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DOUTORAMENTO EM FARMÁCIA
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p53, UM NOVO ALVO MOLECULAR DO ÁCIDO URSODESOXICÓLICO NA MODULAÇÃO DA APOPTOSE EM HEPATÓCITOS

Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para obtenção do grau de Doutor em Farmácia (Bioquímica)

Joana São José Dias Amaral

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De acordo com o disposto no ponto 1 do artigo nº 41 do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, deliberação nº 93/2006, publicada em Diário da República – II Série nº 153 – 5 de Julho de 2003, a Autora desta dissertação declara que participou na concepção e execução do trabalho experimental, interpretação dos resultados obtidos e redacção dos manuscritos.

Aos meus avós "lá em cima"

Aos meus pais e irmã

e a mim...

Contents

Preface

"He flew into the water, and swam towards the beautiful swans. The moment they espied the stranger, they rushed to meet him with outstretched wings. *Kill me!*, cried the poor animal, hanging his head down to the surface of the water, awaiting death. But what did he see in the clear stream below? He saw his own image, no longer an ugly, dark gray bird, but a graceful and beautiful swan. There is no harm in being born in a farmyard when you hatch from a swan's egg." *Hans Christian Andersen, The Ugly Duckling.*

Similar to "The ugly duckling" of our childhood, was the history of p53, a chaotic voyage from the obscure world of oncogenes to the rewarding world of tumor suppressors. In 1979, p53 was discovered independently by Lane and Levine as a cellular protein in complex with the large T antigen (LT) of Simian virus 40. Consistent with its association with LT, p53 was found to cooperate with other oncogene products in *in vitro* transformation assays. This, combined with several observations that p53 was overexpressed in human cancer cell lines, suggested that p53 acted as an oncogene. At that time, who would have imagined that in a few years p53 would achieve the title of "molecule of the year" attributed by Science, in 1993. The popularity of the p53 protein emerged more than a decade after its discovery. A litany of studies demonstrated that normal, wild type p53 could inhibit transformation of cells in culture and that the *p53* gene was mutated in a large percentage of human tumors. In fact, *p53* appeared to be the

most frequently mutated gene in cancer cells, and the first results of *in vitro* transformation assays resulted from artifactual problems related to involuntary cloning of mutant *p53* instead of wild-type. Subsequent work during the 1990's showed that once activated, p53 acted as a sequence-specific transcription factor regulating the expression of several genes involved in a wide variety of cellular responses to stress. By now, it is clearly demonstrated that p53 can specifically modulate cell cycle progression, senescence, and apoptosis. In fact, the apoptotic activity of p53 is considered by most cancer researchers to be signature feature of the tumor suppressor, as well as a key target for cancer therapy. However, the net consequence of widespread p53 activity, and subsequent apoptosis may also be harmful to healthy cells other than tumors, and lead to degeneration of non-renewable cell types such as neurons.

Apoptosis was indeed my motivation. It was in 1972 that the pathologist John Kerr identified within cells structural changes different from the prevailing concepts of cell death. He observed single liver cells, shrinking to half their original volume, losing contact to neighbors, showing aggregated chromatin, and emerging as condensed, membrane-bounded bodies. Shortly after, he named this process, apoptosis from the Greek word αποπτοσισ, whose prefix "apo" (απο) generally means "separation", and the suffix "ptosis" (πτοσισ) the "act of falling off". The complete word can be translated as the falling of leaves from trees in the autumn and refers to the fragmentation of dying cells into characteristic small bodies. The beauty of the apoptosis process is that death is the basis for establishing life. In fact, apoptosis is an integral part of normal embryonic development and tissue homeostasis during adulthood. Sculpture of fingers and toes is a classic example of apoptosis, where cells between the digits dye to separate them. Nevertheless, deregulation of apoptosis can also account for several pathological conditions, ranging from cancer to neurodegenerative disorders. Importantly, in 1998, Rodrigues and co-workers demonstrated that the endogenous

hydrophilic bile acid, ursodeoxycholic acid (UDCA), widely used in the treatment of several liver diseases, was a potent inhibitor of mitochondrial membrane perturbations during the apoptosis process in hepatocytes. In addition, the studies showed that the antiapoptotic function of UDCA was not tissue-dependent, thus highlighting UDCA as a potential therapeutic agent for several diseases associated with high levels of apoptosis. Curiously, UDCA is a major constituent of black bear bile, which has been used in traditional Chinese medicine for the treatment of a litany of diseases for thousands of years. Unfortunately, and despite all efforts from animal rights movements worldwide to avoid it, it has been reported that a few traditional Chinese medicinal solutions still use bile extracted from endangered bears, when there are pharmaceutical-grade alternatives already available.

However, although sometimes strange to occidental eyes, one of the concepts that provide the intellectual framework of Chinese scientific thinking, especially in fields like biology and medicine, suits some of the contents of this thesis. The principle of Yin and Yang describes two opposing and, at the same time, complementary aspects of any one phenomenon. For example, illness is seen as a disturbance in the balance of Yin and Yang and therapy thus depends on accurate diagnosis of the source of the imbalance. In this regard, the p53 apoptotic activity, and apoptosis, in general, may be seen as dualistic mechanisms that when imbalanced may result in disease. Interestingly, “Guardian of the genome”, “Death Star”, and “Good and bad cop” are just a few of the opposing names that have been attributed to p53 over recent years.

When I started my Ph.D. in the laboratory of Professor Cecília M. P. Rodrigues, my main scientific ambition was to elucidate the precise molecular mechanisms underlying the antiapoptotic activity of UDCA. I still remember that, in addition to their outstanding scientific contributions, I found their studies on antiapoptotic UDCA to be a promising and appealing area of therapeutic research.

In addition, as a molecule already in use as first-line treatment in several liver diseases, and with promising clinical use in other apoptosis-related disorders, it becomes imperative to characterize the precise mode of action of UDCA. Although aware that many of the initial questions remain to be answered, the advances achieved with this work are the tasteful rewards of my challenging four-year journey. From the beginning, the purpose of my Ph.D. work, now presented and discussed in this thesis, was to further explore the precise molecular mechanism(s) by which UDCA inhibited apoptosis triggered by p53. In Chapter 1, we begin by giving a general, up-to-date review on the process of apoptosis. In addition, we provide an overview on the activities of p53, specifically as an inducer of apoptotic cell death. We also focus on describing the role of bile acids as modulators of apoptosis. In Chapter 2, we show that modulation of p53 apoptosis pathway by UDCA does not involve direct binding between the bile acid and the DNA binding domain of the tumor suppressor. Possible interactions with other p53 structural domains are also discussed. Chapter 3 demonstrates that UDCA inhibits different levels of p53 activity. Further, Mdm-2 emerges as a crucial player of the antiapoptotic role of the bile acid. The involvement of Mdm-2-dependent ubiquitination and proteasomal degradation of p53 in UDCA-mediated response is discussed in detail as part of Chapter 4. Finally, Chapter 5 integrates all the data and provides a critic and detailed discussion of our overall findings.

The involvement of p53 and apoptosis in the modern world's most common non-infectious diseases, including cancer, neurodegeneration, ischemia or atherosclerosis underscores the importance of understanding this molecule and how it works. As an example, after the paradigm shift of p53 function, more than 45 000 papers have been published on this topic. With this thesis, we hope to contribute to this mass of knowledge on the regulation of apoptosis, further dissecting the beneficial role of UDCA as a strong modulator of intracellular

signaling pathways. Ultimately, these new findings on the molecular mode of action of UDCA may open the door to a wider clinical applicability of this molecule.

Summary

Ursodeoxycholic acid (UDCA) is an endogenous bile acid in clinical use for the treatment of certain liver diseases. There is now strong evidence that the cytoprotective effects of this molecule result, in part, from its ability to reduce the apoptotic threshold in several cell types through modulation of classical mitochondrial pathways. In addition, UDCA modulates upstream molecular targets involved in apoptosis signaling. In these studies, we hypothesized that p53 may represent an important target for UDCA to reduce apoptosis and investigated the molecular mechanisms underlying UDCA protection against p53 triggered hepatocyte apoptosis. Our results using circular dichroism spectroscopy showed that UDCA does not interact directly with the central region of p53, containing the DNA binding domain. Nevertheless, subsequent functional studies revealed that UDCA reduced both transcriptional and DNA binding activity of p53 tumor suppressor, while promoting its nuclear export in primary rat hepatocytes. More importantly, these effects resulted in abrogation of all apoptotic hallmarks induced by p53 overexpression, such as Bax mitochondrial translocation, cytochrome *c* release and caspase-3 activation. Further, we evaluated whether UDCA inhibited p53 via its major repressor, the Mdm-2 protein. Indeed, increased association between p53 and Mdm-2 was detected in hepatocytes preincubated with UDCA. We suggested that by inducing Mdm-2/p53 complex formation, UDCA reduced p53 activity simultaneously blocking its transactivation domain and enhancing its

export to the cytosol. Posttranscriptional silencing of the *mdm-2* gene confirmed the crucial role of this protein in the antiapoptotic function of UDCA. Finally, the fact that proteasomal degradation has been described as the main mechanism by which Mdm-2 inhibits p53 prompted us to investigate the role of UDCA in this pathway. Our data indicated that the bile acid stimulated Mdm-2-dependent ubiquitination of p53, and further increased proteasome activity triggered by wild-type p53. After proteasomal inhibition, UDCA pre-treatment resulted in accumulation of Mdm-2-dependent ubiquitinated p53. It is important to note that the protective function of UDCA was abolished by inhibiting proteasome activity.

In conclusion, the work presented here provides additional insight into the molecular mechanisms underlying the antiapoptotic function of UDCA. This bile acid protects cells from p53-induced apoptosis, by enhancing complex formation between p53 and its inhibitor Mdm-2. Furthermore, by acting as a chaperone-like molecule, UDCA is capable of modulating specific and diverse regulatory events, such as transcription, subcellular localization, and degradation of precise apoptosis-related molecular targets.

Keywords: Apoptosis – Bile acids — Mdm-2 – p53 – Proteasome – Ubiquitin

Sumário

O ácido ursodesoxicólico (UDCA) é um ácido biliar endógeno largamente utilizado no tratamento de diversas doenças hepáticas, como a cirrose biliar primária. Actualmente, sabe-se que o efeito citoprotector do UDCA se deve, em parte, à sua capacidade de inibir uma forma específica de morte programada da célula, designada por apoptose. A modulação da apoptose por parte do UDCA ocorre essencialmente ao nível da mitocôndria. Na verdade, estudos anteriores demonstraram que o UDCA estabiliza a membrana mitocondrial, prevenindo a libertação de factores apoptogénicos para o citoplasma e a consequente activação de caspases, responsáveis últimos pela execução do processo apoptótico. Porém, os mecanismos de sinalização celular iniciados por este ácido biliar são ainda pouco conhecidos. O facto de estar já bem estabelecido que os vários produtos do metabolismo lipídico, incluindo os ácidos biliares, possuem propriedades sinalizadoras, conduziu à hipótese de que a acção anti-apoptótica exercida pelo UDCA poderia envolver a modulação da transcrição génica, ou até mesmo de mecanismos pós-transcricionais, como sejam a activação de vias de sobrevivência e de transdução de sinal. De facto, estudos recentes demonstraram que o UDCA tem a capacidade de modular a expressão de alvos moleculares situados a montante da mitocôndria e que estão envolvidos na sinalização da apoptose, nomeadamente a via apoptótica E2F-1/Mdm-2/p53 induzida pela citocina pró-fibrogénica TGF-β1. Foi ainda demonstrado que, dada a sua semelhança estrutural com o colesterol, este

ácido biliar interage com uma região específica do domínio de ligação dos receptores nucleares de esteróides, nomeadamente com os receptores glucocorticóide e mineralocorticóide, o que lhe permite migrar para o núcleo dos hepatócitos e modular a expressão génica.

No presente estudo, colocou-se a hipótese de que a proteína supressora de tumores, p53, sendo um factor de transcrição que desempenha um papel fundamental na regulação da expressão de vários genes envolvidos no controlo do ciclo celular e da apoptose, poderia representar um alvo importante do UDCA na sua função moduladora da apoptose. Os mecanismos moleculares subjacentes ao papel citoprotector deste ácido biliar contra a apoptose dependente de p53 foram investigados, recorrendo a diversas abordagens metodológicas, tais como sobre--expressão e silenciamento pós-transcricional de genes de interesse, ensaios de imunoprecipitação e imunofluorescência e, ainda, ensaios espectrométricos.

Na primeira parte do trabalho, optou-se por utilizar duas abordagens biofísicas distintas para determinar, *in vitro,* se o UDCA se liga directamente ao domínio da proteína p53 responsável pela ligação ao DNA. A escolha desta região, em detrimento dos outros domínios estruturais da proteína p53, prendeu-se com o facto de ser através desta zona que ocorre a ligação específica entre o factor de transcrição e os seus genes alvo. Utilizou-se a espectrometria por dicroísmo circular, uma técnica amplamente utilizada em estudos de interacção, dada a sua elevada sensibilidade para detectar alterações na estrutura secundária das proteínas, e a anisotropia de fluorescência, outro método espectrométrico altamente sensível às alterações da mobilidade rotacional de um fluoróforo. A formação de estruturas de ligação entre duas moléculas em solução resulta numa restrição do seu movimento rotacional que, por sua vez, se traduz num aumento da anisotropia. Os resultados obtidos por ambas as técnicas não demonstraram a existência de qualquer ligação entre o UDCA e o domínio de ligação da proteína p53 ao DNA. Tanto o espectro de dicroísmo circular, como as experiências de titulação pela

mesma técnica, não revelaram alterações significativas da estrutura secundária da proteína p53 na presença do ácido biliar. Do mesmo modo, a análise da anisotropia não detectou qualquer alteração na mobilidade de p53, ou de UDCA, quando em conjunto. Não se pode excluir, no entanto, a existência de interacção entre o UDCA e outra região da proteína p53. O domínio de transactivação localizado na região N-terminal da proteína, por exemplo, é um local de ligação de inúmeros reguladores de p53, tais como o co-activador p300 e o repressor Mdm-2, constituindo uma região alternativa para a ligação do UDCA.

Numa fase seguinte do trabalho, realizaram-se estudos funcionais em hepatócitos primários de rato, que permitiram concluir que o UDCA reduz a actividade transcricional de p53 e a sua capacidade de ligação ao DNA, ao mesmo tempo que induz a migração do factor de transcrição, do núcleo para o citoplasma. É de salientar que estes efeitos resultaram na inibição de todas as alterações apoptóticas induzidas pela sobre-expressão da forma *wild-type* de p53, nomeadamente a translocação da proteína Bax para a mitocôndria, a libertação de citocromo *c* e a activação de caspase-3. De seguida, pretendeu-se averiguar se a inibição de p53 pelo UDCA era mediada pela proteína Mdm-2. Esta proteína é o principal repressor de p53 que, ao ligar-se à região N-terminal do factor de transcrição, bloqueia o seu domínio de transactivação e ao mesmo tempo promove o seu transporte para o citoplasma. Curiosamente, ensaios de imunoprecipitação revelaram que a presença do UDCA estimula a ligação entre p53 e Mdm-2. Por outro lado, o silenciamento pós-transcricional do gene *mdm-2* provocou uma redução significativa da função protectora do UDCA contra a apoptose dependente de p53, bem como uma acumulação de p53 no núcleo, mesmo na presença do ácido biliar, o que confirma o envolvimento desta proteína na função anti--apoptótica do UDCA.

Por fim, o facto da degradação proteossomal de p53 ser o mecanismo último e principal da sua inibição pelo repressor Mdm-2, serviu de fio condutor para a

parte final deste trabalho. A actividade de ubiquitina ligase característica da proteína Mdm-2 confere-lhe a capacidade de "marcar" a proteína p53 para a via de degradação pelo proteossoma, o que levou ao estudo do envolvimento do UDCA nesta via. A actividade do proteossoma foi medida em extractos proteicos de hepatócitos primários de rato transfectados com um plasmídeo de sobre-expressão de p53 *wild-type*, ou com um plasmídeo vazio para controlo, na presença ou ausência de UDCA. Os resultados revelaram que a actividade do proteossoma nas células que sobre-expressam p53 está aumentada em relação aos respectivos controlos que não sobre-expressam qualquer proteína. Porém, a pré-incubação das células com UDCA resulta numa ainda maior activação do proteossoma no caso dos hepatócitos que sobre-expressam p53, não apresentando, no entanto, qualquer efeito nos hepatócitos transfectados com o plasmídeo controlo. Além do mais, verificou-se que o UDCA diminui o tempo de semi-vida de p53 em células HeLa. De seguida, avaliou-se o papel do UDCA na ubiquitinação da proteína p53. Ensaios de imunoprecipitação demonstraram que a presença do ácido biliar estimula a ubiquitinação de p53 em hepatócitos. Além do mais, a inibição química do proteossoma conduz a uma acumulação significativa de p53 ubiquitinado, o que indica que a degradação proteossomal está, de facto, activa. Resultados obtidos após silenciamento do repressor Mdm-2 permitiram, ainda, concluir que o aumento de p53 ubiquitinado, induzido pelo UDCA, é dependente de Mdm-2. Por fim, verificou-se que um proteossoma activo é fundamental para a acção citoprotectora do UDCA em hepatócitos com níveis aumentados de p53.

Em suma, o presente trabalho revela novos mecanismos moleculares que suportam e melhor caracterizam o papel anti-apoptótico do UDCA. A sobre--expressão de p53 em hepatócitos primários de rato resulta em níveis desregulados de apoptose que, por sua vez, são contrabalançados pela acção protectora do UDCA. Estes estudos demonstram que o ácido biliar tem a capacidade de modular diversos fenómenos de regulação celular, tais como a transcrição, a localização

subcelular e a degradação de alvos moleculares específicos e associados ao processo apoptótico. O recente envolvimento da apoptose dependente de p53 em vários contextos patológicos, como por exemplo nas doenças neurodegenerativas, na aterosclerose e, acima de tudo, nos efeitos secundários graves das actuais terapias contra o cancro, assim como o modo de acção generalizado do UDCA, consolidam o papel deste ácido biliar como possível opção terapêutica, ou coadjuvante, na regulação de níveis desajustados de morte celular, não só no fígado, como em outros órgãos.

Palavras-chave: Ácidos Biliares – Apoptose – Mdm-2 – p53 – Proteossoma – Ubiquitina

Abbreviations

Aβ	amyloid β peptide
AIF	apoptosis-inducing factor
Apaf-1	apoptosis protease-activating factor 1
ATP	adenosine triphosphate
BH	Bcl-2 homology domain
CAD	caspase activated deoxyribonuclease
CAT	chloramphenicol acetyltransferase
CD	circular dichroism
CREB	cAMP-response element-binding protein
ΔΨm	mitochondrial transmembrane potential
DBD	DNA binding domain
DCA	deoxycholic acid
DED	death effector domain
DIABLO	direct IAP binding protein with low pI
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain
FasL	Fas ligand
FITC	fluorescein isothiocyanate
FXR	farnesoid X receptor

GC	glucocorticoid
GR	glucocorticoid receptor
IAP	inhibitor of apoptosis protein
IκB	NF-κB inhibitory protein
IM	mitochondrial inner membrane
JNK	c-Jun N-terminal kinase
LBD	ligand binding domain
MAPK	mitogen-activated protein kinase
Mdm-2	mouse double minute 2
MMP	mitochondrial membrane permeabilization
MPT	mitochondrial permeability transition
MR	mineralocorticoid receptor
NES	nuclear export signal
NF-κB	nuclear factor κB
NSR	nuclear steroid receptor
OM	mitochondrial outer membrane
PBS	phosphate-buffered saline
PI3K	phosphatidylinositide 3'-OH kinase
pRb	retinoblastoma protein
PXR	pregnane X receptor
RING	really interesting new gene
ROS	reactive oxygen species
RSK	ribosomal S6 kinase
siRNA	short interference RNA
Smac	mitochondria-derived activator of caspases
TAD	transactivation domain
tBid	truncated Bid
TGF-β1	transforming growth factor β1

TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TUDCA	tauroursodeoxycholic acid
UDCA	ursodeoxycholic acid
UPP	ubiquitin-proteasome pathway
VDAC	voltage-dependent anion channel

1

General Introduction

1. Apoptotic Cell Death

Early evidence for the existence of two morphological distinct types of cell death came from the pioneering work of Kerr on the effects of hepatic ischemia in hepatocytes (Kerr 1965). Both necrotic and a novel type of cell death were observed. In the latter, cells exhibited histological properties that were very different from necrosis. They included the absence of inflammation, intact lysosomes, and the conversion of scattered individual hepatocytes into small round cytoplasmic masses, some of which contained pyknotic nuclei. In order to highlight its kinetic significance, Kerr named the novel death process "apoptosis", from the Greek word for "falling of the leaves" (Kerr *et al.* 1972).

Electron microscopy examination has further identified the morphological changes during apoptosis to include chromatin condensation, cytoplasmic shrinkage, membrane blebbing, and the generation of small apoptotic vesicles containing intact cytoplasmic organelles as well as nuclear remnants (Kerr *et al.* 1972). These are rapidly eliminated by resident phagocytic and neighboring cells, preventing the release of cellular components into the extracellular space, and consequent inflammatory response. Besides the morphological changes, apoptotic cells undergo a number of distinct biochemical events involving the loss of mitochondrial membrane potential, DNA fragmentation, and protein cleavage (Reed 2002).

Apoptosis has been documented as a prominent player in normal embryonic and postembryonic development, morphogenesis, immune regulation, tissue homeostasis, as well as in pathological and therapeutic settings. Surprisingly, per day, the human body destroys ~ 60 x 10^9 cells through an apoptotic process, in response to physiological, pathogenic, or cytotoxic stimuli, underscoring the

relevance of this orchestrated form of cellular suicide (Reed 2002). Apoptotic cell death is a highly regulated mechanism, which can be viewed as a process to eliminate superfluous, damaged, or mutated cells according to the rule "better death than wrong". However, defects in physiological pathways of apoptosis may occur, contributing to the development of numerous medical illnesses for which adequate therapy or prevention is lacking. In fact, excessive apoptosis can lead to T cell depletion, neurodegenerative diseases, or hepatocellular degeneration, while impaired apoptosis contributes to oncogenesis, autoimmune diseases, and persistent infections (Daniel 2000). Thus, it is not surprising that apoptosis has become a topic of intensive research to identify molecular targets and propose effective therapies in the management of apoptosis-associated disorders.

Apoptosis can be divided into at least three distinct phases: initiation, integration/decision, and execution/degradation (Kroemer *et al.* 1997). The initiation phase is highly heterogeneous and depends on the nature of the death-inducing signal. The integration/decision phase involves the activation of a complex network of apoptotic pathways. During this phase, the "decision to die" is taken and the "point of no return" is trespassed. Finally, the execution/degradation phase culminates in cell death, and is common to distinct types of apoptosis (Kroemer *et al.* 2007). Moreover, the apoptotic process may occur by several molecular pathways. The best characterized and most prominent, however, is the intrinsic pathway, involving the mitochondria (Shi 2002), and the extrinsic pathway, activated by death receptors located at the cellular membrane (Ashkenazi 2002) (Fig. 1). Although apparently independent, these two apoptotic pathways may interact through a delicate coordination and cross-talk involving key proteins that are common to both pathways.

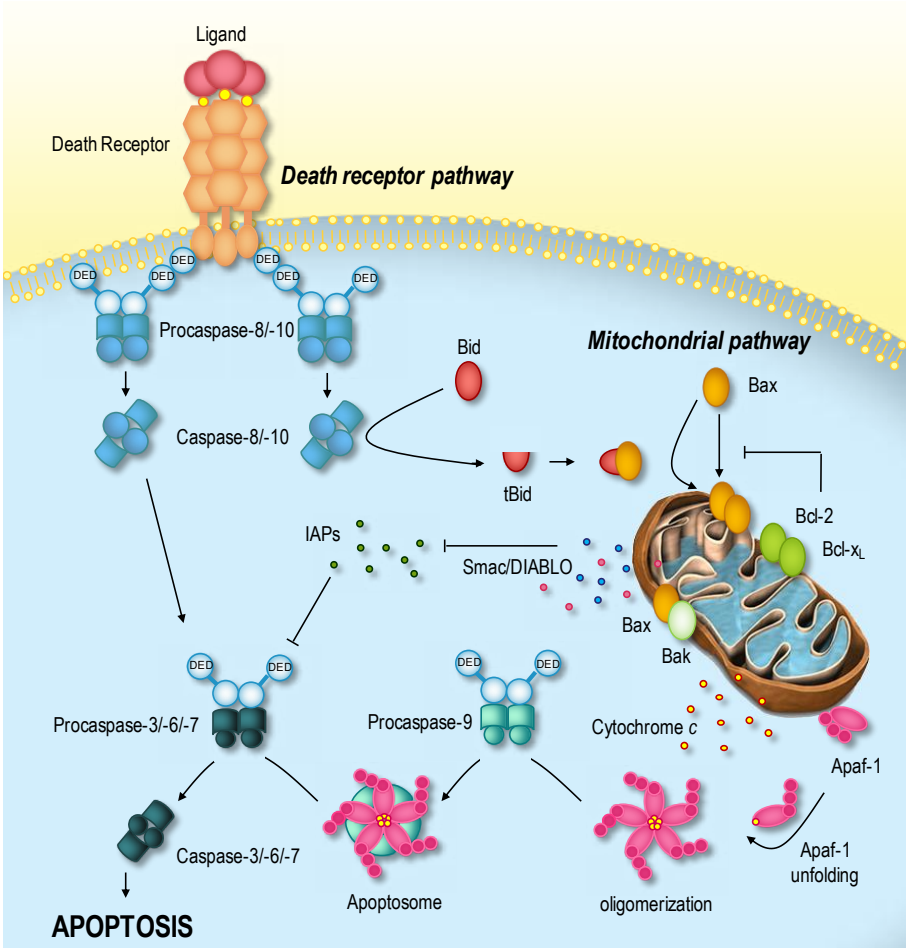


Fig. 1. Schematic overview of extrinsic and intrinsic apoptotic pathways. In the death receptor pathway, after interacting with their ligands, the death receptors recruit adaptor proteins such as FADD and activate caspase-8 and -10. These initiator caspases then cleave effector caspase-3, -6, and -7, which activate key downstream targets and execute the apoptotic process. In the mitochondrial pathway, death stimuli target mitochondria either directly or through transduction by proapoptotic Bax and Bak. Mitochondria then release cytochrome *c*, Smac/DIABLO, and other apoptogenic factors. Cytochrome *c* induces oligomerization of Apaf-1 that recruits and activates procaspase-9. Caspase-9 then

activates effector caspases. The crosstalk between both pathways is mediated by Bid, which is truncated and activated by caspase-8. See text for more complete description. DED, death effector domain.

1.1. Mitochondrial pathway

During the second half of the 20th century, mitochondria were exclusively considered the cell powerhouse; organelles whose particular architecture and biochemical composition would serve the major purpose of maximizing energy production by oxidative phosphorylation. Around 1995, it became clear that mitochondria have a second crucial function, the control of cell death. At first, the idea of mitochondrial cell death control was controversial. On one hand, specialists in bioenergetics wondered how it was possible that the cell's vital forces concentrated in mitochondria could be perverted to serve a lethal purpose. On the other hand, cell death experts were reluctant to recognize that an organelle that does not suffer major alterations in its ultrastructure during apoptosis, compared with the nucleus, can control cell fate (Kroemer *et al.* 2007).

Today, the controversy has been overcome and mitochondria are seen as critical players in both physiological and pathological cell death. Following an apoptotic stimulus, such as oxidative stress, DNA damage, or protein misfolding, levels of calcium are increased, the mitochondrial membrane is rapidly permeabilized, releasing apoptogenic factors from the intermembrane space to the cytoplasm and disrupting the mitochondrial membrane potential, which culminates in cell death (Ricci *et al.* 2003).

1.1.1. Mitochondrial membrane permeabilization

Under physiological conditions, mitochondrial inner membrane (IM) is nearly impermeable to all ions including protons. The charge imbalance that results from the generation of an electrochemical gradient across the IM forms the basis of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$) (Mitchell & Moyle 1965). Although a transient loss of the $\Delta\Psi_m$ may occur in physiological circumstances, a long-lasting or permanent $\Delta\Psi_m$ dissipation is often associated with cell death (Zamzami *et al.* 2005). The permeability of the mitochondrial outer membrane (OM) is also well regulated. It is generally accepted that the OM is freely permeable to small metabolites and solutes, such as ATP, ADP, and respiratory substrates, mainly by the presence of voltage-dependent anion channels (VDAC) (De Pinto & Palmieri 1992).

During apoptosis, the process of mitochondrial membrane permeabilization appears to represent a point of no return for many cell types. Systematic analysis revealed that mitochondria release all soluble proteins, some of them with important proapoptotic functions, from the intermembrane space into the cytoplasm, through the permeabilized OM. Mitochondrial membrane permeabilization (MMP) may occur by the opening of the mitochondrial permeability transition (MPT) pore, or through the formation of specific release channels in the OM, promoted by proapoptotic members of the Bcl-2 family.

The exact molecular composition of the MPT pore is still a matter of debate, although there is growing consensus that it is a multicomponent protein complex that spans the IM and the OM, and not a single protein. The adenine nucleotide translocator (ANT), located in the IM (Brustovetsky & Klingenberg 1996), the VDAC (De Pinto & Palmieri 1992), and cyclophilin D, from the matrix (Crompton *et al.* 1998), were proposed to be part of the MPT pore complex. In addition,

proapoptotic proteins from the Bcl-2 family, namely Bax and Bak, can also engage in a close molecular cooperation with some components of the MPT complex, such as the VDAC and/or the ANT (Tsujimoto & Shimizu 2002), or form themselves pores, further enhancing mitochondrial permeabilization (Wolter *et al.* 1997). MPT pore complex may exhibit several distinct opening states. Ca^{2+}, reactive oxygen species (ROS), proapoptotic Bcl-2 family members, and some chemotherapeutic agents are only a few of the many agents that induce the opening of MPT pore leading to massive mitochondrial swelling.

