinsic apoptosis induction.

The heterogenous impact of p53 on extrinsic cell death induction in tumor cell lines

To further clarify the role of p53 for extrinsic cell death, we studied n = 12 pairs of tumor cell lines expressing p53 and with downregulation / knock-out of p53. Overall, we observed three different phenotypes that were associated with the presence of p53: reduced, unchanged or augmented sensitivity for extrinsic cell death induction by TRAIL (Figure 3A and Additional file 1: Figure S3). Surprisingly, the inhibition or loss of p53 was associated with reduced TRAIL sensitivity in only 2 / 12 cell lines tested, while in 5 / 12 it did not have any impact and in 5 / 12 was associated with augmented cell death induction by TRAIL (Figure 3B). The negative action of p53 on TRAIL sensitivity was observed in hematopoietic, solid and mesenchymal tumor cells. Furthermore, the TRAIL response was independent from the TRAIL sensitivity of the parental cells and inhibition of apoptosis induction by TRAIL was detected in tumor cell lines with wildtype and mutant p53 status (Figure 3C) [16]. In line, the baseline p53 expression level varied widely between the different cell lines without any specific pattern (Figure 3D).

In summary, the impact of p53 on extrinsic cell death induction is much more complex and different actions were observed depending on the individual tumor cell which did not fit in classical categories like tumor entity, drug sensitivity or p53 mutation status of the tumor cell.

The heterogenous impact of p53 on extrinsic cell death induction in xenografted ALL cells

As the inhibitory effect on TRAIL sensitivity was present in nearly half of the tumor cell lines with wildtype p53 status, we chose the ALL xenograft setting to further study the relevance of wildtype p53 for inhibition of extrinsic cell death as they rarely contain p53 mutations [19,20]. We confirmed the wildtype p53 status in all ALL xenograft samples presented (data not shown). Another advantage of the employed experimental setting is that xenograft cells better resemble the in vivo situation as they do not feature non-physiologic alterations or comprise a selection of mutations that do not resemble the patient situation. We studied the impact of p53 on TRAIL sensitivity in xenografted ALL cells using the recently described experimental setting of RNA interference after amplification of primary childhood ALL cells in NOD/SCID mice [3,4,7,21-24]. RNA interference against p53 markedly reduced the p53 expression as described before (Additional file 1: Figure S4 and data not shown) [21]. As identified for the cell lines studies in Figure 3, xenografted ALL cells with knockdown of p53 reacted with three different phenotypes after stimulation with TRAIL: reduced, unchanged or augmented cell death induction (Figure 4A and data not shown). Surprisingly, the distribution of actions of p53 on extrinsic cell death was nearly identical as in the cell lines (Figure 4B). The negative effect of p53 on TRAIL sensitivity was present in B- and T-ALL cells and was detected only in samples obtained at initial diagnosis although the small number of samples at relapse studied does not allow the conclusion that in the situation of relapse the impact of p53 on extrinsic signaling is always not negative. When samples were categorized for their TRAIL response the same observation as in cell lines was detected: the fraction of samples benefiting from the knockdown of p53 was higher in the group of TRAIL resistant samples (Figure 4C). In contrast to the cell line data, the baseline p53 expression level between the different xenograft samples was less inhomogenous and showed a similar distribution for the three categories of the observed TRAIL responses (Figure 4D).

Taken together, the data obtained in established cell lines were confirmed in patient-derived tumor cells and underline the clinical relevance of the described phenotype: heterogeneous impact of p53 on extrinsic cell death induction depending on the individual tumor cell.

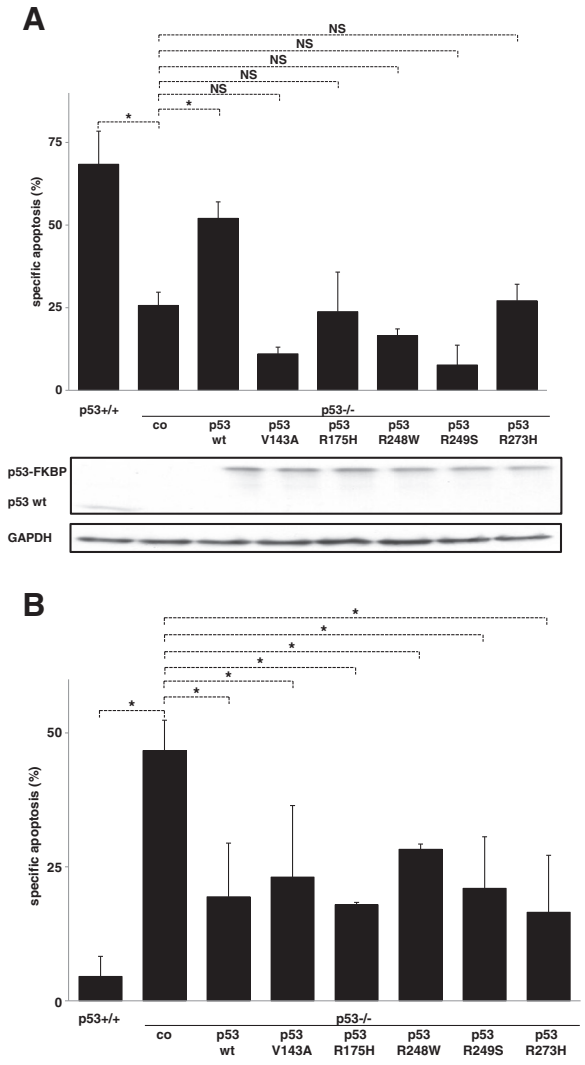

Figure 2 Different impact of mutant p53 on extrinsic and intrinsic cell death induction in HCT116 cells. A,**B**) HCT116 p53-/- cells transfected with the different pCDH constructs containing wildtype p53 or p53 with point mutation at codon 143, 175, 248, 249 or 273 were stimulated with doxorubicin (100 ng/ml, 72 hours, **A**) or TRAIL (100 ng/ml, 48 hours, **B**). Cell death induction, calculation of specific apoptosis, presentation of data and Western Blot analysis were performed as in Figure 1. Statistical analysis was performed using one way RM ANOVA. *p < 0,05, NS = statistically not significant.

Discussion

The data presented here indicate that both overexpression of wild-type or mutant p53 in cancer cells can have negative effects on cell death induction via the extrinsic apoptosis signaling cascade. These findings need to be taken into consideration during designing therapeutic strategies intended to re-introduce p53. While we were able to reproduce the formerly described heterogeneous impact of wild-type and mutant p53 on apoptosis induction by cytotoxic drugs like doxorubicin that act via the intrinsic signaling cascade [10,11], the impact of the p53 status on the extrinsic apoptosis cascade activated by death inducing ligands like TRAIL is much more complex. Investigating a panel of tumor cell lines and xenografted primary tumor cells, we detected three different actions of p53 on extrinsic apoptosis induction and the distribution of the three phenotypes was nearly equal between cell lines and xenograft cells: Promotion of extrinsic apoptosis induction, no change of apoptosis sensitivity and inhibition of cell death in the presence of p53. Surprisingly, the presence of p53 either in wild-type or mutant conformation did not significantly impact the expression level of typically p53-regulated apoptosis proteins like Bax, PUMA or DR5, spontaneous growth or cell cycle distribution [13]. While the impact of p53 on extrinsic apoptosis signaling was so far exclusively / mainly studied for the situation of p53 activation, the data provided here clearly indicate that the p53 status of the tumor cells impacts the response to extrinsic apoptosis stimuli while classical targets of p53 accumulation are not affected. Using RNA interference for p53 knockdown, somatic knockout of p53 and p53 re-expression strategies, the impact of basal p53 expression was studied in detail. Of general importance, we were able to demonstrate that not only protein regulations after p53-activating stimuli but also the p53 status of the tumor cell impacts apoptosis sensitivity both during extrinsic and intrinsic apoptosis induction.

The heterogeneous impact of p53 on cell death induction has attracted much notice during the recent years and further contributed to the complex and so far only rudimentary knowledge of p53 action. Formerly, p53 was thought to act primarily as a transcription factor and that the activity of p53 in wild-type conformation is mainly pro-apoptotic while certain mutants reduce the sensitivity towards apoptotic stimuli. Besides its transcriptional activating function, p53 was also proven for its repression of the transcription of anti-apoptotic proteins like Bcl-2 [25]. The recognition, that mutant p53 retains at least partial pro-apoptotic activities and that p53 in its non-mutated form can inhibit cell death induction, further contributed to the complexity [7,13,16,17]. Thereby, p53 activation within the identical cell can result in apoptosis promotion or inhibition depending on the stimuli and the experimental setting [2-4]. The lack of knowledge is evident, as p53 can also regulate protein expression independent from the direct transcriptional activity i.e. by post-transcriptional regulations and can directly activate the apoptotic machinery at the mitochondrial level [13,26-31]. We suggest that the impact of baseline p53 expression on the apoptosis inducing capacity of single agents and drug combinations should be studied in detail due to the major impact on extrinsic and intrinsic apoptosis sensitivity detected here. Current preclinical and phase I-III studies

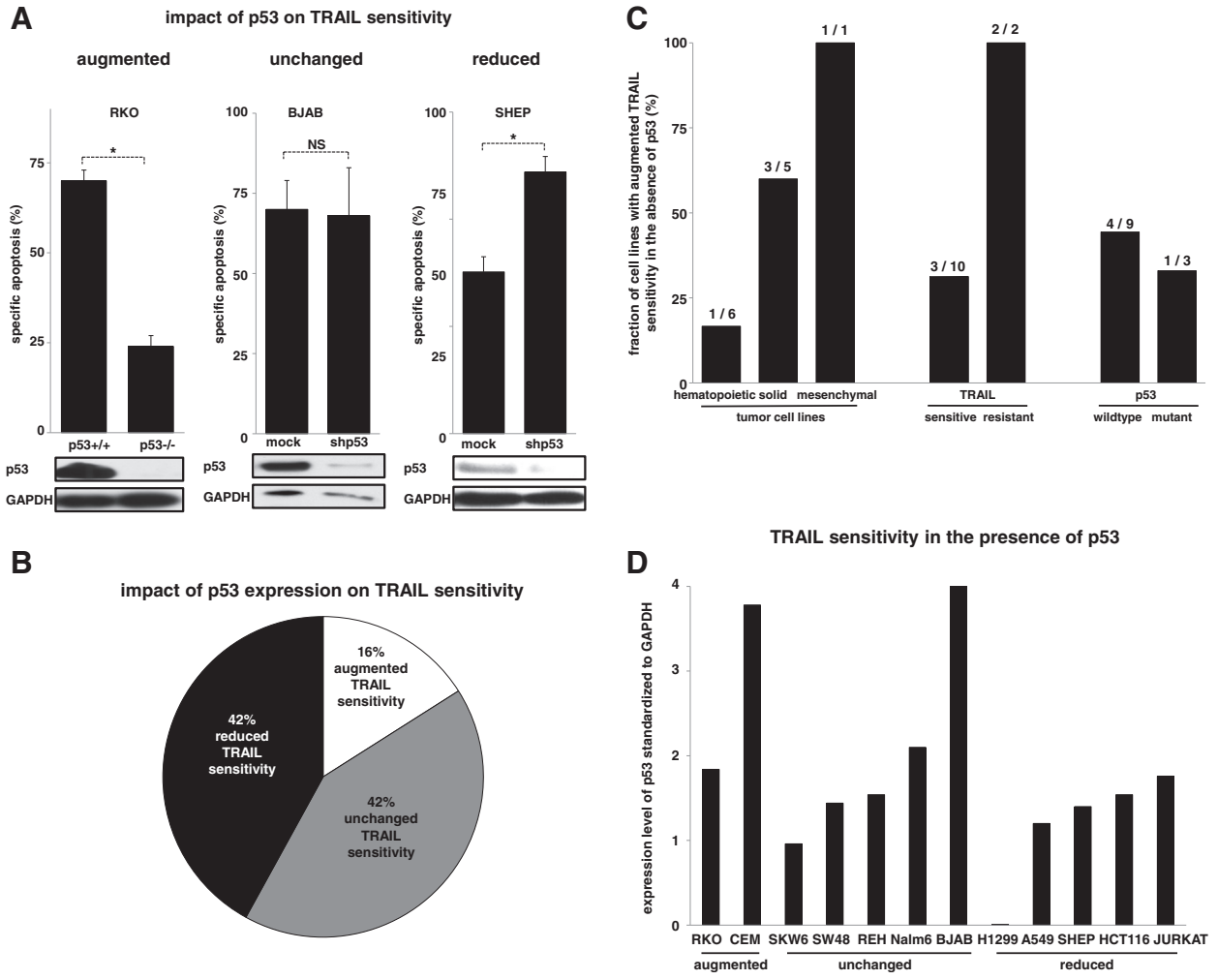

Figure 3 The heterogenous impact of p53 on extrinsic cell death induction in tumor cell lines. A) Parental RKO cells and derivative cells with somatic knockout of p53 (left panel) and BJAB (central panel) and SHEP (right panel) cells stably transfected with shRNA against p53 or a mock sequence were stimulated with TRAIL as in Figure 1A. The impact of baseline p53 expression on extrinsic cell death induction was classified as augmented cell death whenever the presence of p53 was associated with increased TRAIL sensitivity (left panel), as unchanged whenever p53 did not impact cell death by TRAIL (central panel) and as reduced when the presence of p53 diminished apoptosis induction by TRAIL (right panel). **B**) n = 12 different tumor cell lines from Figure 3A and Additional file 1: Figure S3 were classified according to their response to extrinsic cell death induction in dependence of the p53 status. **C**) Tumor cell lines from Figure 3B were categorized in the context of the tumor type, the TRAIL response of the p53 expressing cells and the p53 status of the tumor cells. TRAIL resistance was defined as cell death induction < 10%. **D**) The n = 12 cell lines from Figure 3B were analyzed for the relative p53 content as in Additional file 1: Figure S4. Samples were arranged separated for their TRAIL response in the presence of p53**.** Apoptosis induction in hematopoietic cells was determined by forwardside scatter analysis, in solid tumor cells using Nicoletti staining. Calculation of specific apoptosis, presentation of data and Western Blot analysis were performed as in Figures 1 and 2. Statistical analysis was performed using paired t-test. *p < 0,05, NS = statistically not significant.

testing strategies to activate or modulate p53 functions should consider p53 functions independent from the classical view of protein or cell cycle regulations [8,9,30].

Specific p53 gene mutations displayed a variety of oncogenic properties mostly referred to as gain-of-function. These were mainly categorized according to the properties including tumor formation, augmented tumor cell growth, transformation, invasiveness, metastasis formation and inhibition of DNA repair and differed depending on the specific p53 mutation [14,15,32]. The data presented here clearly indicate a further dimension of complexity as wild-type p53 can act in a pro-apoptotic manner when the intrinsic cell death cascade is activated while it can reduce apoptosis sensitivity via the extrinsic signaling cascade in the identical tumor cell.

