Therapeutic Effect of Adenovirus- and α-fetoprotein Promoter-mediated tBid and Chemotherapeutic Agents in Combination on Orthotopic Hepatocellular Carcinoma in Mice

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Abstract

Hepatocellular carcinoma (HCC) is the third commonest cancer worldwide. However HCC is considered to be highly resistant to chemotherapy. Gene therapies aimed to regulate Bcl-2 proteins may sensitize HCC cells to chemotherapy. Studies have demonstrated that Bid/tBid are crucial in hepatocyte apoptosis. Bid also plays important roles in the development and chemotherapeutic sensitivity of HCC. The objective of this study is to test effect of Ad/AFPtBid and chemotherapeutic agents in combination on an orthotopic HCC model.

The mice bearing orthotopic HCC tumors were treated with Ad/AFPtBid alone or in combination with 5-FU/Dox. Serum AFP levels were measured to mornitor tumor progression. The mice were killed four weeks after treatment. Liver tissues were subjected to immunohistochemical staining of proliferation cell nuclear antigen (PCNA) and TUNEL staining. Another batch of mice was observed for survival rate over a six month period. In addition, possible side effects of Ad/AFPtBid were tested in BALB/c mice. Results demonstrated that Ad/AFPtBid significantly inhibited Hep3B tumor growth. The combination of Ad/AFPtBid with 5-FU was more effective in tumor regression than either agent alone. However, the combination of Ad/AFPtBid with Dox treatment failed to demonstrated better effect than Dox treatment alone because the mice that received Dox exhibited serious

weight loss. Tumor tissues from Ad/AFPtBid alone or combination treatment groups showed a decrease in cells positive for PCNA, and an increase in apoptosis by TUNEL staining, indicating that Ad/AFPtBid induced tumor regression through its pro-apoptotic effect. Inflammatory cell infiltration was also increased. Furthermore, Ad/AFPtBid did not suppress the hepatic tumor formed by non-AFP producing SK-HEP-1 or DLD-1. Finally, Ad/AFPtBid and 5-FU in combination results in better survival rate. No acute toxic effect of Ad/AFPtBid was observed.

It is well established that many apoptosis inducers act in a cell cycle-specific fashion. This leads us to hypothesize that tBid might have phase specific effect. So, we tested the susceptibility of Hep3B cells at G0/G1, S or G2/M phases to tBid. The results revealed that tBid significantly reduced Hep3B cells in G0/G1 phase, increased cells in G2/M phase. On the contrary, 5-FU arrested Hep3B cells in G0/G1 phase, and significantly reduced cells in G2/M phase. The levels of cell cycle-related proteins were altered in line with the result of the cell cycle. This suggests Hep3B cells in G0/G1 phase may be more susceptible to tBid. The complementary effects tBid and 5-FU on different phases of the cell cycle may explain the better therapeutic result if both are used to treat HCC.

In conclusion, (1) Ad/AFPtBid can specifically target and effectively suppress the AFP-producing HCC. (2) Ad/AFPtBid can significantly sensitize HCC to 5-FU, their combination can significantly increase the

anti-tumor effectiveness. (3) Ad/AFPtBid shows little toxicity *in vivo*. (4) The complementary effect of tBid and 5-FU on different phases of the cell cycle may explain the better therapeutic result if both are used to treat HCC. (5) The elucidation of phase specific effect of tBid points to a possible therapeutic option that combines tBid with different phase specific agents to treat HCC.

摘　要

肝細胞癌是世界第三大常見的腫瘤，然而，由于其對化療顯著耐受导致治療效果差。基於調節Bcl-2家族蛋白的基因療法被认为有助於提高肝細胞癌對化療藥物的敏感性。研究表明，促凋亡蛋白Bid/tBid在誘導的肝細胞凋亡，肝細胞癌的發展及其對化療藥物的敏感性中起著重要的作用。本研究的主要目標是檢驗Ad/AFPtBid及其與化療藥物聯合應用對原位肝腫瘤模型的療效。

荷原位肝臟瘤小鼠用Ad/AFPtBid單獨治療或與5-FU/阿黴素聯合應用治療。通過測定小鼠血清AFP水平作為腫瘤進展的標記。治療四周後處死小鼠，對肝臟組織進行免疫組織化學染色檢測增殖細胞核抗原（ PCNA ）的表達，用TUNEL染色方法測定細胞凋亡。另一批小鼠用來觀察腫瘤細胞注射六個月後的存活率。此外，我們還檢測了Ad/AFPtBid 對 BALB/c 小鼠可能产生的副作用。結果表明，Ad/AFPtBid治療顯著抑制Hep3B腫瘤的生長。Ad/AFPtBid與5-FU聯合比兩者單獨應用能更加有效地抑制腫瘤生長。但Ad/AFPtBid與Dox聯合應用並不比Dox單獨應用抑制腫瘤效果好，因爲接受Dox治療的小鼠出現嚴重的體重下降。增殖細胞核抗原在Ad/AFPtBid單獨或聯合治療組腫瘤組織中的表達出現下降，同時TUNEL染色顯示的細胞凋亡增加，提示Ad/AFPtBid通过促凋亡作用抑制腫瘤生長。炎性細胞浸潤也

增加，這可能是tBid介導的細胞出現損害的一個指標。此外，Ad/AFPtBid對不生產AFP的肝癌細胞SK-HEP-1或結腸腺癌細胞DLD-1所形成的肝臟腫瘤沒有抑制作用。最後，用Ad/AFPtBid與5-FU聯合治療顯著提高小鼠的生存率。未觀察到Ad/AFPtBid急性毒性作用。

眾所周知，許多廣泛使用的細胞凋亡誘導劑是以細胞週期特異性方式發揮作用。這使我們假設tBid可能有細胞周期特異性作用。我們測試在G0/G1期， S或G2/M期的Hep3B細胞對tBid的敏感性。tBid顯著減少G0/G1期Hep3B細胞，導致細胞阻滯於G2/M期。細胞周期相關蛋白的表達水平結果與細胞周期的变化相一致。與此相反，5-FU主要引起Hep3B細胞阻滯於G0/G1期，顯著減少G2/M期細胞。這表明處於G0/G1期的Hep3B細胞對tBid更敏感。tBid和5-FU對不同細胞週期作用的互補性，在一定程度上，可以解釋Ad/AFPtBid與5-FU聯合使用治療肝細胞癌能產生更好的效果。

結論

（1）Ad/AFPtBid可以特異靶向並且有效地抑制產生AFP的小鼠原位肝臟腫瘤，但對不產生AFP的肝臟腫瘤無抑制作用。（2）Ad/AFPtBid可顯著提高肝癌細胞對5-FU的敏感性，兩者聯合應用顯著增強抗腫瘤效果。（3）Ad/AFPtBid 在小鼠體內無明顯毒性。（4）tBid和5-FU對不同細胞週期作用的互補性，在一定程度上，可以解釋Ad/AFPtBid與5-FU聯合使用治療肝細胞癌產生更好的效果。（5）tBid對肝癌的細胞

週期特異性作用的闡明提示可聯合應用tBid與作用於不同細胞週期的藥物來更有效的治療肝癌。

Publications

Book Chapter

Shi-Hong Ma, George G. Chen, Paul B.S. Lai. Bcl-2 family members in hepatocellular carcinoma (HCC) - mechanisms and therapeutic potentials. Book Chapter 9 (p219-236) in Apoptosis in Carcinogenesis and Chemotherapy: apoptosis in cancer (2009). Berlin. Springer.

Journal Articles

Shi-Hong Ma, George G. Chen, Johnson Yip, Paul B.S. Lai. Therapeutic effect of α-fetoprotein promoter-mediated tBid and chemotherapeutic agents on orthotopic liver tumor in mice. Gene Therapy. 2009 (Under Review)

Shi-Hong Ma, George G. Chen, Billy C.S. Leung, Rocky L.K. Ho, Paul B.S. Lai. Susceptibility of Hep3B cells in different phases of cell cycle to tBid-mediated apoptosis. Cell Death Differentiation. 2009 (Under Review)

Poster Presentation

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Shi-Hong Ma, George G. Chen, Johnson Yip, Paul B.S. Lai. Therapeutic effect of adenovirus- and alpha-fetoprotein (AFP) promoter-mediated tBid (Ad/AFPtBid) and chemotherapeutic agents in combination on orthoropic hepatocellular (HCC) in mice. In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract nr 3794.