1.1.2. Release of cell death effectors from mitochondria

The opening of the MPT pore and the loss of the $\Delta\Psi_m$ results in the release of two main groups of normally sequestered proapoptotic proteins from the intermembrane space into the cytosol. The first group consists of cytochrome *c*, second mitochondria-derived activator of caspaes/direct IAP binding protein with low pI (Smac/DIABLO), and the serine protease HtrA2/Omi. These proteins activate a family of death-inducing cysteine proteases, termed caspases, which cleave a wide array of cellular substrates, ultimately resulting in cell demise. The second group of proteins includes apoptosis-inducing factor (AIF) and endonuclease G (EndoG), whose function is independent of caspases, and finally DNA fragmentation factor 40 (DFF40) or caspase activated DNase (CAD) (Elmore 2007).

Once in the cytosol, cytochrome *c* oligomerizes with the apoptotic protease-activating factor-1 (Apaf-1), in the presence of dATP, recruiting procaspase-9 to form the apoptosome. This complex formation results in cleavage and activation of caspase-9, which in turn cleaves and activates other caspases that function as downstream effectors of the cell death program finalizing the apoptotic process

(Zou *et al.* 1999). Smac/DIABLO (Du *et al.* 2000) and Htr2/Omi (Martins 2002) are both proteolytically processed within the intermembrane space to yield mature polypeptides, which in turn are reported to promote apoptosis by inhibiting members of the inhibitor of apoptosis protein (IAP) family.

The link between MMP and nuclear degradation has been established by the Kroemer group through the activity of the second group of proapoptotic proteins released from mitochondria (Susin *et al.* 1999). In healthy cells, AIF is required for optimal detoxification of ROS, and assembly or maintenance of the respiratory chain complex I. However, during apoptosis, the mitochondrial localization sequence of AIF is cleaved giving rise to a mature form of the protein that is translocated from the mitochondria to the cytosol, and subsequently to the nucleus. Here, it promotes chromatin condensation and large-scale DNA fragmentation. A similar role is played by EndoG, a mitochondria-specific endonuclease that translocates to the nucleus during apoptosis to cleave single and double stranded DNA and RNA (Li *et al.* 2001). CAD is the last protein being released and it translocates to the nucleus after cleavage by caspase-3, leading to DNA nucleosomal fragmentation and a more pronounced and advanced chromatin condensation (Enari *et al.* 1998).

1.2. Death receptor signaling

The cell fate is deeply influenced by components of the extracellular matrix, which in turn are closely associated with changes in expression and/or functioning of death receptors and their ligands (Fig. 1). Death receptors are type 1 transmembrane proteins with three domains, belonging to the tumor necrosis factor (TNF) receptor superfamily (Locksley *et al.* 2001), including TNF receptor

(TNFR)1, Fas (CD95/Apo-1), TNF-related apoptosis inducing ligand (TRAIL) receptors -1 and -2, death receptor (DR) 3 and DR6. They are usually sequestered in intracellular vesicles associated with the Golgi complex, and are transported to the cytoplasmic membrane after specific stimuli. The members of TNF receptor family share similar cysteine-rich extracellular domains (N-terminal) and have a cytoplasmic domain of about 80 amino acids called the "death domain" (C-terminal) (Ashkenazi & Dixit 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to intracellular signaling pathways. In fact, the triggering point in death receptor signaling is the engagement of transmembrane proteins by their cognate ligands, followed by recruitment of intracellular adaptor molecules, such as the Fas-associated death domain (FADD), forming the death-inducing signaling complex (DISC) (Kischkel *et al.* 1995). Once activated, death receptors induce the cleavage and activation of procaspase-8, and -10 via dimerization of the death effector domain (DED). In type I cells, the apoptotic cascade continues as caspase-8, and -10 directly activate downstream caspases, such as caspase-3 or -7, perpetuating the apoptotic process (Peter & Krammer 2003). In type II signaling cells, such as hepatocytes, the progression of the apoptotic cascade depends entirely on its amplification by mitochondria (Li *et al.* 2002). In this case, caspase-8 cleaves inactive cytoplasmic Bid, exposing an active truncated fragment (tBid) (Scaffidi *et al.* 1998). Once activated, tBid induces conformational changes in proapoptotic Bax and Bak and subsequent translocation to mitochondria (Eskes *et al.* 2000). Moreover, tBid can also inhibit antiapoptotic proteins, such as Bcl-2, (Kim *et al.* 2000), or even directly permeabilize the mitochondrial OM (Goonesinghe *et al.* 2005), inducing the release of cytochrome *c*, which ultimately results in caspase-3 activation and perpetuates the apoptotic process (Fig. 1).

Tumor necrosis α (TNF-α) overlaps with Fas signaling in many aspects. In fact, TNF-R1 activation is followed by recruitment of the adaptor TNF-R-associated protein with DED, which in turn may also recruit FADD as well as other adaptor proteins. Signaling by both receptors plays a pivotal role in liver diseases including alcoholic hepatitis and fulminant hepatic failure (Bird *et al.* 1990, Streetz *et al.* 2000). However, TNF-R1 is unique in its ability to trigger survival signals, including the activation of nuclear factor κB (NF-κB) (Barnhart & Peter 2003), underscoring the relevance and complexity of death receptors.

Interestingly, the activation of caspase-8 by the death receptor pathway, and subsequent activation of Bid, is key to activating the mitochondrial apoptosis machinery, as an amplifying loop of weaker apoptotic signals. This coordinated cross-talk between the extrinsic and intrinsic apoptotic pathways reinforces the critical role of the mitochondrial organelle during programmed cell death.

1.3. Other molecular players in the apoptotic process

The control, regulation and execution of apoptosis involve many proteins, enzymes, and different factors within the cell. Two families of proteins play essential roles in the apoptotic process, the Bcl-2 and caspase families. In fact, to integrate death signals and perform the multiple reactions that culminate in cell death, several components, interactions, and biochemical processes are necessary, in a complex organization that defines the efficiency of apoptosis.

1.3.1. Bcl-2 family

Many studies have indicated that the multifunctional capabilities of Bcl-2 proteins enable them to serve as powerful regulators of the apoptotic cell death program

(Reed 1997). This family gives its name to the gene for B cell leukaemia/lymphoma 2 (*bcl*-2) originally identified as the proto-oncogene involved in the human follicular B cell lymphoma. Curiously, Bcl-2 was found to inhibit cell death, rather than promote proliferation, and it was identified as a mammalian homologue to the apoptosis repressor Ced-9 in *Caenorhabditis elegans (C. elegans)*.

Bcl-2 members can be categorized into three groups, according to their structure and function. The antiapoptotic multidomain proteins (prototypes: Bcl-2, Bcl-X_L) share four conserved Bcl-2 homology (BH) domains (BH1-4); proapoptotic multidomain proteins (prototypes: Bax, Bak), which contain three BH domains (BH1-3); and proapoptotic BH3-only proteins (prototypes: Bid, Bad) (Cory *et al.* 2003). Interestingly, BH domains have been shown to mediate protein interactions forming either homo- or hetero-complexes.

The mechanism(s) by which Bcl-2 family members regulate membrane permeability remains controversial. The three most popular theories postulate that they either form *de novo* protein channels in membranes, interact with and regulate pre-existing mitochondria membrane pores, or alter membrane lipid order to produce pores (Sharpe *et al.* 2004). In healthy cells, Bak is associated with the mitochondrial OM, whereas Bax resides in the cytosol. However, upon induction of apoptosis, Bax undergoes conformational changes, translocates to mitochondria, oligomerizes, and inserts into the OM (Kirsch *et al.* 1999). Here, Bax is thought to form giant protein-permeable pores, alone or in association with Bak or truncated Bid allowing the release of cytochrome *c* (Kuwana *et al.* 2002). Further, Bax and Bak can enhance the loading of the endoplasmic reticulum (ER) calcium store, boosting the calcium load to mitochondria (Scorrano & Korsmeyer 2003).

Curiously, antiapoptotic Bcl-2 resides in the cytoplasmic face of mitochondrial OM, ER, and nuclear envelope, promoting the integrity of membranes, and thereby enhancing cell survival (Yang *et al.* 1997). Both Bcl-2 and its homologue Bcl-X_L heterodimerize with proapoptotic proteins through BH1 and BH2 domains, preventing pore formation and release of apoptogenic factors (Antonsson *et al.* 1997). In addition, Bcl-X_L physically interacts with Apaf-1 to prevent the activation of the caspase cascade and subsequent apoptosis (Hu *et al.* 1998). Finally, some authors suggested that Bax and Bcl-2 also modulate the MPT pore (Vieira *et al.* 2000) while others have demonstrated that Bax does not have a major role in regulating the MPT in isolated mitochondria (De Marchi *et al.* 2004).

BH3-only proteins link the death receptor and the mitochondrial pathways of apoptosis (Luo *et al.* 1998) (Fig. 1). These proapoptotic proteins exert their action by two different mechanisms. They can interact with antiapoptotic proteins, dissociating them from other BH3-only or from BH1-3 proteins, and promoting MMP (e.g. Bad), or they can directly activate BH1-3 proteins to initiate MMP, either by stimulating the translocation of Bax to mitochondrial OM or by local effects on Bak (e.g. tBid). Puma and Noxa are two BH3-only proteins that are also involved in the apoptotic process. Together with Bax, they play an important role in apoptosis induced by the tumor suppressor protein p53 (Miyashita & Reed 1995, Michalak *et al.* 2008). In fact, p53 has been shown to be critical in the regulation of Bcl-2 family proteins, antagonizing the antiapoptotic function of Bcl-X_L, Bak, or Bcl-2 (Mihara *et al.* 2003, Sot *et al.* 2007), through both transcription-dependent and -independent mechanisms.

1.3.2. Caspases

Most morphological changes of apoptotic cells are caused by a set of cysteine proteases that are activated specifically during apoptosis. They cleave a restricted set of target substrates after an aspartate residue, and are termed cysteinyl aspartate-specific proteases or caspases (Thornberry & Lazebnik 1998). These death proteases are part of a large family, which can be thought as the central executioners of the apoptotic pathway. Members of the caspase family are highly conserved through evolution. They are found from humans all the way down to insects, nematodes and hydra (Budihardjo *et al.* 1999, Cikala *et al.* 1999). In 1991, studies in *C. elegans* identified two genes, *ced-3* and *ced-4*, required for apoptosis in the worm: if either gene was inactivated, cell death of the 131 cells during development failed to occur (Ellis *et al.* 1991). The great similarities between CED-3 and caspase-3 established for the first time a connection between caspases and apoptosis (Yuan *et al.* 1993).

To date, at least 14 members of this family have been identified in mammals although not all of them function during apoptosis. Caspases are synthesized as inactive zymogens or precursor forms, and must undergo a process of activation during apoptosis. These zymogens are composed of an N-terminal prodomain and two small caspase subunits, p20 and p10. Usually, caspase activation involves proteolytic cleavage between the p20 and p10 domains, and also between the prodomain and the p20 domain (Wolf & Green 1999). The mature and active enzyme consists of a heterotetramer containing two p20/p10 heterodimers and two active sites (Earnshaw *et al.* 1999). According to their function, caspases are subdivided in upstream proteases, termed initiator caspases (caspase-2, -8, -9 and -10), and their downstream targets known as effector or executioner caspases (caspase-3, -6 and -7) (Thornberry & Lazebnik 1998).

The initiator caspases contain larger prodomains with protein-protein interaction modules, which allow them to bind and associate with their upstream adaptors and regulators. Caspase-2 and -9 contain a caspase activation and recruitment domain (CARD), whereas caspase-8 and -10 contain a death effector domain (DED) (Hofmann *et al.* 1997, Ashkenazi & Dixit 1998). Through these domains initiator caspases interact with adaptor proteins that recruit them to specific "death complexes", which are large multiprotein complexes mediating caspase activation. In mammals, death complexes include the apoptosome, in the mitochondrial pathway, where procaspase-9 is recruited through CARD-CARD interactions to its adaptor Apaf-1, and rapidly processed into active caspase-9 (Thornberry & Lazebnik 1998), and/or the DISC, in the death receptor pathway, where caspase-8 is recruited via binding to its adaptor FADD (Varfolomeev *et al.* 1998). Caspase-10 recruitment during TRAIL and Fas-mediated apoptosis also requires FADD.

Finally, caspase-2 was one of the first caspases discovered, but its physiological function and activation remain obscure. Recently, it was found that activation of caspase-2 involves a large protein complex, the PIDDosome, formed by the p53-induced protein with a death-domain (PIDD) and the receptor interacting protein (RIP)-associated ICH-1/Ced-3-homologous protein with death domain (RAIDD) adaptor protein (Tinel & Tschopp 2004). It is often localized in the cytosol, nucleus, and Golgi, although its protein targets remain largely unclear. Nevertheless, it was shown that caspase-2 is responsible for the mitochondrial OM permeabilization in response to DNA damage in some cells (Zhivotovsky & Orrenius 2005). Further, caspase-2 is involved in response to ER stress- (Upton *et al.* 2008), oxidative stress- (Tamm *et al.* 2008), and p53-mediated apoptosis (Baptiste-Okoh *et al.* 2008). Interestingly, caspase-2 can also function

independently of its protease activity, such as by activation of mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways (Lamkanfi *et al.* 2005).

The extrinsic and intrinsic apoptotic pathways end with the execution phase, considered the final step of apoptosis. Effector caspases are responsible for cleaving specific cellular substrates involved in the apoptotic process, modifying their function. Caspase-3 is the main downstream effector caspase, which can be activated via the death receptor pathway, following activation by caspase-8, and through the mitochondrial pathway, by caspase-9 (Porter & Janicke 1999). In fact, caspase-3 is activated by any of the initiator caspases. It is responsible for cleavage of many substrates, including nuclear lamins and cytoskeletal proteins associated with morphological changes in apoptotic cells (Kothakota *et al.* 1997). In addition, caspase-3 cleaves the inhibitor of caspase-activated DNase (ICAD), promoting the activation of the endonuclease CAD, which induces the characteristic nucleosomal DNA fragmentation (Sakahira *et al.* 1998). Caspase-3 is also responsible for the cleavage of poly(ADP-ribose) polymerase (PARP), inhibiting its capacity to repair DNA (Rosen & Casciola-Rosen 1997).

Caspase-7 is highly homologous to caspase-3, with similar substrate specificity and redundant functions in the majority of general apoptotic events. It can be activated by caspase-8 and caspase-9, and has also a specific role in the ER-stress response pathway (Rao *et al.* 2001). A novel role for caspase-7 was also described recently during cell proliferation at mitosis (Hashimoto *et al.* 2008). Caspase-6, although structurally similar to caspase-3 and -7 has different substrate specificities. Its function and activation are still not entirely understood. However, some caspase-6 substrates have been already described and include lamin A (Takahashi *et al.* 1996). Curiously, caspase-6 proteolytic activity has been recently

implicated in Alzheimer's and Huntington's diseases (Klaiman *et al.* 2008, Warby *et al.* 2008).

Even with apparently similar functions, effector caspase-3, -6 and -7 have different relevance to the apoptotic process. In fact, depletion of caspase-3 in a cell-free apoptotic system inhibited most of the downstream events, including DNA fragmentation and chromatin condensation, while elimination of caspase-6 and -7 did not produce the same effects (Slee *et al.* 2001). Nevertheless, if caspase-3 is missing or not functioning, other effector caspases can compensate the catalytic mechanisms, creating alternative and novel networks.

Of note, certain caspases may also play an essential role in inflammation (Cornelis *et al.* 2007). Activation of inflammatory caspases such as caspase-1 and -5 occurs following the assembly of an intracellular complex designated inflammasome. Caspase activation then leads to activation of proinflammatory cytokines. Finally, caspase-12 also appears to have a distinct role in the ER stress-mediated pathway, which is correlated with disruption of calcium homeostasis (Lamkanfi *et al.* 2004). The full-length human caspase-12 appears to be enzymatically inactive; thus as an alternative to caspase-12 in human, caspase-4 is involved in ER stress-induced cell death pathway. Both murine caspase-12 and human caspase-4 are localized to the ER and cleaved specifically by ER stress (Hitomi *et al.* 2004).

Overall, caspases are prominent players in the apoptosis process due to their role in abrogating survival pathways and activating downstream events that are responsible for cell dismantling and death. Giving their importance and ability to destroy cells, caspases are tightly regulated in normal cell function. The inhibitor of apoptosis proteins (IAPs) are the primary inhibitors of caspase activation, whose homologues have been subsequently described in all eukaryotes, from yeast to

humans (Crook *et al.* 1993). The p53 transcription factor (Erster *et al.* 2004) and CmrA (a cytokine response modifier gene) (Ray *et al.* 1992) can also regulate the activation of caspases. In the last years, several peptide and non-peptide caspase inhibitors have been developed, providing novel therapeutic tools in prevention of apoptosis associated with pathogenic situations, such as neurodegenerative and infectious diseases, and ischemia-reperfusion disorders. In fact, a pancaspase inhibitor was shown to reduce the severity of myocardial reperfusion injury in rat and mouse models of myocardial infarction (Mocanu *et al.* 2000). Further, caspase-1 specific inhibitors have been developed to treat rheumatoid arthritis and other inflammatory conditions (Le & Abbenante 2005). However, other studies did not result in reduced cell death, since the use of caspase inhibitors often sensitizes cells to necrosis and/or autophagy. Moreover, cells can also undergo apoptosis through caspase-independent pathways, involving several other proteases such as cathepsins, calpains, and granzymes. Future advances will doubtlessly lead to the discovery and development of better therapeutic solutions to pathological apoptosis.

1.4. Cell survival signaling

Impaired apoptotic responses resulting in extensive or faulty cell death contribute to a variety of human diseases. However, several molecular pathways protect cells from pathologic apoptosis. The so-called survival signaling pathways include NF-κB, phosphatidylinositol 3-kinase (PI3K), and MAPK pathways.

A wide range of signals trigger cell survival pathways, including cytokines from the TNF superfamily (Gaur & Aggarwal 2003) and bile acids (Schoemaker *et*

al. 2004), among others. Survival stimuli generally trigger intracellular signaling through activation of transmembrane receptors.

Despite its involvement in apoptosis, TNF-α can also stimulate survival pathways via NF-κB-dependent gene expression (Barnhart & Peter 2003). NF-κB is a ubiquitous transcription factor that mediates a variety of proinflammatory responses (Ghosh & Karin 2002). In unstimulated cells, NF-κB is tightly bound to inhibitory protein IκB in the cytoplasm, which prevents its translocation to the nucleus. Following cellular stresses, IκB is phosphorylated by specific kinases, leading to its proteasomal degradation and subsequent dissociation from NF-κB. In the nucleus, NF-κB binds to specific response elements in the promoter of target genes, including proinflammatory cytokines but also antiapoptotic Bcl-2 elements. Thus, activation of NF-κB promotes either cell injury or protection.

The PI3K family of enzymes has also been implicated in controlling cell survival in response to trophic factors and bile acids (Rajesh *et al.* 2005, Ueno *et al.* 2007). In fact, activation of transmembrane receptors results in the recruitment of PI3K isoforms to the inner surface of the plasma membrane as a result of ligand-regulated protein-protein interactions (Toker & Cantley 1997). Downstream effectors of PI3K signaling include a key component in cell survival, the serine/threonine kinase Akt. In turn, Akt targets several proteins to keep cells alive, including apoptosis regulators and transcription factors (Franke *et al.* 2003). It is thought that activation of Akt induces phosphorylation of Bad, which will no longer associate with and inhibit antiapoptotic Bcl-X_L. In addition, Akt phosphorylates and inactivates caspase-9, suppresses translocation of proapoptotic Bax to mitochondria, and activates three transcription factor families, including forkhead, cAMP-response element-binding protein (CREB), and NF-κB, all of which are involved in regulating cell survival. Whereas the phosphorylation of

forkhead family members by Akt negatively regulates death promoting signals, phosphorylation of CREB and IκB kinase (IKK) stimulates survival pathways. Interestingly, IKK phosphorylation by Akt links the PI3K signaling cascade with the NF-κB pathway.

Finally, the MAPK pathway is another important survival pathway. MAPKs are a superfamily of homologous enzymes that integrate and transduce intracellular and extracellular signals into the nucleus. This pathway is activated by the small GTP-binding protein, Ras, and the downstream cascade includes the sequential phosphorylation and activation of kinases Raf, extracellular signal-regulated kinase (ERK), and MAPK/ERK kinase (MEK) (Chong *et al.* 2003). The effect of the MAPK pathway on survival is mediated, at least partly, by activation of the pp90 ribosomal S6 kinase (RSK) family members. Like Akt, RSKs phosphorylate Bad, and both kinases might act synergistically at inhibiting Bad proapoptotic activity. RSKs are also potent activators of CREB, which is known to activate transcription of *bcl-2*, thereby stimulating cell survival directly (Bonni *et al.* 1999). Thus, although there are several survival pathways triggered by different survival signaling, they all converge on the same set of proteins to inhibit the apoptosis program.

Another mechanism through which cells are believed to acquire resistance to apoptosis is by overexpression of IAP proteins. The IAP gene family is highly conserved in a wide range of organisms from insects to humans. The first IAP was discovered in baculovirus, where it was shown to suppress death of viral-infected host cells (Clem *et al.* 1991). To date, the eight known human IAPs are cellular IAP1 (c-IAP1), c-IAP2, neuronal AIP (NAIP), survivin, livin, X chromosome-linked IAP (XIAP), testis-specific IAP (TsIAP) and baculovirus IAP repeat (BIR)-containing ubiquitin conjugating enzyme (BRUCE) (reviewed in Vaux & Silke

2005). In addition to BIR repeats, most IAP proteins contain carboxy-terminal RING (really interesting new gene) domains that function as ubiquitin ligases to promote proteasomal degradation. Unlike the Bcl-2 family of proteins, which exert their regulatory effects through mitochondria, the antiapoptotic activity of IAPs was originally attributed to their ability to bind and inhibit caspases. However, recent studies have demonstrated that under physiological conditions such activity is only inherent to XIAP, while others exhibit weak binding to and inhibition of caspases (Eckelman *et al.* 2006). XIAP was shown to inhibit caspase-3, -7, and -9 (Deveraux *et al.* 1997); however, it was also shown to promote prosurvival signaling by inducing NF-κB and Jun NH_2-terminal kinase (JNK) pathways (Lu *et al.* 2007, Sanna *et al.* 2002).

Importantly, inhibitory activity of IAPs is also subjected to negative control. This control is carried out by Smac/DIABLO. The serine protease HtrA2/Omi also binds and antagonizes a number of IAP proteins. Finally, recent studies have described other functions for IAPs. Survivin and Bruce seem to modulate apoptotic signaling through the control of cell cycle and proliferation, as well as p53 destabilization (Li *et al.* 1999, Ren *et al.* 2005), while c-IAP1 and c-IAP2 are recruited to the type-2 TNF-receptor complex, where they modulate caspase-8 activity (Rothe *et al.* 1995).

2. The p53 Tumor Suppressor Protein: Role in Apoptosis

Throughout their life cycle, organisms constantly face severe DNA damage. Depending on multiple factors, including the source and extent of an insult, DNA damage leads either to cell cycle arrest, in which the cell is given the opportunity to

repair damaged DNA, or to the complete disposal of the cell by apoptosis. One of the main players deciding the fate of a cell following DNA damage is the tumor suppressor protein p53, famously dubbed "The Guardian of the Genome" (Lane 1992).

The p53 protein was first described in 1979 as a cellular protein in complex with the large T antigen of SV40 (Lane & Crawford 1979, Linzer & Levine 1979). The description of this protein and its gene has changed from a virus-associated tumor antigen to an oncogene, and finally to a tumor suppressor gene (Lane & Benchimol 1990). Loss or mutation of p53 is strongly associated with increased susceptibility to cancer, and most functions of p53 have been considered in the light of how p53 might protect from malignant progression (Vogelstein *et al.* 2000). However, it is now becoming clear that this protein also influences other aspects of health and disease apart from cancer development. Indeed, p53 is at the center of an intricate regulatory protein network exhibiting diverse and global functions, in which cell cycle arrest, senescence, and apoptosis are the most characterized (Fig. 2).

It has been reported that p53 dysfunction induces pathological conditions beyond tumorigenesis, specifically associated with deregulated levels of apoptosis (Vousden & Lane 2007). In addition, numerous recent studies have linked p53 to a number of different and sometimes unexpected responses, such as regulation of glycolysis (Matoba *et al.* 2006), autophagy (Tasdemir *et al.* 2008), angiogenesis (Teodoro *et al.* 2006), and differentiation (Murray-Zmijewski *et al.* 2006). p53 functions primarily as a transcription factor with complex functional domains, that both activate or repress the expression of different subsets of genes to accomplish its many biological functions.

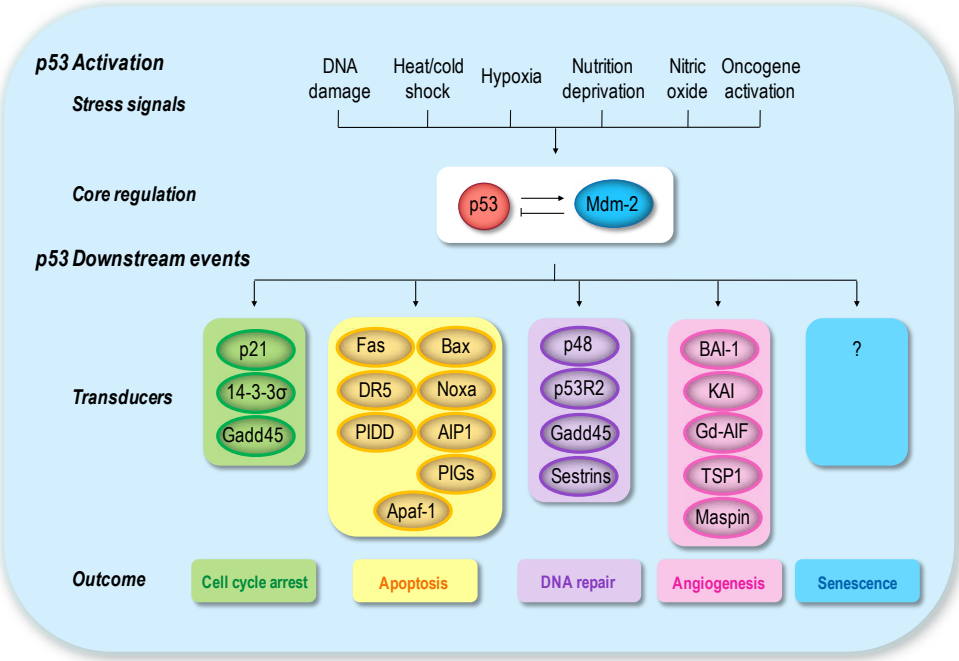


Fig. 2. Schematic diagram of p53 activation and response. Several types of stress signals are detected by the cell and communicated to the p53 protein and its direct regulators. Once activated, p53 functions as a transcriptional modulator of p53-regulated genes. This results in three major outcomes of cell cycle arrest, cellular senescence, or apoptosis. Other p53 target genes are involved in different cellular responses, such as inhibition of angiogenesis or DNA repair. The role of p53 is likely to depend on the context in which the activation occurs.

2.1. Structure and function of p53 domains

The functional complexity of p53 is mirrored in its chemical structure (reviewed in (Joerger & Fersht 2008). Human and murine p53 exist predominantly as tetramers of unusual shape, and to a smaller extent as higher oligomers in solution (Friedman

et al. 1993). It has been shown that p53 is transcriptionally active as a tetramer, with four identical chains of 393 residues, each monomer interacting with five base pairs of its specific DNA consensus sequence (el-Deiry *et al.* 1992).

The p53 protein can be divided into at least four functional domains which include the N-terminal region, the DNA binding and tetramerization domains, and the extreme C-terminus (Fig. 3). The N-terminal region of p53 consists of an acidic natively unfolded transactivation domain (TAD) and a proline-rich region (PRR) (Dawson *et al.* 2003). The TAD is a promiscuous binding site for a multitude of interacting proteins, such as components of the transcription machinery (Thut *et al.* 1995), the transcriptional coactivators p300/CBP (CREB binding protein) (Teufel *et al.* 2007), and the negative regulators Mdm-2 (mouse double minute 2)/Mdm-4 (Kussie *et al.* 1996). The presence of large regions of intrinsic disorder is a recurring motif in the TAD of transcription factors, because it facilitates binding to diverse target proteins with high specificity. Upon binding to the transcription activation complex, regions that are present in the native state become fully folded.

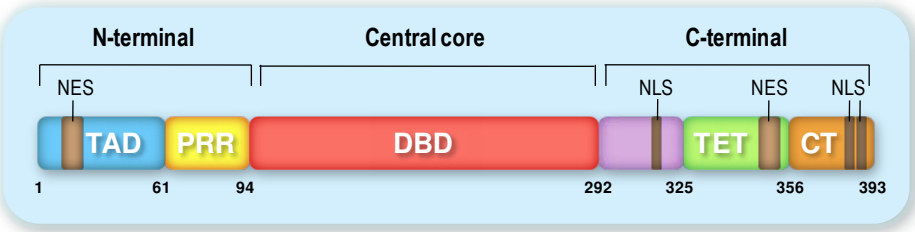


Fig. 3. Schematic diagram of the domain structure of full-length p53 consisting of an N-terminal transactivation domain (TAD), followed by a proline-rich region (PRR), the central DNA binding domain (DBD), the tetramerization domain (TET), and the extreme C-terminus (CT). NES and NLS correspond to nuclear export and nuclear localization signals, respectively.

The exact role of the PRR that links the TAD to the DNA binding domain is still poorly understood. However, the prevalence of prolines in this region is conserved among mammalian p53s, indicating a functional or structural requirement for a certain degree of rigidity. Recent mutational studies on mouse models have revealed that the length of this region is the crucial aspect for p53 activation, whereas putative protein docking sites are dispensable. These findings support a modular role of this domain, potentially as a spacer between the different functional domains (Toledo *et al.* 2007). Some authors also considered this region essential for the proapoptotic activity of p53, as mutants of p53 lacking this region were reported to induce cell cycle arrest but not apoptosis (Baptiste *et al.* 2002).