We had described before, that stimulation with TRAIL can result in four different net effects, namely cell death induction in type A cells, no effect on type 0 cells,

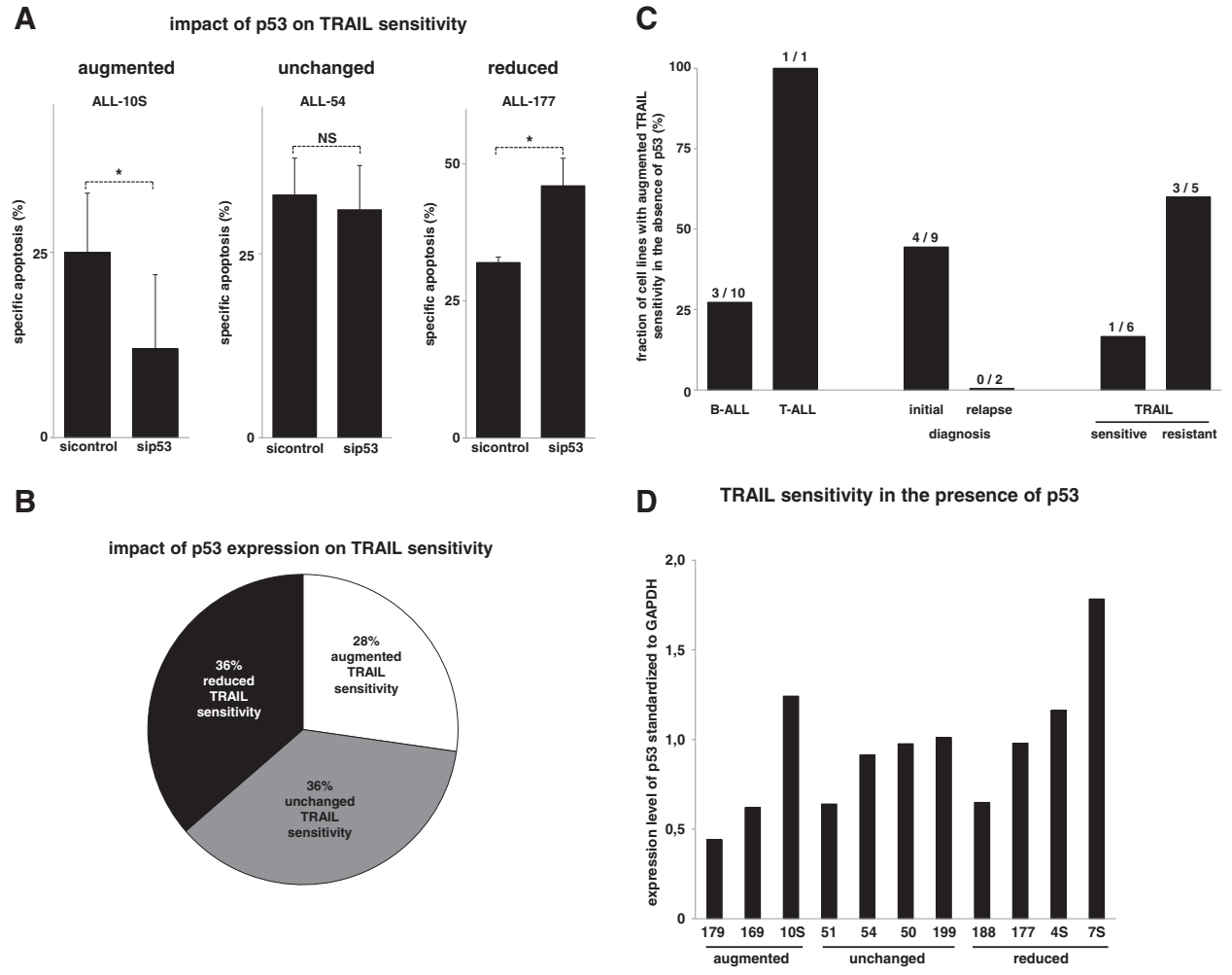

Figure 4 The heterogenous impact of p53 on extrinsic cell death induction in xenografted ALL cells. A) Patient-derived ALL-10S (left panel), ALL-54 (central panel) and ALL-177 (right panel) cells transfected with siRNA against p53 (sip53) or a control sequence (sicontrol) were stimulated with TRAIL (100 ng/ml) for 24 hours. Knockdown-efficiency of p53 by RNA interference is presented in Additional file 1: Figure S4. **B**) Cells from n = 11 different xenograft samples with wildtype p53 status (from Figure 4A and data not shown) were classified as in Figure 3B. **C**) Patient-derived ALL cells from Figure 4B were separated by the type of leukemia, the state of disease and the TRAIL sensitivity in the presence of p53 as in Figure 3C. **D**) Patient-derived ALL cells from Figure 4B were analyzed for the relative p53 content as in Figure 3D. Cell death induction, calculation of specific apoptosis, presentation of data and statistical analysis were performed as in Figure 3. *p < 0,05, paired t-test. NS = statistically not significant.

simultaneous induction of apoptosis and proliferation in type AP cells and selective induction of proliferation in type P cells [33]. The heterogeneous responses described before probably reflect the diversity of each individual tumor and account for the complexity that has to be taken into account when the individual tumor cell is treated at its best. In line, the data presented in this study suggest that the cell death inducing capacity of TRAIL is regulated at many different steps and that besides the proper DISC formation, p53 itself has a major impact on the TRAIL efficacy. While for the proper DISC assembly, the presence and balance of the DISC members is critical, the p53 impact seems to be much more complex as it is not caused by the regulation of classical p53 target proteins, the cell cycle or spontaneous growth [1,33-35].

In summary, future preclinical and clinical studies investigating the implementation of the death inducing ligand TRAIL in clinical combination therapy protocols should not only take into account the critical signaling steps of TRAIL cell death induction but the impact of p53 status of the individual tumor cell on TRAIL sensitivity. The data presented here clearly indicate, that the restoration of p53 function is not always beneficial. Therefore, both strategies, restoration of p53 functionality and inhibition of p53 can be beneficial depending on the individual tumor. We suggest further evaluation of

the therapeutic potential of in vitro drug sensitivity testing within in a similar experimental setting as described here. The results from the in vitro testing to predict the impact of p53 on apoptosis induction should be taken to determine the personalized in vivo treatment and to test the superiority of this approach in comparison to the standard protocol.

Conclusions

The data presented add substantially to the knowledge of p53 functionality. p53 in wild-type status has long been thought to act as a tumor suppressor. Many studies have been performed to take advantage of the activation and restoration of wild-type p53 function which has proven beneficial in many different experimental settings. During the recent years, the complexity of p53 function has become evident. Unfortunately, at least under certain circumstances the advantage can turn into a disadvantage. The molecular data presented here prove that not only p53 activation but the baseline presence of p53 can impact cell death induction. The detailed analyses of p53 in wild-type conformation and of frequent p53 mutations disclose the complexity and the heterogeneous impact on extrinsic and intrinsic apoptosis induction. Surprisingly, even wild-type p53 status can act in an anti-apoptotic manner. The results presented highlight the need for the gain of knowledge and for the consideration of p53 function within the particular context and for the individual tumor to optimize therapy efficacy.

Methods

Materials

TRAIL was prepared as described recently [36]. Alternatively, TRAIL without any modification was obtained from Pepro Tech (Hamburg, Germany) and rendered identical results (data not shown). All further reagents were obtained from Sigma (St. Louis, MO).

For Western Blot, the following antibodies were used: anti-FADD, anti-FLIP and anti-XIAP from BD Biosciences (Franklin Lakes, NJ), anti-Bcl-xL, anti-Bid, anti-cIAP-1 and anti-PUMA from Cell Signaling; anti-Bak, anti-Bax, anti-Bcl-2, anti-cIAP-2, anti-Mcl-1 and anti-p53 from Santa Cruz (Santa Cruz, CA); anti GAPDH from Thermo Fisher (Waltham, MA) and anti NOXA from Calbiochem (San Diego, CA). For flow cytometric determination of TRAIL surface receptor expression, anti-DR4 and anti-DR5 were obtained from AXXORA (Lörrach, Germany) and anti-IgG conjugated to Alx647 from Life Technologies (Darmstadt, Germany).

Cell lines, xenograft ALL cells and transfection experiments

HCT116, RKO and SW48 p53 +/+ and p53- / - cells were obtained from B. Vogelstein (Johns Hopkins University, Baltimore, MD). All further cell lines were obtained from DSMZ (Braunschweig, Germany) and maintained as described [2-4,37,38]. For leukemic cell line experiments, cells were seeded at 0,25 × 10^6/ml, for stimulations with solid tumor cells at 0,05 × 10^6/ml and incubated with TRAIL for 48 hours.

Informed consent was obtained from all patients in written form and studies were approved by the ethical committee of the medical faculty of the Ludwig Maximilians University Munich (LMU 068-08) and the children's hospital of the TU Munich (TU 2115/08). Animal work was approved by the Regierung von Oberbayern (55.2-1-54-2531-2-07). The xenograft mouse model and engraftment, amplification, isolation and standardized procedures of siRNA interference and in vitro stimulation have been described in detail recently [3,4,21]. For the knockdown of p53, siRNA p53 (5′- GGGUUAGUUUACAAUCAGC -3′) was obtained from Ambion (Austin, TX) and as control All Star negative control siRNA from Qiagen (Hilden, Germany). The p53 status of the xenograft cells was determined using next-generation sequencing as described before [39].

Transfection experiments in cell lines where performed using lipofection or lentiviral transduction as described recently [2,3,38]. pCDH p53 constructs were generated by the insertion of p53 in wildtype status into the pCDH plasmid modified as described recently [34] and site specific mutations were generated by QuikChange II site-directed mutagenesis PCR kit from Agilent (Santa Clara, CA). Five different point mutations were generated by site-directed mutagenesis PCR at codon 143, 175, 248, 249 or 273. Shield-1 (Clontech, Saint-Germain-En-Laye, France) was used at 0,3 μM. shRNA p53 and corresponding mock sequences, constructs and protocols for transfection were previously described in detail [2,3,38]. DR4 and DR5 cDNAs obtained from imaGENES GmbH (Berlin, Germany) were cloned into pcDNA3.1 [36].

Apoptosis assays, flow cytometry and Western blot analysis

For leukemia cell lines and xenograft ALL cells, forwardside scatter analysis was performed and verified using the recently described Annexin V – propidium iodid double staining [2]. For all adherent cell lines, cell death induction was determined using Nicoletti staining.

For the determination of TRAIL receptor surface expression, cells were washed in PBS followed by incubation with the primary antibody and by subsequent incubation with a dye-conjugation anti-IgG antibody conjugated to Alx647. The MFI (mean fluorescence intensity) was determined on a LSR II (BD Biosciences) using the Cell Quest Pro software version 3.2.1 (BD Biosciences) for data acquisition and FlowJo software version 8.3. (FlowJo, Ashland, OR) for data analyses.

Western Blot analysis was performed of total cellular lysates as described recently for cell lines and patient-

derived leukemia cells [21,38]. Quantification of western blot analysis of primary samples by AIDA Image Analyzer (Raytest; Straubenhardt, Germany) had been described recently [3].

Statistical analysis

TRAIL resistance was defined as cell death induction of <10% by 100 ng/ml TRAIL.

All data are presented as the mean values of at least three independent experiments ± SEM unless otherwise stated. To test for significant differences, the paired t-test was applied to compare two groups; for multivariate analysis, one way RM ANOVA was used. Statistical significance was accepted with $p < 0,05$.

Availability of supporting data

The data sets supporting the results of this article are included within the additional file 1.

Additional file

Additional file 1: Figure S1. TRAIL sensitivity not affected by TRAIL death receptor overexpression in SHEP cells. A,B)SHEP cells were transiently transfected with DR4 and DR5 expression plasmids. The change in DR4 (left panel) and DR5 (right panel) surface receptor expression was determined 36 hours after transfection as in Figure 1B (A) or cells were stimulated with TRAIL (100 ng/ml, B) for another 24 hours. Cell death induction, calculation of specific apoptosis, presentation of data and statistical analysis were performed as in Figure 1. *p < 0,05, one way RM ANOVA. NS = statistically not significant. **Figure S2.** Different impact of mutant p53 on extrinsic and intrinsic cell death induction in SHEP cells. A,B)pCDH constructs containing the different p53 variants were induced in SHEP cells as in Figure 2. SHEP cells were stimulated with doxorubicin (100 ng/ml, A) or TRAIL (100 ng/ml, B) for 48 hours. Cell death induction, calculation of specific apoptosis, presentation of data and statistical analysis were performed as in Figure 2. *p < 0,05, one way RM ANOVA. NS = statistically not significant. **Figure S3.** The heterogenous impact of p53 on extrinsic cell death induction in tumor cell lines. A-C)n = 8 pairs of cell lines with baseline p53 expression and downregulated p53 by RNA interference against p53, somatic knockout of p53 (SW48) or after transfection with the pCDH p53 wt expression plasmid (H1299) into p53 negative cells were separated according to their TRAIL response as in Figure 3A. TRAIL sensitivity in the presence of p53 was classified as augmented (A), unchanged (B) or reduced (C) efficacy. Stimulation with TRAIL, cell death induction, calculation of specific apoptosis, Western Blot analysis, presentation of data and statistical analysis were performed as in Figure 3. *p < 0,05, paired t-test. NS = statistically not significant. **Figure S4.** p53 knockdown by RNA interference in xenografted ALL cells. ALL-10S, ALL-54 and ALL-177 xenograft cells from Figure 4A were transfected with siRNA against p53. 48 hours later, Western Blot analysis was performed to prove knockdown efficiency. To enable the quantification of RNA interference, the p53 expression was investigated standardized to the expression level of GAPDH. The band density was analyzed using AIDA Image Analyzer and the relative expression level of p53 was calculated as (sample expression of p53 / sample expression of GAPDH) for sicontrol and sip53 transfected cells as described recently [21]. The reduction of p53 expression in sip53 transfected cells was calculated as [(relative p53 expression in sip53 cells / relative p53 expression in sicontrol cells) minus 1].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FW, CB, MG, DMW and HE performed experiments, FW and HE analyzed and interpreted the data and prepared the figures. HE designed the research and wrote the paper. IJ provided scientific support. All authors had a final approval of the manuscript.

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Cell cycle-arrested tumor cells exhibit increased sensitivity towards TRAIL-induced apoptosis

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Resting tumor cells represent a huge challenge during anticancer therapy due to their increased treatment resistance. TNF-related apoptosis-inducing ligand (TRAIL) is a putative future anticancer drug, currently in phases I and II clinical studies. We recently showed that TRAIL is able to target leukemia stem cell surrogates. Here, we tested the ability of TRAIL to target cell cycle-arrested tumor cells. Cell cycle arrest was induced in tumor cell lines and xenografted tumor cells in G0, G1 or G2 using cytotoxic drugs, phase-specific inhibitors or RNA interference against cyclinB and E. Biochemical or molecular arrest at any point of the cell cycle increased TRAIL-induced apoptosis. Accordingly, when cell cycle arrest was disabled by addition of caffeine, the antitumor activity of TRAIL was reduced. Most important for clinical translation, tumor cells from three children with B precursor or T cell acute lymphoblastic leukemia showed increased TRAIL-induced apoptosis upon knockdown of either cyclinB or cyclinE, arresting the cell cycle in G2 or G1, respectively. Taken together and in contrast to most conventional cytotoxic drugs, TRAIL exerts enhanced antitumor activity against cell cycle-arrested tumor cells. Therefore, TRAIL might represent an interesting drug to treat static-tumor disease, for example, during minimal residual disease.
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Resting tumor cells exhibit a severe challenge during anticancer treatment. In minimal residual disease, surviving tumor cells might stay quiescent and induce relapse after a prolonged period of time. Resting tumor cells display enhanced treatment resistance compared with actively cycling tumor cells, as several groups of anticancer drugs directly target the active cell cycle. Cancer stem cells are known to remain resting. To increase the prognosis and cure rate of cancer, anticancer therapy has to remove resting tumor cells.[1–4]

TNF-related apoptosis-inducing ligand (TRAIL) is a promising future anticancer drug due to its tumor selectivity, almost in the absence of side effects in animal trials, and phases I and II clinical studies.[5,6] The intracellular apoptosis signal transduction initiated by TRAIL is well characterized and involves the TRAIL-death receptors, FADD (Fas-associated protein with death domain) as adapter protein and caspases. The apoptosis signal of TRAIL might be amplified by mitochondria, which is regulated by members of the Bcl-2 family. Further regulators of TRAIL-induced apoptosis are the Caspase-8 antagonist FLIP (FLICE inhibitory protein) and members of the IAP-family including XIAP, which antagonize downstream caspases.[7–9]

Although the antitumor effect of TRAIL as a single agent is limited, TRAIL exerts remarkable antitumor activity upon combination with established cytotoxic drugs in phases I and II clinical trials. Cytotoxic drugs like doxorubicin (doxo) or methotrexate (MTX) and others induce synergistic apoptosis upon combination with TRAIL.[7,8,10–14]

Several different mechanisms have been described of how cytotoxic drugs sensitize tumor cells towards TRAIL-induced apoptosis. Among them, the transcription factor p53 is activated by several established cytotoxic drugs and mediates a number of different effects in tumor cells including gene regulation, apoptosis and cell cycle arrest.[15,16] As several proteins mediating or regulating TRAIL-induced apoptosis are p53 target genes, for example, TRAIL receptor 2 (death receptor 5, DR5), p53-mediated gene regulation is suggested to be the main mechanism for mediating synergistic apoptosis of cytotoxic drugs and TRAIL.[6,9,14,17]

We have recently described the importance of p53-mediated cell cycle arrest for inhibiting vinca alkaloid-induced apoptosis.[18] We also described that TRAIL damages stem cell surrogates in patient-derived leukemia cells.[19] As cancer stem cells are often resting, we hypothesized that TRAIL might be able to induce apoptosis in resting tumor cells. Although chemical compounds or drugs in preclinical testing were shown to sensitize tumor cells towards TRAIL-induced apoptosis accompanied by cell cycle arrest,[20–22] no molecular data exist so far and no data on patients' tumor cells are present. Therefore, we studied here how cell cycle arrest influences the ability of TRAIL to induce apoptosis in tumor cells, using molecular approaches in patient-derived tumor cells.