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Abbreviations

%	percentage
°C	degree Celsius
5-FU	5-fluorouracil
Ad/AFPtBid	adenovirus- and α-fetoprotein promoter-mediated tBid
AFP	α-fetoprotein
ALT	alanine aminotransferase
AP	aphidicolin
Apaf-1	apoptotic protease activating factor-1
APS	ammonium persulfate
ASO	antisense oligonucleotides
AST	aspartate aminotransferase
BH domains	Bcl-2 homology domains
Bid	BH-3-interacting domain death agonist
Bik	Bcl-2 interacting killer
Bmf	Bcl-2-modifying factor
Bnip	Bcl-2/adenovirus E1B 19 kDa interacting protein
Bok/Mtd	Bcl-2-related ovarian killer
BSA	bovine serum albumin
BUN	blood urea nitrogen
Cdk2	cyclin-dependent kinase 2
CRE	creatinine

COX-2	cyclooxygenase-2
ddH_2O	double distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dox	doxorubicin
EC_{50}	half maximal effective concentration
ECL	enhanced chemiluminescence
EDTA	ethlenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ET	etoposide
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
GFP	green fluorescence protein
GST	glutathione S-transferase
h	hour
H2B-GFP	histone H2B and green fluorescent protein
HCC	hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hrk/DP5	Harakiri
HY	hydroxyurea
i.p.	intra-peritoneal
kDa	kilodalton

LB medium	luria bertani medium
LBA	LB ampicillin
LYM	lymphocyte
Mcl-1	myeloid cell leukemia-1
mg	milli-gram
min	minutes
ml	milli-liter
mm	milli-meter
mM	milli-molar per liter
MPCs	myeloid precursor cells
MTT	3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyltetrezolium bromide
NEU	neutrophil granulocyte
Nix	Nip3-like protein X
nM	nano-molar per liter
NO	nitric oxide
Noxa	NADPH oxidase activator
PBS	phosphate buffer saline
PCNA	proliferation cell nuclear antigen
PCR	polymerase chain reaction
pfu	plaque forming unit
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PMSF	phenylmethylsulfonyl fluoride

PUMA	p53 upregulated modulator of apoptosis
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
sec	seconds
siRNA	small interference RNA
Spike	small protein with inherent killing effect
TBE	Tris/Borate/EDTA buffer
tBid	truncated Bid
TBST	Tris-Buffered Saline, Tween 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducting ligand
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling
µg	micro-gram
µl	micro-liter
µm	micro-meter
µM	micro-molar per liter
WBC	white blood cell

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Chapter One

General Introduction

1.1 Background

Hepatocellular carcinoma (HCC) is the third commonest cancer that killed men worldwide (WHO, 2007). It is also one of the commonest malignancies in the developing countries of Asia and Africa including China. The incidence and death rates of HCC are also increasing in western countries (NCI, 2007). It is estimated that HCC would be the thirteen leading cause of death by 2030 (WHO, 2008). A major factor that contributes to such a gloomy prospect is lack of effective therapeutic tools. HCC thus remains highly resistant to systemic chemotherapy (Avila *et al.*, 2006). No effective systemic chemotherapy has been demonstrated an unequivocal and significant benefit in terms of survival (Ganne-Carrie and Trinchet, 2004).

Defects in apoptosis signaling contribute to tumorigenesis and chemotherapy resistance of HCC cells. In HCC, the balance between the pro- and anti-apoptotic of Bcl-2 family members is often not properly maintained with a shift in favor of anti-apoptotic members in tumor cells, leading to resistance to cell death and a rapid growth of cells (Mott and Gores, 2007). As shown in Table 1.1, the expression of anti-apoptotic Bcl-xL and Mcl-1 is increased, whereas the expression of pro-apoptotic Bid and Bak proteins is decreased (Chiu *et al.*, 2003, Sieghart *et al.*, 2006, Chen *et al.*, 2001a, Liu *et al.*, 2003). Because of the limitation of the conventional therapies, gene therapy which aims to regulate Bcl-2 family proteins may sensitize HCC cells to chemotherapeutic agents.

The main focus of ongoing research is to clarify the therapeutic efficacy and mechanism of Ad/AFPtBid and its combination with 5-fluorouracil (5-FU) or doxorubicin (Dox) in an orthotopic hepatic tumor model.

This chapter will summarize the recent development in our understanding of Bcl-2 family members in hepatocarcinogenesis and the therapeutic utilization of these molecules to increase the effectiveness in HCC treatment.

Table 1.1 Expression of Bcl-2 family members in liver tissues of HCC and the normal subjects

Function	Structure	Bcl-2 family members	Normal subjects	HCC	
				Expression	References
Anti-apoptotic	Multi-domain	Bcl-2	-	-/+	(Yoon *et al.*, 1998, Chiu *et al.*, 2003, Hussein, 2004, Pizem *et al.*, 2001)
		Bcl-xL	+	↑	(Takehara *et al.*, 2001, Chiu *et al.*, 2003)
		Mcl-1	+	↑	(Sieghart *et al.*, 2006)
		Bcl-w	+	+/ND	(O'Reilly *et al.*, 2001)
		Bcl-B/Nrh/NR13	+	ND	
		Bfl-1/Bcl-2A1/GRS	-	ND	
Pro-apoptotic	Multi-domain	Bax	+	↓/↑	(Guo *et al.*, 2002, Luo *et al.*, 1999)
		Bak	+	↓/ND	(Liu *et al.*, 2003)
		Bcl-xS	+	↓	(Chiu *et al.*, 2003)
		Bok/Mtd	+	ND	
Pro-apoptotic	BH3 domain only	Bid	+	↓	(Chen *et al.*, 2001b)
		BAD	+	Phospho-/+	(Yoo *et al.*, 2006, Chang *et al.*, 2007)
		Bim/Bod	Low level	↑	(Miao *et al.*, 2007)
		Bik/Nbk	ND	-/ND	(Zhao *et al.*, 2007)
		PUMA	ND	ND	
		Noxa	ND	ND	
		Hrk/DP5	-/low level	ND	
		Bmf	+	ND	
		Spike	ND	ND	
		BNip proteins	-/+	ND	

ND = not determined.

1.2 Bcl-2 family members

Apoptosis is widely accepted as a prominent tumor-suppression mechanism. Bcl-2 family proteins have emerged as a dominant regulator of apoptosis in cancer cells. Bcl-2 family proteins are structurally defined by their Bcl-2 homology domains (BH domains) into multi-domains and BH3-only, and functionally categorized into anti-apoptotic and pro-apoptotic. Bcl-2 family members fall into three subgroups. The first group includes Bcl-2, Bcl-xL, Mcl-1 (Myeloid cell leukemia-1), Bcl-w, Bcl-B/Nrh/NR13 and Bfl-1/Bcl-2A1/GRS. These molecules contain multi-BH domains and function to inhibit apoptosis. The second group of Bcl-2 proteins also contains multi-BH domains but the proteins in this group function to promote apoptosis. These proteins include Bax, Bak, Bok/Mtd (Bcl-2-related ovarian killer) and Bcl-xS. The third group contains only a BH3 domain and it includes Bid, BAD, Bik (Bcl-2 interacting killer)/Nbk, Bim/Bod, PUMA (p53 upregulated modulator of apoptosis), Noxa (NADPH oxidase activator), Hrk/DP5 (Harakiri), Bmf (Bcl-2-modifying factor), Spike (small protein with inherent killing effect) and BNip (Bcl-2/adenovirus E1B 19 kDa interacting protein) proteins. The BH3-only proteins can bind and regulate the pro-survival Bcl-2 family members to promote apoptosis.

1.3 Physiological roles of Bcl-2 family members in liver

1.3.1 Anti-apoptotic multi-domain members of the Bcl-2 family

Bcl-2 family members have essential roles in liver homeostasis. Although Bcl-2 is not generally expressed in human hepatocytes (Charlotte *et al.*, 1994), ectopic Bcl-2 expression delays the cell cycle progression of hepatocytes (Vail *et al.*, 2002). While Bcl-xL-/- mice die during embryogenesis with extensive apoptosis in liver (Motoyama *et al.*, 1995). Over-expression of either Bcl-2 or Bcl-xL in mouse livers protects hepatocytes from Fas-induced apoptosis and liver destruction in a dose-dependent manner. The protective effect of Bcl-2 is not associated with the levels of Bcl-xL, Bax, BAD, Bak and p53 (Rodriguez *et al.*, 1996). Bcl-xL is protective without any change in the level of Bax and inhibits hepatic caspase 3-like activity (de la Coste *et al.*, 1999a). Excess Bcl-xL can inhibit p53-induced oligomerization of Bak and the release of cytochrome c when being added to purified mouse liver mitochondria (Mihara *et al.*, 2003).