The DNA binding domain (DBD) is the central region of p53 responsible for sequence-specific DNA binding. It consists of an immunoglobulin-like β-sandwich that provides the basic scaffold for the DNA binding surface. This surface can be divided into two structural motifs: the loop-sheet-helix motif, which binds to the major groove of DNA, and the remaining half, which includes two large loops stabilized by a zinc ion that docks to the DNA minor groove. Zinc loss results in a significant decrease in thermodynamic stability, increases aggregation tendencies, and induces structural fluctuations in neighboring loops causing loss of DNA binding specificity (Duan & Nilsson 2006).

The conformation of the DBD domain is essential for p53 activity as a transcription factor, since it mediates binding to specific double-stranded DNA sequences. Four p53 DBDs, corresponding to tetrameric p53, bind to these responsive elements in a highly cooperative manner of a complex with 4:1 stoichiometry, resulting in bending and twisting of DNA. Binding studies between p53 and several representative response elements revealed varying affinities, showing a loose correlation between affinity for the promoter region and function

of the gene. Nevertheless, it appears that promoters for genes involved in cell cycle arrest and DNA repair bind with higher affinity than those related with apoptosis, but there is no strict correlation (Weinberg *et al.* 2005). Specific key residues in the p53 DBD-DNA interface directly contact with DNA (Lys-120, Ser-241, Arg-248, Arg-273, Ala-276, Cys-277, and Arg-280) (Kitayner *et al.* 2006). Contacts made by Lys-120, Ala-276, and Cys-277 are sequence specific, thereby modulating p53's differential target gene recognition. Posttranslational modifications also provide a second level of regulation of binding selectivity. Acetylation of Lys-120, for instance, may play a role in induction of apoptosis (Sykes *et al.* 2006).

The DBD domain of p53 is connected to a short tetramerization domain that regulates the oligomerization state of the protein, via a flexible linker. The tetrameric structure of the domain is best described as a dimer of primary dimers. Two monomers form a primary dimer, which is stabilized via an antiparallel intermolecular β-sheet and antiparallel helix packing, forming the central hydrophobic core of this dimer. Two such dimers associate through their helices to form a four-helix tetramer. The tetramer interface is largely stabilized by hydrophobic interactions, and truncation of either one of the two key hydrophobic residues is sufficient to shift the oligomerization state toward the formation of stable dimers (Joerger & Fersht 2008). Recent studies on *Drosophila melanogaster* and *C. elegans* p53 homologues suggest a possible evolution of tetrameric mammalian p53 from ancestral dimeric forms (Ou *et al.* 2007).

Finally, at its C-terminus, p53 contains the so-called regulatory domain. This region is intrinsically disordered but may undergo local unfolded-to-folded transitions following binding to other proteins or nonspecific DNA (Bell *et al.* 2002). For example, different unstructured peptides of p53 residues within this

domain adopt stable conformation in complex with several proteins, such as the calcium-dependent dimeric S100B protein, the phospho-cyclin A, and the deubiquitinase HAUSP/USP7, which is also recognized by Mdm-2. The C-terminal region of p53 is also subjected to extensive posttranslational modifications that regulate p53 function and cellular protein levels. Acetylation of C-terminal lysines has been associated with recruitment of coactivators/histone acetyltransferases required for transcriptional activation of target genes (Mujtaba *et al.* 2004). Mutation of all six C-terminal lysines in p53 to arginines, preserving the positive charge but preventing modification at these sites, leads to normal phenotypes in mice. This indicates that C-terminal modifications are not essential but may contribute to fine-tuning the p53 stress response (Krummel *et al.* 2005).

Although p53 individual domains are well characterized, the full-length protein has until recently eluded structural characterization. The low stability of p53 associated with its aggregation tendencies was overcome by the design of a biologically active, stabilized variant of p53. The crystal structure of this variant is virtually identical to the wild type. It revealed that the free protein in solution forms an elongated cross-shaped tetramer with loosely coupled core domain dimers and extended N and C-termini (Tidow *et al.* 2007). The weak assembly of the core domains allows them to interact with regulatory proteins such as members of the Bcl-2 family involved in p53 transcription-independent apoptosis in mitochondria (Chipuk *et al.* 2004). Apart from providing binding promiscuity, the natively unfolded regions in p53 also provide flexible linkers to allow for structural reorganization upon formation of higher-order complexes.

2.2. Activation and stabilization of p53

In broad terms, a wide variety of cellular stress signaling pathways engage the p53 network such as DNA damage, hypoxia, and aberrant proliferative signaling, resulting in marked stabilization and activation of the p53 protein (Vousden & Lu 2002). The regulation of p53 function is extremely complex and has been described at the level of transcription, translation, degradation, structural alterations, and posttranslational modifications (Ashcroft & Vousden 1999). However, at present, there is general agreement that the key mechanism by which p53 is regulated is through control of protein stability.

2.2.1. Regulation by the Mdm-2 protein

In the absence of stress signals, p53 is kept in check to allow normal cell proliferation and/or maintenance of cell viability. The p53 protein is mainly regulated at the posttranslational level by Mdm-2, or in humans Human double minute 2 (Hdm-2), which has been shown to inhibit p53 activity in several ways. To date, it has been demonstrated that Mdm-2 can inhibit p53 by regulating its stability, cellular localization, and ability to activate transcription (Michael & Oren 2003).

Curiously, since Mdm-2 is itself a transcriptional target of p53, an autoregulatory negative feedback loop exists in which p53 activates the expression of its own inhibitor (Barak *et al.* 1993). The importance of Mdm-2 in the control of p53 activity is shown by the observation that Mdm-2 deficiency causes early embryonic lethality in mice which is rescued by knocking out the p53 gene (Jones *et al.* 1995). This indicates that in the absence of Mdm-2, unrestrained p53 activity blocks normal growth and development.

The ubiquitin-proteasome degradation pathway

Ubiquitination is the cellular mechanism responsible for targeting proteins to proteasome degradation. Protein ubiquitination is achieved via the sequential activity of three groups of enzymes, E1, E2, and E3. Mammalian cells have one E1 enzyme that activates ubiquitin in an ATP-dependent manner and transfers it to one of at least 15 different E2 ubiquitin carriers. Finally, ubiquitin is transferred to one of the hundreds of E3 ligases responsible for substrate specificity (Kisselev & Goldberg 2001). Mdm-2 protein contains a RING finger domain and like many RING finger proteins, it can function as an E3 ubiquitin ligase, targeting a number of proteins, including p53 and Mdm-2 itself, for proteasomal degradation. By targeting p53 to the proteasome, Mdm-2 is the key factor which is responsible for maintaining p53 at low cellular concentrations.

The ubiquitin-proteasome pathway (UPP) is the major extralysosomal pathway responsible for intracellular protein degradation in eukaryotes (Glickman & Ciechanover 2002). Moreover, protein turnover mediated by the UPP has many critical regulatory roles, including control by removing abnormal proteins that arise by mutation, metabolic damage, or misfolding (Unno *et al.* 2002).

Eukaryotic proteasomes are large proteolytic complexes, abundant in the cytosol and nucleus. The architecture of the proteasome requires that its active sites exist within the lumen of a cylinder formed by four heptameric rings (Unno et al. 2002). This arrangement prevents well-folded proteins from entering the constricted area, protecting them from degradation. In terms of chemical structure, the eukaryotic proteasome consists of a 26S particle, the accepted physiological form, composed of a 20S core particle complexed at one or both ends with a 19S regulatory cap. The latter modulates the entrance of substrates to the core and is required for recognition of ubiquitin-tagged substrates and unfolded substrates

prior to their entry into the proteolytic chamber (Glickman & Ciechanover 2002). The core of this multimeric protease is a hollow, barrel-shaped protein complex (Kisselev & Goldberg 2001) consisting of four stacked rings, each ring composed of seven α or β subunits, housing duplicate sites of trypsin-, caspase- and chymotrypsin-like peptidase activities.

Several protein mediators of apoptosis are regulated by the UPP, such as members of the Bcl-2 family. Bcl-2 and Mcl-1 are good examples of prosurvival Bcl-2 family members that are degraded via the UPP (Basu & Haldar 2002). Proapoptotic Bax, Bid and Bim protein levels are all also regulated by the UPP. What is unclear from the studies of pro- and antiapoptotic Bcl-2 proteins is the nature of the E3-ligases that regulate their ubiquitination (Thompson *et al.* 2008). The transcriptional factor NF-κB is another regulatory protein, whose mode of activation is controlled by the UPP. The study of signal-induced destruction of IκB has revealed that the UPP plays a crucial role in IκB degradation, thus leading to NF-κB activation (Myung *et al.* 2001).

Subcellular localization of p53

Besides targeting p53 for degradation, Mdm-2 also affects the subcellular localization of the transcription factor. In fact, both Mdm-2 and p53 are nuclear proteins that shuttle constantly between the cytoplasm and the nucleus. The nucleocytoplasmic shuttling is achieved by their intrinsic nuclear localization signal (NLS) and nuclear export signals (NES) sequences. Two NES have been identified in p53, one located within the N-terminal domain and the other located in the C-terminus tetramerization domain (Stommel *et al.* 1999, Zhang & Xiong 2001) (Fig. 3). The well accepted model for the nuclear export of p53 requires Mdm-2-mediated ubiquitination of lysines in both the DBD and the C-terminus of

the tumor suppressor. Mdm-2 binding induces a conformational change in p53, thereby exposing the NES to the nuclear export machinery (Brooks *et al.* 2007). Blocking p53 nuclear export chemically by leptomycin B results in stabilization and nuclear accumulation of the protein (Freedman & Levine 1998), and mutation of the NES sequences reduces, but does not abolish the ability of Mdm-2 to target it for degradation (Yu *et al.* 2000). Degradation can also occur in the nucleus.

Transcriptional activity of p53

Finally, binding of Mdm-2 to the N-terminal TAD of p53 occludes the interaction of p53 with transcriptional activators, namely p300, thus inhibiting the ability of p53 to activate transcription of target genes. Binding to p300 protects p53 from Mdm-2-mediated proteasomal degradation. Conversely, binding of Mdm-2 blocks the TAD and prevents the coactivator or components of the transcription machinery from binding (Joerger & Fersht 2008).

2.2.2. Other regulators of p53

One of the most interesting methods of activating p53 results from the mutational inactivation of a tumor suppressor, such as the retinoblastoma protein (pRb). pRb occupies a central role in regulating the G_1-S transition of the mammalian cell cycle, which is critical for the decision of a cell to proliferate, differentiate or die. The unphosphorylated pRb is typically bounded to the E2F-1 transcription factor, and functions as a growth inhibitory form of the protein, while hyperphosphorylated pRb mediates cell proliferation (Dyson 1998). Inactivation of pRb releases the transcription factor E2F-1, which will lead to induction of apoptosis by both p53-dependent and -independent mechanisms. In fact, E2F-1

can stabilize p53 via the induction of human tumor suppressor protein $p14^{ARF}$ (Bates *et al.* 1998) (Fig. 4). ARF directly binds to Mdm-2 to inhibit its ubiquitin ligase activity, thereby preventing degradation of p53 (Zhang *et al.* 1998).

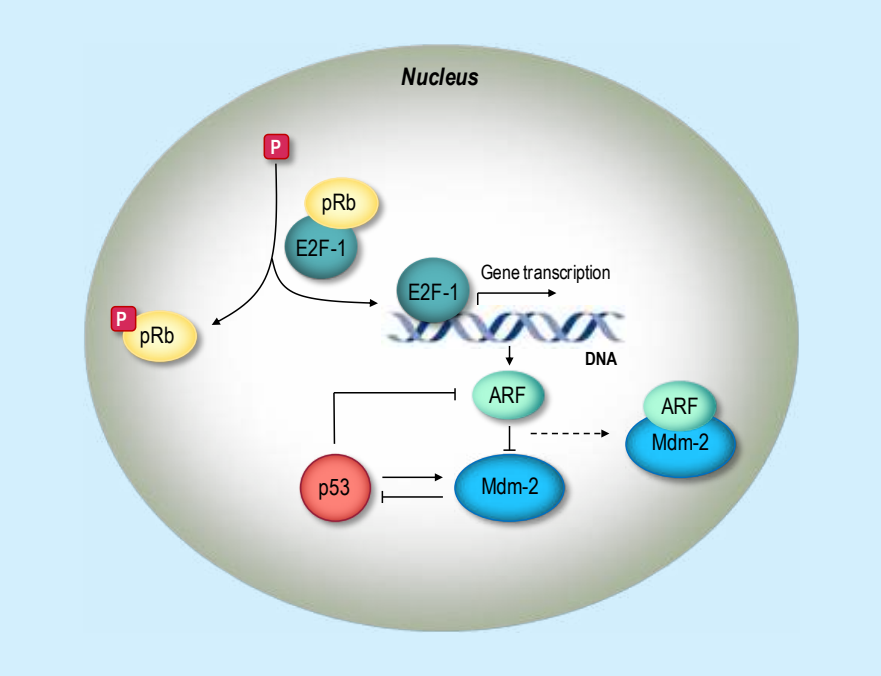


Fig. 4. p53/ARF/Mdm-2 regulatory loop after oncogene activation of p53. E2F-1 is activated after release from phosphorylated pRb. E2F-1 stabilizes p53 via the induction of ARF, which in turn directly binds to Mdm-2, preventing p53 degradation. Conversely, p53 negatively regulates ARF expression.

Although negative control of p53 is essentially exerted by Mdm-2, three additional proteins with E3 ubiquitin ligase activity mediate ubiquitination of p53. These include Prih2 (Leng *et al.* 2003), constitutively photomorphogenic 1 (COP-1) (Dornan *et al.* 2004), and C-terminus of heat-shock cognate (Hsc) 70-interacting

protein (CHIP) (Esser *et al.* 2005). Positive control of p53 is mediated by the transcriptional coactivator protein p300. The balance between p300 coactivation of transcription and E3 ligase ubiquitination controls the transcription flux of the p53 pathway

Activation of p53 is also subject to a complex and diverse array of posttranslational modifications, which markedly influence the expression of p53 target genes. Posttranslational modification of p53 involves covalent addition of a functional group to the protein after translation, and occurs mainly at its N- and C-termini. The number of these types of modifications on p53 appears endless; however, those commonly reported include phosphorylation of serines and/or threonines and acetylation, ubiquitination and sumoylation of lysine residues.

Most sites that are phosphorylated in response to a stress signal generally result in p53 stabilization, accumulation and activation in the nucleus. Phosphorylation of Ser15, Ser20 and Thr18 in the N-terminal region of p53 are the most extensively studied. Ser15 phosphorylation stimulates p53 transactivation, growth arrest, and apoptosis (Shieh *et al.* 1997). However, there are conflicting data on whether Ser15 phosphorylation affects Mdm-2 binding or not (Dumaz & Meek 1999). Phosphorylation of Thr18 and Ser20 does interfere with the interaction of p53 and Mdm-2, preventing the ubiquitination of p53 and thus promoting its stabilization (Chehab *et al.* 1999). There are a number of kinases triggered by different stress stimuli that are responsible for these phosphorylations, such as the ataxia-telangiectasia mutated kinase (ATM), JNK, p38, and others. JNK is responsible for phosphorylation of Thr81, an event that stabilizes and transcriptionally activates p53.

Acetylation of several lysine residues in the C-terminal region of p53 is also important for its protein stability and transcriptional activation. Most data show

that acetylation of p53 by the histone acetylase family members p300/CBP enhance its sequence-specific DNA binding activity; however, this issue remains controversial (Luo *et al.* 2004, Espinosa & Emerson 2001). The levels of p53 acetylation can be regulated via deacetylation by histone deacetylases (HDACs). Deacetylation was shown to repress p53 transcriptional activity, apoptosis, and growth arrest. Mdm-2 may also interfere with the acetylation of p53 by inhibiting p300 and promoting HDAC-1-mediated deacetylation of p53. Lysine residues that can be subjected to acetylation are also targeted by methyl transferases. Further, methylation of p53 has been reported to lead to opposing effects on p53 function (Olsson *et al.* 2007).

Finally, sumoylation and neddylation are modifications similar to ubiquitination in that an isopeptide bond is formed between the target protein and the small ubiquitin-like proteins, Sumo1 and Nedd8, respectively. Both sumoylation and neddylation appear to have repressive effects on p53 (Bode & Dong 2004).

2.3. The p53 apoptotic pathway

One of the most dramatic responses to p53 activation is the induction of apoptosis. Most evidence suggests that the key contribution of p53 to apoptosis is its ability to activate the transcription of various proapoptotic genes, including members of the Bcl-2 family, such as *bax*, *noxa* and *puma* (Miyashita & Reed 1995, Michalak *et al.* 2008). These genes govern the decision to live or die based on the cell type and death stimulus. Under certain conditions, p53 can trigger apoptosis by repressing the expression of antiapoptotic genes, such as *survivin* which may promote caspase activation (Hoffman *et al.* 2002). Although p53 predominantly influences the

mitochondrial pathway of apoptosis, it has also been implicated in the death receptor pathway. In addition to stimulating *fas* transcription in the spleen, thymus, kidney, and lung (Bouvard *et al.* 2000), overexpressed p53 may enhance levels of Fas at the cell surface by promoting trafficking of the death receptor from the Golgi (Bennett *et al.* 1998). p53 also activates DR5/Killer, the death domain-containing receptor for TRAIL. DR5 is induced by p53 in response to DNA damage (Wu *et al.* 1997) and, in turn, promotes cell death through caspase-8. Genes for proapoptotic proteins that may link both apoptotic pathways were also described as transcriptional targets of p53, such as *bid* and *PIDD*. Importantly, p53 is also involved in the activation of the apoptosome via induction of Apaf-1 expression. Apaf-1 induction by p53 is increased by E2F-1, which in turn induces Apaf-1 expression and activates p53, in an ARF-dependent manner (Moroni *et al.* 2001).

Despite the prominence of its transcriptional activities, the contribution of transcription-independent propapoptotic functions of p53 should not be underestimated. Curiously, p53 participates in apoptosis induction by acting in the cytosol, and directly at the mitochondrial level (Fig. 5). Mechanistically, apoptosis induced by mitochondrial p53 differs from that induced by cytosolic p53. Nevertheless, common to both pathways is the non-transcriptional, direct or indirect activation of proapoptotic Bcl-2 family members, leading to permeabilization of the mitochondrial OM, release of cytochrome *c* and activation of caspase-3. In addition both pathways require extranuclear p53 (Moll *et al.* 2005). It is becoming clear that a stress-stabilized cytoplasmic pool of p53 is the major source for mitochondrially translocated p53. However, it is still a matter of debate how this pool is generated. Some authors defend that unstressed cytoplasm contains a mixture of polyubiquitinated p53 that is very unstable and subjected to

immediate proteasomal degradation, and stable monoubiquitinated p53 that remains in the cytoplasm and serves as the source for p53 translocation to mitochondria. In this case the enzymatic E3 ligase activity, rather than the Mdm-2 molecule, acts as an important shuttle for trafficking p53 to mitochondria (Marchenko *et al.* 2007). Upon arrival to mitochondria, p53 undergoes deubiquitination by mitochondrial HAUSP.

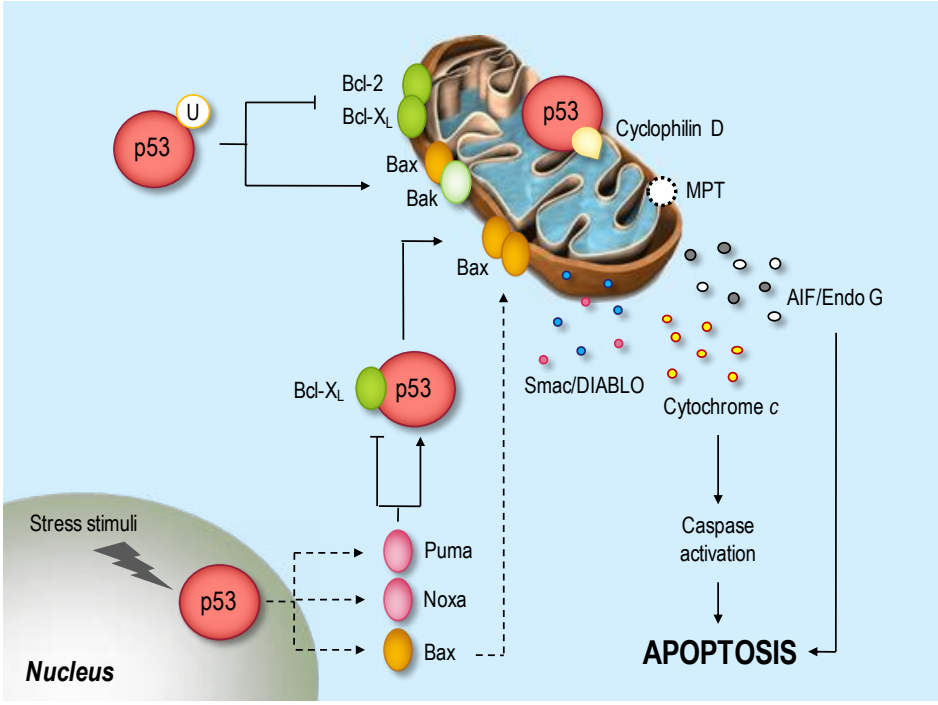


Fig. 5. Cytosolic and mitochondrial p53 apoptotic pathways. In the cytosolic p53 apoptotic pathway, nuclear p53 induces Puma expression, which in turn releases cytosolic p53 held inactive in the cytoplasm through binding to Bcl-X_L. Then, cytosolic p53 induces Bax oligomerization and mitochondrial translocation. Accumulation of p53 in the cytosol as a consequence of normal intracellular transport or stable monoubiquitination is the major

source for mitochondrial p53. In the mitochondria, p53 induces Bax and Bak oligomerization, antagonizes the Bcl-2 and Bcl-X_L antiapoptotic effect, and forms a complex with cyclophilin D in the mitochondrial inner membrane. These changes result in marked disruption of mitochondrial membranes and subsequent release of both soluble and insoluble apoptogenic factors. See text for more complete discussion. MPT, mitochondrial permeability transition; U, ubiquitin.

Alternatively, accumulation of p53 in the cytoplasm and/or mitochondria is simply the consequence of its normal intracellular transport. Given balanced nucleo-cytoplasmic shuttling of p53 and/or stabilization of pre-existing cytoplasmic p53, a threshold level of activated cytosolic or mitochondrial p53 required for induction of apoptosis can be achieved when the total amount of cellular p53 reaches certain levels (Speidel *et al.* 2006).

Once in the mitochondria, p53 effectively liberates the entire store of proapoptotic factors that include not only the soluble factors that trigger caspase activation, but also the insoluble factors responsible for chromatin condensation and endonuclease activities required for breaking down chromatin and DNA. This inescapably results in cell death. Mitochondrial p53 accomplishes this action by inducing Bax and Bak oligomerization, which are the hallmarks of mitochondria undergoing MMP. Moreover, in addition to physically interact with protective Bcl-X_L and Bcl-2, antagonizing their antiapoptotic stabilization of the mitochondria OM in damaged cells, mitochondrial p53 forms a stress-induced endogenous complex with cyclophilin D, a component of the IM. Functionally, these changes are associated with marked disruption of IM and OM integrity (Wolff *et al.* 2008).

Although the major fraction of Bcl-X_L is localized in the mitochondrial OM, some cell types express a portion in the cytosol. In addition to the mitochondrial

p53 action, an alternative cytosolic p53 death pathway was recently reported that directly activates cytosolic Bax. It occurs in those cells expressing Bcl-X_L in the cytosol and was described in UV-treated transformed mouse embryonic fibroblasts (Chipuk *et al.* 2005). It employs a two-step mechanism that involves transcription-dependence and -independence. Upon stress, nuclear p53 transcriptionally induces *puma*. Puma, in turn, liberates cytosolic p53 held inactive in the cytoplasm by a pre-existing soluble p53-Bcl-X_L complex, through binding to Bcl-X_L instead. Then, cytosolic p53 activates monomeric Bax by inducing its homo-oligomerization, followed by Bax mitochondrial translocation (Wolff *et al.* 2008). In contrast to the nuclear and mitochondrial p53 functions that are mediated by the p53 DBD, in the cytosolic p53 apoptotic pathway the N-terminus region is sufficient to mediate Bax activation and apoptosis. The diversity of p53-mediated mechanisms to induce apoptosis is astonishing, ranging from a potent transcription factor in the nucleus, to a "super" BH3-only protein in the mitochondria, acting both as an enabler and activator of BH3-only proteins. Curiously, over the past several years, there is growing evidence to suggest a relatively unforeseen and unexplored facet of p53. In short, the tumor suppressor acts as an active mediator of prosurvival pathways, by transcriptionally activating a multitude of genes whose products efficiently counteract apoptosis (reviewed in Janicke *et al.* 2008).

3. Bile Acids and Apoptosis

Bile acids, the major constituents of bile, are produced in the liver and secreted into the intestine, where they play crucial biological roles such as solubilization of lipids in the intestinal lumen, among many others. However, certain bile acids are cytotoxic molecules implicated in increased cell proliferation and cancer

development in the intestinal tract (Bayerdorffer *et al.* 1993), and/or cell death by necrosis and apoptosis. In fact, apoptosis has been described as a key event during hepatobiliary diseases (Patel & Gores 1995). Curiously, not all bile acids are toxic, and previous studies suggest that this may be related to minor changes in their chemical structure (Hofmann & Roda 1984). In this regard, accumulation of hydrophobic bile acids within the hepatocyte induces cell death of liver cells during cholestasis, while hydrophilic bile acids may be cytoprotective.

3.1. Bile acid metabolism and physiology

Bile acids are detergent molecules synthesized in the liver from neutral sterols by a complex series of chemical reactions (Russell & Setchell 1992). They are a class of acidic steroids with a cyclopentanoperhydrophenanthrene nucleus (ABCD-ring) containing 19 carbons, and most commonly a C5 side chain with a terminal carboxylic acid (Rodrigues *et al.* 2004) (Fig. 6).

In humans and most animal species, bile acids are produced primarily from the cholesterol metabolic pathway (van Berge Henegouwen *et al.* 2000). In fact, the biosynthesis of bile acids from cholesterol is the most significant pathway for the catabolism and removal of cholesterol from the body. The complete synthesis of bile acids requires approximately seventeen enzymes. The expression of these enzymes is tightly regulated by nuclear hormone receptors and other transcription factors, which ensure a steady supply of bile acids to a highly demanding metabolic environment.

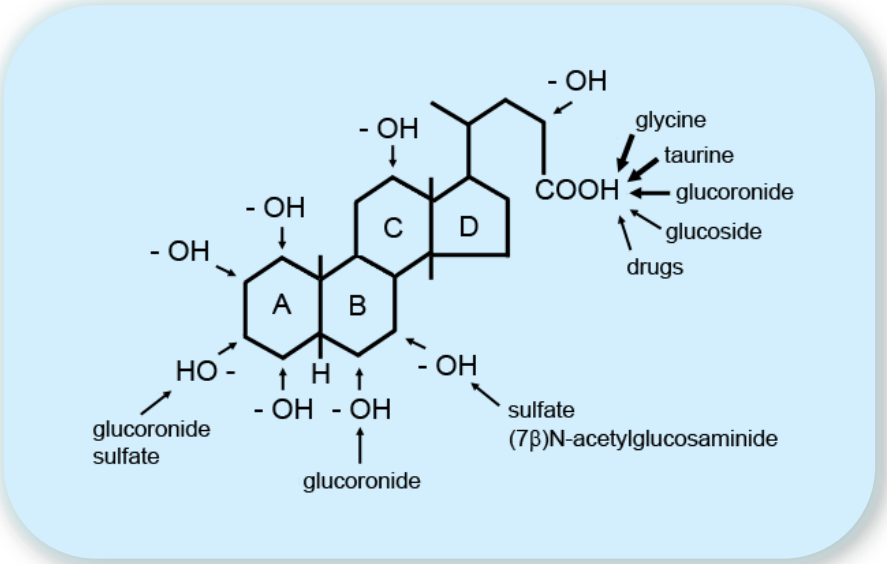


Fig. 6. General bile acid structure. The 5β-cholanoic acid nucleus is the basic structure of C_{24}-bile acids in mammalian species. Positions of the principal functional groups in the ABCD-ring, and possible conjugated derivates are indicated by arrows.

The initial and rate-limiting step in bile acid biosynthesis is the 7α-hydroxylation of cholesterol, catalyzed by the cytochrome P450 enzyme, cholesterol 7α-hydroxylase (CYP7A1). CYP7A1 is located in the ER and its transcription is repressed by primary bile acids but activated in a feed-forward manner by dietary cholesterol (Li *et al.* 1990). Furthermore, the expression of genes that synthesize cholesterol, fatty acids, and bile acids are regulated by intermediates and/or end-products of the bile acid pathway itself. The conversion of 7α-hydroxycholesterol to bile acids involves the addition of hydroxyl groups and the oxidation of the cholesterol side chain. Although the final product is

always an amphipatic molecule with inherent toxicity, different bile acid species have diverse degrees of hydrophobicity, as determined by their biochemical and physicochemical properties. The amphipathic structure allows these water-soluble compounds to interact with proteins and insert into lipid bilayers. These effects could be damaging to cell function and structure, particularly when intracellular concentrations of bile acids exceed certain limits, such as in cholestasis. The proposed mechanisms of hydrophobic bile acid-induced cell damage range from membrane-disruptive effects caused by their detergent properties to induction of cell death. Indeed, bile acids are important signaling molecules that modulate apoptosis and survival transduction pathways.

Primary bile acids, such as cholic and chenodeoxycholic acids, are synthesized in the liver, conjugated with the amino acids glycine or taurine, and then secreted via the bile ducts and gallbladder into the lumen of small intestine (Russell & Setchell 1992). Due to their unique biochemical properties, bile acids act as detergents to emulsify dietary lipids and fat-soluble vitamins, but they can also solubilize bilirubin and other catabolites. While emulsified nutrients are taken up by enterocytes in proximal segments of the gut, bile acids continue to move distally until absorbed in the ileum by the ileal bile acid transporter. Subsequently, bile acids re-enter the liver via the portal vein, are taken up by hepatocytes and resecreted into bile for use in the next feeding cycle (van Berge Henegouwen *et al.* 2000).