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Keywords: cell cycle arrest; TRAIL; apoptosis; DR5; cyclins
Abbreviation: ALL, acute lymphatic leukemia; Bcl-2, B-cell lymphoma 2; Dexa, Dexamethasone; Doxo, Doxorubicin; FADD, Fas-associated protein with death domain; FLIP, FLICE inhibitory protein; IAP, suppressors of apoptosis; MTX, Methotrexate; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis
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Results

Several cytotoxic drugs sensitize towards TRAIL-induced apoptosis and induce p53-typic effects. Numerous conventional cytotoxic drugs of current clinical routine are known to induce cell cycle arrest mediated by the transcription factor p53. In a first approach, cell cycle arrest was induced using cytotoxic drugs.

We used SHEP neuroblastoma cells (printed Figures 1–4) and HCT116 colon cancer cells (Supplementary Figures S2, S4 and S5), both of which express functionally active p53 in wild-type conformation.[23] Additionally, CEM T-ALL leukemia cells were included with mutant, but functionally active, p53 (Supplementary Figure S6)[11,18,23,24] and xenografted ALL leukemia samples (Figures 5 and 6 and Supplementary Figure S7). As described for several cell lines in the literature,[12–14] both SHEP and HCT116 cells displayed prominent synergistic apoptosis induction when TRAIL was combined with doxo (Figure 1a and Supplementary Figure S2A). Apoptosis data were congruent with increased caspase cleavage for the drug combination (Figure 1b). Concomitantly, doxo strongly activated p53 in both cell lines. According to the known different effects of p53, doxo upregulated typical p53 target genes in the TRAIL apoptosis signaling pathway, mainly TRAIL receptor-2 and Caspase-10, and arrested the cell cycle (Figures 1c and d, Supplementary Figures S1A and B, and S2B and C). The upregulation of TRAIL receptor-2 by doxo was higher in cells with cell cycle arrest in G2 (Supplementary Figure S1C). As TRAIL receptor-2 regulation has been reported to be a central determinant of TRAIL sensitivity, overexpression of TRAIL receptor-2 was performed, but did not have an impact on the TRAIL response, arguing against a dominant role of TRAIL receptor-2 expression levels in the regulation of TRAIL sensitivity in our experimental setting (Supplementary Figure S1D). Determination of the phosphorylation status at Serine 10 of Histone H3 revealed that the cell cycle was arrested in G2, but not in M (Figures 1c and d, Supplementary Figures S2D and E). For the combination of doxo plus TRAIL, the fraction of cells in G2 was markedly reduced (Figure 1e). To prevent the cell cycle arrest by cytotoxic drugs, the biochemical inhibitor caffeine was used, as it is highly efficient with nearly absent toxicity and not specific to a certain phase of the cell cycle or chemotherapeutic drug applied. Synergistic apoptosis induction was markedly reduced by pretreatment with caffeine, which also prevented p53 accumulation, reduced the upregulation of TRAIL receptor-2 and Caspase-10, and the cell cycle arrest by doxo (Figures 1a–d, Supplementary Figures S1A and B, and S2A-E).

Similarly, MTX and dexamethasone (dexa) induced superadditive apoptosis with TRAIL (Figures 2a and b), and both drugs arrested the cell cycle in G1 (Figure 2c and data not shown). Whereas MTX increased the expression of TRAIL receptor-2, dexa did not relevantly alter the mean fluorescence intensity (Supplementary Figure S3). Pretreatment with caffeine reduced synergistic apoptosis, TRAIL receptor-2 upregulation and cell cycle arrest (Figures 2a–c, Supplementary Figure S3 and data not shown).

Taken together, several cytotoxic drugs sensitized towards TRAIL-induced apoptosis, induced cell cycle arrest at different phases and upregulated typical p53 target genes, which were all inhibited by caffeine.

Cell cycle inhibitors sensitize for TRAIL-induced apoptosis. Cytotoxic drugs are mainly described to promote TRAIL-induced apoptosis by the regulation of apoptosis protein expression including TRAIL receptor-2 expression.[6,9,14–17] The data presented so far do not allow estimation of the specific contribution of cell cycle arrest. Next, we aimed at discriminating between the different effects of p53 induced by cytotoxic drugs, and asked whether cell cycle arrest itself might be sufficient to sensitize towards TRAIL-induced apoptosis. Towards this aim, we used biochemical cell cycle inhibitors or irradiation, which are known to arrest cells in defined phases of the cell cycle. As published before, FCS withdrawal arrested the cells in G0, mimosine in G1 and irradiation in G2 (Table 1 and Supplementary Table 1).[25] Interestingly, upon arresting the cell cycle in any given phase, both compounds and irradiation significantly sensitized for TRAIL-induced apoptosis (Figure 3a). The analysis with isobolograms showed the synergistic effects clearly (Figure 3b and Supplementary Figure S4). Taken together, biochemical cell cycle arrest was associated with particularly efficient cell death induction by TRAIL.

Knockdown of cyclinB or cyclinE induce cell cycle arrest and sensitize for TRAIL-induced apoptosis. So far, we showed that various drugs, compounds and stimuli induced cell cycle arrest and sensitized towards TRAIL-induced apoptosis. We next asked whether cell cycle arrest was mechanistically responsible for sensitizing towards TRAIL-induced apoptosis. To discriminate between drug-induced cell cycle arrest and further drug-induced, p53-mediated effects such as gene regulation, cell cycle arrest was induced by molecular manipulation using RNA interference.

Cyclins are regulators of the cell cycle that control transition through the different phases of the cell cycle. Whereas expression of cyclinB is a prerequisite for transition from G2 to M, cyclinE controls the slip from G1 to S-phase. Using RNA interference, we studied the impact of downregulation of these cyclines on TRAIL-induced apoptosis.

According to published data, knockdown of cyclinB or cyclinE induced cell cycle arrest in G2 or G1 (Figures 4a–c and Supplementary Figure S5A). As published for the cell cycle arrest in G1 by inhibition of cyclinD1 before, we preferred an siRNA sequence against cyclinE that leads to an incomplete, but statistically significant cell cycle arrest in G1, but does not affect the basal apoptosis rate of transfected cells.[25–27] The expression levels of the apoptosis signaling proteins studied and p53 remained unchanged in cells with knockdown of cyclinB or cyclinE. Of special interest, the expression level of TRAIL receptor-1 and -2 remained unchanged (Figures 4d and e). Knockdown of cyclinB or cyclinE significantly sensitized the solid tumor cell lines SHEP and HCT116 for apoptosis induction by TRAIL (Figure 4f and Supplementary Figure S5B). In line with our previous results for vincristine-induced apoptosis, the slight alteration of cells in G1 was associated with a marked difference in apoptosis induction by TRAIL.[25] Dose–response curves confirmed the general impact of knockdown of cyclinB or E on TRAIL-induced apoptosis (Figure 4g).

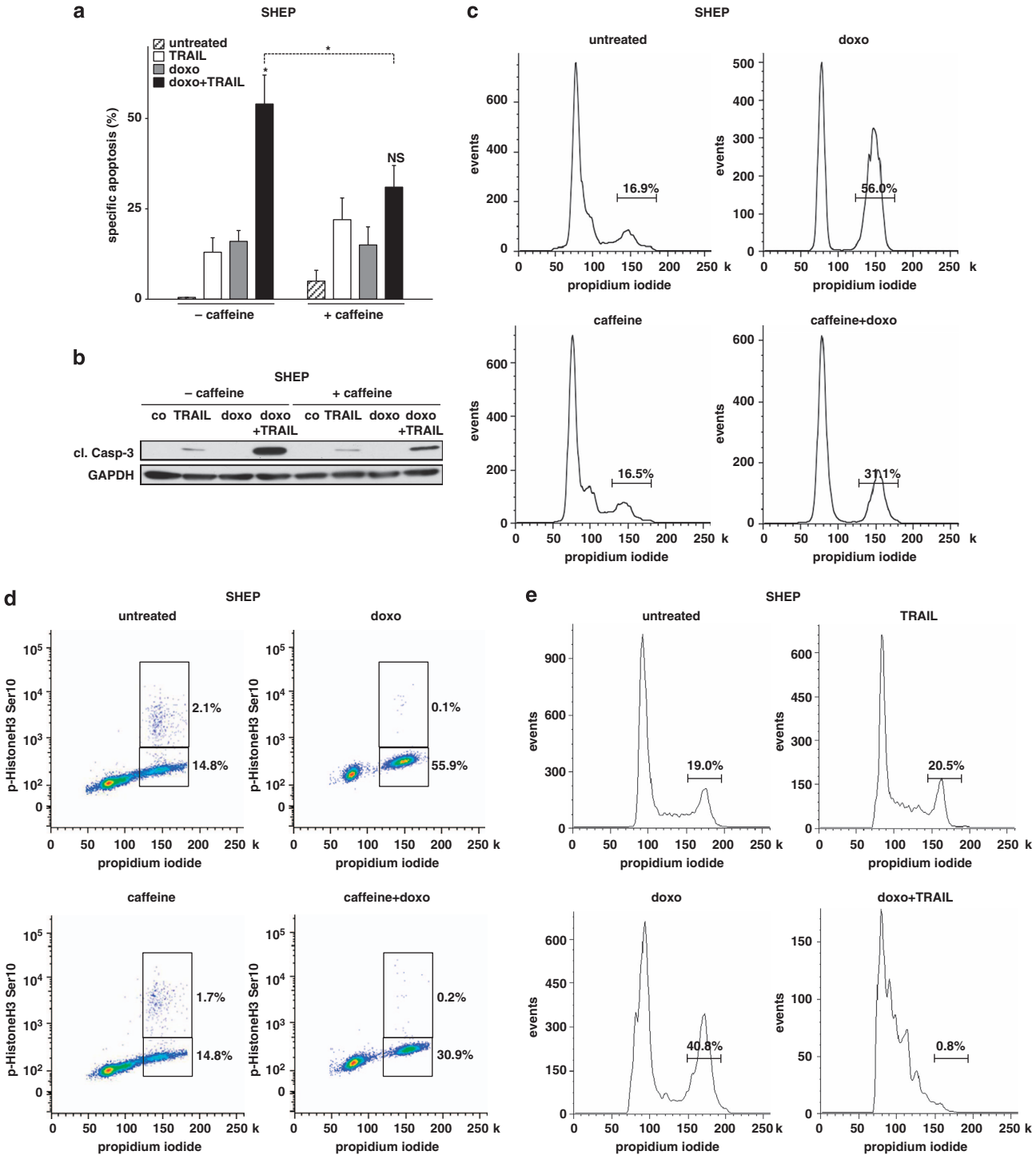

Figure 1 Super-additive activity of doxo and TRAIL in SHEP cells. (**a**) SHEP cells were pretreated with caffeine (300 μg/ml) for 12 h or left untreated, followed by stimulation with doxo (100 ng/ml) for 48 h. TRAIL (100 ng/ml) was added afterwards for another 24 h. (**b**) SHEP cells pretreated and stimulated as in(a) were analyzed for caspase-3 cleavage. co = untreated, cl. Casp-3 = cleaved Caspase-3. (**c**, **d**) SHEP cells treated with caffeine and doxo as in (a) were analyzed for cell cycle distribution using propidium iodide staining (**c**). G2 and M-phase were discriminated by the simultaneous staining for p-HistoneH3Ser10 (**d**). (**e**) SHEP cells were stimulated with doxo (30 ng/ml) for 48 h, followed by TRAIL (10 ng/ml). Cell cycle analysis was performed 24 h after the addition of TRAIL using propidium iodide staining, as in (**c**), gating on living cells. Cell death induction was measured by Nicoletti staining. Statistical analysis was performed comparing the apoptosis induction of the combined stimulation to the addition of cell death induction by doxo and TRAIL alone, and comparing the combinatorial application of pretreated and untreated cells with paired *t*-test. $^{*}P<0{,}05$, NS = statistically not significant

To prove the general significance of the observed phenotype across different tumor entities, the hematopoietic T cell leukemia cell line CEM was additionally studied, which expresses mutant but functionally active p53.[11,18,23,24,28] siRNA against cyclinB and E arrested the cell cycle in G2 and G1, respectively, also to a minor extent compared with the

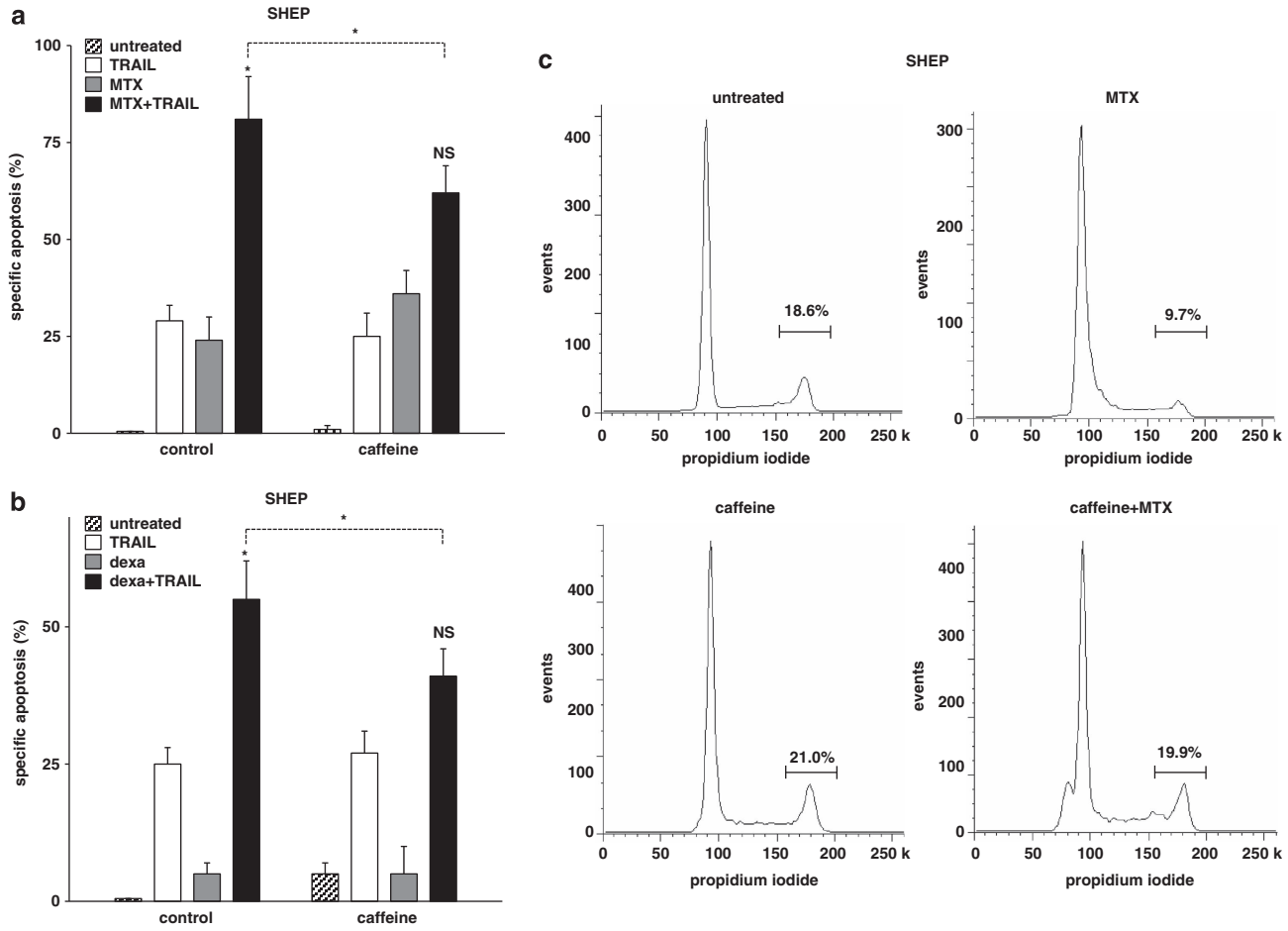

Figure 2 Augmented apoptosis-inducing capacity of TRAIL associated with cell cycle arrest by MTX and dexamethasone. (**a**, **b**) SHEP cells were pretreated with caffeine for 12 h, followed by stimulation with methotrexate (30 μM; (**a**) or dexamethasone (10^{-5} M; (**b**) for 48 h. TRAIL (100 ng/ml) was added afterwards for another 24 h. (c) SHEP cells treated with caffeine and MTX ,as in (a), were analyzed, as in Figure 1b. Determination of apoptosis induction, presentation and analysis of the data and statistical analysis were performed as in Figure 1. *$P<0.05$

solid tumor cell lines (Supplementary Figure S6A). Similar to the solid tumor cell lines studied, cell cycle arrest sensitized towards TRAIL-induced apoptosis in CEM cells (Supplementary Figure S6B).