Mcl-1 is expressed in human liver (Krajewski *et al.*, 1995) and plays a major role in mediating the anti-apoptotic effects of hepatocyte growth factor in primary human hepatocytes (Schulze-Bergkamen *et al.*, 2004). Rapid up-regulation of other anti-apoptotic Bcl-2 family members such as Bfl-1 may also contribute to hepatocyte survival, although Bfl-1 gene is not detected by northern blot in human liver (Schoemaker *et al.*, 2003).

Bcl-B and Bcl-w are expressed in human liver (Ke *et al.*, 2001, Gibson *et al.*, 1996), yet their physiological roles in liver are unknown. A function study showed that Bcl-B suppressed apoptosis by binding Bcl-2, Bcl-xL and Bax but not Bak (Luciano *et al.*, 2007). Bcl-w is functionally similar to Bcl-2 since it enhances cell survival. Bcl-w and Bfl-1 protect against apoptosis induced by over-expression of Bax or BAD but not that induced by Bak or Bik (Holmgreen *et al.*, 1999).

1.3.2 Pro-apoptotic multi-domain members of the Bcl-2 family

A high level of Bax and a moderate level of Bak are expressed in human liver (Krajewski *et al.*, 1994, Krajewski *et al.*, 1996). No histological and functional abnormalities (determined by standard blood chemistries) have been observed in the liver of either $Bak^{-/-}$ or $Bax^{-/-}Bak^{-/-}$ animals (Lindsten *et al.*, 2000).

Bok exhibits a higher level in fetal liver than in adult liver (Lee *et al.*, 2001), till now its physiological role in liver is unknown. Bok is the only member of the Bcl-2 family to have a leucine-rich sequence indicative of a nuclear export signal within its BH3 domain (Bartholomeusz *et al.*, 2006). In the yeast two-hybrid system, Bok interacts strongly with Mcl-1, Bfl-1 and BNip3 but not Bcl-2, Bcl-xL, and Bcl-w (Gao *et al.*, 2005).

1.3.3 Pro-apoptotic BH3 domain only members of the Bcl-2 family

Bid is expressed predominantly in the cytosolic fraction of hepatocytes (Gross *et al.*, 1999). $Bid^{-/-}$ mice apparently develop normal and show normal liver. Bid connects the death receptor apoptosis pathway to the mitochondrial apoptosis pathway (Ding *et al.*, 2004). The cytosolic truncated Bid (tBid) targets mouse liver mitochondria while Bid does not. Anti-Fas antibody results in the appearance of tBid in the cytosol of hepatocytes and tBid translocates to the mitochondria where it stimulates the releases of cytochrome c.

Bid is indispensable for Fas-induced hepatocytes apoptosis and liver injury (Li *et al.*, 2002, Yin *et al.*, 1999). However, the deletion of Bid only delays but does not block TNF-α (tumor necrosis factor-alpha) -mediated apoptosis of hepatocytes and liver injury (Ding *et al.*, 2004, Zhao *et al.*, 2001, Zhao *et al.*, 2003). Hepatocytes require a Bid-dependent mitochondrial amplification loop that releases cytochrome c, oligomerizing Apaf-1 and caspase 9 to activate sufficient effector caspases to execute apoptosis. In both cultured cells and animal models of TNF-α-induced injury, Bid-independent mitochondrial activation could be demonstrated at later time points (Chen *et al.*, 2007). Gene knockout mice model indicated that Bid functions upstream of either Bax or Bak to initiate mitochondrial dysfunction and cell death (Wei *et al.*, 2001). Following Fas activation, Bid is singularly required to oligomerize Bak and release cytochrome c (Wei *et al.*, 2000). Researchers also found that Bid-dependent generation of oxygen

radicals promotes death receptor activation-induced apoptosis in murine primary hepatocytes (Zhao *et al.*, 2003). Transient expression of anti-death Bcl-2 or Bcl-xL reduces the apoptosis of Bid-deficient hepatocytes treated with TNF-α, suggesting that the Bcl-2 family proteins could interact with the Bid-independent mitochondrial activation mechanism. Consistent with this finding, treatment of hepatocytes *in vitro* with TNF-α also cleaves of Mcl-1 on the mitochondria, independent of the status of Bid.

The roles of BAD, Bmf, Bim, Hrk, Spike and BNip in the liver are less clear than Bid. Liver has abundant Bmf, detectable BAD, and very low or undetectable level of Bim and Hrk mRNA in liver ((Inohara *et al.*, 1997, Kitada *et al.*, 1998, U *et al.*, 2001). In the yeast two-hybrid system, Bmf can interact with Mcl-1, pro-survival Bcl-2, Bcl-xL, Bcl-w but not with the pro-apoptotic Bax, Bid and BAD (Puthalakath *et al.*, 2001). Spike has been shown to regulate the interaction between Bcl-xL and Bap31, which is an adapter protein for procaspase 8 and Bcl-xL. No co-immunoprecipitation of Spike with all tested Bcl-2 family members such as Bax, Bcl-xL or Bcl-2 has been observed (Mund *et al.*, 2003).

The BNip proteins include BNip1, BNip2, BNip3, BNIPL-1, BNIPL-2 and Nix (Nip3-like protein X). They are pro-apoptotic members of the Bcl-2 family. The expression of BNip1 and its variants have been observed in liver. Bnip2, a putative pro-apoptotic protein, contains two isoforms BNIP-Sα and BNIP-Sβ. BNIP-Sα but not BNIP-Sβ is expressed in liver. Bnip3

induces necrosis rather than apoptosis. Several human homologues of BNip3 have been reported, all of which are pro-apoptotic molecules: BNip3L, BNip3h and Nix (Zhang *et al.*, 2003). Nix homo-dimerizes similarly to Bnip3 and can overcome the suppressors Bcl-2 and Bcl-xL (Chen *et al.*, 1999). The expression of BNip3 can be suppressed by nitric oxide (NO), and such suppression has been proposed to be a mechanism for NO-induced hepatocyte apoptosis (Zamora *et al.*, 2001). BNIPL-1 and BNIPL-2 are homologous to human Bnip2, can interact with Bcl-2, Cdc42GAP and induce apoptosis (Qin *et al.*, 2003). BNIPL-2 may play its role in apoptosis through regulating the expression of genes associated with cell apoptosis, growth inhibition and cell proliferation (Xie *et al.*, 2004).

1.4 Bcl-2 family members and hepatocarcinogenesis

Given the importance of Bcl-2 family members in normal liver, the relevance of Bcl-2 family members in HCC has been extensively studied.

1.4.1 Anti-apoptotic multi-domain members of the Bcl-2 family

Among this subgroup Bcl-2 and Bcl-xL have been well studied in hepatocytes. In HCC, Bcl-2 is usually absent while Bcl-xL is predominately expressed. Using Northern blot, researchers reported that Bcl-2 and Bcl-xL may play important roles in regulating the apoptosis of normal liver and HCC (Guo *et al.*, 2002). Up-regulation of Bcl-xL is associated with tumorigenesis and resistance to chemotherapeutic treatment, thus becoming a promising target for cancer therapy (Takehara *et al.*, 2001).

The expression and the function of Bcl-w, Bcl-B and Bfl-1 are unknown in HCC.

Some reports showed that the increased level of Bcl-2 RNA was frequently present in HCC (Fiorentino *et al.*, 1999). However, its protein product is either absent (Yoon *et al.*, 1998, Skopelitou *et al.*, 1996) or present only in a very small proportion of tumor cells in HCC tissues (Soini *et al.*, 1996, Hamazaki *et al.*, 1995). The findings suggest a post-translational mechanism of Bcl-2 protein degradation, indicating that Bcl-2 may not play an important role in hepatocarcinogenesis (Ravazoula *et al.*, 2002, Nakopoulou *et al.*, 1999). In contrast, other studies have shown the increased level of Bcl-2 protein in HCC (Hussein, 2004, Pizem *et al.*, 2001) and it may be involved in the development of HCC (Ali *et al.*, 2004). This concept is supported by a study showing that the over-expression of Smad3, a major transforming growth factor-beta (TGF-β) signaling transducer, reduces the susceptibility to hepatocarcinogenesis in a mouse model by reducing the level of Bcl-2 to sensitize hepatocytes to apoptosis (Yang *et al.*, 2006). This observation is in agreement with data demonstrating that excess Bcl-2 expression in TGF-α/Bcl-2 double transgenic mice delays the development of liver tumors induced by the growth factor (Vail *et al.*, 2001) and that Bcl-2 inhibits c-myc-induced liver carcinogenesis (de La Coste *et al.*, 1999b). Furthermore, *in vivo* electroporetic transfer of Bcl-2 antisense oligonucleotide (ASO) into liver can inhibit the development of HCC in rats (Baba *et al.*, 2000). The different expression of Bcl-2 may be related to

the status of p53 since Bcl-2 is remarkably up-regulated in p53-positive HCC tissues, but down-regulated in p53-negative ones (Chiu *et al.*, 2003). Alterations of both p53 and Bcl-2 proteins have been shown during hepatocarcinogensis (Hussein, 2004), and the over-expression of p53 correlates with high levels of proliferation cell nuclear antigen (PCNA), HCC de-differentiation and advanced HCC stages (Hu *et al.*, 2007).