The biliary bile acid pool also includes secondary bile acids, such as deoxycholic (DCA) and lithocholic acids. These bile acids are not formed in the liver, but rather result from the metabolism of primary bile acids by intestinal bacteria. An important example of this metabolism is the formation of ursodeoxycholic acid (UDCA) by oxidation of chenodeoxycholic acid to 7-

oxolithocholic acid, followed by reduction yielding the 7β-isomer. Curiously, in bears, all biliary bile acids are formed in the liver because they are not exposed to bacterial enzymes (Hagey *et al.* 1993).

3.2. Bile acid induction of apoptosis

Accumulation of toxic bile acids is a common feature of several chronic human liver diseases, resulting from interruption in bile flow. This pathological condition, known as cholestasis, can promote liver cell death, leading to cirrhosis (Hofmann 2002). The mechanisms by which bile acids induce apoptosis in hepatocytes are still not entirely known. It was thought that hydrophobic bile acids, such as glycochenodeoxycholic and taurochenodeoxycholic acids, could induce cytotoxicity by acting as detergents on cell membranes. However, other evidence suggests that basic cellular mechanisms of hepatocyte injury might be primarily involved, ultimately causing cell death by either necrosis or apoptosis. Several studies have shown that caspase activation, mitochondrial dysfunction, and cellular distribution of Bcl-2-related proteins determine the fate of hepatocytes in models of cholestasis (Maher 2004). Indeed, pathophysiological concentrations of bile acids induce both ligand-dependent and -independent death receptor pathways and modulate downstream signaling pathways, a combination that strongly sensitizes cells to apoptosis (Fig. 7). Toxic bile acids have been shown to induce apoptosis in a Fas- and TRAIL-dependent manner (Faubion *et al.* 1999, Higuchi *et al.* 2003), involving recruitment of the FADD, activation of caspase-8 and Bid as well as downstream effector caspases. Activation of death receptors by bile acids involves induction of their transport from the Golgi complex, through a Golgi-associated and microtubule-dependent pathway (Sodeman *et al.* 2000). Once at the cell

membrane, spontaneous receptor oligomerization occurs. Alternatively, Fas activation by Fas ligand (FasL) or other agonists may also be increased. Interestingly, this process is stimulated by p53. The transcription factor was shown to transiently increase Fas expression at the cell surface via transport from the Golgi complex, and to induce Fas-FADD binding (Bennett *et al.* 1998). In addition, p53 was implicated in cyclin kinase inhibitor-enhanced, bile acid-induced apoptosis via Fas (Zhang *et al.* 2008).

The activation of death receptors invariably signals the mitochondrial pathway of apoptosis in hepatocytes. In fact, DCA induces significant levels of apoptosis *in vivo* and *in vitro* as well as the MPT in isolated mitochondria (Rodrigues *et al.* 1998a). Further, bile acid-induced apoptosis may also result from disruption of $\Delta\Psi_m$, increased ROS production, translocation of proapoptotic Bax to the mitochondria, and release of cytochrome *c* (Rodrigues *et al.* 2003b).

Finally, bile acids are important signaling molecules that modulate not only apoptosis but also survival transduction pathways, such as the PI3K-Akt and MAPK pathways (Schoemaker *et al.* 2004) (Fig. 7). Curiously, the liver has the ability to limit apoptosis during cholestasis by triggering specific mechanisms. Although it has been shown that Bcl-2, Bcl-X_L, and Bax are expressed in the liver, only cholangiocytes and not hepatocytes normally express antiapoptotic Bcl-2. However, induction of cholestasis by bile duct ligation leads to Bcl-2 expression in hepatocytes, which may represent an adaptative phenomenon to protect hepatocytes. In addition, the activation of NF-κB and subsequent regulation of antiapoptotic genes (Schoemaker *et al.* 2003), as well as the cytoplasmic sequestration of p53 (Oh *et al.* 2002), are complementary mechanisms triggered by the liver to inhibit or modulate apoptosis induced by toxic bile acids.

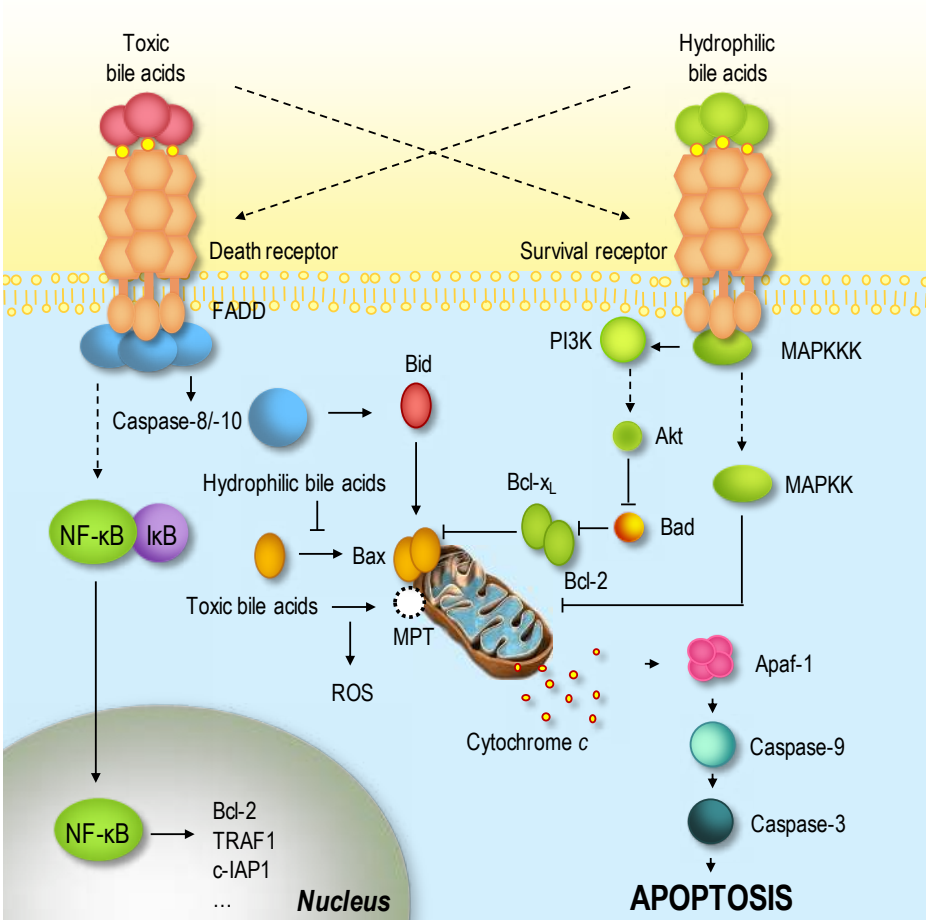


Fig. 7. Schematic representation of death and survival transduction pathways modulated by bile acids. Toxic bile acids induce apoptosis by activating both ligand-dependent and -independent death receptor oligomerization. The activation of death receptors by bile acids invariably signals the mitochondrial pathway of apoptosis in type II cells, such as hepatocytes. In addition, toxic bile acids may directly target mitochondria, either through induction of the MPT and ROS or activation of proapoptotic Bcl-2 family members. Finally, hydrophobic bile acids partially activate death receptor-dependent survival pathways such as the NF-κB. Hydrophilic bile acids do not induce apoptosis as they

simultaneously activate survival signaling pathways such as the MAPK and PI3K, antagonizing Bcl-2 proapototic members and preventing mitochondrial dysfunction and apoptosis. See text for more complete description. MAPKK, MAPK kinase; MAPKKK, MAKK kinase; MPT, mitochondrial permeability transition.

3.3. Ursodeoxycholic acid modulation of apoptosis

In contrast to the toxic effects of hydrophobic bile acids, UDCA is a hydrophilic bile acid that has been widely used as a therapeutic drug for patients with cholestatic liver diseases (Beuers *et al.* 1998, Lazaridis *et al.* 2001). The β orientation of UDCA projects the hydroxyl group toward the hydrophobic surface of the molecule, rendering it hydrophilic and markedly reducing its detergent properties. UDCA is normally present in human bile in a low concentration, representing only 3% of total bile acids. In black bears, however, it is the major biliary bile acid (Hagey *et al.* 1993). Bear bile has been used for centuries in traditional Chinese medicine as a remedy for liver disorders. Subsequently, a number of controlled trials on the use of this bile acid in liver disorders were published in the Western literature (Leuschner *et al.* 1989, Gracielle *et al.* 2002). At the present, it is the only drug approved by the United States Food and Drug Administration for the treatment of primary biliary cirrhosis. The beneficial effects of UDCA in patients with primary biliary cirrhosis provided the first clues on the different biological effects of bile acids in health and disease. Indeed, UDCA is currently used as a therapeutic agent for several hepatobiliary disorders, especially cholestatic liver diseases, becoming a widely prescribed “liver tonic” based solely on empiric observations (Paumgartner & Beuers 2004).

Despite its clinical efficacy, the precise mechanism by which UDCA improves liver function is still not entirely understood. Experimental evidence has suggested three major mechanisms of action, including protection of cholangiocytes against cytotoxicity of hydrophobic bile acids, stimulation of impaired biliary secretion, and/or inhibition of hepatocyte apoptosis (Paumgartner & Beuers 2002). Most likely, UDCA protective function results from a coordinated process involving several effects, depending on the type and stage of the disease.

The antiapoptotic effects of UDCA have been demonstrated both *in vivo* and *in vitro* in rat liver (Benz *et al.* 1998, Rodrigues *et al.* 1998a), and in human hepatocytes (Benz *et al.* 2000). While toxic bile acids fed to rats induce apoptosis in the liver, UDCA inhibits this effect, by preventing formation of ROS and translocation of the proapoptotic protein Bax from the cytosol to the mitochondria. More importantly, these effects were also observed in nonhepatic cells, such as Saos-2, Cos-7 and HeLa cell lines, in response to a variety of agents acting through different apoptotic pathways. In fact, UDCA inhibits DCA-induced apoptosis, as well as apoptosis induced by ethanol, transforming growth factor-β1 (TGF-β1), FasL, and okadaic acid (Rodrigues *et al.* 1998b).

The antiapoptotic effect of UDCA appears to involve the mitochondrial membrane. In fact, UDCA and its amidated conjugates, tauroursodeoxycholic acid (TUDCA) and glycoursodeoxycholic acid (GUDCA), prevent the release of cytochrome *c*, caspase activation, PARP cleavage, and apoptosis-induced changes in transmembrane potential (Rodrigues *et al.* 1999). Furthermore, TUDCA can directly stabilize mitochondrial membranes, having a profound effect on Bax channel formation (Rodrigues *et al.* 2003b). Pretreatment with TUDCA almost completely abolished Bax insertion in the lipid and protein environment of isolated

mitochondrial membranes. Nevertheless, there is no evidence of direct binding between the bile acid and proapoptotic Bax.

UDCA was also shown to partially prevent apoptosis via the death receptor pathway in primary mouse hepatocytes co-cultured with fibroblasts expressing FasL. The protective effect was not associated with reductions in Fas trimerization, but rather appeared to involve a direct effect on the mitochondrial membrane (Azzaroli *et al.* 2002). Although not fully understood, TUDCA may also regulate the ER stress-mediated pathway, reducing calcium efflux and activation of caspase-12, which plays an important role in several liver diseases (Xie *et al.* 2002) (Fig. 8).

Importantly, it has been demonstrated both *in vitro* and *in vivo* that TUDCA benefits certain acute and chronic neurodegenerative disorders that are associated with increased levels of apoptosis. In fact, TUDCA is a potent neuroprotective agent not only in pharmacological and transgenic animal models of Huntington's disease (Keene *et al.* 2002), but also for ischemic and hemorrhagic stroke (Rodrigues *et al.* 2002, Rodrigues *et al.* 2003a). The bile acid improves neurological function, while significantly preserving mitochondrial membrane stability and inhibiting caspase activation. Moreover, TUDCA improved the survival and function of nigral transplants in a rat model of Parkinson's disease (Duan *et al.* 2002), and partially rescued a Parkinson's disease model of *C. elegans* from mitochondrial dysfunction (Ved *et al.* 2005).

Finally, activation of survival pathways may represent important additional mechanisms by which UDCA inhibits apoptosis. For example, UDCA can stimulate the MAPK and the PI3K signaling pathways.

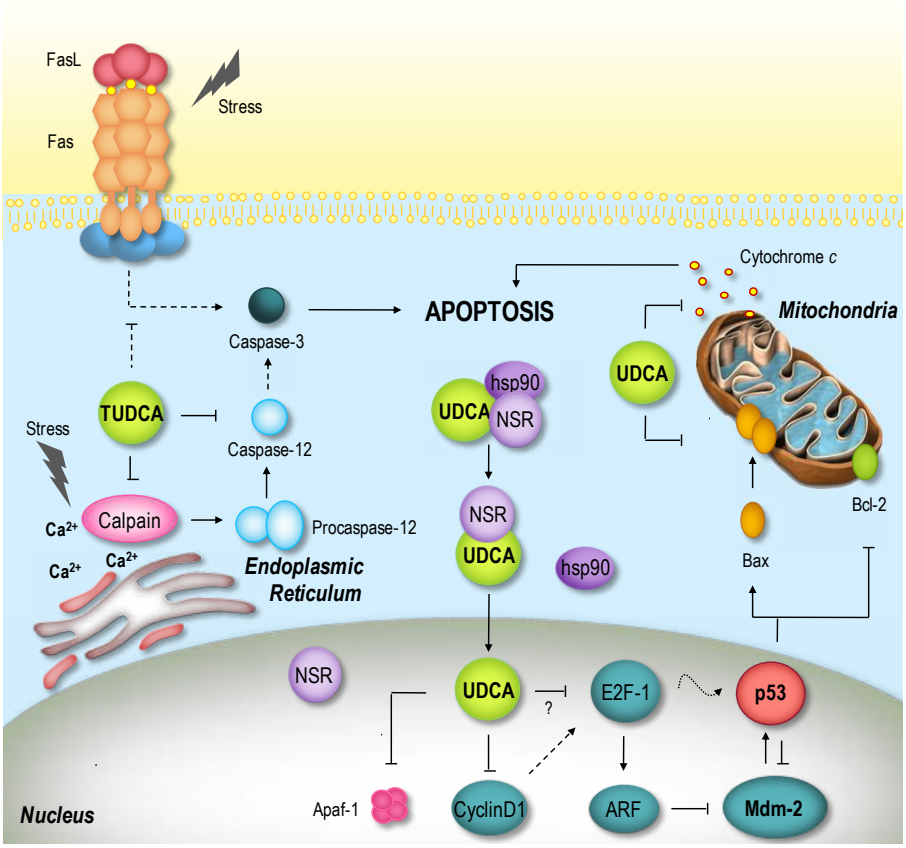


Fig. 8. Proposed mechanisms for the antiapoptotic actions of UDCA and TUDCA. UDCA negatively modulates the mitochondrial pathway by inhibiting Bax translocation, ROS formation, cytochrome *c* release, and caspase-3 activation. UDCA can also interfere with the death receptor pathway, inhibiting caspase-3 activation. Moreover, TUDCA inhibits apoptosis associated with ER stress, by modulating intracellular calcium levels, and inhibiting calpain and caspase-12 activation. UDCA interacts with NSR, leading to NSR/hsp90 dissociation and nuclear translocation of the UDCA/NSR complex. Nuclear trafficking of UDCA allows it to modulate the E2F-1/p53/Bax pathway, thus preventing apoptosis. Finally, UDCA downregulates cyclin D1 and Apaf-1 further inhibiting the mitochondrial apoptotic cascade. See text for more complete description.

In fact, it has recently been demonstrated that the p38/ERK/MAPK and PI3K pathways are involved in protection of TUDCA against glycochenodeoxycholic-induced apoptosis in rat hepatocytes (Schoemaker *et al.* 2004). It appears that the protective effect of TUDCA is independent of caspase inhibition and competition at the cell membrane with toxic bile acids. TUDCA also remarkably inhibited each of the amyloid-β (Aβ)-induced apoptotic events in rat neuronal cells, in part, through activation of the PI3K pathway (Solá *et al.* 2003a). In addition, it protected cardiomyocytes in culture against reperfusion injury in a PI3K/Akt-dependent pathway (Rajesh *et al.* 2005).

It is interesting to note that UDCA may also be cytoprotective through mechanisms other than inhibition of apoptosis. In fact, UDCA has the ability to suppress the NF-κB pathway and subsequent cytokine expression via activation of the glucocorticoid receptor (GR), playing an important anti-inflammatory role in the liver (Miura *et al.* 2001).

3.3.1. Cross-talk between UDCA and nuclear steroid receptors

Many natural ligands of nuclear steroid receptors (NSR) are products of the cholesterol biosynthetic pathway. Thus, as cholesterol-derived molecules and due to their chemical and structural similarities to steroid hormones, bile acids can potentially bind and modulate NSR activation.

Curiously, NSR play a heterogeneous function in apoptosis, which is affected by tissue-specific parameters (McCormick *et al.* 2000). Interestingly, GR was shown to regulate apoptosis through modulation of expression of pro- and antiapoptotic Bcl-2 family members and p53. In the nervous system, GR-induced apoptosis was associated with increased p53 as well as elevated ratios of proapoptotic Bax relative to antiapoptotic Bcl-2 and Bcl-X_L (Almeida *et al.* 2000).

Indeed, in rat hippocampus, it was shown that Bax is essential in GR-mediated death-signaling cascade. GR upregulates other proapoptotic members of the Bcl-2 family, such as Bim and Bad (Bellosillo *et al.* 2002), thus facilitating Bax mitochondrial translocation and subsequent cytochrome *c* release.

At odds with its proapoptotic effects in certain cell types, GR suppresses spontaneous apoptosis of neutrophils as well as TNF-α-mediated apoptosis of mouse fibroblasts (Pagliacci *et al.* 1993). It has been demonstrated that GR inhibits apoptosis during fibrosarcoma development by transcriptionally activating Bcl-X_L (Gascoyne *et al.* 2003), and reducing E2F-1 expression in human osteosarcoma cell lines (Rogatsky *et al.* 1997). Strikingly, GR has profound protective effects against apoptosis in human and rat hepatocytes, as well as in rat hepatoma cell lines (Evans-Storms & Cidlowski 2000, Bailly-Maitre *et al.* 2001).

Interestingly, GR was also found to physically interact with the tumor suppressor p53. The functional interactions between GR and p53 in different physiological conditions may result either in antagonism or complementation between both transcription factors. p53 and GR may repress each other's function either at the transcriptional level or through GR-mediated cytoplasmic anchoring of p53. In contrast, synergy between both transcription factors has also been described in neuronal and rat hepatoma cells (Sengupta & Wasylyk 2004). Indeed, p53 cross-talks not only with GR but also with other steroid-hormone receptors, including androgen and estrogen receptors.

The function of the mineralocorticoid receptor (MR) during apoptosis appears to be mainly protective. In fact, MR inhibits the apoptotic process in most cell types, often preventing GR-induced cell death (Hassan *et al.* 1996). In the brain, stress-induced GR activation leads to an arrest of neurogenesis and increased apoptosis, which in turn is inhibited by MR activation. In addition, MR increases

neuronal survival by decreasing p53 levels as well as the ratio of pro- to antiapoptotic members of the Bcl-2 family (Almeida *et al.* 2000).

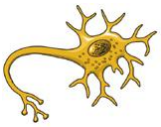
Interestingly, bile acids affect transcription control of gene expression through specific nuclear receptors, such as the farnesoid X receptor (FXR). It has been shown that a number of bile acids, including chenodeoxycholic acid, and to a much lesser extent, DCA and lithocholic acid bind and activate FXR in cultured cells (Makishima *et al.* 1999). FXR is specifically expressed in tissues where bile acids function, such as the liver, intestine, and kidney. As a general regulator of bile acid metabolism, FXR acts in the liver via suppression of CYP7A to reduce synthesis, and in the intestine through activation of intestinal bile acid-binding protein (IBABP) to increase recycling of bile acids (Russell 1999).

The anti-inflammatory properties of UDCA share similarities with glucocorticoid (GC)-mediated immunomodulation. Therefore, the link between UDCA and GR was primarily correlated with anti-inflammatory properties of the bile acid. Interestingly, we have recently shown that GR is required for UDCA antiapoptotic function, by demonstrating that endogenous silencing of GR abolishes the protective effect of UDCA against TGF-β1-induced apoptosis in primary rat hepatocytes (Solá *et al.* 2004). During TGF-β1-induced apoptosis, significant degradation of GR occurs in liver cells. However, pretreatment with UDCA not only markedly upregulated GR, but also increased receptor nuclear translocation (Table 1). In addition, the transcription factor E2F-1, which regulates many genes involved in the control of cell proliferation, was found to be a potent target for UDCA-induced GR activation. Changes in E2F-1 protein levels induced by TGF-β1 were no longer prevented by UDCA in GR-silenced hepatocytes. Curiously, other authors have suggested that GR interferes with the expression of mitogenic factors, such as cyclins, cyclin-dependent kinases, and E2F-1 (Rogatsky

et al. 1997). Moreover, rats fed a combination diet containing DCA and UDCA showed complete inhibition of the drastic increase in steady-state levels of p53 as well as cyclin transcript expression associated with DCA alone (Kren *et al.* 2001).

More recently, new insights have been revealed on how NSR are involved in UDCA antiapoptotic role. Indeed, we further investigated the involvement of GR during UDCA antiapoptotic function and demonstrated that UDCA promotes the dissociation between GR and its molecular chaperone, hsp90, thus inducing subsequent GR translocation into the nucleus of hepatocytes (Solá *et al.* 2005) (Table 1 and Fig. 8). However, when the C-terminal region of GR was deleted, UDCA no longer induced GR/hsp90 dissociation and GR nuclear translocation nor protected against apoptosis, indicating that GR LBD is at least one target domain required for the antiapoptotic function of UDCA. Using a fluorescently labeled UDCA molecule, we clearly demonstrated that UDCA reaches the nucleus of primary rat hepatocytes in a GR-dependent mechanism, which resulted in inhibition of apoptosis-related genes. In fact, similarly to other bile acids, UDCA is detectable within the nuclei of hepatocytes where it may play an important role in controlling gene expression (Setchell *et al.* 1997). Moreover, in liver tissue obtained from patients on oral bile acid therapy, only a small portion of the bile acid was detected in liver tissue. However, a large percentage of that pool was taken up selectively by the nuclei of hepatocytes, and UDCA was identified as the most abundant bile acid (Monte *et al.* 2002).

Table 1. Role of UDCA and/or TUDCA in modulating parameters of GR and MR function in primary rat hepatocytes and cortical neurons.

Cell	Parameter	GR	MR	Reference
Hepatocytes 	total levels	+	+	(Solá *et al.* 2004)
	chaperone association	-	no data	(Solá *et al.* 2005)
	nuclear translocation	+	+	(Solá *et al.* 2004, Solá *et al.* 2005)
	transactivation	+/-	no data	(Solá *et al.* 2005, Tanaka *et al.* 1996, Miura *et al.* 2001, Mitsuyoshi *et al.* 1997)
Neurons 	total levels	no effect	+	(Solá *et al.* 2006)
	chaperone association	no data	-	(Solá *et al.* 2006)
	nuclear translocation	-	+	(Solá *et al.* 2006)
	transactivation	-	+	(Solá *et al.* 2006)

+, activation; -, inhibition.

Nuclear translocation of NSR by bile acids may not always result in transactivation or transrepression of NSR. In fact, bile acids might only require NSR to reach the nucleus, where they themselves regulate gene transcription through modulation of other transcription factors. In this regard, it has been shown that ligand-bound NSR are not permanently localized in the nucleus. Rather, they are continuously and rapidly shuttling between the nucleus and cytoplasm (Madan & DeFranco 1993). Indeed, we showed that the protective role of UDCA does not require GR transactivation in liver cells (Solá *et al.* 2005). UDCA increases the

activation of GC-response elements in hepatocytes that overexpress GR, but does not protect against TGF-β1-induced apoptosis by further increasing GR transactivation (Table 1). Therefore, it is conceivable that UDCA requires NSR for translocation to the cell nucleus as part of a ligand-receptor complex, using a mechanism similar to that used by steroid hormones (Fig. 8).

Interestingly, other studies have demonstrated that UDCA interacts with GR. It has been described that UDCA interacts with GR in the absence of specific ligands, as a novel and selective GR modifier (Tanaka *et al.* 1996). In addition, it was reported that UDCA promotes DNA binding of GR through interaction with its LBD, but without eliciting GR transactivational function (Miura *et al.* 2001). Only under certain conditions, UDCA may also enhance GR-responsive gene expression (Tanaka *et al.* 1996, Mitsuyoshi *et al.* 1997). Although there is no structural evidence for the direct binding of UDCA to GR, it is thought that the bile acid acts at a region of the LBD distinct from that of the classical GR antagonist, dexamethasone, inducing a unique GR conformation that can translocate into the nucleus and bind DNA, but no longer interacts with coactivators to elicit transcription (Miura *et al.* 2001). These data may, in part, explain the anti-inflammatory properties of UDCA. In fact, UDCA repressed NF-κB-dependent transcription and subsequent cytokine expression via the interaction of GR with NF-κB. It is assumed that transrepression of transcription factors, such as activator protein 1 (AP-1) and NF-κB, is the primary mechanism by which GCs mediate their anti-inflammatory activity. It has been demonstrated that different ligands induce various conformations of NSR resulting in unique regulatory properties of the receptors. Indeed, UDCA pretreatment was shown to suppress DNA binding activity of AP-1 during DCA incubation of human colon cancer cells (Im & Martinez 2004). It is therefore possible that UDCA may regulate several

apoptotic-related genes by functionally modulating GR. Other mechanisms of GR activation by UDCA might be membrane related and may involve an unidentified secondary signal that activates other signal transduction pathways.

UDCA has also been shown to regulate activity of other NSR. In fact, UDCA is a relatively strong pregnane X receptor (PXR) agonist and a weaker FXR agonist. Although UDCA does not itself bind FXR (Makishima *et al.* 1999), it does inhibit receptor activation by more hydrophobic bile acid species (Howard *et al.* 2000). Other studies have shown that UDCA activates PXR to induce CYP3A4, a bile acid-metabolizing enzyme in human hepatocytes. It is possible then that the antifibrotic effects of UDCA are also mediated via PXR.

Interestingly, MR is a potent inhibitor of apoptosis in many cell types, including neuronal cells; whereas GR activation has been correlated with neuronal pathologies. Although MR and GR share similar ligands and target the same genes, they may also form heteromeric complexes in which MR represses GR function. In fact, these heterodimers are found in dynamic association with a still growing number of chaperone proteins and other factors mediating their actions. Thus, although there is no evidence that MR and UDCA interact directly, it is conceivable that UDCA-mediated modulation of GR may also affect MR.

The antiapoptotic action of MR has been extensively described in neuronal cells, but less is known about its role during hepatocyte apoptosis. Interestingly, our results have suggested a relevant mechanistic function for MR during hepatocyte apoptosis (Table 1). We demonstrated that much like GR, MR contributes to the protective effect of UDCA against the E2F-1/Mdm-2/p53 apoptotic pathway induced by TGF-β1 in primary rat hepatocytes (Solá *et al.* 2004). However, in the presence of Aβ, TUDCA appears to differentially modulate MR and GR nuclear translocation in rat cortical neurons (Solá *et al.*

2006) (Table 1). In fact, TUDCA preferentially interacted with MR LBD, thus promoting MR dissociation from its cytosolic chaperones and translocation to the nucleus. It is possible that specific co-modulators may somehow induce TUDCA-mediated MR nuclear translocation in neuronal cells, while reducing GR nuclear traffic. Surprisingly, in contrast with hepatocytes, pretreatment of PC12 cells with TUDCA significantly modulated Aβ-induced changes in NSR transactivation. Indeed, the transcriptional activity of MR was increased while GR transactivation was reduced by TUDCA (Solá *et al.* 2006).

3.3.2. Modulation of cell cycle-related proteins by UDCA

Tissue homeostasis depends on the perfect balance between cell death and proliferation. Therefore, it is somehow expected that both processes intimately connect with each other, sharing key molecular regulators.

DNA microarray analysis showed that UDCA can significantly modulate the expression of 96 different genes, most of them involved in apoptosis, but also in cell cycle regulation and proliferation (Castro *et al.* 2005). Cyclin D1 was one of the target genes found to be downregulated in rat hepatocytes in response to UDCA incubations. Cyclin D1 is well known for regulating the G_1 phase of the cell cycle. However, elevated levels of this protein can also lead to growth suppression and apoptosis. Recent evidence showed cyclin D1 facilitate apoptosis of primary rat hepatocytes exposed to DCA, while pre-incubation with UDCA and TUDCA significantly abrogated their proapoptotic effects (Castro *et al.* 2007). Moreover, modulation of cyclin D1 expression appears to contribute to the antiapoptotic effects of the bile acid, in part through a p53-dependent mechanism.

It is known that elevated levels of cyclin D1 result in pRb phosphorylation which no longer binds to and inhibits E2F-1. Released E2F-1, in turn, directly or

in combination with other proteins may activate genes necessary for apoptosis, eventually leading to p53 activation (Bates *et al.* 1998). Interestingly, although E2F-1 is mainly implicated in the control of cell proliferation, it can also regulate apoptosis by at least three different mechanisms. First, E2F-1 stabilizes p53 by induction of ARF, a possible direct target of E2F-1, which binds to Mdm-2 and prevents p53 degradation (Zhang *et al.* 1998). E2F-1 is also able to upregulate transcription of p73, a homologue of p53 that shares the ability to induce apoptosis (Irwin *et al.* 2000). Finally, E2F-1 inhibits antiapoptotic factors such as NF-κB, thus promoting cell death (Phillips *et al.* 1999). Curiously, previous findings have shown that UDCA modulates the TGF-β1-induced E2F-1/p53/Bax apoptotic pathway (Solá *et al.* 2003b). It appears that UDCA abrogates E2F-1-induced p53 and p53-associated Bax expression, independently of its effect on mitochondria and/or caspases. Therefore, cyclin D1 may represent an alternative, upstream target of this pathway by activating E2F-1 (Fig. 8).