These data show that cell cycle arrest itself sensitizes towards TRAIL-induced apoptosis in the clear absence of protein regulation.

Patient-derived tumor cells are sensitized for TRAIL-induced apoptosis by cytotoxic drugs. Established cell lines might have acquired additional, non-physiologic mutations upon prolonged culture *in vitro*. For example, the majority of leukemic cell lines inherit mutations in p53, which are rarely found in leukemia patients.[23,29–31] To exclude a culture-specific artifact and to go beyond cell line work, tumor cells from patients were studied. Towards this aim, we used primary tumor cells from children with acute leukemia. As these cells are notoriously reluctant towards *in vitro* growth, primary cells were passaged through immunocompromised mice,[11,32] where they remain largely genetically stable.[33]

Three different ALL samples were stimulated with doxo and TRAIL, with and without pretreatment with caffeine. Whereas doxo partially arrested the cells in G2, caffeine markedly reduced the G2 arrest (Figure 5a and Supplementary Figure S7A). On a functional level and in accordance to data obtained in cell lines, doxo and TRAIL induced synergistic apoptosis, which was inhibited by pretreatment with caffeine (Figure 5b and Supplementary Figures S7B and C).

Patient-derived tumor cells are sensitized towards TRAIL-induced apoptosis by knockdown of cyclinB or cyclinE. To prove that cell cycle arrest was capable to sensitize towards TRAIL-induced apoptosis, patient-derived ALL cells were transfected with siRNA targeting cyclinB or E, using our recently described technique.[11,24,32] Whereas siRNA against cyclinB accumulated cells in G2, siRNA against cyclinE increased the fraction of cells in G1 (Figure 6a and data not shown). Concomitantly, knockdown of either cyclinB or cyclinE augmented TRAIL-induced apoptosis in ALL cells of all three patients (Figure 6b and Supplementary Figures S7D and E).

Thus, cell cycle arrest augmented TRAIL-induced apoptosis not only in cell line cells, but also in tumor cells derived from various children with B precursor ALL. Taken together and in

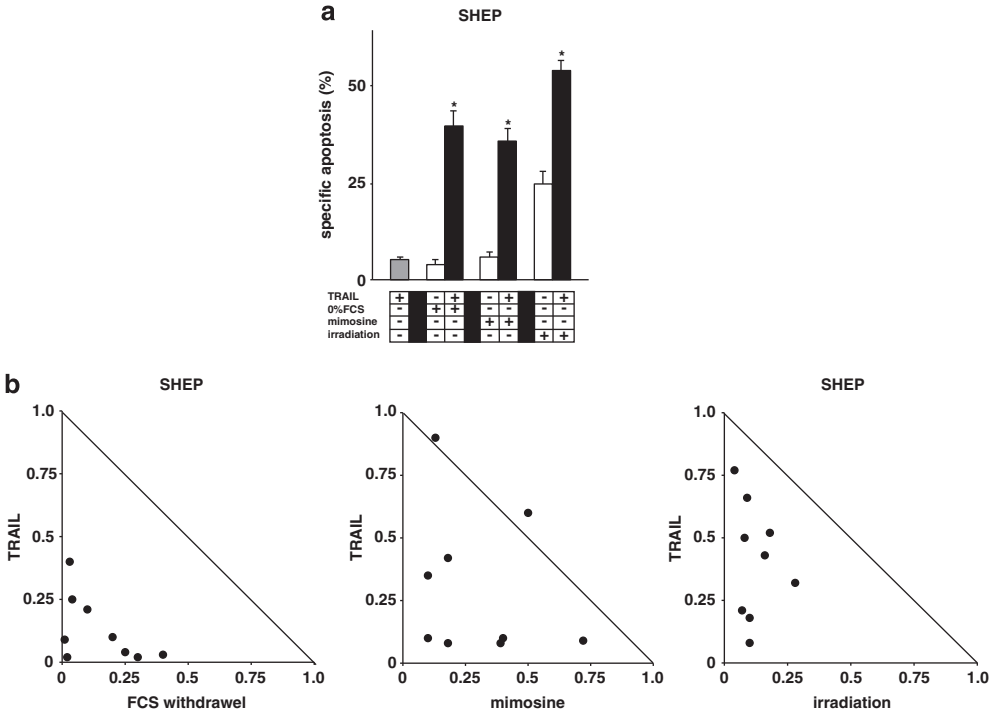

Figure 3 Biochemical cell cycle arrest sensitizes for TRAIL. (**a**) SHEP cells were pretreated with FCS withdrawal, preincubated with mimosine (100 μM) or irradiated with 30 Gy for 24 h. TRAIL (100 ng/ml) was added for another 24 h. (**b**) Isobolograms were applied to the experimental setting from (**a**) to test for synergistic effects of FCS withdrawal (0, 0,1 and 1% FCS), mimosine (10, 30 and 100 μM) treatment or irradiation (3, 6 and 10 Gy) plus TRAIL (3, 10 and 30 ng/ml). Determination of apoptosis induction, presentation and analysis of the data, and statistical analysis were performed as in Figure 1. •Each data point represents the dose-equation of the applied combinatorial treatment. *$P < 0.05$

Table 1 Cell cycle distribution in SHEP cells after cell cycle inhibition

Cell cycle distribution	*G0 (%)*	*G1 (%)*	*G2 (%)*	*M (%)*
Control	0.2 ± 0.2	67.8 ± 3.6	18.8 ± 4.5	2.8 ± 0.8
0% FCS	30.9 ± 0.9	52.6 ± 2.9	9.8 ± 0.5	0.2 ± 0.1
Mimosine	0.1 ± 0.1	85.0 ± 3.5	10.9 ± 1.0	0.1 ± 0.1
Irradiation	1.1 ± 0.3	15.8 ± 2.3	75.2 ± 3.8	0

SHEP cells were treated by withdrawal of FCS (0% FCS), with mimosine (100 μM) or irradiated (30 Gy) for 24 h. Cell cycle distribution was analyzed with propidium iodide in combination with cyclinD1 staining to discriminate G0 and G1 phases, and with p-Histone H3 staining to separate the arrest in G2 and M-phase. Data are presented as mean ± S.E.M. of three independent experiments.

contrast to conventional chemotherapeutics, TRAIL induces apoptosis more efficiently in tumor cells during cell cycle arrest compared with actively cycling tumor cells.

Discussion

Our data show that TRAIL induces apoptosis more efficiently if tumor cells undergo cell cycle arrest compared with actively cycling tumor cells. For the first time, we obtained mechanistic proof that cell cycle arrest itself sensitizes tumor cells towards TRAIL-induced apoptosis, including patients' tumor cells. This finding was obtained by inducing cell cycle arrest by (i) conventional cytotoxic drugs; (ii) known cell cycle arrestors or (iii) molecularly by knockdown of certain cyclines. Knockdown-induced cell cycle arrest sensitized towards TRAIL-induced apoptosis in cell lines of various different tumor entities, as well as in patient-derived leukemia cells.

Therapeutic targeting of cells in cell cycle arrest is of high clinical importance. Cancer stem cells are known for their low cycling activity and chemoresistance. Static-tumor diseases are especially difficult to treat, for example, during minimal residual disease or in low-grade tumors. Insufficient treatment of static-tumor disease often results in tumor relapse. Our finding might suggest testing TRAIL in static-tumor disease *in vivo* as TRAIL seems to be especially efficient against resting tumor cells.

As TRAIL induces limited apoptosis in most primary tumor cells when given alone, the combined use of TRAIL together with conventional cytotoxic drugs has been intensively studied over the last years. Several different conventional anticancer drugs strongly sensitize tumor cells towards TRAIL-induced apoptosis. In search for underlying signaling mechanisms, p53 and its downstream effects were studied intensively. Most cytotoxic drugs accumulate and activate p53. p53-mediated gene regulation of signaling mediators of TRAIL-induced apoptosis such as TRAIL receptor-2 was thought to be responsible for drug-induced sensitization towards TRAIL-induced apoptosis. These considerations were used to optimize combinatorial approaches involving TRAIL.[6,8,9,14,17,34]

Besides protein regulations, p53 induces cell cycle arrest. Although p53 is mutated in many tumor cells, leading to altered p53 function, induction of cell cycle arrest is not affected by loss of DNA-binding capacity in most p53 mutants.[34,35] Our

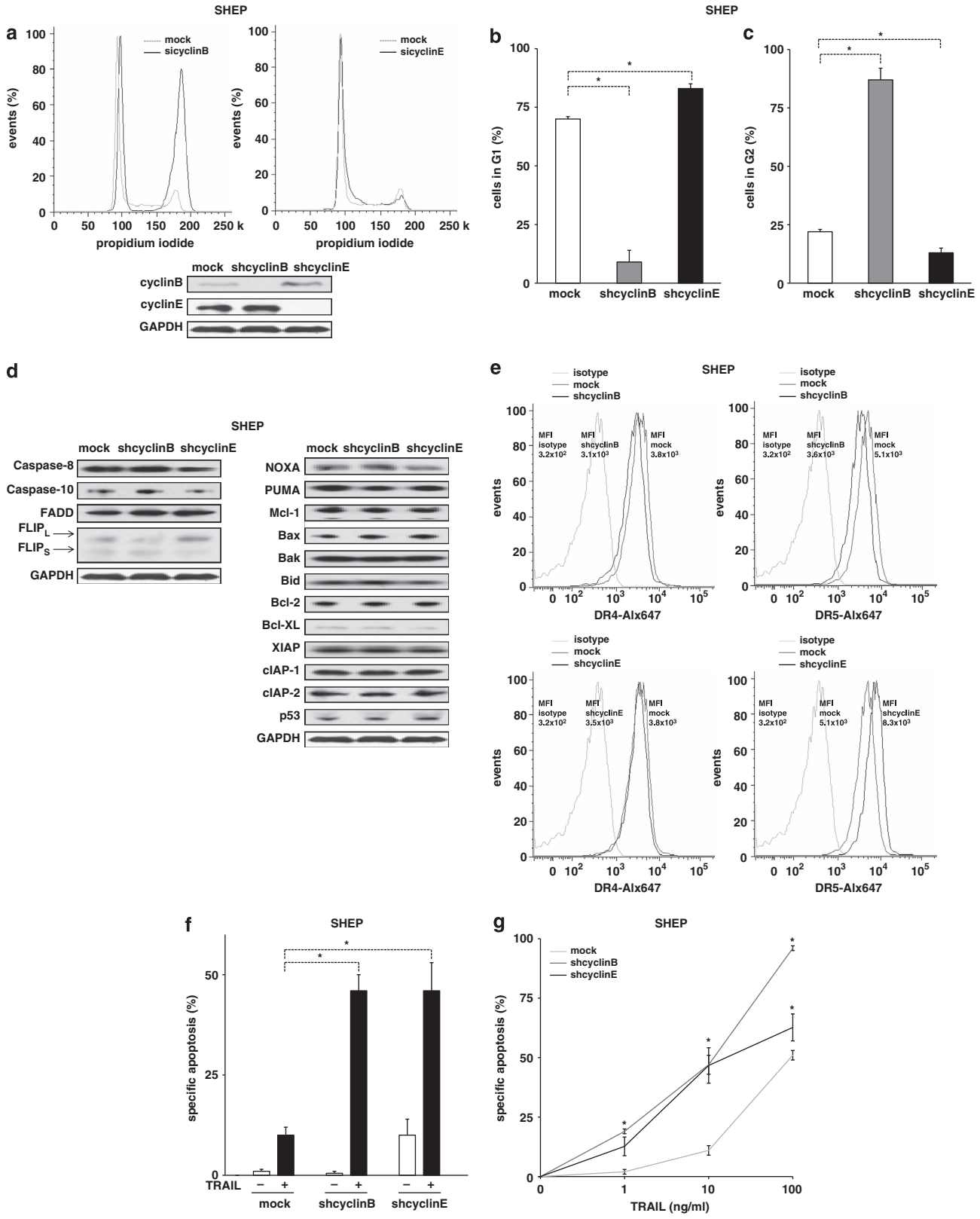

Figure 4 Molecular cell cycle arrest sensitizes for TRAIL. (**a**) SHEP cells transfected with shRNA against cyclinB or cyclinE were analyzed for the cell cycle distribution, as in Figure 1c. (**b**–**e**) SHEP cells from (**a**) were analyzed for cell cycle distribution in G1 (**b**) and G2 (**c**), apoptosis protein expression (**d**) and TRAIL-death receptor-1 and -2 expression (**e**) using the experimental setting from Figures 1c and d and Supplementary Figures 1A and 1B. (**f**) SHEP cells from (**a**) were stimulated with TRAIL (10 ng/ml). (**g**) TRAIL dose–response curves were performed in parental SHEP cells, and cells with knockdown of cyclinB or cyclinE from (**a**). Determination of cell cycle distribution and apoptosis induction, presentation and analysis of the data, and statistical analysis were performed as in Figure 1. For multivariate analysis, RM ANOVA was used. *$P < 0.05$

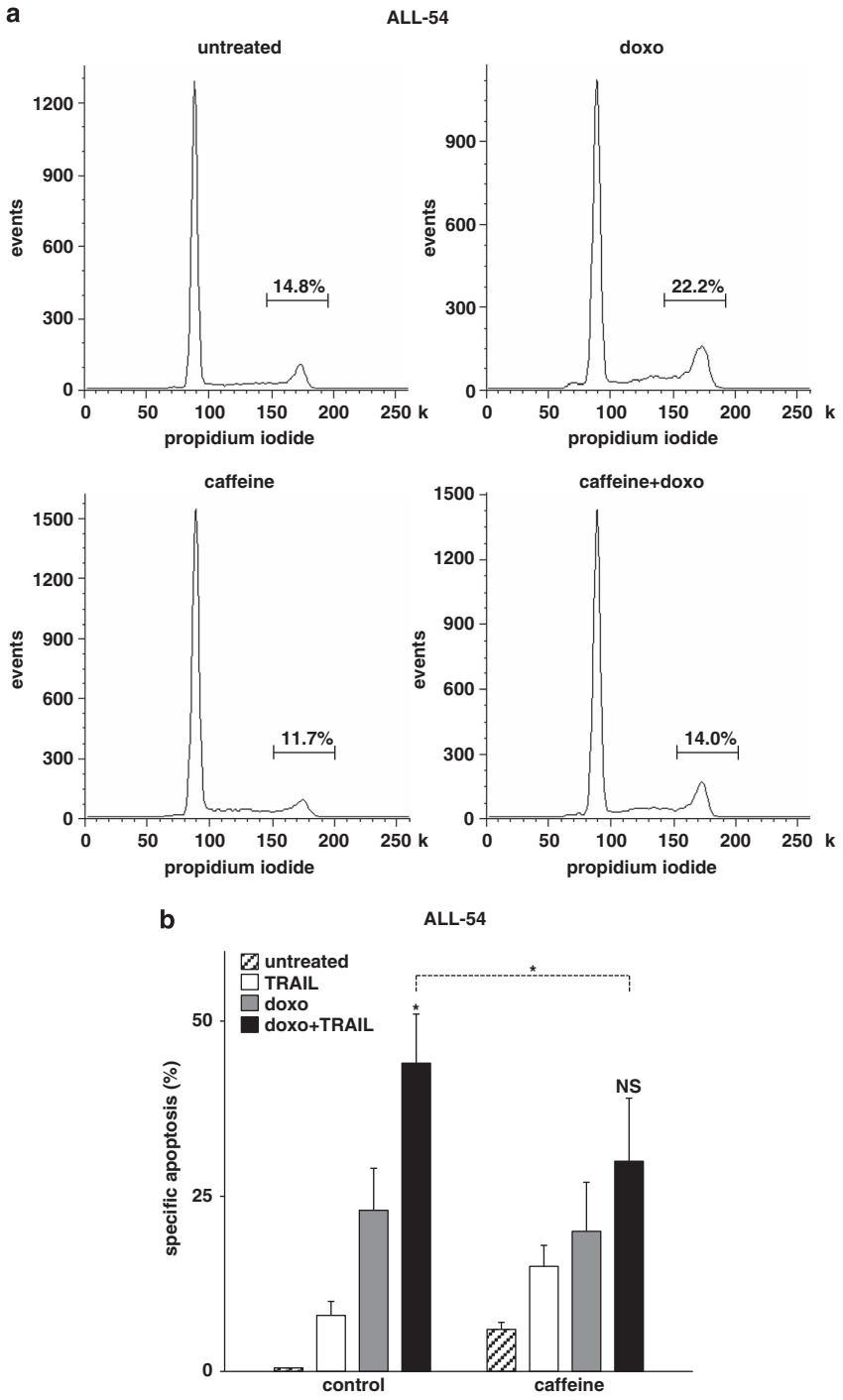

Figure 5 Doxo-induced cell cycle arrest associated with efficient TRAIL apoptosis induction in xenografted ALL cells. (**a**, **b**) Xenografted pre-B ALL-54 cells were pretreated with caffeine (100 μg/ml) for 12 h, followed by stimulation with doxo (30 ng/ml). After 24 h of incubation with doxo, cell cycle analysis was performed (**a**) or cells were stimulated with TRAIL (10 ng/ml) for another 24 h (**b**). Determination of cell cycle distribution and apoptosis induction, presentation and analysis of the data and statistical analysis were performed, as in Figure 1. $^*P<0.05$

data show that in addition to the dominant p53-mediated gene regulation, p53-mediated cell cycle arrest represents a mechanism by which cytotoxic drugs sensitize tumor cells towards TRAIL-induced apoptosis mediated by p53.