Bcl-x can alternatively produce two distinct proteins, anti-apoptotic Bcl-xL and pro-apoptotic Bcl-xS (Yang *et al.*, 2004). Bcl-xL is expressed at high levels in HepG2, Hep3B, and Huh7 cell lines and in a great percentage of murine and human HCC tissues. Bcl-xL can be up-regulated in HCC regardless of p53 status (Chiu *et al.*, 2003). Bcl-xL is located not only in the cytoplasm but also in the nuclei of some HCC cells, suggesting that it is involved in the progression of HCC cells *in vivo* (Watanabe *et al.*, 2002). Increased expression of Bcl-xL in human HCC cells inhibits apoptosis produced by serum starvation, and p53 activation (Takehara *et al.*, 2001). The function of Bcl-xL can be modulated through deamidation at asparagine residues in a post-translational manner, and human HCC cells may acquire resistance to apoptosis and a survival advantage by suppressing the deamidation (Takehara and Takahashi, 2003).

Mcl-1 interacts with tBid and, thereby, inhibits intrinsic as well as extrinsic apoptotic signaling (Clohessy *et al.*, 2006). Mcl-1 protein is considerably over-expressed in Huh7, Hep3B, HepG2 and human HCC tissues, and it

correlates with Bcl-xL expression in HCC tissues (Sieghart *et al.*, 2006). Enhancing cell survival Bcl-w is also expressed in HepG2 (O'Reilly *et al.*, 2001).

The expression of Bcl-2 fails to correlate with the natural prognosis or survival (Garcia *et al.*, 2002). However, Bcl-xL is a significant prognostic factor for disease progression and poorer survival in human HCC (Watanabe *et al.*, 2004). There is no correlation between the levels of Bcl-2 and Bcl-xL and age, gender, differentiation or stage of tumor in HCC patients (Guo *et al.*, 2002). However, the induction of anti-apoptotic Bcl-2 related proteins at the early stage of differentiation is important for the maintenance of tumor cell differentiation by antagonizing pro-apoptotic molecules such as Bax (Wakabayashi *et al.*, 2000). Although Bfl-1 gene may be involved in cancer progression by promoting cell survival and its level is over-expressed in other digestive cancers, Bfl-1 gene is not detectable in human hepatic tumors (Choi *et al.*, 1995).

1.4.2 Pro-apoptotic multi-domain members of the Bcl-2 family

Bax, Bak, Bcl-xS and Bok function either by inactivating pro-apoptotic Bcl-2 family members or by forming pores in the mitochondria. Most of studies indicated that the expression of these pro-apoptotic proteins is decreased in HCC. For example, Bax levels were frequently down-regulated with non-functional p53 protein over-expressed in HCC samples (Guo *et al.*, 2002, Beerheide *et al.*, 2000). The level of Bak is down-

regulated in human hepatoma cell line BEL-7402 (Liu *et al.*, 2003). Bcl-xS is remarkably down-regulated in p53 positive HCC (Chiu *et al.*, 2003). However, there are some reports showing that the level of Bax was not reduced in human HCC QGY-7703 cells and that its level was even up-regulated in HCC regardless of the p53 status (Luo *et al.*, 1999). The reason for the controversial results is unknown.

The survival rate in the patients with Bax-expressing HCC appears to be better (Osada *et al.*, 2004, Garcia *et al.*, 2002). Immunostaining intensity of Bax tends to be positively correlated with overall survival (Garcia *et al.*, 2002). The level of Bax does not correlate with either age or gender in HCC patients (Guo *et al.*, 2002). Bax may be used to identify the patient prognosis of the lower grade histological cases but another study fails to show such a relationship (Guo *et al.*, 2002, Osada *et al.*, 2004). Although the ratio of Bcl-2 to Bax is generally regarded as a critical index for determining whether a cell is likely undergone apoptosis, this ratio correlates with neither apoptosis nor clinical markers in HCC (Ikeguchi *et al.*, 2002).

1.4.3 Pro-apoptotic BH3 domain only members of the Bcl-2 family

BH3 domain only members of the Bcl-2 family promote apoptosis by binding anti-apoptotic Bcl-2 family members, leading to inhibit their activity. It can also interact with Bax or Bak to regulate cell death. Bid and BAD are found to be down-regulated or inactive in HCC. However,

surprisingly the level of Bim is increased. The expression and function of other recently discovered members of this subgroup in HCC are unknown.

The expression and the regulation of Bid may play crucial roles not only in liver tumorigenesis but also in the chemotherapeutic treatment of HCC. The level of Bid shows to be decreased in HCC except in poorly differentiated HCC in which cells may undergo a process of apoptosis or necrosis ((Miao *et al.*, 2004, Miao *et al.*, 2006, Chen *et al.*, 2001a). The decreased Bid may be related to hepatitis B virus X protein or hepatitis C virus polyprotein (Chen *et al.*, 2001b, Disson *et al.*, 2004). Bid can block the inhibitory effect of Bcl-2 on Fas-mediated apoptosis of HCC cell line BEL-7404 cells through oligomerising Bak to release cytochrome c (Chang and Xu, 2000). In SK-HEP1 cells TNF-related apoptosis-inducting ligand (TRAIL) is known to induce the translocation of Bax, which subsequently leads to the cleavage of Bid ((Kim *et al.*, 2002b).

BAD is a pro-apoptotic Bcl-2 family protein that regulates the intrinsic apoptosis pathway. Phosphorylation of BAD inhibits apoptosis whereas de-phosphorylation of BAD promotes it. In FaO hepatoma cells, TGF-β1 can induce cleavage of BAD at its N terminus to generate a 15-kDa truncated protein, leading to apoptosis (Kim *et al.*, 2002a). A mutant BAD can prevent caspase 3 from cleavage and thus block TGF-β1-induced apoptosis (Kim *et al.*, 2002a). The loss of phospho-BAD expression, but not *BAD* gene mutation has been shown to be a feature of HCC and the loss of

phospho-BAD expression may play a role in hepatocarcinogenesis (Yoo *et al.*, 2006). However, this seems not to be the case in all HCC, since the phospho-BAD has been observed in HBV-infected HCC (Chang *et al.*, 2007).

Bim mRNA and protein are strongly expressed in HCC (Miao *et al.*, 2007). Bim EL, L, S, a1, a2, a3, b2, b4 and b6 are abundant isoforms according to their mRNA levels. However, only Bim EL, L and S proteins could be clearly detected in HCC. Although the pathological role of such an increase in HCC is unclear, over-expression of Bim EL, L, S and all alpha isoforms (containing BH3 domain in their translation products) appears to have a role in the regulation of apoptosis in HCC, which may contribute to not only the growth of tumor cells but also the sensitivity of HCC cells to 5-FU. Further study is necessary to identify whether the phosphorylation of Bim EL in Hep3B contributes to the sensitivity or resistance of HCC to chemotherapy (Miao *et al.*, 2007).

Unlike other BH3-only proteins, Bik is the first BH3-only protein to be identified as an endoplasmic reticulum (ER)-resident, from which it induces apoptosis (Mathai *et al.*, 2005, Boyd *et al.*, 1995, Zou *et al.*, 2002). Bik could apparently induce apoptosis in Hep3B cells through activating caspase 12-dependent signal transduction pathway. Bik activates caspase 9 and depolarization of mitochondrial membrane potential, which is decreased concomitantly with caspase 12 silenced (Zhao *et al.*, 2007). Bik

is not normally detected in Hep3B and HepG2 cells, however after treatment with 5-azaC or butyrate an increase of Bik mRNA expression can be observed (Wang *et al.*, 1998), suggesting that the pro-apoptotic role of this molecule is secondary to other stimuli.

Hrk/DP5 activates apoptosis and interacts selectively with Bcl-2 and Bcl-xL but not Bcl-xS (Boyd *et al.*, 1995). Methylation of Hrk silences the gene expression. However, the methylation around the transcription start site was not detected in HCC cell lines and in 20 HCC primary tumors (Obata *et al.*, 2003).