The tumor suppressor p53 is also a target of bile acid cytoprotective functions in both hepatic and non-hepatic cells. In addition to its modulation in rat hepatocytes, it was described that TUDCA was able to inhibit the E2F-1/p53/Bax apoptotic pathway induced by Aβ in neuronal PC12 cells (Ramalho *et al.* 2004). TUDCA was also shown to counteract the increased expression of p53 in Alzheimer's disease mutant neuroblastoma cells (Ramalho *et al.* 2006). Moreover, recent studies have demonstrated a neuroprotective role of UDCA against neurotoxicity induced by the anticancer therapeutic drug, cisplatin (Park *et al.* 2008). Interestingly, it was suggested that UDCA blocks cisplatin-induced neuronal apoptosis via the downregulation of the p53 signaling pathway.

Although p53 has been mainly considered as a tumor suppressor, protecting cells against cancer development, it also contributes for deleterious cell loss in the

severe side effects of chemotherapies, as well as in several pathological conditions (Zamzami & Kroemer 2005, Vousden & Lane 2007). UDCA, acting as a pleiotropic agent at inhibiting the apoptosis process, with minimal toxicity associated, represents a potent therapeutic approach to several disorders associated with higher susceptibility to apoptotic cell death.

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Objectives

The main objective of the studies presented in this thesis was to characterize the molecular mechanisms by which UDCA inhibits the p53 apoptotic cascade in hepatocytes. Because one of the most prominent functions of p53 is as a transcription factor, we first analyzed whether UDCA was able to bind directly to the DNA binding domain of p53, thereby reducing its activity. Since there was no evidence of such interaction, we next investigated possible secondary and perhaps indirect mechanisms of p53 regulation where UDCA could play a role. In this regard, Mdm-2, which is the main repressor of p53 activity, emerged as a potential protein that deserved further evaluation. Finally, we determined whether UDCA affected p53 degradation by the proteasome. Under certain pathological conditions, small molecules such as UDCA could alter the stability and modulate several molecular targets, including p53, thus emerging as excellent candidates for therapeutic use.

The specific questions addressed in this thesis are:

1. Does UDCA bind directly to the DNA binding domain of p53 to prevent p53-induced apoptosis?
2. Is UDCA capable of reducing the transcriptional and DNA binding activities of p53, thereby reducing its proapoptotic function? If so, does it function via the Mdm-2 repressor of p53?

3. Is the Mdm-2-ubiquitin-proteasome degradation pathway involved in UDCA protective function against p53-induced apoptosis?

Ultimately, our goal is to achieve a better understanding of the precise molecular mechanisms underlying the UDCA antiapoptotic function. The identification of specific targets and modulators will help design novel and more effective therapeutic strategies for human pathological conditions associated with high levels of apoptosis.

2

No evidence of direct binding between ursodeoxycholic acid and p53 DNA-binding domain

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Abstract

Ursodeoxycholic acid (UDCA) is used increasingly for the treatment of cholestatic liver diseases. Among other cytoprotective effects, this endogenous bile acid is a potent inhibitor of apoptosis, interfering with both intrinsic and extrinsic apoptotic pathways. In previous studies, we have demonstrated that the TGF-β1-induced E2F-1/Mdm-2/p53 apoptotic pathway was an upstream molecular target of UDCA. The tumor suppressor p53 is a well described transcription that induces the expression of multiple different proapoptotic gene products. Its regulation involves a variety of signaling proteins and small molecules, and occurs at multiple levels, including transcription, translation, and posttranslation. In this study, we have investigated the possible interaction between p53 core domain, also referred to as the DNA binding domain, and UDCA by using different biophysical techniques. Our results showed no evidence of direct binding between the bile acid and p53 core domain.

Introduction

The tumor suppressor protein p53 is a transcription factor that functions to maintain genome integrity. It exerts its role by inducing or repressing the expression of a network of genes involved in cell cycle control, senescence, and apoptosis, in response to diverse stress stimuli. Although there is evidence that p53 can mediate apoptosis by transcription-independent mechanisms, numerous apoptotic genes are transcriptionally activated by the tumor suppressor. These include members of the Bcl-2 family, such as Bax, or the BH3-only proteins Noxa and Puma (Miyashita & Reed 1995, Oda *et al.* 2000, Nakano & Vousden 2001). The potent regulatory functions of p53 are usually under tight control. In normal cells, p53 is present in almost undetectable levels due to constitutive ubiquitination by Mdm-2, an E3 ligase that targets p53 to proteasomal degradation. Posttranslational modifications, such as phosphorylation, acetylation, sumoylation, among others are also important mechanisms for p53 regulation.

p53 complexity starts in its structure. The monomer with 393 aminoacid residues can be divided in 3 functional domains. The N-terminal domain (1-93) is intrinsically disordered, and includes the transactivation domain (TAD) and the proline-rich region, followed by a structured core region that constitutes the DNA-binding domain (DBD) (102-292). The C-terminal domain consists of two small domains; a tetramerization domain that regulates the oligomerization state of p53 (320-356), and a natively unfolded regulatory domain (363-393) that binds DNA nonspecifically (Joerger & Fersht 2008). Apart from the complex structure of each monomer, p53 is only active as a tetramer and its structure and dynamics in that particular oligomeric state are crucial to understand its function.

Ursodeoxycholic acid (UDCA) is an endogenous hydrophilic bile acid, widely used in the treatment of certain cholestatic disorders of the liver. The therapeutic effects of UDCA may result, in part, from its ability to inhibit liver cell apoptosis. It has been demonstrated that this bile acid plays a unique role in modulating the apoptotic threshold in both hepatic and nonhepatic cells, in response to a variety of agents acting through different apoptotic pathways (Rodrigues *et al.* 1998). The precise mechanism(s) by which UDCA prevents cell death remains speculative and involves molecular targets other than mitochondria. Previous studies indicated that UDCA inhibits the E2F-1/Mdm-2/p53 apoptotic cascade induced by TGF-β1 (Solá *et al.* 2003, Solá *et al.* 2004). The ability of E2F-1 to induce apoptosis involves stabilization of p53 via transcription of $p14^{ARF}$, which markedly inhibits the p53 repressor, Mdm-2 (Phillips & Vousden 2001). UDCA, in turn, counteracts TGF-β1 effect by abrogating E2F-1-induced p53, thereby modulating the expression of apoptosis-related p53 target genes. This mechanism appears to be dependent of nuclear steroid receptors (NSR) (Solá *et al.* 2004). In fact, we and others have shown that UDCA and its taurine-conjugated derivative, tauroursodeoxycholic acid, interfere with NSR (Tanaka & Makino 1992, Solá *et al.* 2005, Solá *et al.* 2006). More importantly, these bile acids promote dissociation of NSR from their cytosolic chaperone, Hsp90, and translocate into the nucleus as a ligand-receptor complex to reduce apoptosis.

In the present study, we have used complementary biophysical techniques to determine whether UDCA binds to or alters the structural stability and folding properties of the p53 DBD. This domain is unstable, with a melting temperature of ~ 41-44ºC (Canadillas *et al.* 2006). The binding of UDCA could compromise p53 function either by destabilizing its structure or by inducing structural fluctuations that could inhibit its DNA-binding specificity.

Experimental Procedures

Materials and plasmids

Chemicals, reagents and enzymes were of the highest purity grade commercially available. Chromatography materials were from Amersham Pharmacia Biotech (Uppsala, Sweden). For protein expression, p53 DBD plasmid coding for amino acids 94-312 of human wild-type p53 was transformed into *E.coli* BL21 competent cells.

Gene expression and protein

The DBD of p53 was expressed as soluble protein as described by Bell *et al*. (Bell *et al.* 2002). In brief, bacteria were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicilin, at 37ºC, to an A_{600} of 0.5 followed by overnight induction at 22ºC with 1 mM isopropyl-β-D-thiogalactoside (IPTG). After induction, cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl, pH 6.8, 5 mM dithiothereitol (DTT), 1 mM benzamidine, complete protease inhibitor tablets (Complete™; Roche Applied Science, Mannheim, Germany), 200 units of benzonase (Merck, Darmstadt, Germany), and disrupted by high-pressure dispersion using a Thermo IEC French Press (Needham Heights, MA). Purification of p53 DBD was performed at 4ºC using a combination of cation exchange chromatography on a HiTrap SP Fast Flow column and affinity chromatography on a Heparin HiTrap column (GE Healthcare Life Sciences, Freiburg, Germany) in 50 mM sodium phosphate, pH 7,0, 150 mM KCl, and 5 mM DTT. The purity of the protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). p53 DBD was found to be stable in solution and maintained its spectroscopic properties and melting temperature after

flash-freezing in liquid nitrogen. Protein concentration was determined spectrophotometrically by using the extinction coefficient of 0.649 for p53 DBD calculated for a 1 mg/ml solution in a 1 cm cuvette at 280 nm.

Spectroscopic methods

Anisotropy measurements were performed at 10ºC, using a SLM Aminco spectrofluorimeter (Spectronic Instruments, Inc., Rochester, NY) with cell stirring. Far-UV circular dichroism (CD) spectra were recorded typically at 0.2 nm resolution on a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) fitted with a cell holder thermostatically controlled by a Peltier.

Thermal denaturation

Thermal unfolding was followed by monitoring the intrinsic tryptophan fluorescence (λ_{em} = 340) and the ellipticity ($\Delta\varepsilon_{mrw}$ at 222 nm) variations. In all experiments, a heating rate of 1ºC/min was used, and the temperature was changed from 10 to 90ºC. Data were analyzed according to a two-state model described by the following equation:

$$y = \frac{(y_f + m_f \cdot T) + (y_u + m_u \cdot T) \cdot \exp\left(\frac{\Delta H_m}{RT} \cdot \frac{T - T_m}{T_m}\right)}{1 + \exp\left\{\frac{\Delta H_m}{RT} \cdot \frac{T - T_m}{T_m}\right\}}$$

where y is the spectroscopic signal observed, m_f and m_u are the slopes of the pre- and post-transition baselines, y_f and y_u correspond to the value of y for folded and unfolded forms, T_m is the midpoint of the thermal unfolding curve, and ΔH_m is the enthalpy change for unfolding at T_m. The fits to the unfolding transitions were made using Origin (MicroCal Software Inc, Northampton, MA)

Binding studies

All measurements were performed with fluorescein isothiocyanate (FITC)-tagged UDCA. The interaction between p53 DBD and FITC-UDCA was evaluated by using both far-UV CD and fluorescence anisotropy spectroscopy, at a constant temperature of 10ºC, in buffer containing 20 mM NaP, 50 mM NaCl, and 2 mM DTT, pH 7.5. Far-UV CD measurements were performed with 100 μM p53 DBD, before and after the addition of the bile acid at stoichiometric ratio 1:1, using a 1 mm cell path length (bandwidth 2 nm). The spectra were recorded from 260 to 190 nm and accumulated 10 times. For titration experiments, aliquots of 3 μl of titrant (500 μM FITC-UDCA) were sequentially added to p53 DBD peptide in solution (0.22 mg/ml), until the concentration reached p53 DBD 1:54 UDCA. All spectra were corrected for buffer contributions and protein concentration. Ellipticities were followed at 208 and 222 nm.

For anisotropy experiments, p53 DBD (initial concentration 30 μM) was titrated with FITC-UDCA (500 μM) with increments of 5 μl, waiting ~ 5 min between each addition. Similarly, FITC-UDCA (initial concentration 5 μM) was titrated with p53 DBD (50 μM), additions of 10 μl every 5 min. When the FITC-UDCA was titrated, fluorescence anisotropy was measured on excitation at 480 nm (bandwith 8 nm) and emission at 520 nm (bandwith 8 nm). When the protein was titrated fluorescence anisotropy was measured on excitation at 280 nm (bandwith 8 nm) and emission at 340 nm (bandwith 8 nm).

Results

Characterization of p53 DNA-binding domain

p53 DBD was expressed in *E.coli* with a low yield of ~ 3 mg of purified protein per 3 g of wet culture (Fig. 1). The fold and the stability of the purified p53 DBD was evaluated by far-UV CD to confirm that the protein used in the binding assays was correctly folded. The far-UV CD spectrum is shown in Figure 2A and is in agreement with previously published data by Klein *et al.* (Klein *et al.* 2001). As expected, the far-UV CD spectrum of the DNA binding domain of p53 showed two negative peaks (208 and 222 nm), which reflect its α-helical content. As a result of the β-sheet contributions, the 208 nm peak was broader and the 222 nm peak was less intense. To evaluate the protein stability, a thermal denaturation was performed following the far-UV CD signal at 222 nm (Fig. 2B). A melting temperature (T_m) of 41.5 ± 0.3ºC was obtained, in agreement with previously published data (Bell *et al.* 2002).

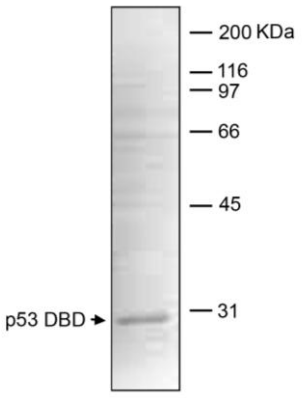

Fig. 1. Purity of p53 DNA-binding domain. p53 DBD purified from *E.coli* bacteria was subjected to electrophoresis on 12.5% polyacrylamide gels and stained with Coomassie brilliant blue.

Evaluation of UDCA interaction with p53 DNA-binding domain

To investigate a possible interaction between UDCA and p53 DBD, we used both far-UV CD and fluorescence anisotropy to monitor structural changes that might occur as a consequence of bile acid presence. First, we followed the titration of p53 DBD with FITC-UDCA by far-UV CD (208 nm and 222 nm). Adding the bile acid has no effect on the protein spectrum, even for a stoichiometry of 1 p53 DBD to 54 FITC-UDCA (Fig. 2A and 3A), meaning that FITC-UDCA does not affect the p53 DBD secondary structure. Similarly, thermodynamic stability of p53 DBD is not significantly affected by the presence of FITC-UDCA.

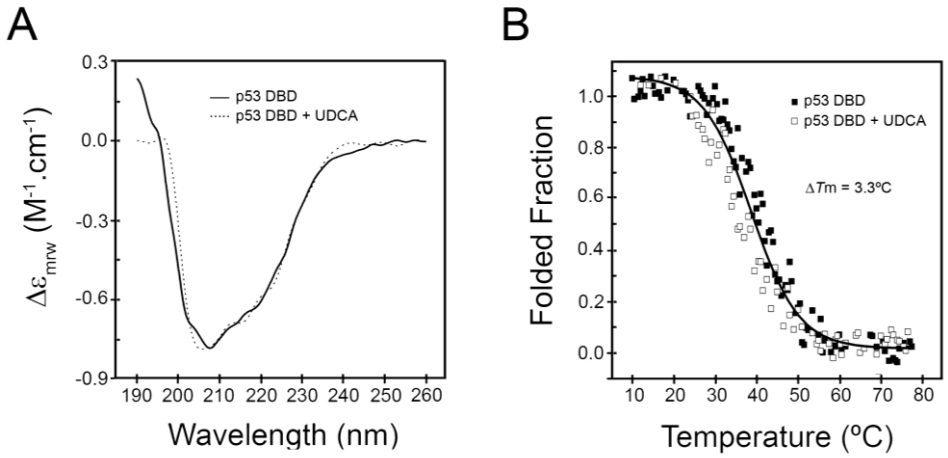

Fig. 2. Effect of UDCA in the structural properties of p53 DNA-binding domain. (A) Far-UV CD spectra of p53 DBD (0.22 mg/ml), with or without UDCA. (B) Far-UV CD analysis of thermal denaturation transitions of p53 DBD alone (■), or in the presence of UDCA (□). See "Experimental Procedures" for details.

The presence of FITC-UDCA induced a shift of -3.3 ± 1ºC in the T_m of p53 DBD (Fig. 2B). However, the presence of the bile acid significantly reduced the signal/noise, thus increasing the uncertainty in the T_m calculation so that the shift observed could be considered smaller and negligible. Similar titrations were performed following changes in fluorescence anisotropy. A stock solution of 500 µM FITC-UDCA was titrated with 30 µM of p53 DBD peptide in solution. However, no changes in anisotropy of the labeled-bile acid were detected (Fig. 3B). The same results were obtained when titrating FITC-UDCA with increasing amounts of p53 DBD (data not shown).

Taken together, these results do not support a direct interaction between anti-apoptotic UDCA and the DNA-binding domain of human p53, *in vitro*.

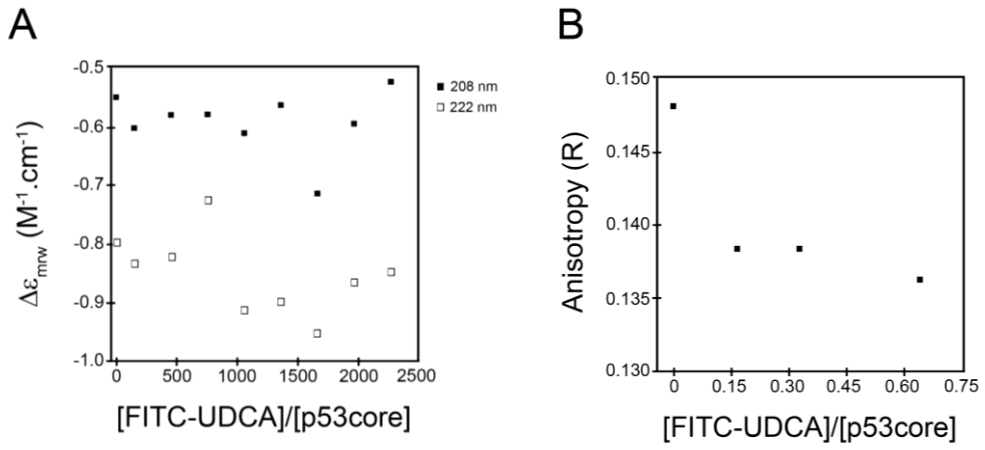

Fig. 3. Titration of p53 DBD with FITC-UDCA. Titration of p53 DBD protein with increasing amounts of FITC-UDCA, analyzed by far-UV CD (A) or fluorescence anisotropy spectroscopy (B). See "Experimental Procedures" for details.

Discussion

The mechanisms of bile acid effects on cell survival and apoptosis are not entirely understood. We have previously reported that UDCA prevents apoptosis in several cell types by inhibiting the mitochondrial pathway (Botla *et al.* 1995, Rodrigues *et al.* 1998, Rodrigues *et al.* 1999) and modulating the E2F-1/Mdm-2/p53 apoptotic cascade (Solá *et al.* 2003). In addition, bile acids have been detected in nuclei of several cell types (Setchell *et al.* 1997, Kren *et al.* 2001, Monte *et al.* 2002, Solá *et al.* 2006). We have suggested that UDCA, as a cholesterol-derived molecule, could reach the nucleus through mechanisms similar to those used by steroid hormones. It appears that NSR are specifically required for the nuclear trafficking of UDCA, ultimately protecting cells from undergoing apoptosis (Solá *et al.* 2005, Solá *et al.* 2006). Therefore, it is conceivable that bile acids may act as molecular modulators of apoptosis-related gene expression, by interfering directly with DNA, or by interacting with transcription factors and other nuclear proteins involved in the apoptotic machinery.

In this study, we attempted to use different biophysical approaches to determine whether UDCA binds directly to recombinant p53 DBD. p53 has a multidomain structure comprising a folded region with DNA-binding and tetramerization domains, and natively unfolded regions with molecular recognition features that provide binding promiscuity (Joerger & Fersht 2008). Specific binding to p53-responsive genes occurs through the DNA-binding domain of p53. Thus, we sought to examine the effect of UDCA in the structure and conformation of this precise region.

Far-UV CD spectroscopy is widely used in the study of molecular interactions due to its remarkable sensitivity to changes in protein conformation,

whatever their origin. The far-UV bands derive mainly from peptide bond absorption, reflecting the secondary structure of the protein (Martin & Bayley 2002). Fluorescence anisotropy, in turn, detects the rotational mobility of a fluorophore, which is also a reliable and sensitive spectroscopic method to locally evaluate the existence of physical interactions. Unbounded molecules can freely rotate in solution, while formation of binding structures restricts their motion, thereby increasing anisotropy (Royer 1995).

Results from both CD and anisotropy do not support a direct binding between UDCA and the p53 DBD. CD spectra and titration experiments do not indicate significant changes of p53 secondary structure in the presence of UDCA. Similarly, data of anisotropy showed no alterations in the mobility of either p53, or UDCA, when together. Although no binding between UDCA and p53 DBD has been detected, we cannot exclude a possible interaction with other regions of p53.

The N-terminal region of p53 contains the TAD, considered to be a binding site for a multitude of interacting proteins, such as components of the transcription machinery (Lu & Levine 1995, Di Lello *et al.* 2006), the transcriptional coactivators p300/CBP (CREB-binding protein) (Antonsson *et al.* 1997, Teufel *et al.* 2007), and the negative regulator Mdm-2 (Kussie *et al.* 1996, Schon *et al.* 2002). Upon binding, intrinsically disordered regions of the p53 TAD rigidify and become fully folded. This is the case for the p53 fragment comprising residues 15-29 after binding to a hydrophobic cleft in the N-terminus of Mdm-2 (Popowicz *et al.* 2007). Moreover, it has been reported that Mdm-2 also undergoes extensive conformational changes along its N-terminal region, upon binding to p53 (Schon *et al.* 2002). Interestingly, the Mdm-2 protein is the major repressor of p53, and UDCA has been shown to increase its levels in rat hepatocytes undergoing TGF-β1-induced apoptosis (Solá *et al.* 2003). Thus, the N-terminus of p53, or more

specifically, the TAD domain, could also be a potential target region for UDCA binding/interaction. The bile acid may additionally interact with Mdm-2 itself, in the p53-binding cleft of bounded or unbounded Mdm-2, to somehow stabilize the complex. The p53-binding cleft is the most flexible region of the Mdm-2 protein, presenting enough plasticity to adapt to diverse topologies of incoming ligands (Espinoza-Fonseca & Trujillo-Ferrara 2006). In fact, the number of newly discovered small-molecules that bind the Mdm-2-p53 binding cleft is continuously increasing over the years (Vassilev *et al.* 2004, Grasberger *et al.* 2005, Dudkina & Lindsley 2007, Dey *et al.* 2008).

Interaction between UDCA and the extreme C-terminus of p53 should also be analyzed. The C-terminal region of p53, called regulatory domain, is subjected to extensive posttranslational modifications to regulate p53 function, including acetylation, ubiquitination, phosphorylation, and methylation. Finally, three putative nuclear localization signals (Liang & Clarke 1999) and one nuclear export signal (Stommel *et al.* 1999) sequences have been identified within the C-terminal region of p53. Subcellular localization of p53 is determinant for its activity, since stabilized p53 remains active in the nucleus, whereas translocation to the cytoplasm is required for Mdm-2-mediated p53 degradation (O'Keefe *et al.* 2003). Thus, UDCA could inhibit the p53 apoptotic pathway not by directly binding to p53 but by interfering with other proteins and molecular pathways involved in the regulation of this complex protein.

The multifaceted network of p53 regulation opens the door for an endless world of hypothesis in which UDCA could participate and play a role. Although, we could not detect direct binding between the p53 DBD and UDCA, our findings contributed to the characterization of the interaction between these two molecules.

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3

p53 is a key molecular target of ursodeoxycholic acid in regulating apoptosis

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Abstract

p53 plays an important role in regulating expression of genes that mediate cell cycle progression and/or apoptosis. In addition, we have previously shown that the hydrophilic bile acid ursodeoxycholic acid (UDCA) prevents TGF-β1-induced p53 stabilization and apoptosis in primary rat hepatocytes. Therefore, we hypothesized that p53 may represent an important target in bile acid-induced modulation of apoptosis and cell survival. In this study, we demonstrated that UDCA reduces p53 transcriptional activity, thereby preventing its ability to induce Bax expression, mitochondrial translocation, cytochrome *c* release and apoptosis in primary rat hepatocytes. More importantly, bile acid inhibition of p53-induced apoptosis was associated with decreased p53 DNA-binding activity. Subcellular localization of p53 was also altered by UDCA. Both events appear to be related with increased association between p53 and its direct repressor, Mdm-2. In conclusion, these results further clarify the antiapoptotic mechanism of UDCA and suggest that modulation of Mdm-2/p53 interaction is a prime target for this bile acid.

Introduction

Ursodeoxycholic acid (UDCA), a hydrophilic bile acid with low intrinsic toxicity has been successfully used in the treatment of cholestatic liver diseases. Its therapeutic effects have been attributed to several mechanisms, including the ability to stimulate hepatobiliary secretion and inhibit liver cell apoptosis (Poupon *et al.* 1991, Paumgartner & Beuers 2002). We and others have shown that UDCA reduces the apoptotic threshold in several cell types through modulation of classical mitochondrial pathways (Botla *et al.* 1995, Rodrigues *et al.* 1999). Further, reduction of cell death by UDCA may involve alternate and upstream molecular targets of the E2F-1/Mdm-2/p53 apoptotic pathway (Solá *et al.* 2003b, Solá *et al.* 2004).

The tumor suppressor protein p53 is a transcription factor that plays an important role in regulating expression of genes that mediate cell cycle arrest and/or apoptosis in response to a wide variety of cellular stress factors (Vogelstein *et al.* 2000, Vousden & Lu 2002). Although its role in suppressing cell cycle progression has been extensively described (el-Deiry *et al.* 1993, Harper *et al.* 1993), less is known about the mechanisms by which p53 induces apoptosis. Curiously, recent studies have revealed that p53 can mediate apoptosis by a transcription-independent process (Chipuk & Green 2003). Nevertheless, cells in which wild-type p53 was replaced by a transcriptionally inactive mutant showed loss of both cell cycle arrest and apoptotic functions, supporting the idea that transcriptional activity is of paramount importance in these cellular responses (Chao *et al.* 2000, Jimenez *et al.* 2000). In fact, it is thought that p53 signals apoptosis through its activity as a sequence-specific transcriptional activator of proapoptotic target genes, such as *bax*, *Noxa,* or *PUMA* (Miyashita & Reed 1995,

Oda *et al.* 2000, Nakano & Vousden 2001). These proteins are translocated to mitochondria, where they promote loss of the mitochondrial membrane potential and cytochrome *c* release, thus activating the Apaf-1/caspase-9 apoptotic cascade (Bossy-Wetzel & Green 1999). Indeed, *Apaf*-1 itself has also been described as a transcriptional target for p53 (Moroni *et al.* 2001).

Interestingly, in unstressed conditions, p53 is a short-lived protein. Pivotal to its regulation is the function of the mouse double minute 2 protein (Mdm-2) (Freedman *et al.* 1999). p53 is tightly regulated by a negative feedback loop where the tumor suppressor induces Mdm-2 transcription, which in turn binds to p53 and inhibits its function (Haupt *et al.* 1997, Kubbutat *et al.* 1997). The Mdm-2 protein has been shown to inhibit p53 activity by binding to its transactivation domain, targeting it to ubiquitination, transporting it to the cytoplasm and promoting its degradation by the proteasome. The precise mechanism(s) involved in p53 stabilization with response to stress remains unclear. It has been shown that it involves a series of post-translational modifications to both p53 and Mdm-2, which in turn may facilitate the dissociation of the Mdm-2/p53 complex (Lavin & Gueven 2006).

We have previously demonstrated that UDCA decreases E2F-1 transcriptional activation, thus preventing the downstream events of TGF-β1-induced cell death associated with Mdm-2 degradation and p53 stabilization (Solá *et al*. 2003b). Moreover, microarray analysis revealed that UDCA incubation increased an expressed sequence tag (EST) highly similar to Mdm-2 protein while decreasing another EST for p53-apoptosis-associated target (Castro *et al.* 2005) in primary rat hepatocytes. Finally, UDCA was shown to alter Mdm-2 protein levels in several cell types (Solá *et al.* 2003b, Ramalho *et al.* 2004). Here we further explore the molecular events underlying the cytoprotective role of UDCA in p53-

induced apoptosis of hepatocytes. Our results indicate that UDCA inhibits p53 transactivation and its DNA-binding activity in hepatocytes by preventing nuclear accumulation of this tumor suppressor protein, in part, through a p53/Mdm-2 binding-dependent mechanism.

Experimental Procedures

Hepatocyte isolation and cell culture

Rat primary hepatocytes were isolated from male Sprague-Dawley rats (100–150 g) by collagenase perfusion as described previously (Mariash *et al.* 1986). In brief, rats were anesthetized with phenobarbitol and the livers perfused with 0.05% collagenase. Hepatocyte suspensions were obtained by passing collagenase digested livers though 125 μm gauze and washing cells in William's E medium supplemented with 26 mM sodium bicarbonate, 0.01 units/ml insulin, 2 mM L-glutamine, 10 nM dexamethasone, 100 units/ml penicillin, 100 units/ml streptomycin (Sigma-Aldrich Corp., St. Louis, MO), and 20% heat-inactivated fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA). Cell viability was determined by trypan blue exclusion and was typically 80–85%. After isolation, hepatocytes were resuspended in William's E medium and plated on Primaria™ tissue culture dishes (BD Biosciences, San Jose, CA) at either 5 x 10^4 cells/cm^2 for cell viability assays, or 2.5 x 10^5 cells/cm^2 for all other experiments. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 4 h to allow attachment. Plates were then washed with medium to remove dead cells, and incubated in William's E medium containing 10% heat-inactivated FBS.

Transfections and CAT assays

Transfections were performed using two expression plasmids pCMV-$p53_{wt}$ and pCMV-$p53_{179}$, and a *bax* promoter-driven chloramphenicol acetyltransferase (CAT) reporter construct. The *bax*-CAT construct consisted of a 371 base pair SmaI/SacI fragment of human *bax* gene subcloned into the HindIII site of the promoterless CAT plasmid, pUCSV0CAT (Miyashita & Reed 1995). Overexpression plasmids were generated by cloning either wild-type p53 (pCMV-$p53_{wt}$) or a mutant inactive form of human p53 (pCMV-$p53_{179}$(His-179→Glu), all under CMV enhancer/promoter control (Unger *et al.* 1992). Twelve hours after plating, hepatocytes were treated with vehicle or 100 μM of UDCA, and co-transfected with 2 μg of both expression and reporter plasmids using Lipofectamine 2000 (Invitrogen). For normalization, cells were cotransfected with 0.5 μg of the luciferase reporter construct, PGL3-Control vector (Promega Corp., Madison, WI). Transfection efficiencies of ~ 70% were determined in primary rat hepatocytes using a reporter plasmid expressing β-galactosidase, and did not differ between reporter and expression plasmids. At 48 h post-transfection, attached cells were harvested for CAT ELISA (Roche Applied Science, Indianapolis, IN) and luciferase assays (Promega), according to the manufacturers' instructions. In parallel experiments, hepatocytes were pre-treated with vehicle or 100 μM of UDCA, twelve hours prior to transfection with 8 μg of each expression plasmid. Cells overexpressing either wild-type or mutant p53 were harvested for protein extraction and immunoblot analysis. Nuclear, mitochondrial and cytosolic protein fractions, or total proteins were prepared at 36 h or 60 h after transfection, respectively. Attached cells were fixed for morphologic evaluation of apoptosis and culture medium used for lactate dehydrogenase (LDH) viability assays.