We have recently described that anthracyclines and vinca alkaloids are less effective when applied simultaneously as anthracyclines induce cell cycle arrest, whereas vinca alkaloids require active cell cycling for antitumor efficiency.[18] In contrast, cell cycle arrest is beneficial for TRAIL. The data presented here widen the therapeutic potential for TRAIL to all phases of the cell cycle. Our data add to the controversial discussion, whether or when cell cycle arrest is beneficial, irrelevant or detrimental during anticancer therapy, for example, using TRAIL.[18,20–22,34–38]

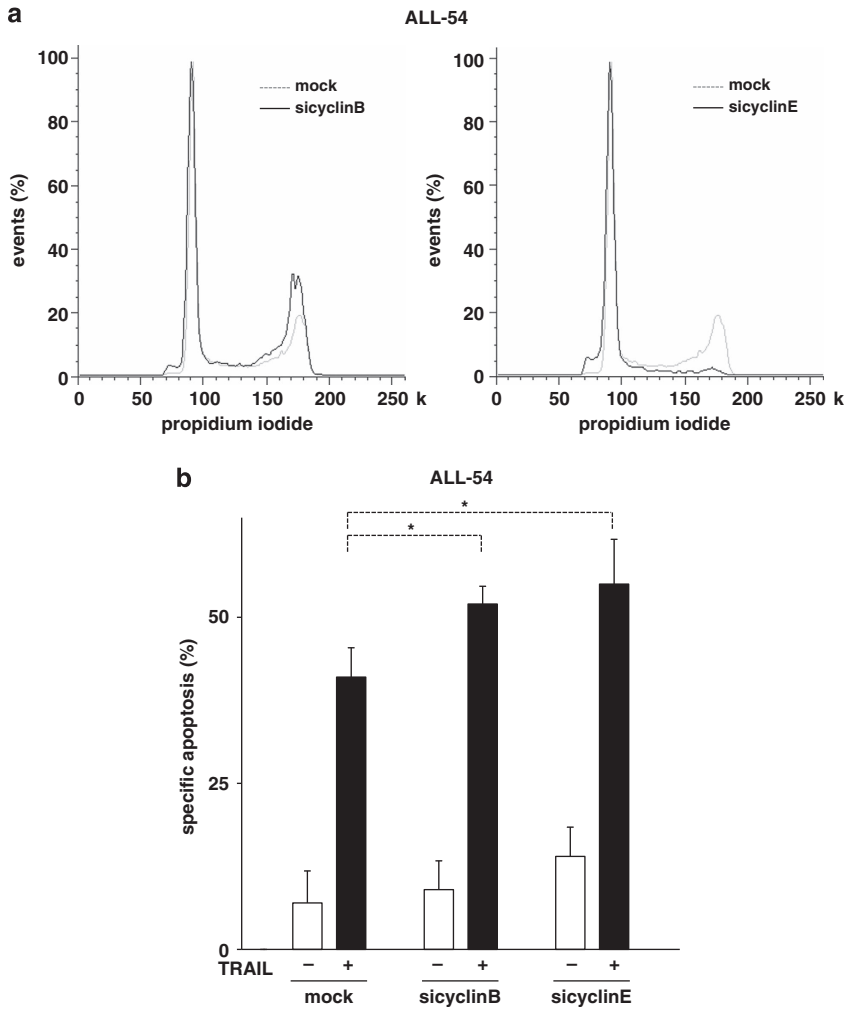

Figure 6 Molecular cell cycle arrest in G1 or G2 promotes apoptosis induction by TRAIL in xenografted ALL cells. (**a**, **b**) ALL-54 cells were transiently transfected with siRNA against cyclinB, cyclinE or a mock sequence using single nucleofection, as described in Materials and Methods. Cells were investigated for cell cycle distribution 24 h after transfection (**a**) or were stimulated with TRAIL (10 ng/ml) for another 24 h (**b**). Determination of cell cycle distribution and apoptosis induction, presentation and analysis of the data, and statistical analysis were performed as in Figures 1 and 4. $*P<0.05$

Increased activity of TRAIL against resting tumor cells might explain why TRAIL is especially effective in combination with cytostatic drugs, which induce cell cycle arrest. Our data highlight TRAIL as a promising candidate in contrast to others for the combination with cytostatic drugs.[18,20,37,39] Future polychemotherapy protocols might position TRAIL in close relation to cell cycle in order to gain highest antitumor efficiency by TRAIL, based on the molecular understanding of drug–drug interactions.

Materials and Methods

Materials. TRAIL was obtained from Pepro Tech (Hamburg, Germany). Alternatively, trimerized TRAIL was produced as described recently, rendering identical results.[39] Caffeine and L-mimosine were obtained from Calbiochem (Darmstadt, Germany); all further reagents were obtained from Sigma (St. Louis, MO, USA).

For flow cytometric analysis, the following antibodies were used: anti-cyclinD1 from BD Biosciences (San Jose, SA, USA) and anti-p-HistoneH3Ser10 from Cell Signaling Technology (Danvers, MA, USA), and anti-DR4 and anti-DR5 from Alexis Corp. (Lausen, Switzerland), Alx647-conjugated secondary anti-mouse antibody was obtained from Invitrogen (Darmstadt, Germany); for western blot: anti-FADD, anti-FLIP and anti-XIAP from BD Biosciences, anti-Bcl-xL, anti-Bid, anti-cIAP-1 and anti-PUMA from Cell Signaling; anti-Bak, anti-Bax, anti-Bcl-2, anti-cIAP-2 and anti-p53 from Santa Cruz (Santa Cruz, CA, USA); anti-Caspase-10 from MBL International (Woburn, MA, USA); anti-GAPDH from Thermo Fisher (Waltham, MA, USA); anti-NOXA from Calbiochem (San Diego, CA, USA), and anti BIM and anti Caspase-8 from Alexis Corp.

Cell lines, xenograft ALL cells and transfection experiments. HCT116 p53 $+/+$ were obtained from B. Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD, USA). All further cell lines were obtained from DSMZ (Braunschweig, Germany). For leukemic cell line experiments, cells were seeded at 0.25×10^6/ml for stimulations of solid tumor cells at 0.05×10^6/ml. Tumor cells were incubated with caffeine and chemotherapeutic drugs, as indicated in the corresponding figure legends.

Informed consent was obtained from all patients in written form, and studies were approved by the ethical committee of the medical faculty of the Ludwig Maximilians University Munich (LMU 068-08) and the Children's Hospital of the TU Munich (TU 2115/08). Animal work was approved by the Regierung von Oberbayern (55.2-1-54-2531-2-07). The xenograft mouse model, patient characteristics and engraftment, amplification, isolation and standardized procedures of *in vitro* stimulation have been described in detail recently.[11,25,32]

Transfection experiments in HCT116 was performed with lipofectamine 2000 (Life Technologies, Grand Island, NJ, USA) according to the manufacturers'

instructions. Lentiviral transduction of SHEP cells was described recently.[18,24,28] Nucleofection of CEM cells was performed with one million cells per reaction, and of xenograft ALL cells with five million cells per reaction using Amaxa Nucleofector technology (Lonza, Cologne, Germany) and program C16.[32] Lipofection in HCT116 and nucleofection in CEM cells was performed three times consecutively every 12 h. Lentiviral transduction was performed annealing the following sense and corresponding antisense oligonucleotides for the generation of pGreen-Puro shcyclinB 5′-GTCGGATCCGAAATGTACCCTCCAGAAATTGAATTCGTTTCTGGA GGGTACATTTCTTTTTAAGCTTAGT-3′ and 5′-GATCCAAGTGCTACTGCCGCA GTATCTTCA AGAGAGATACTGCGGCAGTAGCACTTTTTTTC-3′ for the construct containing shcyclinE.[24] DR4 and DR5 cDNAs were obtained from imaGENES GmbH (Berlin, Germany) and were cloned into pcDNA3.1.[39] For the transient knockdown of cyclinB or cyclinE in HCT116, CEM and xenograft ALL cells, siRNA against cyclinB (5′-GAAAUGUACCCUCCAGAAAtt-3′, 20 μM) and siRNA against cyclinE 5′-AAGTGCTACTGCCGCAGTATCtt-3′, 20 μM) were obtained from MWG Biotech (Ebersberg, Germany). All Star negative control siRNA from Qiagen (Hilden, Germany) was used for control transfection.

Apoptosis assays, flow cytometric analyses and western blot. Determination of cell death induction was performed with forward side scatter analyses for leukemia cells and with Nicoletti staining for solid tumor cells. Apoptotic cell death was confirmed in selected experiments with Annexin V–propidium iodide double staining as described.[11,18,28] To discriminate between G2 and M arrest, double staining with p-Histone H3 and propidium iodide was performed; to separate G0 and G1 phase, double staining with cyclinD1 and propidium iodide was performed, as described recently.[18,25] After cell fixation in 70% ethanol overnight, cells were resuspended in PBS with 0.25% Triton X, followed by incubation with the specific antibodies overnight in PBS supplemented with 1% BSA. After three washing steps, cells were incubated with RNAse A (100 μg/ml) at 37 °C and propidium iodide was added at 10 μg/ml directly before flow cytometric analysis. For the detection of TRAIL-death receptors, cells were incubated with the specific primary antibody, followed by incubation with an anti-mouse IgG1-specific antibody conjugated to Alx647 (Life Technologies). To exclude dead cells, death receptor staining was followed by Annexin V (BD Biosciences)/propidium iodide (1 μg/ml) double staining. LSR II (BD Biosciences) was used for the determination of cell cycle distribution and TRAIL receptor expression gating on living cells, and data were analyzed using Flow Jo software version 8.8.6 (Ashland, OR, USA). Western blot analysis was performed using a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% Triton X, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate and 1 mM Na_3VO_4 supplemented with proteinase inhibitor cocktail set I (Merck, Darmstadt, Germany) according to the manufacturer's instructions.

Statistical analysis. Specific apoptosis was calculated as ((apoptosis of stimulated cells at end minus apoptosis of unstimulated cells at end) divided by (100 minus apoptosis of unstimulated cells at end) times 100).

All data are presented as the mean values of at least three independent experiments ± S.E.M., unless otherwise stated. Isobolograms were performed with CompuSyn software version 1.0 (ComboSyn Inc., Paramus, NJ, USA). To test for significant differences, the paired *t*-test was applied to compare two groups; for multivariate analysis, one-way RM ANOVA was used. Statistical significance was accepted with $P<0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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Optimized anti–tumor effects of anthracyclines plus Vinca alkaloids using a novel, mechanism-based application schedule

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Application of anthracyclines and Vinca alkaloids on the same day represents a hallmark of polychemotherapy protocols for hematopoietic malignancies. Here we show, for the first time, that both drugs might act most efficiently if they are applied on different days. Proof-of-concept studies in 18 cell lines revealed that anthracyclines inhibited cell death by Vinca alkaloids in 83% of cell lines. Importantly, in a preclinical mouse model, doxorubicin reduced the anti–tumor effect of vincristine. Both drugs acted in a sequence-dependent manner and the strongest anti–tumor effect was obtained if both drugs were applied on different days. Most notably for clinical relevance, in 34% of 35 fresh primary childhood leukemia cells tested in vitro, doxorubicin reduced the anti–tumor effect of vincristine. As underlying mechanism, doxorubicin activated p53, p53 induced cell-cycle arrest, and cell-cycle arrest disabled inactivation of antiapoptotic Bcl-2 family members by vincristine; therefore, vincristine was unable to activate downstream apoptosis signaling. As molecular proof, antagonism was rescued by knockdown of p53, whereas knockdown of cyclin A inhibited vincristine-induced apoptosis. Our data suggest evaluating anthracyclines and Vinca alkaloids on different days in future trials. Selecting drug combinations based on mechanistic understanding represents a novel conceptional strategy for potent polychemotherapy protocols. (*Blood*. 2011;118(23):6123-6131)

Introduction

Chemotherapeutic drugs, such as anthracyclines and Vinca alkaloids, are used during anti–tumor therapy with the aim of clearing tumor cells on induction of cell death. As monotherapy is poorly effective, chemotherapeutic drugs are combined in polychemotherapy protocols to increase anti–tumor efficiency.[1-3]

Combinations of different chemotherapeutic drugs have been optimized heuristically in clinical multicenter trials. Because of substantial efforts required to realize clinical trials, only a limited number of drug combinations and application parameters could be optimized clinically. For example, anthracyclines and Vinca alkaloids are widely coapplied on the same day in many different anti–cancer protocols to treat hematopoietic malignancies and some forms of solid tumors. Nevertheless, the application schedule and sequence of these 2 classes of chemotherapeutic drugs were never optimized in clinical trials.[4,5] The rationale for using both drugs in combination was based on very limited (< 10) animal experiments back in the 1970s, which were performed exclusively on rat and mouse tumor cells, as far as we know.[6,7]

For certain chemotherapeutic drug combinations, anti–tumor efficiency highly depends on the application schedule, as shown in few clinical trials and numerous preclinical studies.[8-13] Among others, sequence dependency was proven for the combination of asparaginase and methotrexate for anti–leukemia therapy.[8,10,12-16]

On a molecular level, the effects and signaling pathways induced by many chemotherapeutic drugs have not been analyzed in depth, and the consequences of their combinatorial application remain unclear.[17] As far as we know, no mechanistic data exist so far to explain the sequence dependency of any clinically proven drug combination on a molecular level.

Here we aimed at optimizing the anti–tumor effect of the drug combination of anthracyclines and Vinca alkaloids based on an understanding of their signaling interactions. We detected a strongly sequence-dependent anti–tumor effect of both drugs and characterized the intracellular signaling mechanisms responsible for sequence dependency in leukemia cells. We introduced an optimized, mechanism-based application schedule, which might improve the effectiveness of both drugs in clinical trials for hematopoietic malignancies.

Methods

Materials

For Western blot, the following antibodies were used: anti–Bcl-xL, anti–Casp-2, anti–cleaved Casp-3, anti–cleaved Casp-6, anti–cleaved Casp-7, anti–cleaved PARP, and anti–phospho-histone H3 (Ser10) from Cell

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Signaling Technology; anti–Bcl-2, anti–cleaved Casp-1, and anti–p53 from Santa Cruz Biotechnology; anti–cyclin A and anti–GAPDH from Thermo Fisher; anti–Casp-9 from Transduction Laboratories; anti–Casp-10 from MBL; and anti–caspase-8 from Alexis Corp. For flow cytometric analysis, annexin V was obtained from BD Biosciences and anti–p-Histon H3 Ser 10 from Cell Signaling Technology. Vincristine (VCR) and all biochemical inhibitors were obtained from Calbiochem with the exception of 2,3-dichlorophenoxypropylaminoethanol (DCPE; Biomol International LP). All other reagents were obtained from Sigma-Aldrich.

Cell lines, transfection experiments, and primary samples

All leukemia cell lines were obtained from DSMZ and were maintained as previously described.[18,19] For all cell line experiments, cells were seeded at 0.2×10^6/mL and incubated with chemotherapeutic drugs at peak plasma concentration for 48 hours unless otherwise stated. CFUs were performed with a starting cell density of 0.02×10^6/mL using methylcellulose base media supplemented with 2mM L-glutamine, 10mM HEPES buffer solution, and 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen). Transfection experiments were performed using the Cell Line Nucleofector kit V (Lonza Walkersville) according to the manufacturer's instructions and shRNA against p53 and mock plasmids as previously described.[19] Alternatively, lentiviral transduction was performed targeting the identical p53 sequence or cyclin A with the following oligonucleotides annealed: sense sequence 5′-GATCCGAAATGTACCCTCCAGAAATTGAATTCGTTTCTGGAGGGTACATTTCTTTTTG-3′, antisense sequence 5′-AATTCAAAAAGAAATGTACCCTCCAGAAACGAATTCAATTTCTGGAGGGTACATTTCG-3′.