PUMA and Noxa are BH3-only proteins that are involved in p53-dependent apoptosis (Nakano and Vousden, 2001, Hijikata *et al.*, 1990). Previous studies reported that Noxa induces apoptosis in various cell lines (Oda *et al.*, 2000). Bnip3 and its homologue Nix are hypoxically regulated in many tumor types (Sowter *et al.*, 2001). Unfortunately, the roles of these BH3-only proteins are unknown in HCC.

1.5 Bcl-2 family members in HCC treatment

1.5.1 Bcl-2 family members as targets for anti-HCC therapies

Over-expression of Bcl-2 protein protects several human HCC cells from various apoptotic stimuli such as TRAIL (Guo and Xu, 2001), FAS (Takahashi *et al.*, 1999) and TGF-β (Huang and Chou, 1998). Therefore, a

high level of Bcl-2 may raise the apoptotic threshold and cause HCC cells less likely to be killed. In contrast, a decrease in the expression of Bcl-2 protein promotes cell death in HCC cells, which is the mechanism responsible for apoptosis of HCC cells induced by TNF-α and TGF-β (Li *et al.*, 2001a). Bcl-2 and Bcl-xL play important roles in the resistance to chemical therapy in HCC. Bcl-xL inhibits staurosporine-induced apoptosis in human HCC. Primary cultured HCC cells, resistant to paclitaxel, are known to express high levels of the anti-apoptotic Bcl-2 and Bcl-xL proteins. The level of Bcl-xL can be further increased upon paclitaxel treatment (Chun and Lee, 2004). Constitutive expression of Bcl-2 can render HCC QGY-7703 cells more resistant to Taxol and doxorubicin. Contrarily, a decreased level of Bcl-2 renders the tumor cells to be more sensitive to the drugs (Luo *et al.*, 1999).

Antisense oligonucleotides (ASO) strategy has been used to target Mcl-1 to down-regulated its level, leading to an increase in apoptosis or a decrease in cell viability in HCC cells. In combination with cisplatin or doxorubicin, Mcl-1 ASO has an additive effect (Sieghart *et al.*, 2006, Fleischer *et al.*, 2006). Similar to ASO strategy, small interference RNA (siRNA) can mediate the inhibition of Mcl-1, resulting in significantly enhanced susceptibility of HCC cells to chemotherapy or TRAIL-mediated apoptosis. The regulation of Mcl-1 involve in phosphoinositide 3-kinase (PI3K) since the inhibition of PI3K reduces expression of Mcl-1 in Hep3B cells (Fleischer *et al.*, 2006). Therefore, treatment of HCC cells with PI3K

inhibitors and chemotherapeutics in combination can greatly enhance the apoptosis induced. The important role of Mcl-1 in HCC treatment is further demonstrated by the Mcl-1 over-expression experiment, in which the over-expression of Mcl-1 reduces apoptosis of Huh-7 cells induced by chemotherapeutic agents or TRAIL (Wirth *et al.*, 2005). Therefore, Mcl-1-expressing HCC cells are resistant to apoptosis and usually show low sensitivity to chemotherapeutic drugs.

Up-regulation of Bax expression either by p21/ceramide or by the introduction of high level p53 in Hep3B cells induces apoptosis. JNK- and p38 kinase-mediated phosphorylation of Bax leads to apoptosis of human HepG2 cells (Kim *et al.*, 2006). These results demonstrate Bax may participate in inducing p53-dependent apoptosis in HCC (Kang *et al.*, 1999, Lai *et al.*, 2007). Over-expression of Bax sensitizes HCC-9204 cell to adriamycin-induced apoptosis (Zheng *et al.*, 2005). Also, an inverse relationship of Bax expression with doxorubicin resistance has been shown in HCC (Hu *et al.*, 2004). The over-expression of Bak leads to apoptosis in HCC-9204 cells and sensitizes the cells to apoptosis induced by doxorubicin (Li *et al.*, 2000). Transfer of Bcl-xS plasmid is effective in preventing and inhibiting rat HCC induced by N-nitrosomorpholine (Baba *et al.*, 2001). Over-expression of BNIPL-1 suppresses Hep3B cell growth probably though cell cycle arrest or apoptotic cell death pathway (Xie *et al.*, 2005).

1.5.2 Strategies to target Bcl-2 expression in HCC

In HCC, resistance to currently used chemotherapeutic drugs is partly mediated by over-expression of the cell survival proteins. Interaction between anti-apoptotic and pro-apoptotic members of the Bcl-2 family should determine cell fate. Strategies which aim to down-regulate anti-apoptotic Bcl-2 proteins or interfere with its anti-apoptotic function are likely to sensitize cancer cells to chemotherapeutic agents as well as lower the dose of therapy required to kill the cancer cells. Therapeutic strategies currently in use or potentially useful to modulate apoptosis in HCC are summarized in Fig 1.1.

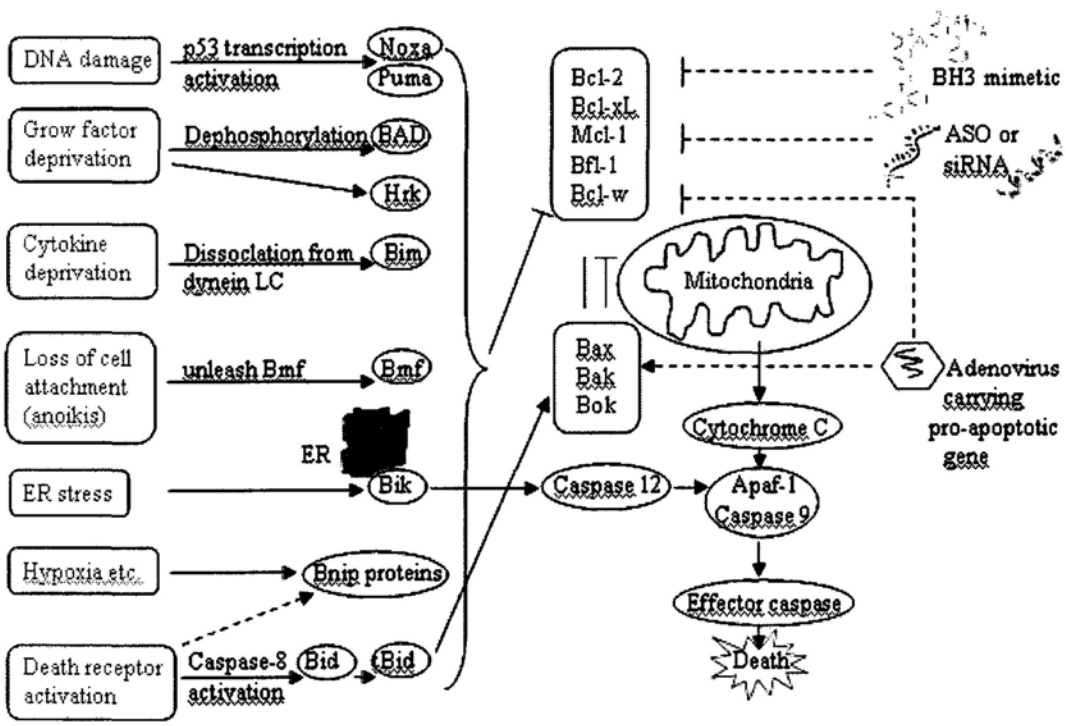


Fig 1.1 Signaling of Bcl-2 family members in hepatocytes and potential strategies for HCC treatments.

Note: Bcl-2 and Bfl-1 were not expressed, Noxa, Puma and Bik were not determined in hepatocytes.

ASO and siRNA present a genetic approach to inhibit the anti-apoptotic function of Bcl-2 family members. Results from human phase I clinical trials of Genasense (G3139), ASO of Bcl-2, have demonstrated good efficacy with low toxicity in non-Hodgkins lymphoma patients (Webb *et al.*, 1997, Waters *et al.*, 2000). However a recently report of a phase I-II study of G3139 in combination with doxorubicin in 21 patients with advanced HCC fails to have a positive result and the failure of the treatment is likely due to the low level of Bcl-2 in the patients studied (Knox *et al.*, 2008). Taken together, it appears that anti-Bcl-2 treatment is not suitable for all HCC and HCC with a high level of Bcl-2 likely responds well to the treatment. Therefore, it is critical to determine the level of Bcl-2 before the treatment.