Short interference-mediated silencing of the *mdm-2* gene

A pool of 4 short interference RNA (siRNA) nucleotides designed to knock down *mdm-2* gene expression in rats was purchased from Dharmacon (Waltham, MA). A control siRNA containing a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA was used as control. Four hours after p53 overexpression, the culture medium was changed and hepatocytes were transfected using INTERFERin™ Transfection Reagent for siRNA (Polyplus Transfections, Illkirch, France), according to the manufacturer's instructions for additional 48 h. The final concentration of siRNAs was 10 nM. UDCA was re-added to the cultures after the first 4 h of silencing. Floating and attached cells were harvested for preparation of total, nuclear, and cytosolic protein extracts, which were then subjected to immunoblot analysis. Attached cells were fixed for Hoechst staining.

Measurement of cell death and caspase activity

Cell viability was measured by the LDH (Sigma-Aldrich) viability assay according to the manufacturer's instructions. In addition, Hoechst labeling of cells was used to detect apoptotic nuclei. Briefly, the medium was gently removed to prevent detachment of cells. Attached hepatocytes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma-Aldrich) at 5 μg/ml in PBS for 5 min, washed with PBS and mounted using PBS:glycerol (3:1, v/v). Fluorescent nuclei were scored blindly and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation

of condensed chromatin. Five random microscopic fields per sample of ~ 100 nuclei were counted and mean values expressed as the percentage of apoptotic nuclei. In addition, caspase activity was determined in cytosolic protein extracts after harvesting and homogenization of cells in isolation buffer containing 10 mM Tris-HCl buffer, pH 7.6, 5 mM $MgCl_2$, 1.5 mM KAc, 2 mM dithiothreitol, and protease inhibitor mixture tablets (Complete™; Roche Applied Science, Mannheim, Germany). General caspase-3-like activity was determined by enzymatic cleavage of chromophore *p*-nitroanilide (pNA) from the substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; Sigma-Aldrich). The proteolytic reaction was carried out in isolation buffer containing 50 μg cytosolic protein and 50 μM DEVD-pNA. The reaction mixtures were incubated at 37°C for 1 h, and the formation of p-NA was measured at 405 nm using a 96-well plate reader. In addition, caspase-3 cleavage was determined by immunoblotting.

p53-DNA binding ELISA assay

The TransAM™ p53 transcription factor assay kit (Active Motif, Carlsbad, CA) was used according to the manufacturer's protocol. Nuclear extracts were diluted to 2 μg/mL of total protein with Lysis Buffer. Extracts were applied to plates containing immobilized 20-mer oligonucleotide with a p53 consensus binding site (5'-GGACATGCCCGGGCATGTCC-3'). After 1 h incubation at room temperature, plates were washed and incubated with diluted p53 antibody (1:1000) for an additional 1 h. Diluted anti-rabbit horseradish peroxidase-conjugated antibody (1:1000) was then added to previously washed plates and developing solution was added and incubated for 5-8 min to allow color development. The reaction was stopped and absorbance read at 450 nm with a reference wavelength

of 650 nm. In addition, both nuclear and cytosolic protein fractions were analyzed for p53 by immunoblotting.

Bax translocation and cytochrome *c* release

Cellular distribution of Bax and cytochrome *c* was determined using mitochondrial and cytosolic protein extracts. Cells were harvested and centrifuged at 600*g* for 5 min at 4°C. The pellets were washed once in ice-cold PBS and resuspended with 3 volumes of isolation buffer containing 20 mM HEPES/KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, supplemented with protease inhibitor cocktail tablets, in 250 mM sucrose. After chilling on ice for 15 min, cells were disrupted by 40 strokes of a glass homogenizer, and homogenates were centrifuged twice at 2,500*g* for 10 min at 4°C to remove unbroken cells and nuclei. The mitochondrial fraction was then centrifuged at 12,000*g* for 30 min at 4°C, and the pellet resuspended in isolation buffer and frozen at -80°C. For cytosolic proteins, the 12,000*g* supernatants were removed, filtered sequentially through 0.2 and 0.1 µm Ultrafree MC filters (Millipore, Bedford, MA) to remove other cellular organelles, and frozen at -80°C.

Immunoblotting

For Bax and cytochrome *c* detection, 40 µg of mitochondrial and cytosolic proteins were separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition, steady-state levels of Bax and p53, as well as p53 cellular distribution and caspase-3 cleavage were also determined. Following electrophoretic transfer onto nitrocellulose membranes, the immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. After blocking with a 5% milk solution, the membranes were incubated overnight at 4ºC with primary mouse monoclonal

antibodies reactive to Bax (B-9), p53 (Pab 240), p-p53 (mSer 20), caspase-3 (H-227), cytochrome *c* oxidase subunit II (K-20), lamin A/C (346) (Santa Cruz Biotechnology, Santa Cruz, CA), p-p53 (pSer 15, Ab-3; Calbiochem, Darmstadt, Germany) and cytochrome *c* (7H8.2C12; PharMingen, San Diego, CA), and finally with secondary goat anti-mouse or anti-rabbit IgG antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 3 h at room temperature. The membranes were processed for protein detection using the SuperSignal substrate (Pierce Biotechnology, Rockford, IL). β-actin (AC-15, Sigma-Aldrich), lamin A/C, and cytochrome *c* oxidase were used as loading controls for total, nuclear, and mitochondrial proteins, respectively. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories) according to the manufacturer's specifications.

Immunoprecipitation

Binding of p53 to Mdm-2 was detected by immunoprecipitation analysis. In brief, whole cell extracts were prepared by lysing cells in M-PER Mammalian Protein Extraction Reagent (Pierce). Immunoprecipitation experiments were carried out using a primary mouse monoclonal antibody to Mdm-2 (SMP 14; Santa Cruz Biotechnology) and the Ezview Red Protein G Affinity Gel (Sigma-Aldrich). Typically, 200 μg of lysate were incubated with 1 μg of Mdm-2 antibody overnight at 4ºC. Immunoblots were then probed with the mouse monoclonal anti-p53 antibody. Mdm-2 expression was determined in the same membrane after stripping off the immune complex for the detection of p53. Immunoprecipitation assays using high-detergent conditions as well as immunoblot analysis showed absence of nonspecific binding of the Mdm-2 antibody to p53. In addition,

immunoprecipitation assays using the mouse monoclonal antibody reactive to β-actin demonstrated no association with either p53 or Mdm-2.

Immunofluorescence

Primary rat hepatocytes overexpressing wild-type p53 in the presence or absence of UDCA were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 30 min. Following fixation, cells were blocked for 1 h in PBS containing 0.1% Triton-X-100, 1% FBS and 10% normal donkey serum. Cells were then sequentially incubated with monoclonal antibody to p53 (Santa Cruz Biotechnology) at a dilution of 1:200 in blocking solution, overnight at 4ºC. Subsequently, after three washes with PBS, cells were incubated with AMCA-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) for 2 h along with fluorescein isothiocyanate (FITC)-tagged UDCA. Subcellular localization of p53 and UDCA was visualized using an Axioskop fluorescence microscope (Carl Zeiss, Jena, Germany).

Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the Quantity One Version 4.6 densitometric analysis program (Bio-Rad Laboratories). Statistical analysis was performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

Results

UDCA reduces p53 transactivation

In addition to its role in cell cycle progression and senescence, p53 also induces apoptosis. Further, we have previously shown that UDCA prevented TGF-β1-induced p53 stabilization and apoptosis in primary rat hepatocytes (Solá *et al.* 2003b). We now hypothesize that p53 may represent an important target in bile acid-induced modulation of apoptosis. Immunoblot analysis confirmed that total p53 expression was increased in cells transfected with pCMV-$p53_{wt}$ and pCMV-$p53_{179}$ overexpressing plasmids as compared with endogenous levels of pCMV-transfected cells (Fig. 1A). Our results showed significant levels of apoptosis in cultured primary rat hepatocytes after transfection with wild-type p53 overexpression plasmid, as assessed by changes in nuclear morphology (Fig. 1B), caspase activity and processing (Fig. 1C) ($p < 0.01$). Mutant p53 only slightly increased cell death (data not shown), implying a transcription-independent role of p53 in apoptosis. Notably, pretreatment with UDCA reduced the levels of apoptosis to those of controls transfected with mutant p53 ($p < 0.01$), while inhibiting caspase-3-like activity and caspase-3 processing by 90% ($p < 0.05$). In addition, camptothecin, a topoisomerase I inhibitor, known to induce p53 accumulation, resulted in ~ 25% hepatocyte apoptosis; and this was inhibited with UDCA by ~ 80% ($p < 0.01$). Similar results were obtained with the LDH viability assay (data not shown).

It is well established that p53 binds to specific sequences within the promoter of various genes to modulate their expression. Proapoptotic Bax was the first member of the Bcl-2 gene family shown to be induced by p53 (Miyashita & Reed 1995, Thornborrow *et al.* 2002).

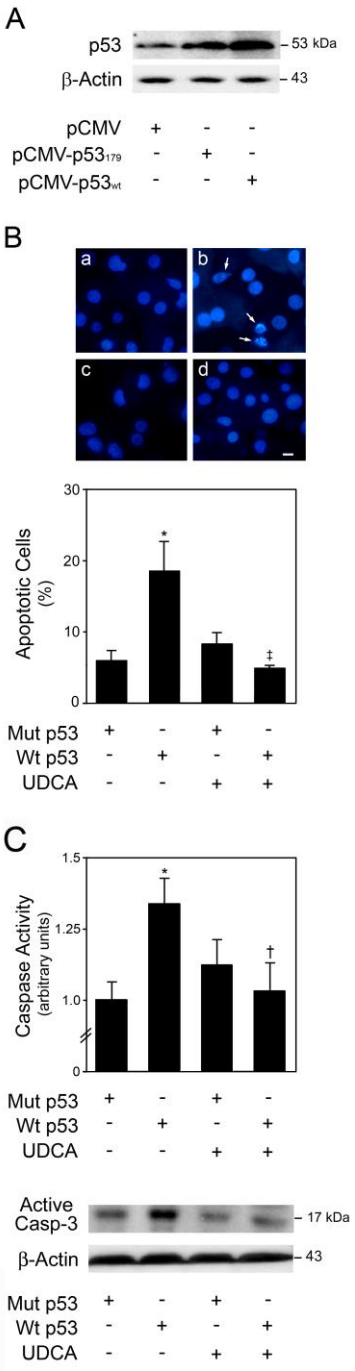

Fig. 1. UDCA inhibits apoptosis induced by p53 overexpression in primary rat hepatocytes. Cells were transfected with either mutant p53 (pCMV-$p53_{179}$) or wild-type p53 (pCMV-$p53_{wt}$) overexpression plasmids. Vehicle or 100 µM UDCA were included in the culture medium 12 h prior to transfection. At 36 h after transfection, cells were fixed and stained for morphological assessment of apoptosis, and cytosolic proteins were extracted for caspase activity and processing assays as described in "Experimental Procedures". (A) Representative immunoblot of p53 total levels. β-actin was used to control for lane loading. (B) Fluorescent microscopy of Hoechst staining (top) in cells transfected with either mutant p53 (a; control) or wild-type p53 (b), and in cells transfected with mutant p53 plus UDCA (c) or wild-type p53 plus UDCA (d). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation (arrows). Scale bar: 10 µm. Percent of apoptosis in control hepatocytes and those overexpressing wild-type p53, ± UDCA (bottom). (C) DEVD-specific caspase activity (top) and representative immunoblot of active caspase-3 (bottom) in cytosolic protein extracts after overexpression of p53, ± UDCA. The results are expressed as mean ± SEM of at least 3 different experiments. *$p < 0.01$ from control; †$p < 0.05$ and ‡$p < 0.01$ from wild-type p53 alone.

Therefore, primary rat hepatocytes were cotransfected with a reporter gene construct containing the *bax* gene promoter to drive transcription of CAT in combination with wild-type or mutant p53 overexpression plasmids. At 48 h after transfection, cell death was associated with a strong transactivation of the *bax* gene promoter by > 5-fold compared to cells overexpressing mutant p53 ($p < 0.01$) (Fig. 2A).

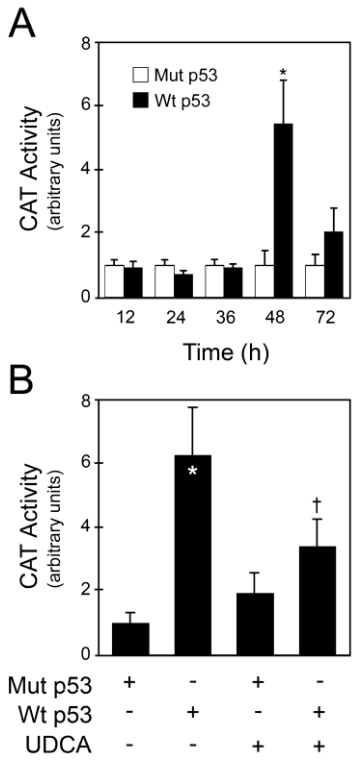

Fig. 2. UDCA prevents p53 transcriptional activation in primary rat hepatocytes. Cells were cotransfected with a *bax* promoter-driven CAT construct and a luciferase control plasmid in combination with either mutant p53 (pCMV-$p53_{179}$) or wild-type p53 (pCMV-$p53_{wt}$) overexpression plasmids. Vehicle or 100 µM UDCA were included in the culture medium at the time of transfections. At the indicated time points after transfection, cells were harvested for the CAT ELISA and luciferase assays as described in "Experimental Procedures". (A) *bax* promoter activity in cells overexpressing either mutant or wild-type p53 for the indicated times. (B) *bax* promoter activity in cells overexpressing either mutant or wild-type p53, ± UDCA for 48h. CAT activity (absorbance/mg) was normalized to control luciferase expression, and the results are expressed as mean ± SEM arbitrary units for at least 5 different experiments. *$p < 0.01$ from control; †$p < 0.05$ from wild-type p53 alone. Mut, mutant; Wt, wild-type.

Notably, when hepatocytes were incubated with UDCA, p53-induced *bax* transcription was reduced by ~ 50% ($p < 0.05$) (Fig. 2B). Taurine- and glycine-conjugated derivatives of UDCA were similarly protective. In contrast, treatment with hydrophobic bile acids, such as deoxycholic, lithocholic and chenodeoxycholic acids, resulted in increased *bax* transcriptional activation up to 25-fold (data not shown). This may explain the toxicity and increased levels of apoptosis seen in hepatocytes incubated with these bile acids. Thus, UDCA is a specific, strong repressor of p53-driven *bax* transcription.

UDCA prevents p53-induced Bax expression and translocation

It has been demonstrated that UDCA plays an important role in preventing apoptosis through the mitochondrial pathway (Botla *et al*. 1995, Rodrigues *et al*. 1999). To further characterize the mechanism by which UDCA modulates p53-mediated apoptosis, we analyzed the expression and subcellular distribution of its downstream target, Bax. Total protein extracts were prepared from primary rat hepatocytes overexpressing either wild-type or mutant p53 in the presence or absence of the bile acid. In addition, at 60 h post-transfection, hepatocytes overexpressing wild-type p53 showed an almost 50% increase in Bax protein compared with control cells expressing mutant p53 ($p < 0.05$) (Fig. 3A). Notably, the levels of total p53 remained unaltered. In contrast, pretreatment with UDCA completely abolished p53-driven Bax expression ($p < 0.01$). As expected, cells transfected with wild-type p53 showed significantly increased Bax translocation from the cytosol to mitochondria, which in turn was significantly prevented by UDCA ($p < 0.05$) (Fig. 3B). The release of cytochrome *c* into the cytosol was consistent with the observed changes in Bax. Thus, these results suggest that UDCA prevents hepatocyte apoptosis associated with wild-type p53

overexpression by abrogating p53 transcriptional activity. This then leads to reduced total levels of Bax in the cell, and decreased Bax translocation to the mitochondria.

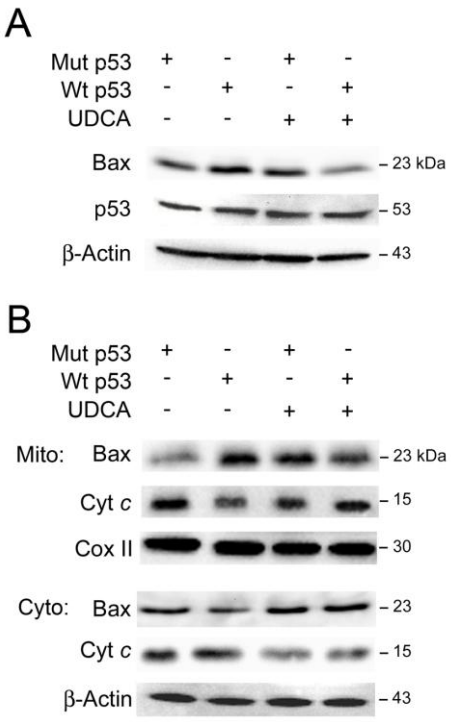

Fig. 3. UDCA modulates p53-induced Bax expression, mitochondrial translocation and cytochrome *c* release. Cells were transfected with either mutant p53 (pCMV-$p53_{179}$) or wild-type p53 (pCMV-$p53_{wt}$) overexpression plasmids. Vehicle or 100 μM UDCA were included in the culture medium at 12 h prior to transfection. Total, mitochondrial and cytosolic protein fractions were processed for immunoblot analysis as described in "Experimental Procedures". (A) Representative immunoblots of total Bax and p53, 60 h after transfection. Blots were normalized to endogenous β-actin. (B) Representative immunoblots of Bax and cytochrome *c* in the mitochondria and cytosol, 36 h after transfection. Blots were normalized to cytochrome *c* oxidase II (CoxII) or β-actin.

UDCA inhibits DNA-binding activity of p53

Activation of p53 involves stabilization of the protein and enhancement of its DNA-binding activity (Haupt *et al.* 2003). When stabilized, p53 accumulates in

the nucleus and regulates the expression of numerous proapoptotic genes by binding to their promoter sequences (Yu & Zhang 2005). To further investigate the mechanism by which UDCA inhibits p53 transactivation, we evaluated DNA-binding activity of p53 in hepatocytes with or without UDCA. Nuclear extracts were prepared 48 h after transfection with p53 overexpression plasmids, and DNA-bound p53 was assayed in the nuclear lysates. As shown in Figure 4, levels of DNA-bound p53 were 6-fold increased in cells overexpressing wild-type p53 relative to the mutant form ($p < 0.01$). Notably, when cells were pretreated with UDCA, there was a reduction of ~ 50% in DNA binding activity of p53 ($p < 0.05$). The presence of the bile acid in cells with mutant p53 did not alter the transcription factor binding activity. This suggests that UDCA-mediated decrease in p53-DNA binding may result from either a direct interference or indirect inhibition of p53 stabilization and/or accumulation in the nucleus. To address this question, we prepared nuclear protein extracts from both wild-type and mutant transfected cells, incubated the extracts with UDCA, and analyzed p53-DNA binding activity. Curiously, the results showed that the bile acid was unable to prevent p53-DNA binding by directly interfering with nuclear proteins and/or DNA (Fig. 4). In addition, preliminary circular dichroism results suggest that UDCA does not interact directly with the p53 core DNA-binding domain (data not shown). However, it is possible that UDCA requires cooperation with other cellular factors to reduce nuclear p53 stability, thus compromising its transcriptional activity.

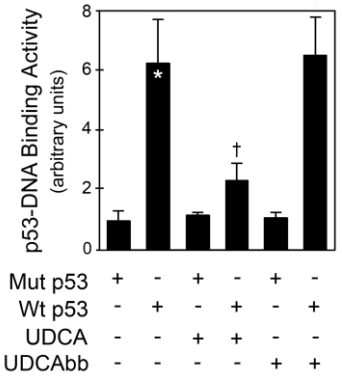

Fig. 4. UDCA inhibits p53-DNA binding activity. Cells were transfected with either mutant p53 (pCMV-$p53_{179}$) or wild-type p53 (pCMV-$p53_{wt}$) overexpression plasmids. Vehicle or 100 μM UDCA were included in the culture medium at 12 h prior to transfection. At 36 h after transfection, nuclear protein extracts were prepared as described in "Experimental Procedures". The level of p53 present in nuclear lysates that can bind to its DNA consensus recognition sequence was determined by the TransAM™ p53 ELISA and expressed as fold change relative to the control. In parallel experiments, nuclear protein extracts were prepared from both wild-type and mutant transfected cells, incubated with UDCA, and analyzed p53-DNA binding activity. The results are expressed as mean ± SEM of at least 3 different experiments. *$p < 0.01$ from control; †$p < 0.05$ from wild-type p53 alone. UDCAbb, UDCA added in the binding buffer.

UDCA represses p53 transcriptional activity via increased binding to Mdm-2

The p53 inhibitor Mdm-2 is a key regulator of p53 abundance and activity. It inhibits the transcriptional activity of p53 and, more importantly, promotes its shuttling to the cytoplasm and subsequent degradation by the proteasome (Haupt *et al.* 1997, Kubbutat *et al.* 1997). Moreover, we have previously reported that UDCA prevented the decrease of Mdm-2 protein levels associated with TGF-β1-induced cell death (Solá *et al*. 2003b). To investigate whether Mdm-2 was an important regulatory factor in the antiapoptotic function of UDCA, we performed

posttranscriptional *mdm-2* gene silencing experiments. As a control for the specificity of siRNA, cells were transfected with a non-specific pool of siRNAs. Western blot analysis confirmed that Mdm-2 expression decreased by ~ 70% after transfection with specific Mdm-2 siRNAs (Fig. 5A).

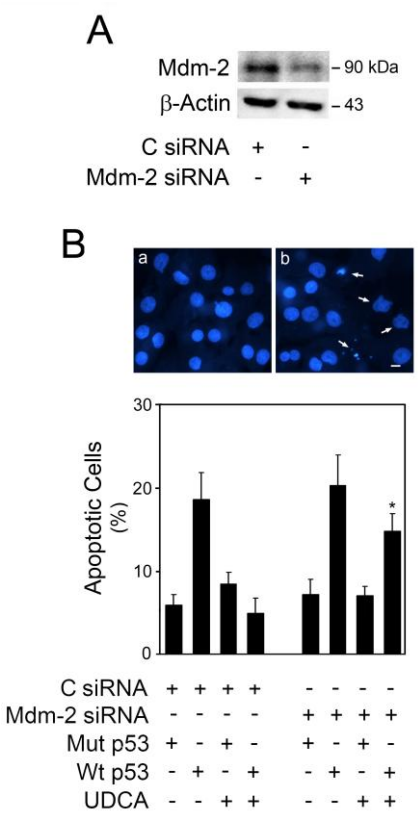

Fig. 5. The antiapoptotic activity of UDCA is mediated via Mdm-2. Cells were transfected with either mutant p53 (pCMV-$p53_{179}$) or wild-type p53 (pCMV-$p53_{wt}$) overexpression plasmids. Vehicle or 100 µM UDCA was included in the culture medium at 12 h prior to transfection. In addition, cells were transfected for 48 h with either control or *mdm-2* siRNA 4 h after p53 overexpression. Total proteins were extracted and subjected to Western blot analysis of Mdm-2. Cells were fixed and stained for morphological evidence of apoptosis. (A) Representative immunoblot of Mdm-2. β-actin was used to control for lane loading. (B) Fluorescent microscopy of Hoechst staining (top) in cells transfected with control (a) or *mdm-2* (b) siRNA after overexpression of wild-type p53 and treatment with UDCA. Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation (arrows). Scale bar: 10 µm. Percent apoptosis is indicated at bottom. Results are expressed as mean ± SEM for at least 3 different experiments. $*p < 0.01$ from corresponding control siRNA transfected cells.

Notably, Mdm-2 silencing reduced UDCA protection against p53-induced nuclear fragmentation by ~ 60% ($p < 0.01$) (Fig. 5B). Caspase activity was similarly reduced (data not shown). Thus, it appears that Mdm-2 plays a key role during UDCA-mediated modulation of p53-induced apoptosis.

Next, we investigated whether UDCA-mediated p53 inhibition was associated with an increase of p53 repression by Mdm-2. Complex formation of p53 and its inhibitor Mdm-2 was analyzed by immunoprecipitation assays after transfection with both wild-type and mutant p53 plasmids, in the presence or absence of the bile acid (Fig. 6A). Overexpression of wild-type p53 induced a 2-fold increase of p53/Mdm-2 dissociation compared with controls ($p < 0.01$). Notably, pre-incubation of hepatocytes with UDCA completely abolished this increase ($p < 0.05$), indicating that UDCA protection is associated with a marked Mdm-2-dependent reduction of free p53 nuclear levels. In fact, both immunofluorescence and immunoblot analysis revealed marked differences in the subcellular distribution of p53 between cells overexpressing wild-type p53 in the presence or absence of UDCA (Fig. 6B and C). Untreated hepatocytes exhibited a predominant nuclear staining of p53. In contrast, when cells were preincubated with the bile acid, p53 appeared localized primarily in the cytoplasm, confirming that UDCA-induced p53/Mdm-2 complex formation results in increased p53 nuclear export. In both cases, a punctuate pattern of p53 was detected in the cytosol, which may indicate that p53 is also localized to mitochondria. The bile acid appeared diffusely in the cytosol and nucleus of hepatocytes. Moreover, we analyzed the phosphorylation status of two serine residues of the N-terminal domain of p53, Ser15 and Ser20. Both are thought to be involved in p53 apoptotic activity and its dissociation from Mdm-2 (Unger *et al.* 1999, Thompson *et al.* 2004).

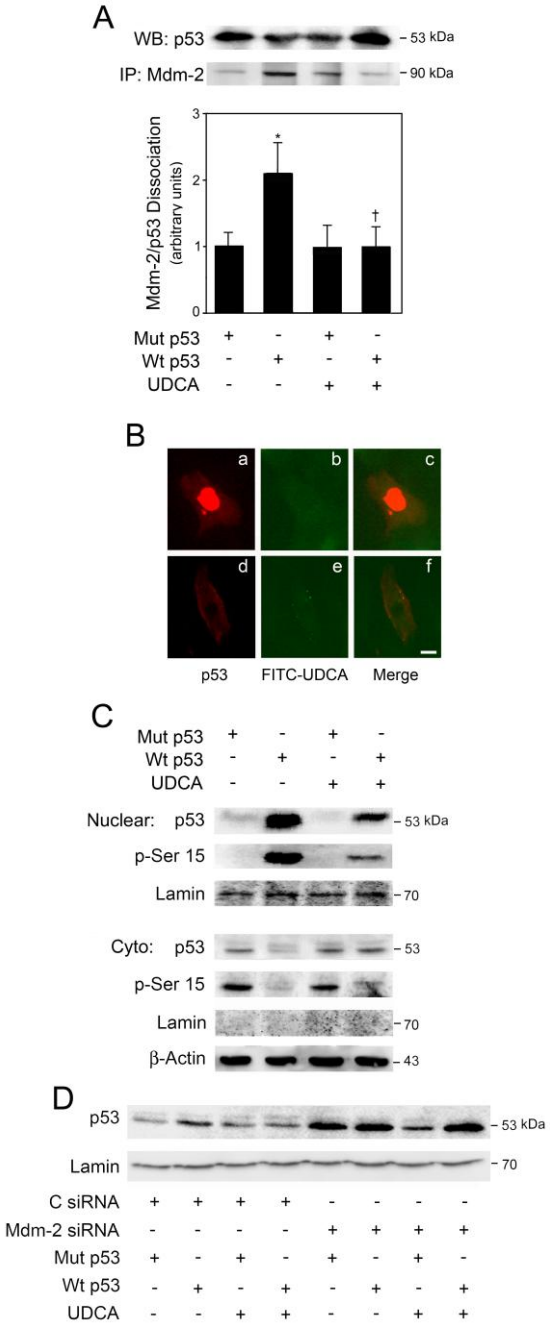

Fig. 6. UDCA induces Mdm-2/p53 association and p53 nuclear export. Cells were transfected with either mutant p53 (pCMV-p53$_{179}$) or wild-type p53 (pCMV-p53$_{wt}$) overexpression plasmids. Vehicle or 100 μM UDCA were included in the culture medium at 12 h prior to transfection. At 36 h after transfection, total proteins were extracted for immunoprecipitation (IP), and nuclear and cytosolic protein fractions for Western blot (WB) analysis. In addition, cells were labeled with a fluorescent UDCA molecule (FITC-UDCA) and fixed for immunofluorescence analysis as described in "Experimental Procedures". (A) Immunoprecipitation analysis of Mdm-2/p53 dissociation. Representative immunoblots with p53- and Mdm-2-specific antibodies (top), and histogram of Mdm-2/p53 dissociation (bottom). All densitometry values for p53 were normalized to respective Mdm-2 expression, and the results are expressed as mean ± SEM arbitrary units for at least 3 different experiments. *p < 0.01 from control;

†$p < 0.05$ from cells overexpressing wild-type p53 alone. (B) Subcellular localization of p53 in FITC-UDCA-labeled primary rat hepatocytes. Fluorescent microscopy of p53 staining in cells transfected with wild-type p53 and treated with either vehicle (a, b, c) or UDCA (d, e, f). Scale bar: 10 µm. (C) p53 translocation from the nucleus to the cytosol and phosphorylation status of Phospho-Ser15-p53 . Representative immunoblots of nuclear and cytosolic p53. Blots were normalized to endogenous lamin or β-actin. (D) Effect of Mdm-2 silencing on nuclear p53. Cells were transfected for 48 h with either control or *mdm-2* siRNA 4 h after the initial transfection with p53 overexpression plasmids. Representative Immunoblot of nuclear p53. Blot was normalized to endogenous lamin.