Primary leukemia blasts were obtained from 35 children treated for acute leukemia at the Ludwig Maximilian University Children's Hospital and the Children's Hospital of TU Munich during 2005 and 2008. Samples were obtained, isolated, and stimulated simultaneously with doxorubicin and VCR as previously described.[19,20]

Animal trial

The animal trial was approved by the Bavarian federal government, and animal care was in accordance with institution guidelines. Female NSG mice 8 to 12 weeks of age were obtained from Charles River. Mice were subcutaneously injected with 0.1 mL PBS containing 2.8×10^6 CEM cells into both flanks. After 10 days, all animals had developed tumors with diameters ranging between 2 and 6 mm. The animals were distributed into groups (control, n = 8 animals; doxorubicin, n = 8; VCR, n = 16; doxorubicin + VCR, n = 19) and treated with intravenous injections of doxorubicin solution (0.3 mg/kg body weight; MEDAC) and/or the intravenous administration of VCR solution (0.9 mg/kg body weight; TEVA) at day 0 and 8. Tumor size (2 dimensions) was determined at the beginning of treatment and was followed for 15 days. The relative change in tumor size was calculated for each animal.

Cell imaging, flow cytometric analysis, apoptosis assays, and Western blot analysis

For biochemical inhibition, cell lines were pretreated for 8 hours or irradiated 24 hours before further stimulation for another 48 hours. The release of cytochrome c was detected as recently described,[18] with loss of mitochondrial membrane potential using $DiOC_6$ staining. Cell-cycle analysis was performed using propidium iodide staining. To discriminate between G_2 and M arrest, double staining for p-histone H3 and propidium iodide was performed. In brief, cells were fixed by 70% ethanol, resuspended in PBS with 0.25% Triton X, followed by incubation with specific antibody in PBS with 1% BSA and addition of RNAse A (100 μg/mL) and propidium iodide 20 μg/mL after 3 washing steps. Apoptosis was measured by forward side scatter analysis and precision of this technique confirmed by annexin V and propidium iodide double staining according to the manufacturer's instructions using FACscan or LSR II flow cytometry and Cell Quest Pro Version 3.2.1 (BD Biosciences) and FlowJo Version 8.3 (TreeStar) software. Western blot analysis of total cellular protein or of cytosolic and nuclear fractions was performed as previously described, and 10 μg of protein was loaded.[19]

Statistical analysis

Specific apoptosis was calculated as [(apoptosis of stimulated cells at end minus apoptosis of unstimulated cells at end) divided by (100 − apoptosis of unstimulated cells at end) × 100], specific survival as [100 − specific apoptosis induction]. In Figure 2C, doxorubicin resistance was defined as specific apoptosis of < 10%. In Figure 2C, fractional product method (FP)[21] was used to discriminate between synergistic and antagonistic apoptosis induction after combined application of doxorubicin and VCR. FP values ≤ −0.1 were defined as relevant antagonism, and FP values ≥ 0.1 as relevant synergism. For primary samples, the expected apoptosis induction of independent application of doxorubicin and VCR was calculated as [(1 − (survival after simulation with doxorubicin × survival after stimulation with VCR)) × 100]. Alternatively, median effect blots were used performed by CompuSyn Version 1.0 software.

For cell line experiments, data are presented as the mean values of at least 3 independent experiments ± SEM unless otherwise stated. To test for significant differences, the paired *t* test was applied; for multivariate analysis, 1-way rank-sum ANOVA was used. Significance was set at $P < .05$. For animal trials, Mann-Whitney rank-sum test or Student *t* test was applied for $P < .01$.

Results

Anthracyclines and Vinca alkaloids are applied on the same day in several polychemotherapy protocols for hematopoietic malignancies. Here, we aimed at optimizing their anti–tumor effect based on the understanding of the responsible signaling interaction.

Inhibition of Vinca alkaloid-induced apoptosis by anthracyclines

The T-cell leukemia cell lines CEM (data presented in Figures 1-7 and supplemental Figures 1-3, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) and JURKAT (data presented in supplemental Figure 4) are highly sensitive toward VCR-induced apoptosis and partially sensitive toward doxorubicin-induced apoptosis. To our surprise and as a completely new finding, doxorubicin inhibited VCR-induced apoptosis in both cell lines, when both drugs were given simultaneously (Figure 1A). The net effect of the combinatorial use of both drugs resulted in antagonism as measured by various techniques, including morphology, forward side scatter analysis in FACscan, annexin V staining, uptake of propidium iodide, and DNA fragmentation (supplemental Figure 1). Both median effect plots and the fractional product method confirmed antagonistic interaction between doxorubicin and VCR (Figure 1B; and data not shown). More detailed studies revealed that doxorubicin inhibited VCR-induced apoptosis over a long period of time, in a dose-dependent manner, and enabled the survival of colony-forming tumor cells otherwise erased by VCR (Table 1; supplemental Table 1). The antiapoptotic effect of doxorubicin was not because of direct drug-drug interaction as it persisted after medium exchange. The antiapoptotic effect was not restricted to doxorubicin but also observed with other anthracyclines, including daunorubicin, epirubicin, and idarubicin. Reciprocally, doxorubicin attenuated induction of cell death not only by VCR, but also by vinblastine and vinorelbine, suggesting a general inhibitory and antiapoptotic effect of

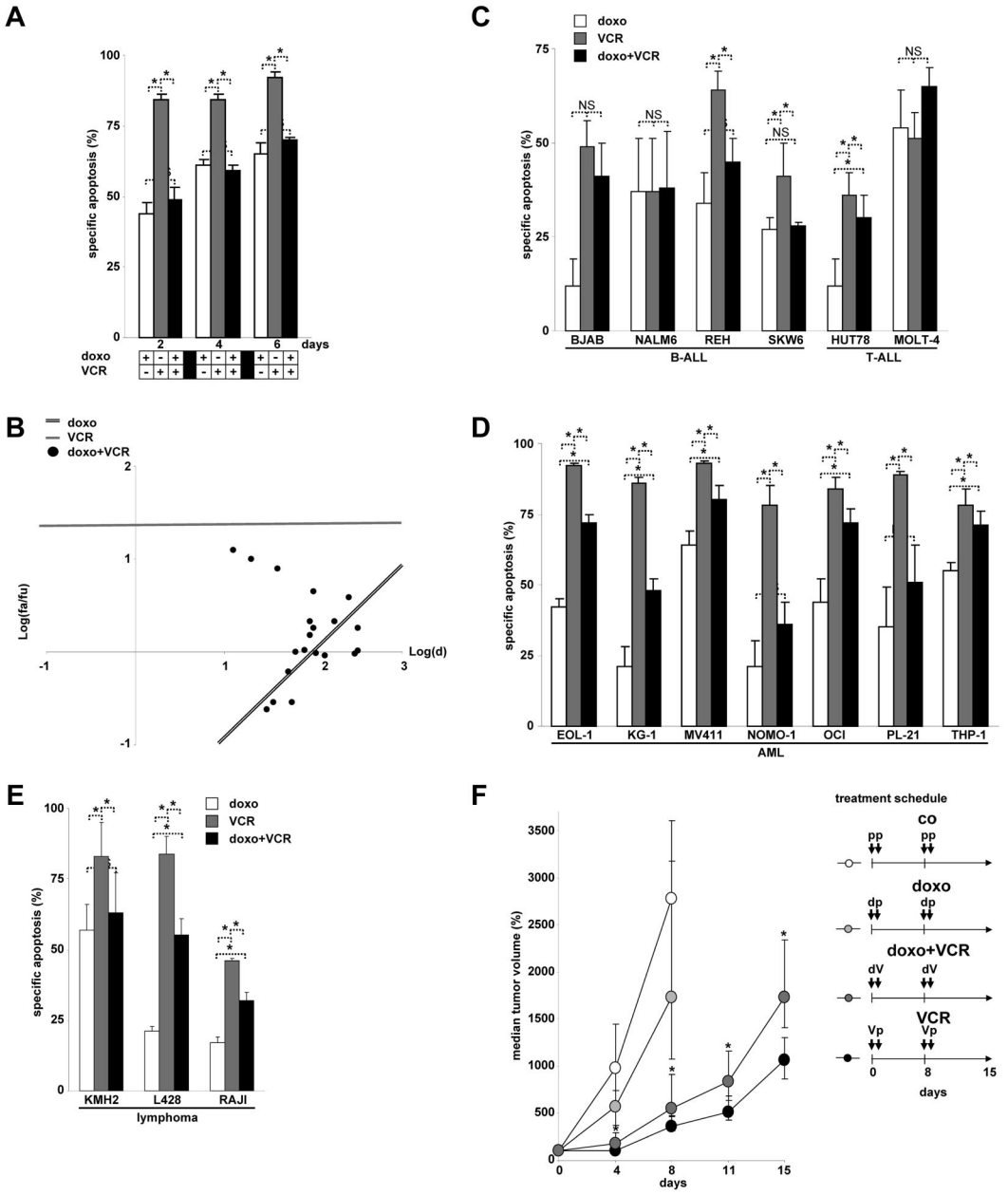

Figure 1. Inhibition of Vinca alkaloid-induced apoptosis by anthracyclines. (A) CEM leukemia cells were simultaneously stimulated with doxorubicin (doxo, 100 ng/mL) and VCR (300 ng/mL) for time periods indicated. *$P < .05$ (ANOVA). NS indicates not significant. (B) Corresponding data from panel A for 48-hour incubation time and simultaneous application were analyzed for a total of n = 20 combinations by median effect plots investigating a range of drug concentrations (doxorubicin 10, 30, 60, and 100 ng/mL; VCR 3, 10, 30, 100, and 300 ng/mL). fa indicates apoptotic fraction; fu, fraction of cells alive; and d, drug dosage. (C-E) Further n = 6 B-ALL and T-ALL (C), n = 7 acute myeloid leukemia (D), and n = 3 lymphoma cell lines (E) were stimulated with doxorubicin and VCR for 48 hours as in panel A. *$P < .05$ (ANOVA). NS indicates not significant. (F) Xenograft study of CEM leukemia cells subcutaneously implanted into NSG mice was performed as described in "Animal trial." Mice were treated as shown in the treatment schedule with doxorubicin (0.3 mg/kg) and/or VCR (0.9 mg/kg) or placebo as shown. Tumor size was measured in 2 dimensions, and tumor volume was calculated. Statistical analysis using Mann-Whitney rank-sum test was performed comparing VCR and combinatorial treatment (doxorubicin + VCR) at each measurement point (*$P < .01$) and revealed that doxorubicin followed by VCR 1 day later significantly inhibited the effect of VCR alone. The 25th and 75th quartiles are shown. p indicates placebo; d, doxorubicin; and V, VCR.

anthracyclines toward Vinca alkaloid-induced cell death (data not shown for all).

Cell lines of different hematopoietic tumors were tested, including 8 B-acute lymphoblastic leukemia (B-ALL) and T-ALL cell lines, 7 acute myeloid leukemia cell lines, and 3 lymphoma cell lines. All lines expressed functionally active p53.[22] Overall, in 15 of 18 (83%) cell lines, a negative interaction between doxorubicin and VCR was detected, when both drugs were applied together, as their mutual apoptosis induction was lower than expected from the activity from the single drugs (Figure 1A,C-E; supplemental Figure 4A). When hematopoietic subtypes were analyzed, inhibition of VCR-induced apoptosis was present in 63% of ALL and 100% of acute myeloid leukemia and lymphoma cell lines.

To test the described phenotype within the complex in vivo situation, NSG mice were xenografted subcutaneously with human CEM T-ALL leukemia cells. CEM cells bearing mice were treated with either doxorubicin or VCR alone or both drugs simultaneously. Similar to the in vitro data, doxorubicin significantly inhibited the anti–tumor effect of VCR in vivo, when doxorubicin was applied together with VCR (Figure 1F).

In summary, doxorubicin frequently and severely inhibited VCR-induced apoptosis in hematopoietic tumor cells in vitro and in vivo when both drugs were applied simultaneously.

Table 1. New colony formation 7 days after stimulation with doxorubicin and VCR in CEM cells

	Control	Doxorubicin	VCR	Doxorubicin + VCR
No. of colonies	165 ± 15	70 ± 11	2 ± 1	40 ± 4*

Data are mean of 3 independent experiments ± SEM. CEM cells were stimulated with doxorubicin (30 ng/mL) and/or VCR (30 ng/mL) or left untreated (Control), as in Figure 1A using 96-well plates. Consecutive light microscopy pictures were taken for 7 days to detect new colony formation. Drug concentrations were reduced compared with the experimental setting in Figure 1, according to lower cell density.

*$P < .05$, comparing new colony formation after VCR alone with the combined doxorubicin + VCR treatment.

Antagonistic effect of doxorubicin and VCR on primary leukemic tumor cells

To approximate the clinical situation and move beyond established cell lines, 35 fresh primary tumor samples were investigated. Cells were obtained from children with acute leukemia and isolated from diagnostic bone marrow aspirations before the onset of anti–cancer treatment. Fresh primary leukemia cells were stimulated in vitro with each drug alone or both drugs simultaneously, applying a range of clinically relevant drug concentrations. Doxorubicin inhibited VCR-induced apoptosis in several primary leukemia tumor cells (Figure 2A) and over a broad range of concentrations (Figure 2B).

Next, we aimed to estimate the effect of the different application schedules of both drugs on a number of primary leukemia cells. Unfortunately, these cells allow only one single short-time experiment in vitro. To estimate how efficient the 2 drugs might have been if they had been given one after the other, independently from each other, independent apoptosis induction was calculated out of the data obtained by single-agent stimulation using the equation by Webb (Figure 2C, white and gray bars; details of calculations in "Statistical analysis"). This equation allows the most precise calculation on limited numbers of experiments[21] (Figure 2C white and gray bars; details of calculations in "Statistical analysis"). For comparison, apoptosis induction by the drug combination is depicted as black dots in Figure 2C. In 11% of samples, apoptosis induction was increased by simultaneous application of doxorubicin and VCR compared with independent application yielding synergistic apoptosis. In contrast, simultaneous application of

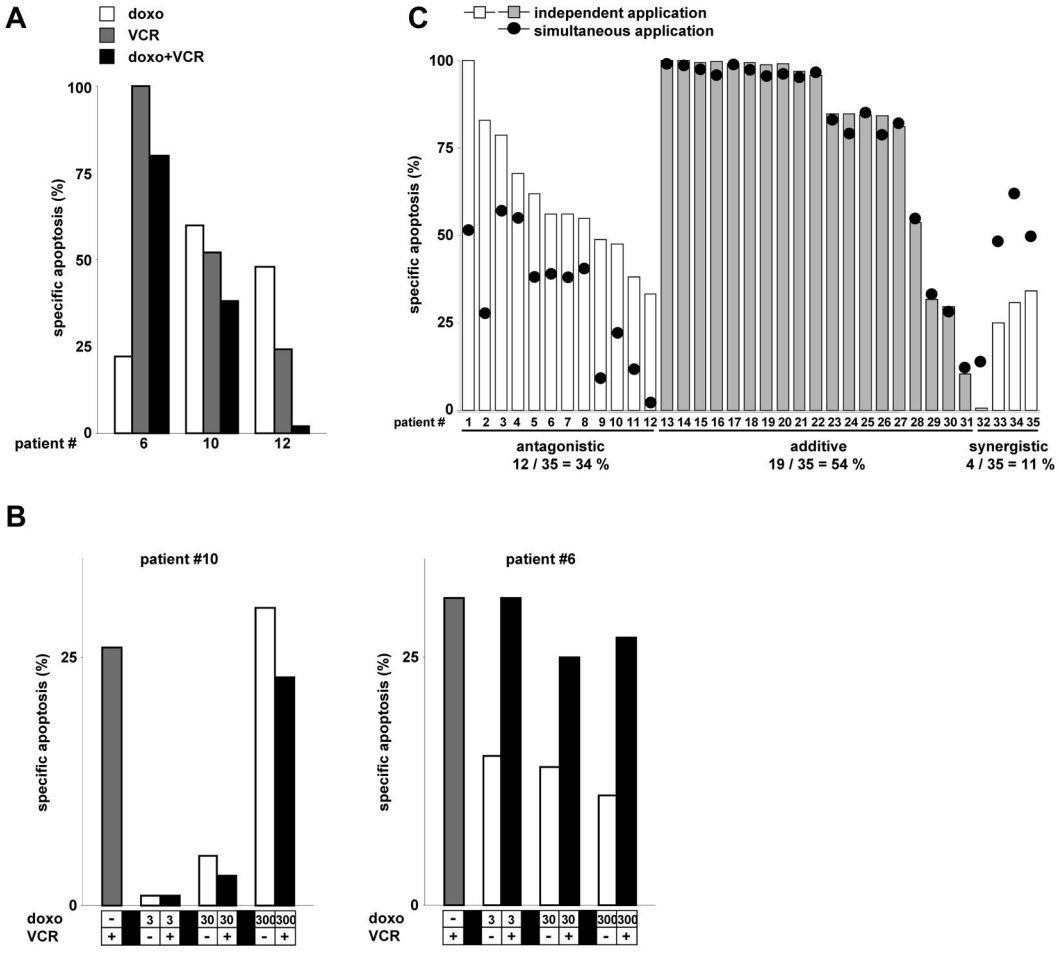

Figure 2. Antagonistic effect of doxorubicin and VCR on primary leukemic tumor cells. (A) Three primary leukemia samples (patients 6, 10, and 12 from panel C) were simultaneously stimulated with doxorubicin (300 ng/mL) and VCR (300 ng/mL). Apoptosis induction was measured after 48 hours, when spontaneous apoptosis had reached 40%, otherwise after 72 hours. (B) Patient sample 10 (left panel, VCR 300 ng/mL) and patient 6 (right panel, VCR 30 ng/mL) from panel A were stimulated with doxorubicin and VCR (as indicated). (C) Thirty-five primary leukemia samples were stimulated with either doxorubicin or VCR alone or simultaneously with doxorubicin and VCR as in panel A. All samples were sensitive for doxorubicin (specific apoptosis > 10%) besides samples 29 to 32. Measured apoptosis for the combination of doxorubicin and VCR is depicted as black dots (called "simultaneous application"). The expected apoptosis induction, if doxorubicin and VCR were given independently, was calculated from the results obtained with each drug alone as described in "Statistical analysis" and is shown as white and gray bars (called "independent application"). Definition of antagonistic, additive, and synergistic apoptosis is described in "Statistical analysis."