Adenoviral gene therapy is an effective mode of gene delivery that has been demonstrated to be safe in clinical trials of gene therapy. Adenovirus- and α Alpha-fetoprotein (AFP) promoter-mediated tBid (Ad/AFPtBid) have been shown to significantly and specifically kill AFP-producing Hep3B cells *in vitro* and in mice subcutaneously implanted with HCC cells (Miao *et al.*, 2006). Adenovirus that carries a lethal gene PUMA under the control of a beta-catenin/Tcf-responsive promoter (AdTOP-PUMA) has also been

demonstrated to efficiently kill HepG2 HCC cells (Dvory-Sobol *et al.*, 2006).

The binding pocket formed by the BH1, 2 and 3 domains of Bcl-2 and related survival molecules has great potential as a target for small molecule inhibitors. Antimycin A has emerged as a Bcl-2 groove binder upon screening mitochondrial respiration inhibitors for pro-apoptotic activity in hepatocytes with graded expression of Bcl-xL (Kim *et al.*, 2001, Tzung *et al.*, 2001). Antimycin A interacts with the BH3-binding hydrophobic groove of Bcl-xL and can also compete with a Bak BH3 peptide for binding to recombinant Bcl-2. BH3 mimetics ABT-737 is a small molecule that binds within the hydrophobic cleft of Bcl-2, Bcl-xL, and Bcl-w and kills cells in a fashion dependent on Bax and Bak. A combination of sorafenib (BAY 43-9006) with ABT-737 is effective in the treatment of HCC (Lin *et al.*, 2007). Likewise, sorafenib, a kinase inhibitor, has a beneficial effect in the treatment of HCC (Abou-Alfa *et al.*, 2006), likely in part by down-regulating Mcl-1. Finally, cyclooxygenase-2 (COX-2) can act in HCC cells to increase Mcl-1 protein expression (Kern *et al.*, 2006), and thus the inhibition of COX-2 can decrease Mcl-1 protein expression to sensitize tumor cells to apoptosis.

1.6 Perspective

Physiological apoptosis is associated with liver homeostasis. In contrast, deficient apoptosis contributes to the development of liver cancer.

Members of the Bcl-2 family serve as critical regulators of apoptosis, acting to either inhibit or promote cell death. Aberrant expression patterns of some members of this family have been reported in HCC. However the roles of some Bcl-2 proteins such as Bcl-B, Bfl-1, Bok, Hrk and Bmf in HCC are unknown. A proper balance between anti-apoptotic and pro-apoptotic molecules is required for normal hepatocytes, and the imbalance caused by either an increase in the anti-apoptotic Bcl-2 members or a decrease in pro-apoptotic members may contribute to hepatocarcinogenesis as well as to the sensitivity of HCC to anti-tumor treatments. Therefore, the balance between pro- and anti-apoptotic Bcl-2 family members determines HCC outcome. Our understanding of Bcl-2 family members has resulted in the development of some novel and promising treatments for HCC.

Chapter Two

Therapeutic effect of adenovirus- and α-fetoprotein promoter-mediated tBid and chemotherapeutic agents in combination on orthotopic hepatocellular carcinoma in mice

2.1 Introduction

HCC is the third commonest cancer that killed men worldwide (WHO, 2007). It is estimated that HCC would be the thirteenth leading cause of death by 2030 (WHO, 2008). A major factor that contributes to such a gloomy prospect is the lack of effective therapeutic tools. No effective systemic chemotherapy has demonstrated an unequivocal and significant benefit in terms of survival (Ganne-Carrie and Trinchet, 2004). Defects in apoptosis signaling contribute to the chemotherapy resistance. In HCC, there is an imbalance between the pro- and anti-apoptotic of Bcl-2 family members (Mott and Gores, 2007). Because of the limitation of the conventional therapies, gene therapy which aims to up-regulate pro-apoptotic Bcl-2 proteins or interfere with function of anti-apoptotic Bcl-2 proteins may sensitize HCC cells to chemotherapeutic agents as well as lower the dose of therapy required to kill the HCC cells.

Studies have demonstrated that BH3 domain-only protein Bid and its activated form tBid are crucial in hepatocyte apoptosis induced by Fas/TNF-R1 signals (Ding *et al.*, 2004, Li *et al.*, 2002, Yin *et al.*, 1999, Zhao *et al.*, 2003). Bid-dependent generation of oxygen radicals promotes death receptor activation-induced apoptosis in murine primary hepatocytes (Zhao *et al.*, 2003). Gene knockout mouse model indicated that Bid functions upstream of either Bax or Bak to initiate mitochondrial dysfunction and cell death (Wei *et al.*, 2001, Wei *et al.*, 2000). Others have shown that Bid can block the inhibitory effect of Bcl-2 on Fas-mediated

apoptosis of HCC cells through oligomerising Bak to release cytochrome c (Chang and Xu, 2000). In SK-HEP1 cells TRAIL is known to induce the translocation of Bax, which subsequently leads to the cleavage of Bid (Kim *et al.*, 2002b).

Bid also plays important roles in the development and chemotherapeutic sensitivity of HCC. Our previous experiments have shown that the level of Bid decreased in HCC tissues of patients except in poorly differentiated HCC in which cells may undergo a process of apoptosis or necrosis (Chen *et al.*, 2001a). The application of Ad/AFPtBid significantly and specifically kills AFP-producing Hep3B cells *in vitro* and in mice subcutaneously implanted with them. Furthermore, over-expression of Bid especially tBid significantly sensitized Hep3B cells to two commonly used HCC chemotherapeutic agents 5-fluorouracil (5-FU) and doxorubicin (Dox) *in vitro* (Miao *et al.*, 2006, Miao *et al.*, 2004). It appears that the combination of gene therapy with existing chemotherapy and radiotherapy is likely to be more effective than separate approach alone (Schmitz *et al.*, 2002, Moon *et al.*, 2003).

The objective of this study is to test therapeutic efficacy of Ad/AFPtBid and its combination with 5-FU or Dox in an orthotopic hepatic tumor model, which represents naturally occurring HCC (Yao *et al.*, 2003). Results demonstrated that Ad/AFPtBid in combination with 5-FU significantly arrested the growth of liver tumor.

2.2 Materials and Methods

2.2.1 Cell culture

The human HCC cell lines Hep3B, SK-HEP-1 and human colorectal adenocarcinoma cell line DLD-1 were obtained from American Type Culture Collection (Rockville, MD). Hep3B and SK-HEP-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA), DLD-1 was maintained in RPMI medium 1640 (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco™, Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2. Exponentially growing cells were collected by trypsin-EDTA (Invitrogen, Carlsbad, CA) and washed once with phosphate buffer saline (PBS). The cell pellet was suspended in PBS and kept in ice before the intrahepatic injection.

2.2.2 Establish of orthotopic tumor model

Male BALB/c athymic (*nu/nu*) nude mice (4-5 weeks) were provided by the animal house of the Chinese University of Hong Kong and acclimated to laboratory conditions one week before tumor implantation. Nude mice were kept under pathogen-free conditions, fed standard food, and given free access to sterilized water. Housing and all procedures were performed according to protocols approved by the Animal Experimentation Ethics Committee (AEEC) of our institute. The institutional guideline for the

welfare of animals was strictly followed during the study (AEEC No.: 06/006/ERG and 04/042/ERG; No. and date of licence to conduct experiments: (08-99) in DH/HA&P/8/2/1 Pt.2 9/12/2008-8/12/2010 issued by Department of health, the government of the Hong Kong special administrative region).

The procedure for establishment of orthotopic liver tumor model followed the previous description (Yao *et al.*, 2003). Hep3B cells were injected into the liver of nude mice to establish xenografts. Briefly, the mice were anesthetized by intra-peritoneal (i.p.) injection of 80 mg/kg Ketamine and 10 mg/kg Xylazine (Alfasan, Woerden, Netherlands). After anaesthesia, nude mice were placed in a supine position. A small transverse incision below the sternum was made to expose the liver. Hep3B cells (2×10^6), suspended in 50 μl of PBS, were slowly injected into the upper left lobe of the liver, so that a transparent bleb of cells could be seen through the liver capsule. After injection, a small piece of sterile gauze was placed on the injection site, and light pressure was applied for 1 min to prevent bleeding. The abdomen was then closed with a 6-0 silk suture. Gross tumors were formed four weeks after tumor cell implantation. Two independent experiments were performed with non-AFP producing SK-HEP-1 and DLD-1 cells. Each mouse was inoculated with 2×10^6 SK-HEP-1 or 3.5×10^6 DLD-1 cells in liver as control experiments.