Expression of p53 phosphorylated Ser20 was low or undetectable (data not shown). However, a significant increase in phosphorylation at Ser15 was detected in hepatocytes overexpressing wild-type p53 (Fig. 6C). This increase was greatly inhibited in hepatocytes pretreated with UDCA, reinforcing the notion that this bile acid affects binding of p53 and Mdm-2. Finally, silencing of Mdm-2 resulted in increased accumulation of p53 in the nucleus, even in the presence of UDCA (Fig. 6D), thus confirming the crucial role of Mdm-2 in the bile acid protective effect.

Taken together, these results strongly suggest that UDCA inhibits p53 transcriptional activation in part by inducing Mdm-2/p53 association, which in turn results in increased p53 degradation.

Discussion

The precise molecular mechanisms by which UDCA modulates cell survival and apoptosis are still a matter of debate. We have previously reported that UDCA is a pleiotropic agent that prevents apoptosis in primary rat hepatocytes by inhibiting the mitochondrial pathway (Rodrigues *et al.* 1998, Rodrigues *et al*. 1999), and

interfering with the E2F1/Mdm-2/p53 apoptotic cascade (Solá *et al*. 2003b). The results presented here suggest that UDCA modulates p53-induced cell death by altering p53 transactivation and DNA-binding activity, and preventing its accumulation in the nucleus.

The tumor suppressor p53 is a transcription factor that coordinates a complex network of cellular proteins. In response to diverse stress factors, p53 is activated and induces the expression of different subsets of genes leading to cell cycle arrest, DNA repair, senescence, or apoptosis. However, the mechanism(s) by which p53 mediates the apoptotic process is still a matter of intense study.

Numerous studies have recently described the importance of p53 transcriptional regulation in both the intrinsic and extrinsic pathways of apoptosis (Balint & Vousden 2001). In fact, several target gene products have pivotal roles in p53-dependent apoptosis. Proapoptotic Bax is one example of a direct and evolutionary conserved transcriptional target of p53 (Miyashita & Reed 1995, Thornborrow *et al.* 2002). During apoptosis, cytosolic Bax is translocated to the outer mitochondrial membrane where it induces cytochrome *c* release (Jurgensmeier *et al.* 1998, Pastorino *et al.* 1998). Once in the cytoplasm, cytochrome *c* functions as a cofactor with apoptosis protease-activating factor 1 (Apaf-1) to promote the cleavage of procaspase-9, initiating apoptosis (Green 2000). Recently, it has been demonstrated that following hypoxia or DNA damage, a small fraction of p53 translocates to mitochondria, where it interacts with Bak, Bcl-X_L, or Bcl-2 and antagonizes their antiapoptotic function (Leu *et al.* 2004, Mihara *et al.* 2003). Cytosolic p53 was also shown to induce apoptosis through direct activation of Bax (Chipuk *et al.* 2004). Nevertheless, most data suggest that the activities of p53 are due to its sequence-specific transcriptional activation.

Our data show that UDCA inhibits p53-induced apoptosis of primary rat hepatocytes. In fact, wild-type p53 overexpression resulted in high levels of apoptosis, which were associated with strong transactivation of the *bax* gene promoter, Bax mitochondrial translocation, cytochrome *c* release and caspase-3 activation. Notably, preincubation with UDCA abrogated all apoptotic changes. We have previously shown that UDCA prevented the increase of Bax protein levels induced by p53 (Solá *et al*. 2003b). Here we demonstrate that UDCA preincubation leads to a significant decrease of p53-driven *bax* promoter activation, thus via modulation of p53 transcriptional activity. Moreover, the presence of UDCA in hepatocytes overexpressing wild-type p53 resulted in down-regulation of Bax but not in a reduction of p53 total protein levels, reinforcing the notion that the transcription activity of p53 is impaired in cells pretreated with UDCA.

It has been reported that bile acids are detectable within the nuclei of rat hepatocytes (Setchell *et al.* 1997, Monte *et al.* 2002) where they may play an important role in controlling gene expression. More recently, nuclear translocation of UDCA mediated by nuclear steroid receptors was shown to be essential for its antiapoptotic properties (Solá *et al.* 2005). Once in the nucleus, UDCA might regulate gene expression by interacting directly with chromatin. Interestingly, ^{32}P-labeling analysis of DNA-reactive bile acids provided evidence of DNA-adduct formation (Hamada *et al.* 1994). Conversely, other authors have reported that bile acids do not bind covalently to DNA (Scates *et al.* 1995). It is also possible that UDCA interacts with nuclear proteins, such as transcription factors, thereby indirectly modulating the expression of several genes. In this regard, it has recently been shown that UDCA suppresses DCA-induced DNA binding activity of activator protein-1 (AP-1) in a human colon cancer cell line (Im & Martinez

2004). In this report we show that UDCA reduces the ability of p53 to bind DNA, through at least two possible mechanisms. First, UDCA may interact directly with p53 preventing an effective p53-DNA binding; or second, UDCA induces p53 destabilization in the nucleus, which may involve second messenger signals. While direct interaction between p53 and the UDCA molecule remains to be proven, UDCA appears to inhibit the ability of p53 to bind DNA through indirect mechanism(s). Moreover, UDCA was previously shown to prevent TGF-β1-induced p53 stabilization and Mdm-2 degradation. Therefore, it seems more plausible that UDCA is affecting p53 nuclear stabilization possibly via its repressor Mdm-2.

Mdm-2 regulates p53 through negative feedback loops that include inhibition of the transcriptional activity of p53, and its degradation via the ubiquitin-proteasome pathway. Mdm-2 also plays a role in regulating the subcellular localization of the tumor suppressor protein. The ubiquitin ligase activity of Mdm-2 contributes to the efficient nuclear export of p53 (Boyd *et al.* 2000, Geyer *et al.* 2000), which depends on the nuclear export sequence identified in the C-terminus of p53 (Stommel *et al.* 1999). Here we report that UDCA increases the association between p53 and its repressor Mdm-2. In addition, UDCA induces p53 nuclear export to cytoplasm, which is strongly correlated with Mdm-2 levels. All together, our results clearly show that inhibition of p53 by its direct repressor Mdm-2 is an essential step of UDCA regulation. By inducing Mdm-2/p53 complex formation, UDCA reduces p53 activity by simultaneously blocking its transactivation domain and enhancing its export to cytosol. However, the precise mechanism by which UDCA induces Mdm-2/p53 binding remains to be determined.

The binding of p53-derived peptides to Mdm-2 results in extensive conformational changes along the N-terminal domain of the repressor (Schon *et al.* 2002). Furthermore, various peptidic and nonpeptidic agents have been reported to bind to the Mdm-2/p53 transactivation domain-binding cleft (Vassilev *et al.* 2004, Grasberger *et al.* 2005). Although the majority of these molecular agents are inhibitors of Mdm-2/p53 interaction, it is plausible that other ligands favor this interaction. In fact, molecular dynamics simulation studies revealed that the p53-binding cleft of Mdm-2 is highly flexible and adaptable to differential binding of ligands that could potentially induce global conformational changes and biological function (Espinoza-Fonseca & Trujillo-Ferrara 2006). UDCA was already shown to interact with several proteins, namely the glucocorticoid receptor, GR (Solá *et al*. 2005, Tanaka & Makino 1992), or the p65 subunit of NF-κB (Miura *et al.* 2001). Interestingly, we have recently shown that GR is required for UDCA anti-apoptotic function, by facilitating its translocation into the nucleus (Solá *et al.* 2004). Furthermore, GR and p53 were found to repress each other's function either at the transcriptional level or through GR-mediated cytoplasmic anchoring of p53 (Sengupta & Wasylyk 2004). In fact, GR itself could be cofactor in the modulation of p53/Mdm-2 binding by UDCA. Other nuclear steroid receptors are also regulated by UDCA. This bile acid is a relatively strong agonist of the pregnane X receptor (PXR) and a weaker agonist of the farnesoid X receptor (FXR). Although UDCA does not itself bind FXR, it does inhibit receptor activation by more hydrophobic bile acid species, such as chenodeoxycholic, deoxycholic and lithocholic acids, which may also contribute to its protective effects (Amaral *et al.* 2007). Finally, the taurine-conjugated derivative of UDCA has been described as a chemical chaperone that reduces endoplasmic reticulum stress and restores glucose homeostasis in a mouse model of type 2 diabetes

(Ozcan *et al.* 2006). Therefore, it is possible that UDCA interacts with the Mdm-2/p53 transactivation domain-binding cleft, thus stabilizing the complex.

Alternatively, UDCA may stimulate Mdm-2/p53 binding via the phosphatidylinositide 3'-OH kinase (PI3K) survival pathway. PI3K in turn activates the serine/threonine kinase Akt, which then phosphorylates a range of targets that function to promote cell survival, including Mdm-2 (Mayo & Donner 2001). Phosphorylation of Mdm-2 by Akt results in activation and nuclear accumulation of the Mdm-2 protein and consequent destabilization of p53 which may serve to protect cells from p53-induced apoptosis (Ogawara *et al.* 2002). Interestingly, UDCA has previously been suggested to protect from apoptosis in several cell types by stimulating PI3K/Akt-dependent survival signaling (Solá *et al.* 2003a, Im *et al.* 2005, Rajesh *et al.* 2005).

In conclusion, our studies show that Mdm-2/p53/Bax apoptotic pathway is a prime target of UDCA modulation. Further, bile acid inhibition of p53-induced apoptosis involves Mdm-2-dependent shuttling of p53 to the cytoplasm and decreased p53 transcriptional and DNA-binding activities. It is of paramount importance to investigate the precise mechanism by which UDCA interacts with p53 and/or Mdm-2 in an effort to develop novel effective therapeutics for apoptosis-related liver diseases.

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4

Ursodeoxycholic acid modulates the ubiquitin-proteasome degradation pathway of p53

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Abstract

The tumor suppressor p53 is a transcription factor that regulates the expression of genes involved in cell cycle arrest, senescence, and apoptosis. It is a labile protein, tightly regulated by the Mdm-2-ubiquitin-proteasome degradation pathway. In previous studies, we demonstrated that p53/Mdm-2 interaction is a prime target of ursodeoxycholic acid (UDCA) for regulating apoptosis in primary rat hepatocytes. Here, we further explore the role of UDCA in downregulating p53 by Mdm-2. The results showed that UDCA further increases cellular proteasomal activity triggered by wild-type p53 overexpression. In addition, p53 half-life was decreased following exposure of HeLa cells to UDCA. More importantly, immunoprecipitation assays revealed that UDCA promotes p53 ubiquitination leading to increased p53 proteasomal degradation. In this regard, proteasome inhibition after UDCA pre-treatment resulted in accumulation of ubiquitinated p53. The involvement of Mdm-2 in UDCA-mediated response was confirmed by siRNA-mediated gene silencing experiments. Finally, the protective effect of UDCA against p53-induced apoptosis was abolished after inhibition of proteasome activity. In conclusion, these findings suggest that UDCA protects cells from p53-induced apoptosis by promoting p53 degradation via the Mdm-2-ubiquitin-proteasome pathway.

Introduction

Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid that has been widely used as a therapeutic drug for patients with cholestatic liver diseases. Recently, it became evident that this bile acid is capable of exerting different effects at the cellular, subcellular, and molecular levels. In fact, we and others have demonstrated that UDCA is cytoprotective in hepatic and nonhepatic cells by regulating the immune response, activating survival transduction signals, and modulating classical mitochondrial pathways of apoptosis (Rodrigues *et al.* 1998, Rodrigues *et al.* 1999, Bellentani 2005, Lindor 2007). UDCA mitigates the apoptotic process by inhibiting mitochondrial membrane depolarization and pore formation, cytochrome *c* release, caspase activation and poly(ADP)-ribose polymerase cleavage (Rodrigues *et al*. 1999). Further, inhibition of apoptosis by UDCA may involve alternate and upstream molecular targets, such as E2F-1 and p53 transcription factors (Solá *et al.* 2003b, Solá *et al.* 2004, Amaral *et al.* 2007a).

The p53 protein is a transcription factor that is activated in response to a wide variety of cellular insults, such as DNA damage, hypoxia or oxidative stress. Once activated, p53 modulates the expression of genes involved in cell cycle arrest and repair, senescence or apoptosis (Vogelstein *et al.* 2000, Riley *et al.* 2008). A tight regulation of p53 function is critical for normal cell growth and development. In fact, p53 is a rapid turnover protein that is maintained at low, often undetectable, levels under stress-free conditions. p53 is regulated mainly by its rate of degradation which, in turn, is mediated by the Mdm-2-ubiquitin-proteasome pathway of degradation (Ciechanover *et al.* 1991, Haupt *et al.* 1997, Brooks & Gu 2006).

The ubiquitin-proteasome pathway (UPP) is the major extra lysosomal mechanism for control of protein degradation. The proteasome is abundant in cytosol and nucleus, and it is considered central to many different aspects of cellular physiology by removing aberrant and misfolded proteins (Berkers *et al.* 2005). The majority of proteins that are destined for degradation by UPP are marked by the covalent attachment of multiple ubiquitin molecules, thus providing a recognition signal for the 26S proteasome. Protein ubiquitination is, in turn, achieved via the sequential activity of three different sets of enzymes; E1, E2 and E3, which shuttle ubiquitin, and eventually link it to specific protein substrates (Myung *et al.* 2001).

Regulation of p53 function is one of the most complex processes within the cell, still far from being fully understood. One of p53 major regulators, the Mdm-2 protein, is the product of a p53-inducible gene, thus establishing a negative feedback loop in which p53 increases expression of its own inhibitor. Importantly, Mdm-2 has also been defined as an ubiquitin E3 ligase, playing a key role in ubiquitin-mediated pathways where it serves as the specific recognition factor. Mdm-2 binds to and ubiquitinates the N-terminus of p53, targeting it for proteasomal degradation (Fang *et al.* 2000, Michael & Oren 2003).

We have recently shown that UDCA prevented p53-induced apoptosis by decreasing p53 transcriptional and DNA-binding activities in primary rat hepatocytes, which appears to be related to increased association between p53 and its direct repressor, Mdm-2. In addition, posttranscriptional silencing of Mdm-2 partially reduced the cytoprotective effect of UDCA against p53 triggered apoptosis, thereby indicating a key role of the repressor in UDCA-mediated response (Amaral *et al.* 2007a). In the present study, we further investigated the role of UDCA in modulating the p53 apoptotic pathway, specifically at the level of

p53 protein stability and proteasome-mediated degradation. Our results demonstrate that exposure of cells to UDCA decreased the half-life of p53, while increasing proteasome activity. More importantly, when we chemically inhibited proteasome activity, pre-treatment with UDCA resulted in increased accumulation of ubiquitinated p53, and absence of protection against p53-induced apoptosis. These findings suggest that UDCA inhibits the p53 apoptotic pathway by promoting UPP degradation in the complex network of p53 regulation.

Experimental Procedures

Cell Cultures

Rat primary hepatocytes were isolated from male Sprague-Dawley rats (100–150 g) by collagenase perfusion as described previously (Mariash *et al.* 1986). In brief, rats were anesthetized with phenobarbitol and the livers perfused with 0.05% collagenase. Hepatocyte suspensions were obtained by passing collagenase digested livers though 125 μm gauze and washing cells in William's E medium supplemented with 26 mM sodium bicarbonate, 0.01 units/ml insulin, 2 mM L-glutamine, 10 nM dexamethasone, 100 units/ml penicillin, 100 units/ml streptomycin (Sigma-Aldrich Corp., St. Louis, MO), and 20% heat-inactivated fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA). Cell viability was determined by trypan blue exclusion and was typically 80–85%. After isolation, hepatocytes were resuspended in William's E medium and plated on Primaria™ tissue culture dishes (BD Biosciences, San Jose, CA) at either 5 x 10^4 cells/cm^2 for morphologic evaluation of apoptosis, or 2.5 x 10^5 cells/cm^2 for all other experiments. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 4 h to allow attachment. Plates were then washed with medium to remove

dead cells, and incubated in William's E medium containing 10% heat-inactivated FBS.

HeLa human cervical carcinoma cell line was cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin and 2 mM glutamine, and maintained at 37ºC in an humidified atmosphere of 5% CO_2. Cells were plated on tissue culture dishes at 2.5 x 10^5 cells/ml.

Plasmids, transfections and *in vitro* treatments

Transient transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were treated with vehicle or 100 µM of UDCA, 12 h prior to transfection with DNA encoding wild-type p53 (pCMV-$p53_{wt}$) (Unger *et al.* 1992), or p53 lacking the ubiquitination site at amino acids 13-19 (p53 ΔI) (Marston *et al.* 1994), alone or in combination with HA-tagged ubiquitin plasmid (HA-Ub) (Treier *et al.* 1994). In proteasome activity experiments and morphologic evaluation of apoptosis, hepatocytes were transfected with 4 µg of pCMV-$p53_{wt}$. Immunoprecipitation assays were performed in hepatocytes overexpressing 2 µg of either pCMV-$p53_{wt}$ or p53 ΔI along with 6 µg of HA-Ub. UDCA was removed from culture medium at the time of transfection, and then re-added 4 to 6 h later. For normalization, cells were cotransfected with the luciferase reporter construct, PGL3-Control vector (Promega Corp., Madison, WI). Where indicated, the proteasome inhibitor MG132 (Calbiochem, Darmstadt, Germany) was added to a final concentration of 10 µM for 12 h. At 48 h post-transfection, cells were harvested for luciferase assays (Promega), protein extraction and immunoblot analysis. Attached cells were fixed for morphologic evaluation of apoptosis.

Proteasome activity assay

Proteasome activity was determined in primary rat hepatocytes transfected with either wild-type p53 or empty plasmid for 48 h, in the presence or absence of UDCA. In brief, cells were washed twice with cold phosphate-buffered saline (PBS), pH 7.4, pelleted and lysed in homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP, and 250 mM sucrose. Membrane fractions, nuclei and cell debris were removed from supernatant by centrifugation at 16,000*g* for 5 min, 4ºC. The proteolytic reaction was carried out with 10 μg of cell lysate diluted in substrate buffer containing 20 mM Hepes, pH 8.2, 0.5 mM EDTA, 1% DMSO, 1 mM ATP, and 60 μM of the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-MCA (Calbiochem), which belongs to the class of chymotrypsin substrate III (adapted from Berkers *et al*. 2005). Fluorescence was measured at 380 nm excitation and 460 nm emissions, at 37ºC, by using a fluorescent microplate reader (TECAN Infinite M200; Tecan Trading AG, Switzerland).

Protein decay rate analysis

HeLa cells were plated onto 35 mm Petri dishes at 2.5 x 10^5 cells/ml. Eight hours after plating, cells were treated with vehicle or 100 μM of UDCA for additional 12 h. Cells were chased with cycloheximide (Sigma-Aldrich) at a final concentration of 100 μg/ml. At the indicated time points after the treatment with cycloheximide, cells were harvested, and whole-cell lysates were processed for immunoblot analysis with the anti-p53 DO-1 antibody. β-actin was used as protein loading control.

Short interference-mediated silencing of the *mdm-2* gene

A pool of 4 short interference RNA (siRNA) nucleotides designed to knock down *mdm-2* gene expression in humans was purchased from Dharmacon (Waltham, MA). A control siRNA containing a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA was used as control. Wild-type p53 and HA-Ub overexpression, as well as *mdm-2* gene silencing were performed simultaneously by cotransfecting HeLa cells with Lipofectamine 2000 (Invitrogen) for 48 h, according to the manufacturer's protocol. Total DNA (4 μg) in each transfection was equalized by the addition of pCMV-empty vector. The final concentration of siRNAs was 100 nM. UDCA was removed from culture medium at the time of transfection, and then re-added 4 to 6 h later. Where indicated, the proteasome inhibitor MG132 (Calbiochem) was added to a final concentration of 10 μM for 12 h. Floating and attached cells were harvested for preparation of total protein extracts, which were then subjected to immunoblot analysis.

Measurement of cell death and caspase activity

Hoechst labeling of cells was used to detect apoptotic nuclei. Briefly, the medium was gently removed to prevent detachment of cells. Attached hepatocytes were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma-Aldrich) at 5 μg/ml in PBS for 5 min, washed with PBS and mounted using PBS:glycerol (3:1, v/v). Fluorescent nuclei were scored blindly and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Five random microscopic fields per sample of ~ 100

nuclei were counted and mean values expressed as the percentage of apoptotic nuclei. In addition, caspase activity was determined in cytosolic protein extracts after harvesting and homogenization of cells in isolation buffer containing 10 mM Tris-HCl buffer, pH 7.6, 5 mM $MgCl_2$, 1.5 mM KAc, 2 mM dithiothreitol, and protease inhibitor mixture tablets (Complete™; Roche Applied Science, Mannheim, Germany). General caspase-3-like activity was determined by enzymatic cleavage of chromophore *p*-nitroanilide (pNA) from the substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; Sigma-Aldrich). The proteolytic reaction was carried out in isolation buffer containing 50 μg cytosolic protein and 50 μM DEVD-pNA. The reaction mixtures were incubated at 37°C for 1 h, and the formation of p-NA was measured at 405 nm using a 96-well plate reader.

Immunoblotting

For p53 detection, 40 to 80 μg of total proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following electrophoretic transfer onto nitrocellulose membranes, the immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. After blocking with a 5% milk solution, the membranes were incubated overnight at 4ºC with primary mouse monoclonal antibodies reactive to p53 (DO-1) and Mdm-2 (SMP 14) (Santa Cruz Biotechnology, Santa Cruz, CA), or primary rabbit polyclonal antibody reactive to HA (Zymed, Invitrogen), and finally with anti-mouse or anti-rabbit IgG antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) for 3 h at room temperature. The membranes were processed for protein detection using the SuperSignal substrate (Pierce Biotechnology, Rockford, IL). β-actin (AC-15, Sigma-Aldrich) was used as loading control. Protein concentrations were

determined using the Bio-Rad protein assay kit (Bio-Rad) according to the manufacturer's specifications.

Immunoprecipitation

Binding of p53 to ubiquitin was detected by immunoprecipitation analysis. In brief, whole cell extracts were prepared by lysing cells in M-PER Mammalian Protein Extraction Reagent (Pierce). Immunoprecipitation experiments were carried out using a primary rabbit polyclonal antibody to HA (Zymed, Invitrogen) and the Ezview Red Protein G Affinity Gel (Sigma-Aldrich). Typically, 200 μg of lysate were incubated with 5 μg of anti-HA antibody overnight at 4ºC. Immunoblots were then probed with the mouse monoclonal anti-p53 antibody. HA expression was determined in the same membrane after stripping off the immune complex for the detection of p53.

Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the Quantity One Version 4.6 densitometric analysis program (Bio-Rad). Statistical analysis was performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

Results

UDCA increases proteasome activity in hepatocytes overexpressing wild-type p53

p53 function is regulated primarily through control of protein stability and degradation. Specifically, Mdm-2 is a key regulator of p53 by promoting its proteasome-mediated degradation (Lavin & Gueven 2006). Recently we have demonstrated that modulation of p53/Mdm-2 interaction is a prime target of UDCA in modulating apoptosis (Amaral *et al*. 2007a). We have now evaluated whether this effect was associated with increased p53 degradation by the proteasome. Primary rat hepatocytes were transfected with pCMV-$p53_{wt}$ or pCMV-empty expression plasmids, in the presence or absence of UDCA, and cell lysates were processed for proteasomal analysis. Cellular proteasome activity was significantly increased in hepatocytes transfected with pCMV-$p53_{wt}$ and pre-treated with the bile acid compared to untreated cells (Fig. 1; $p < 0.05$). The proteasome inhibitor MG132 dramatically decreased the proteasome activity, and was used as positive control. In addition, UDCA had no effect on the proteasome activity of hepatocytes transfected with the empty vector (data not shown). Thus, our results suggest that UDCA-induced proteasome activation is not a global effect, but rather a specific and directed mechanism to degrade deregulated levels of p53.

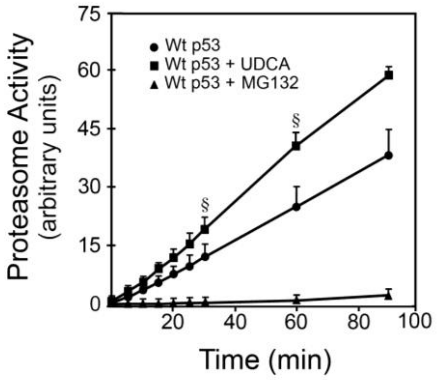

Fig. 1. UDCA stimulates cellular proteasome activity of primary rat hepatocytes overexpressing wild-type p53. Cells were transfected with wild-type p53 (pCMV-$p53_{wt}$) overexpression plasmid. Vehicle or 100 μM UDCA was included in the culture medium 12 h prior to transfection. At 48 h after transfection, cells were collected and processed for proteasome activity as described under "Experimental Procedures". Cells treated with 10 μM of MG132 12 h before preparation of lysates were used as a positive control. The results are expressed as mean ± SEM of at least 3 different experiments. §$p < 0.05$ from wild-type p53 alone. Wt, wild-type.

p53 half-life is decreased after exposure to UDCA

To determine whether UDCA could modulate p53 turnover, we analyzed the half-life of p53, in the presence or absence of the bile acid, using cycloheximide to block *de novo* protein synthesis. Eight hours after plating, HeLa cells expressing endogenous wild-type p53 were exposed to UDCA for an additional 12 h. At the indicated time points, whole-cell lysates were subjected to immunoblotting against p53. When cells were pre-treated with UDCA, the degradation rate of p53 was faster than that in cells with no treatment (Fig. 2) ($p < 0.05$). In contrast, UDCA had no effect in the half-life of NF-κB, a regulatory protein whose activity is

controlled by the UPP (data not shown). These data are consistent with the UDCA-mediated increase of proteasome activity, further suggesting an involvement of the bile acid in p53 protein stability and degradation.

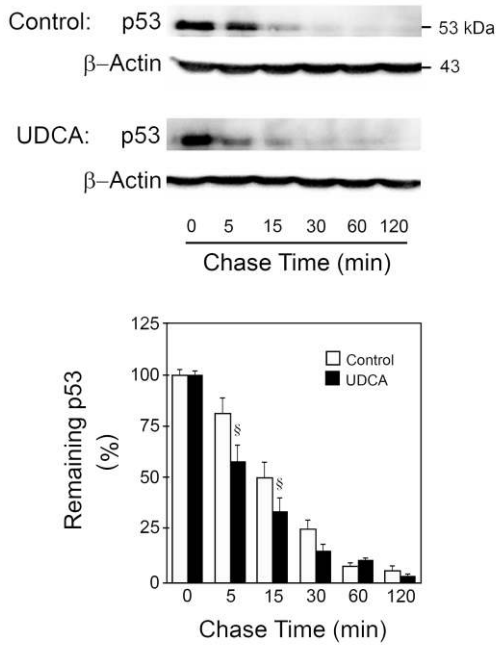

Fig. 2. UDCA decreases the stability of p53. HeLa cells were treated with vehicle or 100 μM UDCA for 12 h, and then incubated with cycloheximide (100 μg/ml). Whole-cell lysates were analyzed for p53 by immunoblotting at the indicated time points. Blots were normalized to endogenous β-actin. The results are expressed as mean ± SEM arbitrary units for at least 5 different experiments. §$p < 0.05$ from control.

UDCA enhances Mdm-2-dependent ubiquitination of p53

Mdm-2 has been defined as an E3 ubiquitin ligase. Besides direct ubiquitination of p53, Mdm-2 also contributes to its nuclear export, which appears to be important for efficient degradation of p53 (Yu *et al.* 2000, Lavin & Gueven 2006). In fact, it was suggested that ubiquitination of p53 by Mdm-2 may serve to both target p53 to proteasome degradation, and export to the cytoplasm (Geyer *et al.* 2000). We have previously reported that UDCA promoted p53/Mdm-2 association, thus leading to

increased p53 nuclear export (Amaral *et al*. 2007a). Therefore, we sought to examine whether pre-treatment of hepatocytes with UDCA affected ubiquitination of p53. Complex formation between p53 and ubiquitin was analyzed by immunoprecipitation after transfection of hepatocytes with wild-type p53 or p53 ΔI, a mutant form of p53 lacking the ubiquitination site, in combination with HA-Ub, with or without UDCA (Fig. 3).

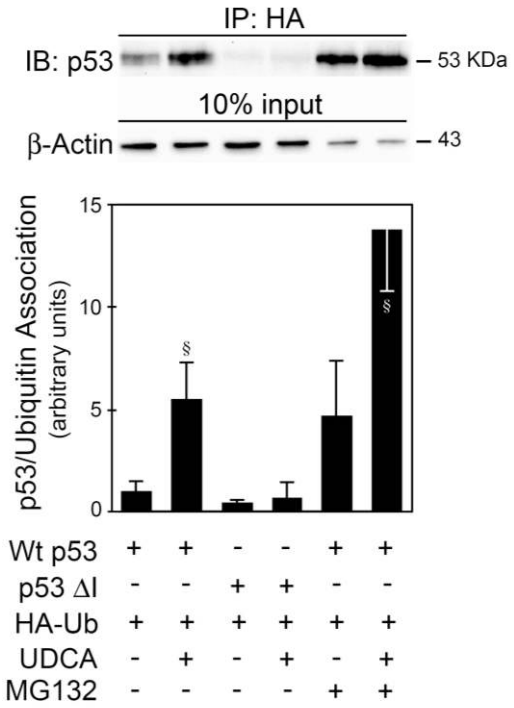

Fig. 3. UDCA induces p53/ubiquitination. Primary rat hepatocytes were cotransfected with either wild-type p53 (pCMV-$p53_{wt}$) or mutant p53 lacking the ubiquitination site (p53 ΔI) in combination with HA-Ub expression plasmid. Vehicle or 100 μM UDCA was included in the culture medium 12 h prior to transfection. When indicated, 10 μM MG132 was added to cell cultures 12 h before harvest. At 48 h post-transfection, cell lysates were analyzed for ubiquitinated p53 by immunoprecipitation using an antibody against HA. Representative immunoblot using p53 DO-1 specific antibody (*top*), and histogram of p53/ubiquitin association (*bottom*). The results are expressed as mean ± SEM arbitrary units for at least 3 different experiments. §$p < 0.05$ from cells overexpressing wild-type p53 alone. IP, immunoprecipitation; IB, Immunoblotting; Wt, wild-type.