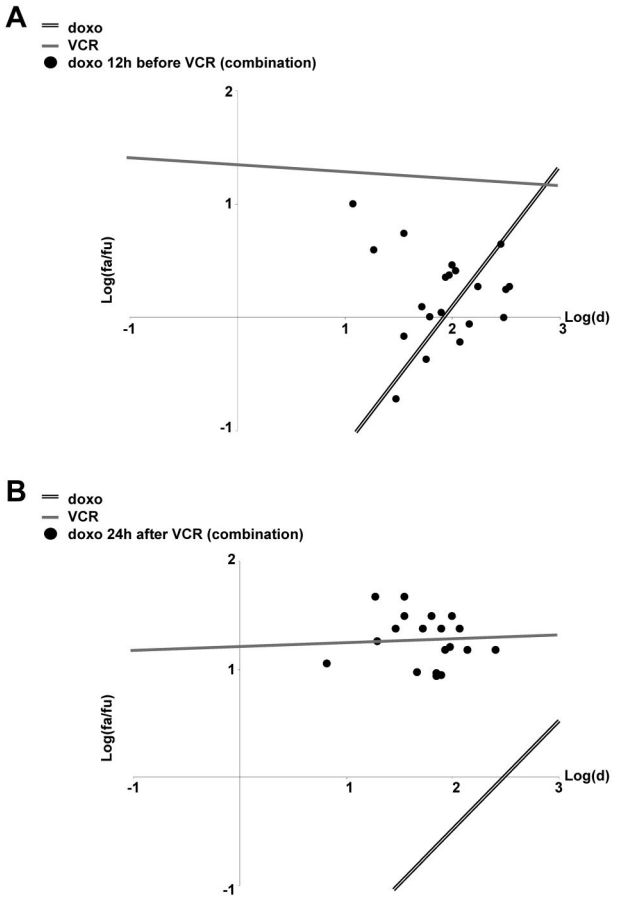

Figure 3. Sequence-dependent effects of the drug combination doxorubicin/VCR. (A-B) CEM cells were stimulated and analyzed as in Figure 1B, but now doxorubicin was applied 12 hours before VCR (A) or 24 hours after VCR (B).

doxorubicin and VCR was less effective than independent application, yielding antagonistic apoptosis: When both drugs were given at peak plasma concentration, doxorubicin inhibited VCR-induced apoptosis in 34% (12 of 35) of samples, which increased to 54% (19 of 35) of samples when lower concentrations of doxorubicin were included (data not shown). Within the small cohort, no correlation of inhibition of VCR-induced apoptosis by doxorubicin with genetic alterations could be detected (data not shown).

In summary, doxorubicin frequently and markedly inhibited VCR-induced apoptosis in primary tumor cells from children. Importantly, doxorubicin exerted its antiapoptotic effect on leukemia cells obtained from those children who received simultaneous application of doxorubicin and VCR within induction therapy of the ongoing polychemotherapy trial.

Sequence dependency of the antagonism in vitro and in vivo

When 2 or more chemotherapeutic drugs are combined, sequence-dependent effects were revealed for a number of drug combinations both in vitro as well as in clinical studies.[8,10,12-16] Therefore, we studied whether the antagonism between doxorubicin and VCR might be sequence dependent. Indeed, when VCR was given first, 1 day before doxorubicin, VCR induced significant apoptosis. In contrast, when doxorubicin was given first or together with VCR, doxorubicin markedly inhibited apoptosis induction by VCR (Figures 1B,3). At least in part, doxorubicin reduced VCR-induced apoptosis, even when given after VCR (supplemental Figure 4E). Thus, the efficiency of the combinatory treatment of doxorubicin and VCR depends on the sequence of application and whether doxorubicin encounters active VCR.

Taken together, doxorubicin inhibited apoptosis induction by VCR depending on the application schedule. These data add the clinical routine combination of anthracyclines and Vinca alkaloids to the drug combinations with sequence-dependent anti–tumor effects.

Signaling mechanism of VCR and inhibition by doxorubicin

To characterize underlying signaling mechanisms responsible for the negative drug interaction, doxorubicin and VCR were studied in parallel on CEM and JURKAT leukemia cell lines.

First, we aimed to characterize the effects of doxorubicin on VCR-induced apoptosis signaling events. The apoptosis signaling cascade activated by VCR is not completely characterized, but it involves incomplete spindle formation, cell-cycle arrest, activation of p53 and NF-κB, phosphorylation of antiapoptotic Bcl-2 members, and initiation of the downstream intrinsic apoptosis signaling cascade, among others.[23] In a candidate approach, we analyzed the expression levels and functions of putative players.

VCR activates the intrinsic apoptosis signaling cascade. The Bcl-2 and IAP-member families contain important antagonists of this signal transduction. Their expression levels remained unchanged by treatment with doxorubicin, VCR, or the combination of both (supplemental Figure 2A). During VCR-induced apoptosis, antiapoptotic Bcl-2 and Bcl-xL become inactivated by phosphorylation.[23] Apoptosis induction by VCR alone highly depended on phosphorylation of antiapoptotic Bcl-2 members, as overexpression of phosphorylation-deficient mutants of Bcl-2 and Bcl-xL markedly reduced apoptosis induction by VCR[24,25] (supplemental Figure 2B). For the combined application of doxorubicin and VCR, the phosphorylation of Bcl-2 and Bcl-xL by VCR was markedly inhibited by doxorubicin (Figure 4A). Thus, failure to phosphorylate and inactivate antiapoptotic Bcl-2 and Bcl-xL was identified as the most proximal signaling step within the VCR-induced apoptotic signaling cascade, which was inhibited by doxorubicin. After lack of phosphorylation and inactivation of Bcl-2 and Bcl-xL, all downstream cell death signaling steps, which were rapidly activated on VCR-induced apoptosis, were inhibited by doxorubicin, including loss of mitochondrial membrane potential, release of cytochrome c, cleavage of caspases, and cleavage of PARP (Figure 4B).

To study the role of Bcl-2 members in apoptosis inhibition in more detail, DCPE was added, which is known to down-regulate Bcl-2 family members by as yet unknown mechanisms.[26] DCPE reduced the expression levels of anti–apoptotic Bcl-2 and Bcl-xL as shown by Western blot (Figure 4C). Thereby, DCPE enabled to override the inhibitory effect of doxorubicin on VCR-mediated apoptosis (Figure 4C). Treatment with 2 phosphatase inhibitors, calphostin c and okadaic acid, caused phosphorylation of Bcl-2 and Bcl-xL independently from VCR by blocking dephosphorylation (Western blots in Figure 4C; and data not shown). Calphostin c and okadaic acid restored VCR-induced apoptosis in the presence of doxorubicin, supporting an important role for the stabilization of antiapoptotic Bcl-2 members in doxorubicin-induced inhibition of apoptosis (Figure 4C; and data not shown).

Taken together, these results show that doxorubicin stabilized anti–apoptotic Bcl-2 family members by preventing their phosphorylation, which was reversed by treatment with DCPE, calphostin c, and okadaic acid. Doxorubicin inhibited VCR-induced Bcl-2 member phosphorylation and thereby disabled the downstream intrinsic apoptosis signaling cascade of VCR (Figure 6E).

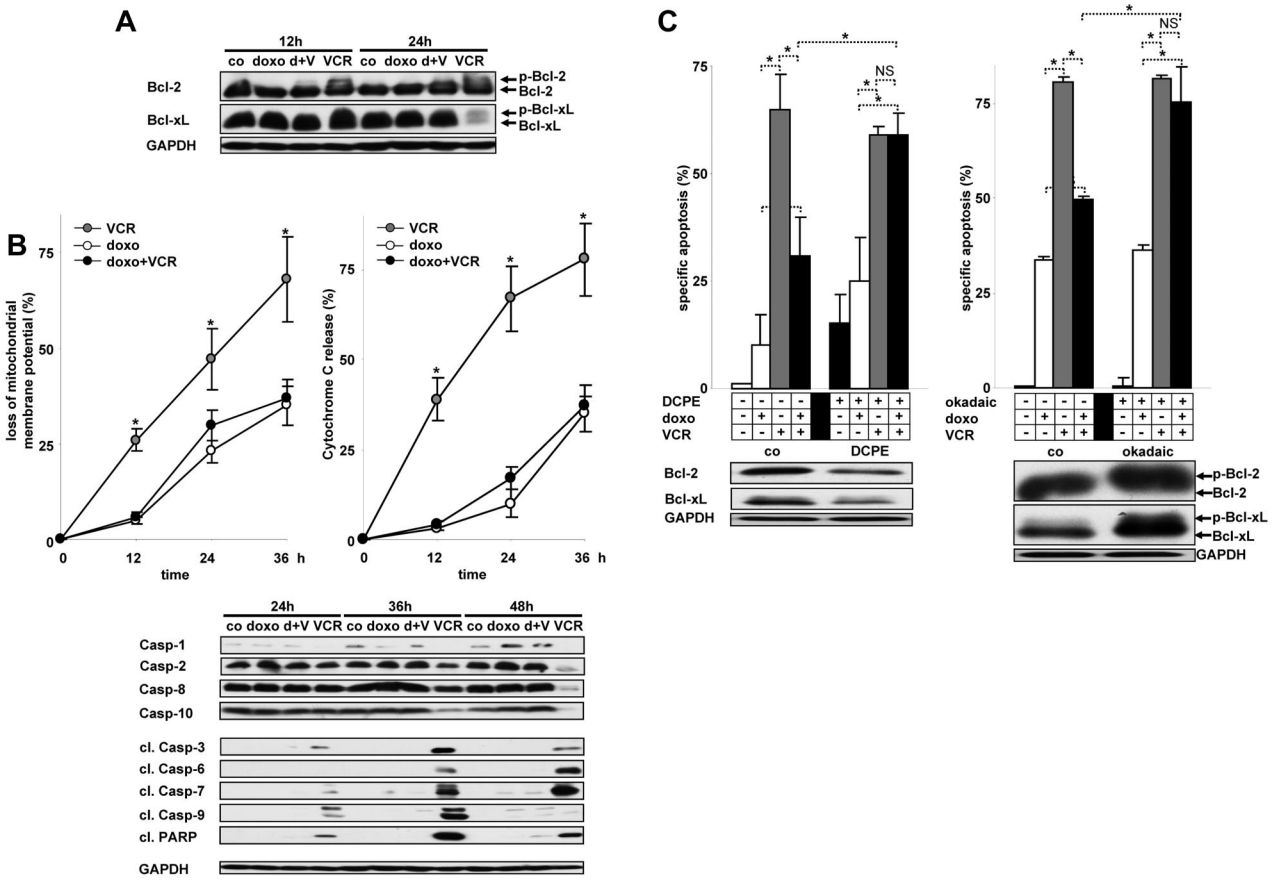

Figure 4. Signaling mechanism of VCR and inhibition by doxorubicin. (A) Western blot of total cellular protein was performed on CEM cells stimulated with doxorubicin and VCR as in Figure 1A. GAPDH served as a loading control. (B) CEM cells were stimulated with doxorubicin and VCR. Loss of mitochondrial membrane potential (left panel) and cytochrome c release (right panel) was measured by FACScan. Caspase cleavage was detected by Western blot (bottom panel). $*P < .05$ (ANOVA), comparing stimulation with VCR alone with doxorubicin alone or combined stimulation with doxorubicin + VCR. (C) CEM cells were treated with 2,3-DCPE (10μM, left panel) or the phosphatase inhibitor okadaic acid (okadaic, 0.03 ng/mL, right panel) for 8 hours, followed by doxorubicin together with VCR for another 48 hours as indicated. Western blot of total cellular protein was performed after 56 hours. $*P < .05$ (ANOVA). NS indicates not significant. The concentrations of doxorubicin and VCR, measurement of apoptosis, presentation of data, and statistical analysis were performed as described in Figure 1A. Casp indicates caspase; co, unstimulated control cells; cl., cleaved; d, doxorubicin; p, phosphorylated; h, hour; and V, VCR.

Signaling mechanism of doxorubicin and impact on VCR-induced apoptosis

Next, we searched for signaling molecules that mediate the antiapoptotic function of doxorubicin. In tumor cells, anthracyclines are known to intercalate into DNA, induce formation of free radicals, and inhibit topoisomerase II among other actions.[27] Anthracyclines induce DNA damage, activate ataxia teleangiectasia mutated kinase, induce p53, and lead to cell-cycle arrest or apoptosis.[27,28]

The transcription factor p53 is activated by DNA-damage and signals pleiotrophic effects, such as DNA repair, cell-cycle arrest, and cell death.[29] In our 2 cell lines investigated, p53 is present and doxorubicin induced the accumulation of nuclear p53 both in the presence and absence of VCR (Figure 5A; data not shown). The down-regulation of p53 protein levels restored VCR-induced apoptosis in the presence of doxorubicin, indicating an important role for p53 in this process (Figure 5B).

Activated p53 induces cell-cycle arrest, which might participate in apoptosis inhibition. Both VCR and doxorubicin caused a marked accumulation of cells in G_2/M (Figure 6A; supplemental Figure 3A). Further cell-cycle discrimination revealed that doxorubicin arrested the cell cycle in G_2, whereas VCR arrested the cell cycle in M (Figure 6B). No signs of cellular senescence were detected (data not shown). When drugs were combined, cell-cycle arrest was enhanced and $> 80\%$ of the cells were arrested in the G_2 for the entire observation period available in cell culture (Figure 6A; and data not shown). Cell-cycle arrest in G_2 by doxorubicin was reduced in p53 knockdown cells (supplemental Figure 3B).

To prevent cell-cycle arrest induced by doxorubicin and VCR, cells were pretreated with caffeine or KU-55933, an inhibitor of ataxia teleangiectasia mutated kinase. Both compounds alleviated the block in the presence of both doxorubicin and VCR (supplemental Figure 3C; data not shown) and sensitized the cells for VCR-induced apoptosis (Figure 6C; and data not shown).

To arrest the cell cycle on a molecular level, cyclin A was knocked down by RNA interference, which induced a major fraction of cells in G_2 (Figure 6D). Knockdown of cyclin A and concomitant cell-cycle arrest significantly inhibited VCR-induced apoptosis (Figure 6D) proving that cell-cycle arrest disabled VCR-induced apoptosis.