2.2.3 Administration of Ad/AFPtBid and chemotherapeutic agents to tumor-bearing mice

As shown in Fig 2.1, four weeks after Hep3B cell implantation, the mice were randomized into six groups: (i) Ad/AFPLacZ, (ii) Ad/AFPtBid, (iii) 5-FU and Ad/AFPLacZ, (iv) 5-FU and Ad/AFPtBid, (v) Dox and Ad/AFPLacZ, and (vi) Dox and Ad/AFPtBid. The first group was treated with Ad/AFPLacZ as control. The administration of 1×10^{10} plaque forming unit (pfu) of Ad/AFPtBid via tail vein was applied every 2 days for a total of 3 times under sterile conditions. 5-FU (Sigma-Aldrich, St. Louis, MO) was administrated i.p. at 40 mg/kg for 3 consecutive days after the first injection of Ad/AFPtBid. Dox (Toronto Research Chemicals, North York, ON, Canada) was administrated i.p. at a single dose of 10 mg/kg immediately after the first injection of Ad/AFPtBid. Mice were continuously monitored for their activity until they were euthanized and humanly killed four weeks after treatment. The liver was removed intactly and examined for tumors. Tumors were measured in two orthogonal dimensions by external caliper and volume was estimated by the formula [length (mm) × width $(mm)^2$]/2 (Carlsson *et al.*, 1983). Liver tissues were subjected to H&E staining and immunohistochemical detection of PCNA and AFP. *In situ* determination of apoptosis was performed using Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) staining (Miao *et al.*, 2006). Another batch of mice as grouped above was observed for survival over a six-month period after tumor cell injection. Mortality was recorded for all animals.

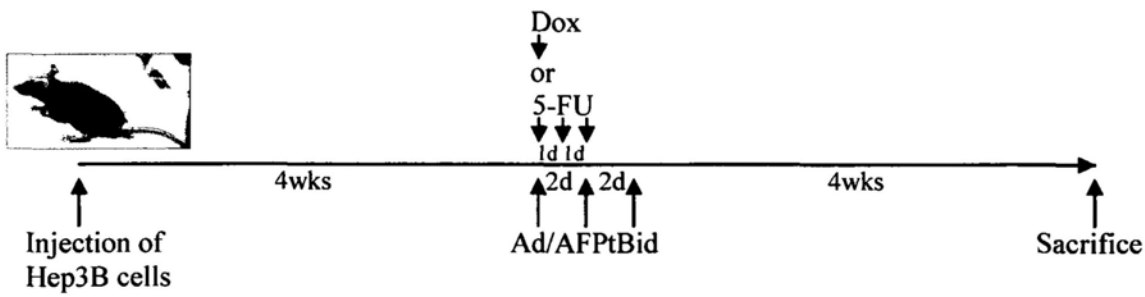


Fig 2.1 Experimental schedule.

2.2.4 Serum AFP Measurement

Blood samples (10 μl) were collected from the tail vein weekly. Serum samples were kept at –80°C and the levels of AFP in the serum were determined by ELISA kit (GenWay Biotech, San Diego, CA) to monitor tumor progression.

2.2.5 Immunohistochemical Staining

The preparation of the tissues and the immunohistochemical staining of PCNA and AFP were performed according to the manufacturer's instructions (The Vector® M.O.M.™ Immunodetection Kit, Vector Laboratories, Burlingame, CA). Briefly, tissues were fixed in 10% neutral buffered formalin solution, underwent tissue processing and embedded in paraffin. Formalin fixation before embedding was less than 30h throughout, which is important for preserving the antigenic determinants. Tissues were sectioned at 5 μm thickness. Staining methods were as follows. Tissue sections were deparaffinized and rehydrated through three changes of

xylene and graded alcohol. After tissue sections were boiled in citrate-based antigen unmasking solution and cooled, the endogenous peroxidase activity in tissue sections was quenched with 3% hydrogen peroxide solution for 5 min. Working solution of M.O.M™ mouse Ig blocking reagent was used to block tissue sections for 1h. Afterwards, preparations were incubated with a primary antibody for 30 min at room temperature. Antibodies against PCNA and AFP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody of PCNA and AFP were used at a working dilution of 1:600 and 1:40 respectively. After tissue sections were washed with PBS, a biotinylated anti-mouse IgG was applied for 30 min. After washing with PBS, ABC reagent conjugated with horseradish peroxidase (VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA) was applied for 30 min. Finally, staining was visualized by DAB substrate (DAB Substrate Kit, Zymed Laboratories, South San Francisco, CA). The reaction was stopped by rinsing the sections in tap water. All incubations were done in a humidified environment at room temperature. Finally, sections were counterstained with Mayer's hematoxylin. After dehydration through graded alcohol and being cleared with xylene, they were mounted with DPX permanent mountant. Photos were captured using Nikon microscope equipped with 3CCD camera (DC-330, DAGE-MTI, Canada). Images were analyzed by the MetaMorph Imaging System (Universal Imaging Corp., PA).

2.2.6 Detection of *in situ* apoptosis by TUNEL staining

TUNEL assay was performed according to the manufacture's instructions (Phoenix Flow Systems, Inc., San Diego, CA). Briefly, after deparaffinization and rehydration, tissue sections were incubated with proteinase K for 20 min at room temperature and washed in PBS. The slides were incubated with a mixture of TdT enzyme and Br-dUTP for 60 min at 37°C. After being washed in PBS, the slides were incubated with Biotin~PRB-1 antibody solution for 60 min at room temperature and washed in PBS. The slides were incubated with conjugate for 30 min. To detect TUNEL positive signals, the slides were incubated with a mixture of DAB substrate and hydrogen peroxide/Urea. Slides were examined and the images were recorded by a bright field light microscope. Dark brown staining represented positive reaction. All the experiments were performed in triplicate.

2.2.7 The acute toxicity of Ad/AFPtBid in mice

Six-week old male BALB/c mice were randomized into five groups and each group contained 5 mice. Ad/AFPtBid was administered by (i) single dose of 1×10^{10} pfu, (ii) single dose of 1×10^{11} pfu, (iii) 3 doses of 1×10^{10} pfu every 2 days, and (iv) 3 doses of 1×10^{11} pfu every 2 days. Ad/AFPLacZ was given as control. Three doses of 1×10^{10} pfu adenovirus every 2 days were previously determined to confer clinical benefit to the animals (Miao *et al.*, 2006). Mice were monitored daily for their activity (digestive, respiratory and circulation systems) and weighed every day. On

day 4 and 7 after Ad/AFPtBid administration, blood was collected from tail vein for hematology analysis. At the same time, retro-orbital blood collection was also performed for the assessment of hepatic enzymes Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (CRE) (Beijing King Hawk Pharmaceutical Co., Ltd.). Microscopic examinations of H&E staining of livers were performed to observe the pathological change.

2.2.8 Statistical analysis

The difference in mean of the tumor volume between groups was compared for statistical significance, using the ANOVA test by SPSS 15.0. $P < 0.05$ was considered statistically significant. The survival rate of animals after various treatments was calculated using Kaplain Meier survival analysis.

2.3 Results

2.3.1 Liver tumors formed from intra-hepatic injection of tumor cells

Four weeks after tumor cell implantation, a small tumor (2×3 mm) could be observed on the surface of the lobe where tumor cells were injected. By the end of the study, as shown in Table 2.1, tumors formed in most of the mice (43/59, 72.88%) with Hep3B cell implantation were limited to the injected lobe as a single node, and a clear boundary between the tumor and normal tissues was obviously seen (Fig 2.2). For the rest of the mice (16/59, 27.12%) Hep3B tumors were not limited to the injected lobe but were

found in other lobes as well. With SK-HEP-1 and DLD-1 cell implantation, 50% (8/16) and 40% (8/20) of mice had more than one tumor node in liver respectively. No metastases of Hep3B and DLD-1 to other organs were observed in any of the animal tested. Metastases to abdominal cavity were observed in 31.25% (5/16) mice with SK-HEP-1 tumors (Fig 2.2C).

Table 2.1 Tumor profiles of mice implanted with different cancer cells

Cell line	Ad/AFPLacZ	Ad/AFPtBid	5-FU+ Ad/AFPLacZ	5-FU+ Ad/AFPtBid	Dox+ Ad/AFPLacZ	Dox+ Ad/AFPtBid
Hep3B	8/17	6/22	1/5	2/5	1/5	1/5
SK-HEP-1	4/8	4/8	—	—	—	—
DLD-1	3/10	5/10	—	—	—	—

Note: Denominator represented the number of mice with tumor, numerator referred to the number of mice with more than one tumor node.