Surprisingly, UDCA pre-treatment resulted in a 5-fold increase in ubiquitin-conjugated p53 compared to control cells ($p < 0.05$). When protein degradation was inhibited by MG132, we observed an accumulation of ubiquitinated p53, although the effect of UDCA was still detectable. As expected, p53 ubiquitination in hepatocytes expressing the mutated form of p53 lacking the ubiquitination site was essentially undetectable.

In parallel experiments, we evaluated the effect of UDCA using HeLa cells cotransfected with wild-type p53, in combination with the expression plasmid for HA-tagged ubiquitin. To inhibit degradation of ubiquitinated proteins, cells were treated with the proteasome inhibitor MG132 for 12 h before preparation of whole-cell lysates. High molecular weight p53 conjugates corresponding to proteins modified with endogenous and exogenously expressed ubiquitin were detected in extended exposures of blots (Fig. 4). Notably, p53 conjugates were significantly increased upon pre-treatment with UDCA (Fig. 4, lanes 3 and 4), in agreement with previous immunoprecipitation results in hepatocytes.

We performed *mdm-2* gene silencing experiments to confirm involvement of Mdm-2 in UDCA-induced ubiquitination of p53. Western blot analysis showed that Mdm-2 expression decreased by ~ 70% after transfection with Mdm-2-specific siRNA (Fig. 4, compare lanes 1-4 with lanes 5-8), compared with cells transfected with control-siRNA. Silencing of Mdm-2 decreased ubiquitination of p53 slightly, and more importantly, abolished UDCA function (Fig. 4, lanes 7 and 8), highlighting the importance of this protein in UDCA effects. Taken together, these results further clarify the role of UDCA in the regulation of p53, suggesting that this bile acid enhances Mdm-2-dependent p53 ubiquitination.

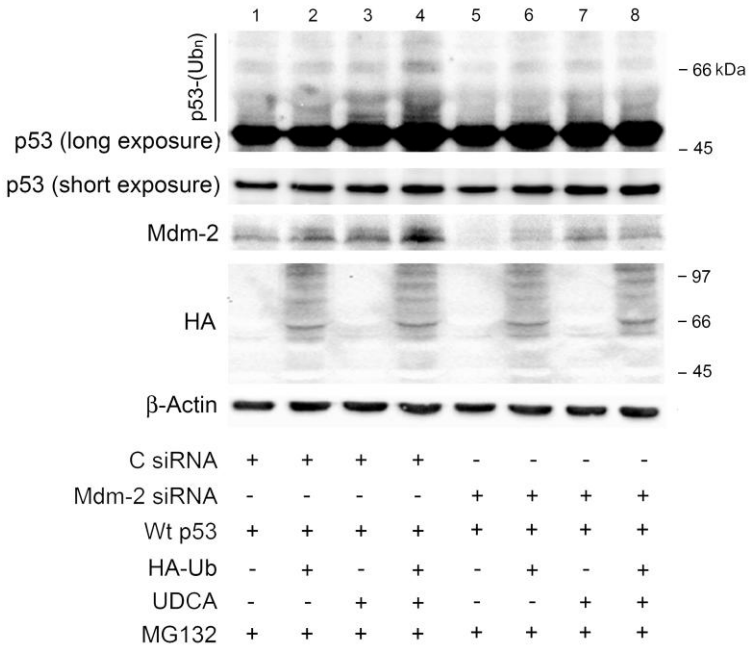

Fig. 4. UDCA enhances ubiquitination of p53 by Mdm-2. HeLa cells were cotransfected with wild-type p53 (pCMV-$p53_{wt}$) in combination with HA-Ub plasmids and simultaneously with either control or *mdm-2* siRNA, followed by exposure to 10 μM MG132. Vehicle or 100 μM UDCA was included in the culture medium at 12 h prior to transfection. At 48 h post-transfection total proteins were extracted and subjected to Western blot analysis of p53, Mdm-2, and HA. β-actin was used to control for lane loading. Similar results were obtained for at least 3 different experiments.

Ubiquitination and proteasomal degradation are essential for UDCA protection against p53-induced apoptosis

Accumulation of p53 leads to induction of apoptosis (Yonish-Rouach *et al.* 1991). We have already shown that UDCA protected hepatocytes from p53-mediated apoptosis (Amaral *et al*. 2007a). However, proteasome inhibition in hepatocytes

overexpressing wild-type p53, abolished UDCA protection, as assessed by changes in nuclear morphology and caspase activity ($p < 0.01$) (Fig. 5A and B).

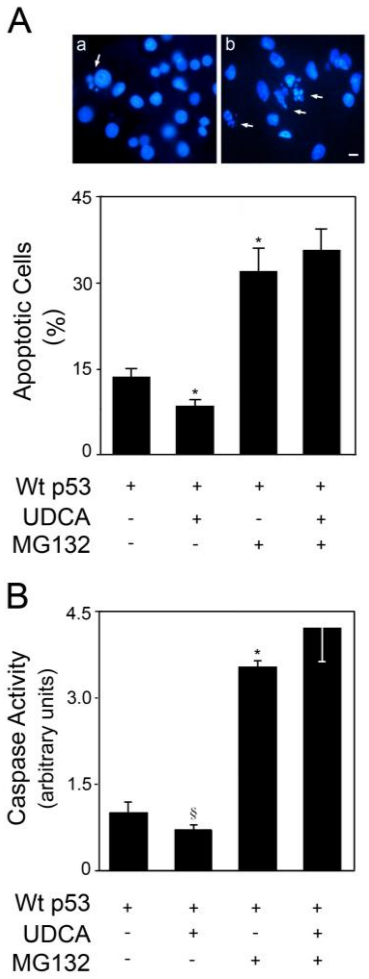

Fig. 5. The antiapoptotic activity of UDCA against p53-induced cell death is reduced by proteasome inhibition. Primary rat hepatocytes were transfected with wild-type p53 (pCMV-$p53_{wt}$) overexpression plasmid. Vehicle or 100 µM UDCA was included in the culture medium at 12 h prior to transfection. When indicated, 10 µM MG132 was added to cell cultures. At 48 h after transfection, cells were fixed and stained for morphological assessment of apoptosis, and cytosolic proteins were extracted for caspase activity as described under "Experimental Procedures". *A*, fluorescent microscopy of Hoechst staining (*top*) in cells transfected with wild-type p53 and treated with UDCA (a) or with both UDCA and MG132 (*b*). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation (*arrows*). Scale bar, 10 µm. Percent nuclear fragmentation in hepatocytes overexpressing wild-type p53 ± UDCA, in the presence or absence of MG132 (*bottom*). *B*, DEVD-specific caspase activity in cytosolic protein extracts after overexpression of wild-type p53 ± UDCA, in the presence or absence of MG132. The results are expressed as mean ± SEM arbitrary units for at least 3 different experiments. **p* < 0.01 and §*p* < 0.05 from cells overexpressing wild-type p53 alone. Wt, wild-type.

Apoptosis was significantly increased in cells exposed to MG132 compared with non-treated cells ($p < 0.01$). Finally, similar results were obtained when we transfected hepatocytes with p53 ΔI plasmid encoding for a mutant form of p53 that does not undergo ubiquitination (data not show). These data strongly suggest that both ubiquitination and proteasomal degradation are essential steps for UDCA modulation of the p53 apoptotic pathway.

Discussion

It is well established that UDCA has an important role in preventing deregulated levels of apoptosis in many different cell types. We have demonstrated that UDCA prevents apoptosis by inhibiting the mitochondrial pathway (Rodrigues *et al.* 1998, Rodrigues *et al*. 1999) and interfering with the E2F-1/p53/Bax apoptotic cascade (Solá *et al*. 2003b, Solá *et al*. 2004). Furthermore, UDCA's antiapoptotic effects involve the activation of nuclear steroid receptors coupled with the inhibition of p53, via Mdm-2 (Amaral *et al*. 2007a, Solá *et al.* 2005, Amaral *et al.* 2007b). Despite the progress made in understanding UDCA mode of action, the precise mechanisms by which the bile acid affects cell survival and apoptosis are still unresolved.

In this report, we further clarify how UDCA intervenes in the complexity of p53 regulation. p53 is one of the most intensively studied proteins, whose levels and activity are largely controlled by Mdm-2 and the UPP. Our results suggest that ubiquitination and functional proteasome degradation are crucial processes for UDCA modulation of p53-induced cell death. The presence of UDCA stimulates Mdm-2-dependent ubiquitination of p53 and increases proteasome activity of cells with augmented levels of this protein. More importantly, if we prevent p53 from

undergoing ubiquitination or inhibit proteasome activity, the protective function of UDCA is abolished.

The proteasome plays a central role in degradation of regulatory, misfolded and damaged proteins (Hershko & Ciechanover 1998). Over the past few years, apoptosis-pathway molecules have been identified as substrates and targets for proteasome degradation, including members of the Bcl-2 family (Chang *et al.* 1998), inhibitor of apoptosis protein family (Yang & Li 2000), inhibitor of κB (Orian *et al.* 1995, Jesenberger & Jentsch 2002), and p53 (Ciechanover *et al*. 1991, Maki *et al*. 1996). Therefore it is not surprising that altered function of this degradation pathway will affect cell survival. In fact, it has long been known that inhibition of proteasome function induces apoptosis, depending on cell type and conditions (Sadoul *et al.* 1996, Drexler 1997, Meriin *et al.* 1998). This characteristic transformed certain proteasome inhibitors into potential antitumor pharmaceuticals. For example, bortezomib has been approved for the treatment of multiple myeloma (Hideshima *et al.* 2001). However, there are also several diseases related to failure of the proteasome in removing abnormal proteins. This is the case of degenerative and conformational diseases such as Alzheimer's, Parkinson's, or Huntington's diseases (Kitada *et al.* 1998, Keller *et al.* 2000). Curiously, this is not the first time bile acids are described as modulators of UPP. Indeed, others have shown that bile acids may suppress p53 and p63 by stimulating their proteasome-mediated degradation (Qiao *et al.* 2001, Roman *et al.* 2007). In addition, the taurine-conjugated derivative of UDCA, TUDCA is neuroprotective in a transgenic animal model of Huntington's disease, by reducing number and size of ubiquitinated neuronal intranuclear huntingtin inclusions, among other effects (Keene *et al.* 2002). How bile acids affect proteasome activity is still unknown. There are no evidences of direct binding between these molecules and proteasome

subunits. Instead, it is plausible that they influence proteasome activity indirectly, by interfering with ubiquitination of UPP substrates. In fact, our results do not show a global activation of proteasome by UDCA, but a specific and directed effect as result of p53 overexpression. Moreover, UDCA and TUDCA regulate transcription of several genes, in particular apoptosis-, cell cycle- and proliferation-related genes (Castro *et al.* 2005). Interestingly, some of these genes are also substrates of UPP, such as cyclin D1 (Feng *et al.* 2007). Proteasomal degradation of cyclin D1 has been highlighted as a cancer chemopreventive mechanism, and UDCA prevented DCA-induced cyclin D1 and cell death (Castro *et al.* 2007). It would be interesting to investigate whether UDCA could affect ubiquitin-dependent proteolysis of cyclin D1.

We have recently demonstrated that UDCA facilitates binding between p53 and Mdm-2, thus promoting p53 nuclear export (Amaral *et al*. 2007a). However, exposure of nuclear export signals by binding of Mdm-2 is insufficient to promote p53 nuclear export in the absence of Mdm-2 ubiquitination (Nie *et al.* 2007). Here we further demonstrate that UDCA also promotes p53 ubiquitination by Mdm-2, which strengthens the role of the bile acid in the degradation pathway of p53. One potential mechanism by which UDCA induces p53 ubiquitination by Mdm-2 is direct binding to either or both proteins. Another is stimulation of the phosphatidylinositide 3'OH kinase/Akt survival pathway. Akt was shown to enhance the ubiquitin-promoting function of Mdm-2 (Ogawara *et al.* 2002), and UDCA stimulates this pathway in several cell types (Solá *et al.* 2003a, Im & Martinez 2004, Rajesh *et al.* 2005).

Finally, although turnover of p53 is regulated primarily by Mdm-2, the complexity underlying regulation of p53 stabilization and activation is enormous and involves other cellular factors (Lavin & Gueven 2006). For instance, Prih2

(Leng *et al.* 2003), COP-1 (Dornan *et al.* 2004), and CHIP (Esser *et al.* 2005) are additional proteins with E3 ubiquitin ligase activity that mediate ubiquitination of p53. Mdm-4 (also known as MdmX) is an Mdm-2 structurally related protein that is also critical to restrain p53 activity (Marine *et al.* 2006). Furthermore, activation of p53 is subjected to a complex and diverse array of posttranslational modifications, such as phosphorylation, acetylation, and methylation (Olsson *et al.* 2007). Interaction with molecular chaperones, including Hsp70 and Hsp90, is other additional factor regulating p53 stability (Muller *et al.* 2008). Complexes between Hsp90 and wild-type p53 have been recognized, and it is currently believed that this chaperone acts as a positive regulator of p53, through inhibition of its ubiquitination and degradation by Mdm-2. Hsp90 and Mdm-2 have opposing effects on p53 conformation. While Mdm-2 binding drives wild-type p53 into a mutant conformation, Hsp90 opposes this effect by maintaining p53 in a wild-type and active conformation (Sasaki *et al.* 2007). Since UDCA promotes dissociation of nuclear steroid receptors and Hsp90 (Solá *et al*. 2005, Solá *et al.* 2006), it could similarly affect p53 by preventing its stabilization by Hsp90.

In conclusion, our results provide additional insight into the molecular mechanisms underlying UDCA function. This hydrophilic bile acid appears to protect cells from p53-induced apoptosis, at least in part, by increasing p53 ubiquitination and degradation by the proteasome. The identification and characterization of upstream events involving apoptosis modulation by bile acids may result in the development of novel therapeutic options for diseases associated with deregulated apoptosis.

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5

Concluding Remarks

The studies presented in this thesis contribute to better understand the molecular mechanisms underlying the antiapoptotic role of ursodeoxycholic acid (UDCA) against p53-induced apoptosis. Briefly, we demonstrated that the bile acid reduces p53 transcriptional and DNA binding activities by interfering with stabilization and degradation of the transcription factor, in part, through mechanisms dependent on Mdm-2 function. These findings into the molecular functioning of UDCA provide new research tools for future studies surrounding the modulation of apoptosis in human diseases. In this final Chapter, we integrate the new insights of our work with the important role of bile acids as signaling molecules that modulate complex protein networks, such as p53 regulation. We further discuss possible controversies from our results regarding the inhibition of a tumor suppressor, and highlight the importance of our findings in the context of therapeutic intervention for apoptosis-related disorders.

UDCA is an endogenous hydrophilic bile acid with widespread clinical use for treatment of chronic liver diseases. The paradox of UDCA as well as many other drugs used worldwide such as glucocorticoids is that the strong evidence of therapeutic efficacy is not supported by in-depth knowledge of mechanisms of action. Nevertheless, during the last five years great progress has been made to ascertain the biochemical and molecular mechanisms underlying UDCA therapeutic effects. It is now well established that inhibition of liver cell apoptosis is one of the main routes for the protective effect of UDCA. Indeed, it has been shown that UDCA acts as a pleiotropic agent playing a unique role in modulating the classic mitochondrial pathway of apoptosis in different cell types (Botla *et al.* 1995, Rodrigues *et al.* 1998). Notably, UDCA was shown to modulate mitochondrial membrane perturbation, pore formation, Bax translocation, cytochrome *c* release, caspase activation and subsequent substrate cleavage (Rodrigues *et al.* 1999). By now, the challenge is to identify the primary molecular targets that mediate UDCA signaling to the mitochondria. Importantly, bile acids

are cholesterol-derived molecules that may function as hormone-like ligands that interact with nuclear receptors, enroute to the nucleus (De Fabiani *et al.* 2004). These findings have opened new avenues for UDCA protection from apoptosis. Once in the nucleus, the bile acid could possibly interfere with the transcription of the genome. In fact, we have demonstrated that nuclear trafficking of UDCA by nuclear steroid receptors, but not nuclear receptor transactivation is critical for the antiapoptotic function of the bile acid in hepatocytes (Solá *et al.* 2005). Moreover, microarray studies revealed that UDCA *per se* modulates gene transcription of primary rat hepatocytes, in particular, cell cycle- and apoptosis-related genes (Castro *et al.* 2005). UDCA was shown to reduce transcriptional activation and expression of cyclin D1 in hepatocytes incubated with deoxycholic acid, which appears to contribute to the antiapoptotic effects of the hydrophilic bile acid (Castro *et al.* 2005, Castro *et al.* 2007). Curiously, the results suggested that p53-dependent signaling was, in part, responsible. Further, UDCA specifically modulated the expression of E2F-1, Mdm-2, p53, Bax, and Bcl-2 proteins, independently of its effect on mitochondria and/or caspases (Solá *et al.* 2003).

Since p53 was repeatedly suggested to be involved in modulation of apoptosis by UDCA, and due to its strong impact in a wide variety of human diseases, we have decided to further explore the regulation of p53 by UDCA. The complexity of the structural organization of p53 makes it a highly flexible transcription factor, with affinity for several specific DNA sequences, and numerous activators and/or repressors (Joerger & Fersht 2008). Given the challenge of studying all the functional domains of p53, we have initiated our studies by evaluating the ability of UDCA to bind directly to the DNA binding domain (DBD) of p53, responsible for sequence-specific DNA binding. As no significant changes were detected in the secondary structure of p53 DBD, or in its anisotropy, we have then dedicated our attention to possible indirect mechanisms of p53 regulation by UDCA.

Our data confirmed that overexpression of wild-type p53 triggers the apoptosis process, which was associated with strong transactivation of the *bax* gene. Notably, pretreatment of primary rat hepatocytes with UDCA resulted in significant decreases of p53-driven *bax* promoter activation, revealing that the bile acid was efficient at modulating p53 transcriptional activity. In addition, the presence of UDCA abrogated all apoptotic changes induced by p53 overexpression, such as Bax mitochondrial translocation, cytochrome *c* release and caspase-3 activation. More importantly, we have also demonstrated that UDCA reduced the DNA binding activity of p53, while promoting its nuclear export. Since Mdm-2 is the main repressor of p53, controlling its subcellular localization and ability to regulate the transcription of target genes (Lavin & Gueven 2006), we evaluated whether UDCA was inhibiting p53 via an Mdm-2-dependent mechanism. Indeed, increased association between p53 and Mdm-2 was detected in hepatocytes preincubated with UDCA. By inducing Mdm-2/p53 complex formation, UDCA reduced p53 activity simultaneously blocking its transactivation domain and enhancing its export to cytosol (Fig. 1). When we performed posttranscriptional silencing of the *mdm-2* gene, the protective effect of UDCA against p53-induced apoptosis was partially reduced and p53 accumulated in the nucleus, thereby confirming the crucial role of Mdm-2 in the antiapoptotic function of UDCA. These novel findings suggested that UDCA may act as a chaperone-like molecule stabilizing the Mdm-2/p53 complex. Curiously, a recent study published in a prestigious scientific journal, described TUDCA as a chemical chaperone of the endoplasmic reticulum (ER) in mice, alleviating ER stress, and thereby acting as a potent antidiabetic agent, with potential application in the treatment of type 2 diabetes (Ozcan *et al.* 2006).

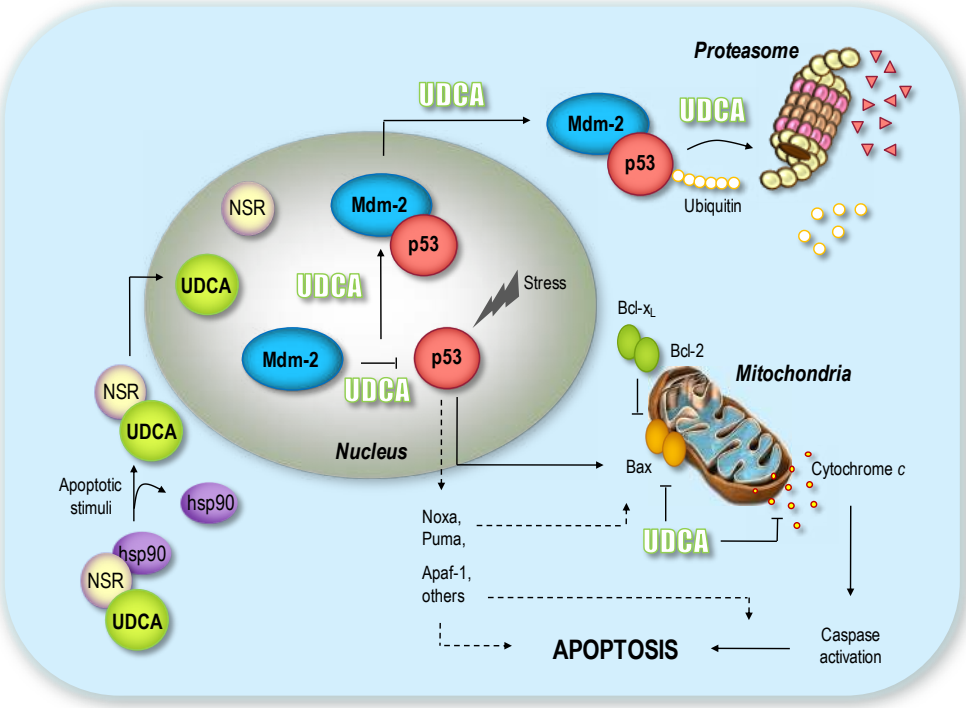

Fig. 1. Proposed model for the antiapoptotic function of UDCA. Activation of p53 may under certain circumstances result in apoptosis by inducing the expression of its proapoptotic target genes, or alternatively by acting directly at the mitochondrial level. Importantly, UDCA reaches hepatocyte nuclei, in part through a NSR-dependent mechanism. Once in the nucleus, UDCA inhibits p53-induced apoptosis by inducing Mdm-2/p53 complex formation. This, results in reduction of p53 transcriptional activity and enhanced export to the cytosol. Further, the bile acid stimulates Mdm-2-dependent ubiquitination of p53, and further increases wild-type p53-triggered proteasome activity, which appears to be essential for the protective function of UDCA. Finally, UDCA may directly interact with mitochondria function inhibiting Bcl-2 family proapoptotic members and apoptosis. Hsp90, heat shock protein 90; NSR, nuclear steroid receptor.

It is generally accepted that p53 function is regulated mainly through control of protein stability. Therefore, in the last part of this thesis our efforts were

directed to clarify the involvement of UDCA in Mdm-2-mediated ubiquitination and degradation of p53. Our results showed that the bile acid stimulated Mdm-2-dependent ubiquitination of p53, and further increased proteasome activity triggered by wild-type p53. It is important to note that the protective function of UDCA was abolished by inhibiting proteasome activity.

Taken together these studies provide relevant information about the molecular mode of action of antiapoptotic UDCA, suggesting that the finely tuned, complex control of p53 by Mdm-2 is a key step for UDCA modulation of deregulated p53-induced cell death (Fig. 1). However, these studies may also raise some controversy.

As a key player in the cellular response to stress, p53 is most notably recognized as the major roadblock to tumorigenesis. In this regard, several approaches for restoring p53 function in tumor cells are presently being pursued. For cancer cells that retain wild-type p53, but are unable to activate the tumor suppressor, a number of compounds have been described that specifically target Mdm-2 ubiquitination and the physical interaction between p53 and its repressor (Vassilev *et al.* 2004, Yang *et al.* 2005, Dudkina & Lindsley 2007). Nevertheless, the induction of p53 is not without cost. The net consequence of such widespread p53 activity is widespread apoptosis responsible for the classic symptoms of radiation sickness and side effects of cancer therapy. Acute ablation of Mdm-2 activity elicits profound p53 activation, rapidly triggering lethal pathologies in classical radiosensitive tissues (Ringshausen *et al.* 2006). Importantly, the scientific community is aware of the "guardian angel's dark side", and interest in inhibiting p53 continuous to increase over the years (Komarov *et al.* 1999, Gudkov & Komarova 2005, Strom *et al.* 2006). The obvious application for this contrasting approach would be to protect normal cells from the side effects of chemotherapy. Would UDCA be able to differentiate "normal" and tumor cells? Or would it favor tumor development? These questions remain to be clarified.

Since the antiapoptotic function of UDCA is triggered by toxic stimuli, such as hydrophobic bile acids, TGF-β1, or p53 overexpression, it is conceivable that the bile acid acts in those cells where p53 activation results in imbalanced apoptosis, but not in cancer cells where activated p53 is counteracting excessive proliferation. More importantly, we cannot ignore the fact that UDCA has been the first-line treatment of several liver diseases for decades, without increasing patients' predisposition to cancer. On the contrary, a recent study examined the effects of prolonged UDCA administration on the prevalence and recurrence of colorectal adenoma in 114 patients with primary biliary cirrhosis (Serfaty *et al.* 2003). The results revealed that long-term administration of UDCA not only did not increase the risk of colon adenoma, but also exerted some chemopreventive activity against colon carcinogenesis, by significantly decreasing the rates of colorectal adenoma recurrence following removal. Other authors have presented data linking UDCA to a lower rate of colorectal dysplasia, based on their observation of patients with ulcerative colitis and primary sclerosing cholangitis (Tung *et al.* 2001). In addition, UDCA was also shown to prevent tumor development in a rat azoxymethane model of colon cancer, in part by inhibiting the growth-enhancing alterations in cyclin D1 and E-cadherin induced by this carcinogen (Earnest *et al.* 1994, Wali *et al.* 2002a, Wali *et al.* 2002b). Finally, it has recently been reported that UDCA abrogated cisplatin-induced apoptosis of mouse sensory neurons via downregulation of the p53 signaling pathway (Park *et al.* 2008). Cisplatin is one of the most effective anticancer chemotherapeutic drugs; however, it brings about the serious side effect of sensory neuropathy in cancer patients who receive the drug.

There is growing evidence of a role for p53 in other diseases beyond cancer. For example, induction of p53 during ischemia has been shown to contribute to damage through activation of apoptosis. Temporary inhibition of p53 function under these conditions might be highly beneficial in the prevention of injury to the liver, brain and kidneys (Georgiev *et al.* 2006, Fiskum *et al.* 2004, Dagher 2004),

or in treatment following myocardial infarction (Matsusaka *et al.* 2006). Inhibition of p53 also protected rat liver tissue against endotoxin-induced apoptotic and necrotic cell death (Schafer *et al.* 2003). This is particularly important since a significant cause of mortality in patients with gram-negative sepsis is the development of liver dysfunction and liver failure.

p53 also plays a role in AIDS pathogenesis. The glycoprotein complex of human immunodeficiency virus-1 (HIV-1) was shown to activate p53 through a complex pathway that involves several kinases (Castedo *et al.* 2005). In addition, the tumor suppressor is also linked to AIDS-associated neurodegeneration (Garden & Morrison 2005) and other neurological disorders associated with increased levels of apoptosis, including stroke, Parkinson's, Alzheimer's, and Huntington's diseases (Nair 2006, Culmsee & Mattson 2005, Feng *et al.* 2006). Preclinical data suggested that inhibitors of p53 may be effective therapeutic agents for neurodegenerative conditions (Culmsee & Mattson 2005). Curiously, it has been demonstrated that water soluble tauroursodeoxycholic acid (TUDCA) prevents neurodegeneration both *in vitro* and *in vivo* (Duan *et al.* 2002, Keene *et al.* 2002, Rodrigues *et al.* 2002, Rodrigues *et al.* 2003, Ramalho *et al.* 2004, Ramalho *et al.* 2006). Further, UDCA is currently in phase I/II clinical trials in patients with amyotrophic lateral sclerosis.

As a final note, it is important to look beyond the role of p53 just as a tumor suppressor to consider the real possibility that p53 is a critical stress point implicated in the pathogenesis of the Western world's most common non-infectious diseases, including atherosclerosis (Guevara *et al.* 1999), neurodegeneration, and cancer. It is still puzzling as to whether UDCA has the ability to physically interact with p53 in regions other than the DBD, or with Mdm-2, or even with both proteins in complex. At present, most of the knowledge is confined to the bench, but as we discover more of the underlying mechanisms involved, UDCA is expected to become increasingly important at the bedside. The

next decade will almost certainly unveil the remarkable role of this bile acid in the life and death struggle of different cells and organ systems.

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Curriculum Vitæ

Joana São José Dias Amaral was born in Lisbon, Portugal, on 20 February 1979. In 1997, she graduated from High School and started her studies in Biology at the Faculty of Sciences, University of Lisbon, Portugal. In 2000, she was awarded an Erasmus university students' mobility program fellowship, which allowed her to spend a 6-month period of her BSc degree at the Faculty of Biology, Complutense University of Madrid, Spain. In 2002, she graduated in Biology after completing a thesis on the characterization of a model for the study of hereditary and environmental susceptibility factors of neurodegenerative diseases, based on the expression of N-acetyltransferase 2 recombinants in mammalian cells. These studies were supervised by Professor Maria Celeste Lechner at the Unidade de Biologia Molecular, Faculty of Pharmacy, University of Lisbon, Portugal. In that same year, she was awarded a research fellowship supported by the project POCTI/BIO/36203/99, from Fundação para a Ciência e Tecnologia (FCT), Lisbon, Portugal. The studies were directed to ascertain the role of an *Escherichia coli* morphogene, *bolA*, on the control of cell division and adaptation to stress, supervised by Professor Cecília Maria Arraiano at the Instituto de Tecnologia Química e Biológica (ITQB), New University of Lisbon, Portugal. During this period, she performed some studies at the Centro de Biología Molecular Severo Ochoa, Faculty of Sciences, Autonomous University of Madrid, Spain, under the guidance of Professor Miguel Ángel de Pedro. In the academic year of 2004, she

was awarded a Ph.D. scholarship (BD/17799/2004) by FCT to undertake a project leading to this thesis, under the supervision of Professor Cecília M. P. Rodrigues at the Centro de Patogénese Molecular, Faculty of Pharmacy, University of Lisbon, Portugal.

List of Publications

Articles in International Journals

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