Taken together, these results show that p53 and cell-cycle arrest mediate the antiapoptotic function of doxorubicin. Doxorubicin inhibited VCR-induced apoptotic signaling upstream of Bcl-2/Bcl-xL. Lack of p53, addition of 2 cell-cycle stimulators, caffeine or KU55933, or addition of agents antagonizing Bcl-2/Bcl-xL, such as calphostin c, okadaic acid, and DCPE, alleviated the antiapoptotic function of doxorubicin toward VCR-induced apoptosis, whereas knockdown of cyclin A inhibited VCR-induced apoptosis similarly to doxorubicin as illustrated in a schematic chart (Figure 6E).

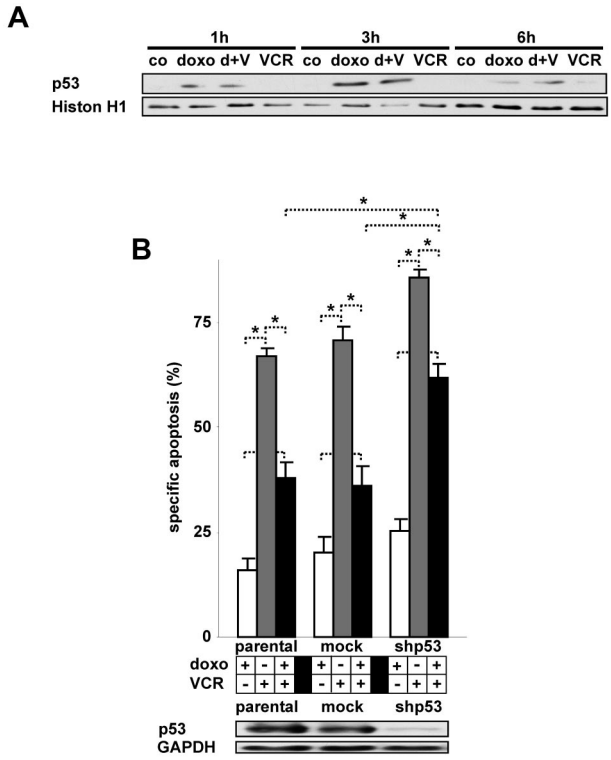

Figure 5. Activation of p53 by doxorubicin and its impact on VCR-induced apoptosis. (A) Nuclear extracts of CEM cells simultaneously stimulated with doxorubicin and VCR as indicated were analyzed by Western blot. Histone H1 served as a loading control. (B) Parental CEM cells stably transfected with shRNA targeting p53 (shp53) or a control mock shRNA sequence were stimulated with doxorubicin and VCR simultaneously. $*P < .05$ (ANOVA). NS indicates not significant. Western blot was performed of total cellular protein. The concentrations of doxorubicin and VCR, measurement of apoptosis, presentation of data, and statistical analysis were performed as described in Figure 1A. d indicates doxo; V, VCR; h, hour; and co, unstimulated control cells.

Cell-cycle arrest–based antagonistic interaction between irradiation and VCR

So far, our mechanistic studies revealed that cell-cycle arrest mediated doxorubicin-induced inhibition of VCR-induced apoptosis. Based on these new mechanistic insights, we hypothesized that further cell-cycle arresting drugs and stimuli would inhibit VCR-induced apoptosis similarly to doxorubicin.

Indeed, irradiation induced cell-cycle arrest in G_2[30,31] and significantly inhibited VCR-induced cell death (Figure 7). Similarly to the phenotype observed in G_2 arrested cells, cell-cycle arrest in G_0 or G_1 by dexamethasone, serum starvation, or L-mimosine hampered apoptosis induction by VCR (data not shown). These data show that VCR is generally unable to induce cell death in cell-cycle–arrested tumor cells and that VCR requires active cell cycling for effective induction of apoptosis. Thus, our new understanding of the mechanisms of VCR-induced apoptosis enabled the identification of numerous antagonizing stimuli.

Taken together, we have identified cell-cycle arrest as a new general mechanism responsible for sequence-dependent effects of drug combinations with VCR: If VCR is given first and activates the proapoptotic signaling cascade before induction of cell-cycle arrest by any second stimulus, VCR potently induces apoptosis. If a cell-cycle arresting, cytostatic stimulus is given shortly before VCR, the proapoptotic efficacy of VCR is markedly reduced.

Discussion

In several different polychemotherapy protocols for hematopoietic tumors, anthracyclines and Vinca alkaloids are given on the same day. Our data show that the anti–tumor effect of this drug combination might be enhanced if both drugs are given on different days.

In vitro, the drug combination was found to be highly sequence-dependent both in tumor cell lines and in primary, patient-derived tumor cells. Sequence dependency was also found in an in vivo trial. Using complex signaling studies and knockdown strategies, we delineate the underlying signaling mechanism for antagonistic apoptosis: Doxorubicin activates p53, p53 induces cell-cycle arrest, and cell-cycle arrest inhibits the downstream apoptosis signaling pathway otherwise activated by VCR. Characterization of this signaling mechanism enabled to identify further antagonistic drug combinations (eg, irradiation and VCR).

Back in the 1970s, clinicians decided to combine anthracyclines and Vinca alkaloids during polychemotherapy based on few (< 10) animal experiments performed on rat and mouse tumor cells.[6,7] In these animal trials, high concentrations of doxorubicin potentiated VCR-induced anti–tumor efficiency. Regarding the combination of doxorubicin and VCR; and as far as we know, no systematic evaluation of application schedules was ever performed on human tumor cells either in vitro or in small animal models in vivo or in clinical trials in patients.

In contrast, in our study, we used human leukemia cell lines in vitro and in vivo and primary tumor cells of children with acute leukemia ex vivo and found that anthracyclines markedly inhibited Vinca alkaloid-induced apoptosis. Here, independent application of doxorubicin and VCR was more effective against most tumor cells than combined application in vitro. To study the biologic effects and signaling mechanisms of chemotherapeutic drugs, we used drug concentrations in vitro, which are achieved in the plasma of patients during anti–tumor therapy in vivo (peak plasma concentration = ppc as maximum).[32] The difference between the previous data and our data might rely on factors, such as interspecies differences, drug concentrations used, different read-outs, and the small number of cell lines tested in those former studies.

It will be interesting to evaluate whether the independent application of anthracyclines and Vinca alkaloids will increase treatment efficiency in cancer patients, especially as doxorubicin and VCR showed antagonistic effects in the primary tumor cells of one-third of children treated with this combination.

The newly discovered antiapoptotic function of doxorubicin is in line with our previous data showing that TRAIL, a chemotherapeutic drug currently in phase 1 and 2 clinical trials, displays antiapoptotic and even pro-proliferative features in apoptosis-resistant tumor cells.[18-20]

There is a long-lasting conceptual discussion of whether cytotoxic and cytostatic drugs should be combined.[33] The term "cytostatic drugs" has been used for drugs that act against tumor cells by inducing cell-cycle arrest. In our study, anthracyclines arrested the cell cycle and behaved like cytostatic drugs in the cell lines investigated. On the other hand, several chemotherapeutic drugs require active cell cycling for induction of apoptosis and cell-cycle arrest reduces or abrogates the anti–tumor efficiency of these drugs.[16,33] In our experiments, Vinca alkaloids acted like drugs depending on active cell cycling. Our data allow the hypothesis that cytostatic drugs and drugs depending on active cell cycling might not be combined, as they exert optimal anti–tumor efficiency if given independently.

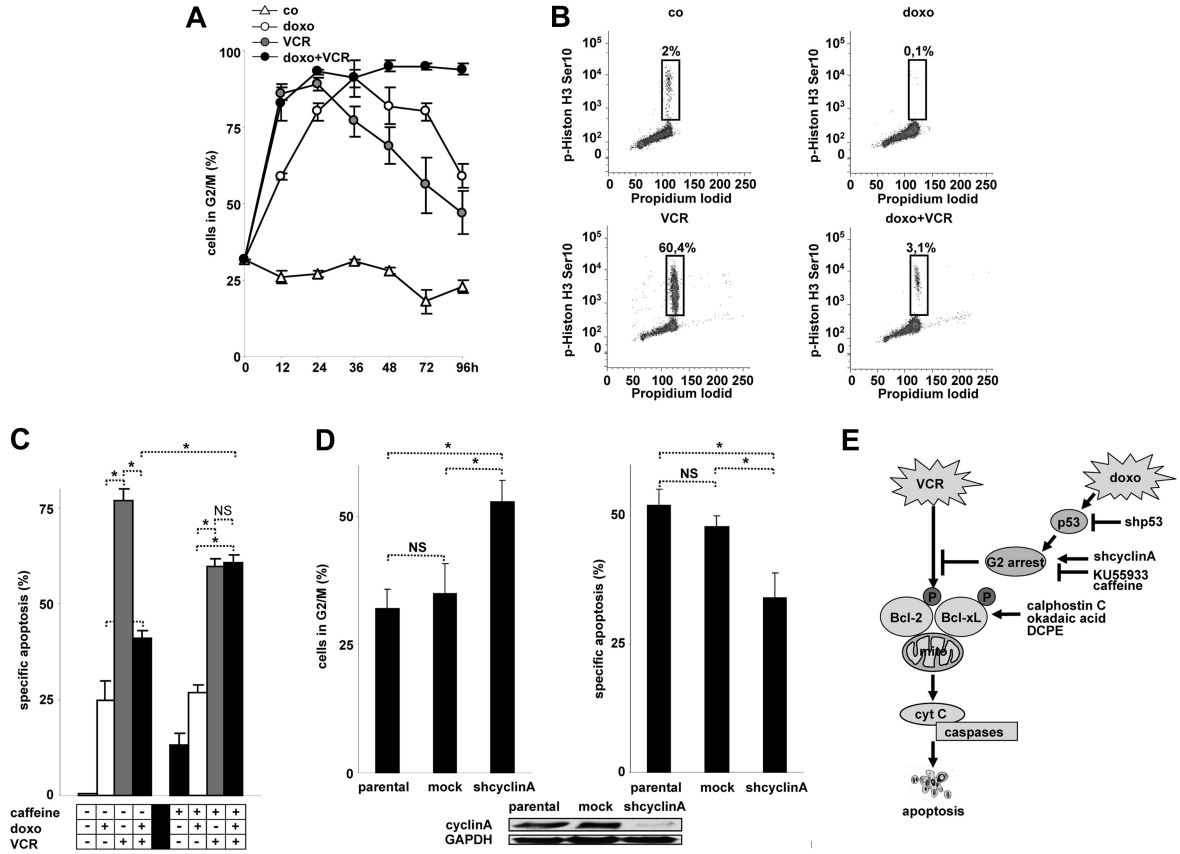

Figure 6. Arrest of the cell cycle by doxorubicin and its impact on VCR-induced apoptosis. (A-B) Cell-cycle analysis was performed using propidium iodide staining of DNA in CEM cells (A). Corresponding cell-cycle histograms are presented in supplemental Figure 3A. To discriminate between G_2 and M arrest, double staining for phospho-histone H3 (Ser10) and propidium iodide was performed after 24 hours (B). co indicates unstimulated control cells. (C) CEM cells were preincubated with caffeine (300 μg/mL) for 8 hours, followed by doxorubicin together with VCR for 48 hours. *$P < .05$ (ANOVA). NS indicates not significant. (D) CEM cells were stably transfected with a shRNA targeting cyclin A (shcyclin A) or a control mock sequence and were analyzed for cell-cycle distribution of spontaneously growing cells (left panel) or for apoptosis induction by VCR (3 ng/mL) after 48 hours (right panel). *$P < .05$ (ANOVA). NS indicates not significant. (E) Scheme summarizing the data presented in Figures 4 to 6: Doxorubicin-mediated activation of p53 and G_2 arrest inhibits VCR-induced cell death, abrogating VCR-induced phosphorylation of Bcl-2 family members, the distal apoptosis signaling pathway, and cell death. The concentrations of doxorubicin and VCR, measurement of apoptosis, presentation of data, and statistical analysis were performed as described in Figure 1A.

Vinca alkaloids are not the only class of chemotherapeutic drugs that depends on active cell cycling.[16] More studies are required to identify those drugs, which share the molecular mechanism of Vinca alkaloids and should therefore not be combined with cytostatic drugs.

p53 was shown by others[34] and by us[19] to predominantly act as a proapoptotic mediator. In contrast to its mainly proapoptotic functions, in our study p53 acted as an apoptosis inhibitor toward VCR-induced apoptosis. Although cell lines contained different p53 conformations and mutations, no correlation was found between mutations of p53 and interaction between doxorubicin and VCR.[22]

The present work clearly underlines the significance to reevaluate the inhibitory effect within the multiple known functions of p53.[35] Because many chemotherapeutic drugs activate p53, the new anti–apoptotic effect might not be restricted to anthracyclines and irradiation, but might involve additional established chemotherapeutic drugs that activate p53. Our data allow the hypothesis that further stimuli of p53 will disable VCR-induced apoptosis.

It is well known that (1) numerous chemotherapeutic drugs activate p53, (2) p53 can induce cell-cycle arrest in tumor cells, and (3) certain chemotherapeutic drugs need active cell cycling for the induction of cell death.[16,19,23,29,31] The new antiapoptotic function of anthracyclines, although clinically and empirically surprising, is not surprising once the underlying molecular mechanisms are considered.

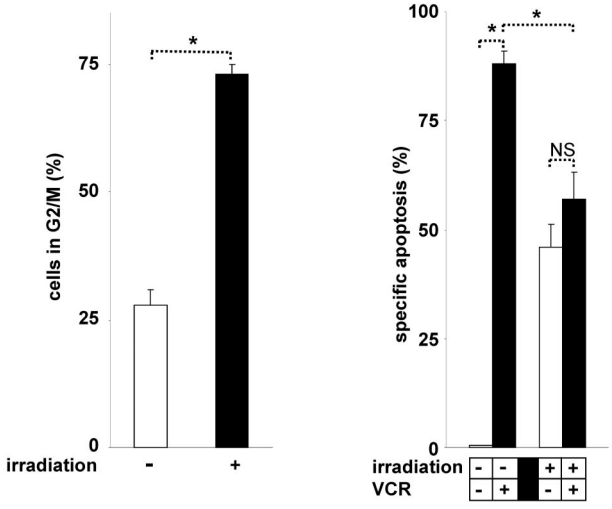

Figure 7. Cell-cycle arrest–based antagonistic interaction between irradiation and VCR. CEM cells were irradiated with 6 Gy for 24 hours. Cell-cycle analysis was performed (left panel), and cells were stimulated with VCR for another 48 hours (right panel). *$P < .05$ (ANOVA). NS indicates not significant. The concentration of VCR, measurement of apoptosis, presentation of data, and statistical analysis were performed as described in Figure 1A.

Our data represent the first characterization of a molecular mechanism responsible for sequence-dependent anti–tumor effects of chemotherapeutic drugs in routine clinical use. Our data clearly reinforce the need for the detailed mechanistic understanding of signaling pathways and pathway cross-talks. "Targeted therapies" represent a promising concept to optimize anti–tumor therapy based on the mechanistic understanding of target proteins and cellular signaling. In parallel, our data encourage to widen the concept and search for "targeted drug combinations" in which the positive or negative interaction of drugs is characterized on a molecular level. These new mechanistic insights will enable the design of more effective polychemotherapy protocols to treat hematopoietic malignancies.

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Authorship

Contribution: H.E., D.S., C.M., and F.W. performed experiments; S.H. provided phosphorylation-deficient Bcl-xL expression plasmids; U.G. and M.N. provided primary patient samples; H.E. and I.J. designed the research, provided administrative support, analyzed and interpreted the data, prepared the figures, and wrote the paper; and all authors gave final approval of the manuscript.

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Publikationsliste

Publikationen in peer reviewed Journalen

Ehrhardt H, Schrembs D, Pfeiffer S, Wachter F, Grunert M, Jeremias I.
Activation of the DNA damage response by antitumor therapy counteracts the activity of vinca alkaloids.
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Abstrakt

Wachter F, Grunert M, Ehrhardt H, Jeremias I.
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Poster

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TRAIL preferentially affects cell cycle-arrested tumor cells including stem- and progenitor cells from patients with acute lymphoblastic leukemia.
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TRAIL targets cell cycle arrested tumor cells.
Poster 20th Euroconference on Apoptosis, European Cell Death Organization, Rome, Italy.

Eidesstattliche Versicherung

Wachter, Franziska

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

Der konträre Einfluss von p53 auf die extrinsische und intrinsische Apoptoseinduktion in Tumorzellen

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Selbitz, den 16.12.2013

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