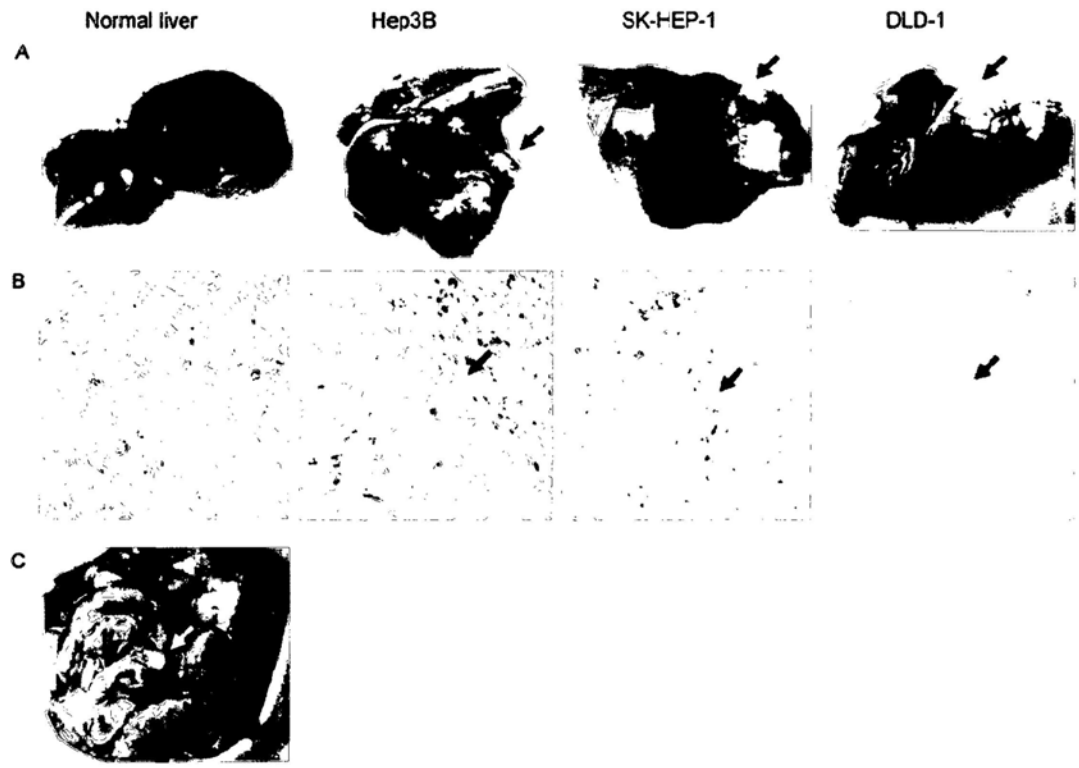


Fig 2.2 Orthotopic tumor in livers of the nude mice.

A, Tumor formed in the liver of nude mice. 2×10^6 Hep3B, 2×10^6 SK-HEP-1 or 3.5×10^6 DLD-1 cells were injected directly into the liver of the nude mice. In normal liver (control), PBS was injected in place of tumor cell implantation. Pictures were taken eight weeks after cell implantation. **B**, H&E staining of liver tumors. Original magnification, ×200. Eight weeks after cell implantation, the implanted lobes of nude mice were collected, fixed with 4% paraformaldehyde, and embedded in paraffin blocks for H&E staining. **C**, Metastases of SK-HEP-1 tumor to the abdominal cavity.

2.3.2 Serum AFP as a surrogate marker for Hep3B tumor progression

AFP was detected by ELISA in cultured Hep3B cells but not in cultured SK-HEP-1 or DLD-1 cells (Table 2.2). *In vivo*, the determination of AFP was started at 5 weeks after Hep3B cell injection and it was weekly monitored until the end of the experiment. The AFP level was elevated with the increased period of tumor cell implantation, suggesting that the AFP level was correlated with Hep3B tumor growth in mice. AFP was significantly reduced following the combination of Ad/AFPtBid and 5-FU or Dox treatments compared with the control group (Fig 2.3A). AFP was also detectable in Hep3B tumor tissues by immunohistochemical staining (Fig 2.3B). However, AFP was not detectable in both blood samples and liver tissue sections from normal mice and mice bearing SK-HEP-1 or DLD-1 tumors.

Table 2.2 AFP production by different tumor cells

	AFP (ng/ml, 6×10^5 cells/48h)	
Cell line	cell lysate	culture media
Hep3B	9.3 ± 0.4	40.8 ± 0.2
SK-HEP-1	0	0
DLD-1	0	0

Note: 6×10^5 cells cultured 48h in 3 ml media, whole cell lysate and culture media were collected for AFP analysis. The assay was performed in duplicates. Values are mean ± SD of three independent experiments.

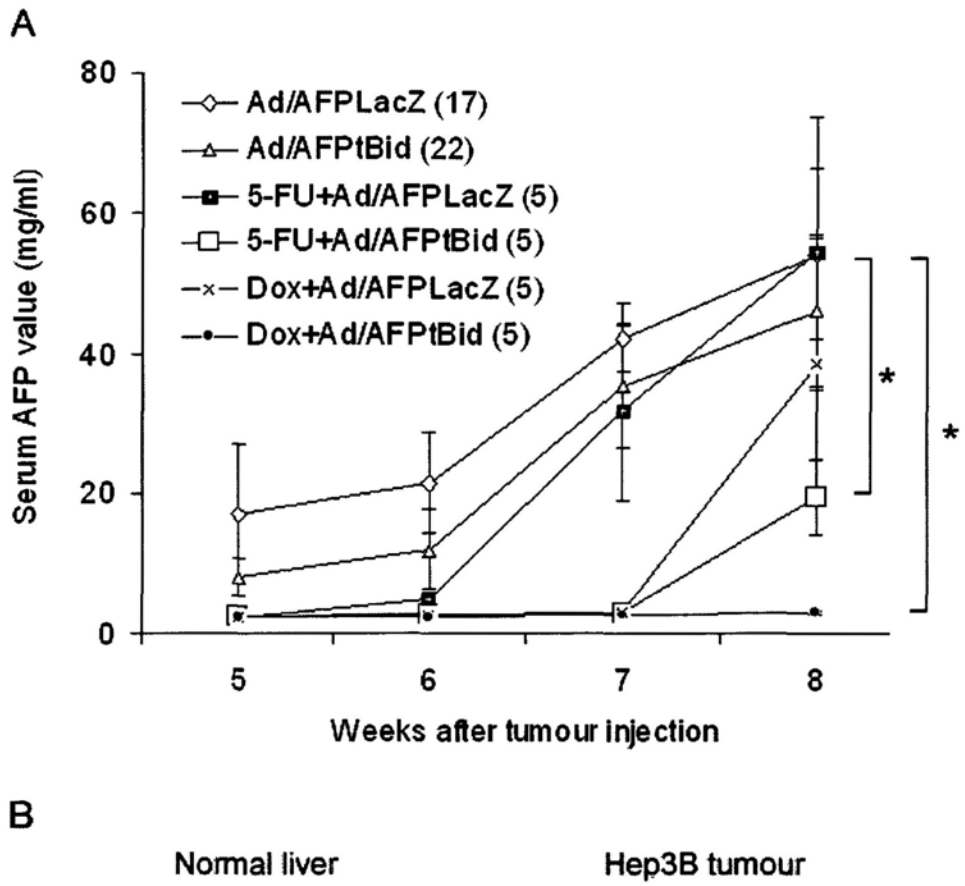


Fig 2.3 Serum AFP as a marker for Hep3B tumor progression.

A, AFP value during Hep3B tumor growth. Note: number in bracket of all figures indicated mice of each group. Values are mean ± SE with * Indicates $P < 0.05$ compared with Ad/AFPLacZ treated group. Error bars, SE (standard error). **B**, Immunohistochemical staining of AFP. Original

magnification, ×200. Strong staining was observed in Hep3B tumors compared with absence in normal liver tissues.

2.3.3 Combination therapy of Ad/AFPtBid with 5-FU or Dox on Hep3B tumor

To evaluate the effect of Ad/AFPtBid and chemotherapeutic agents in combination on orthotopic tumors, Ad/AFPtBid was used alone or in combination with 5-FU or Dox.

The extent of growth delay of the Hep3B xenograft tumors achieved from various treatments is illustrated in Fig 2.4A. Tumor growth was significantly reduced following all treatments compared with the control group. The combination of Ad/AFPtBid and 5-FU was more effective than either agent alone. The tumor grew rapidly in the control animal treated with Ad/AFPlacZ and it reached the volume of approximately 1000 mm^3 at the end of the study. On the other hand, the average size of the tumor was about 380 mm^3 for the Ad/AFPtBid treated group and 11 mm^3 for the combination of Ad/AFPtBid with 5-FU treated group. The average size of the tumor was about 27 mm^3 for the combination of Ad/AFPlacZ and Dox treated group and 17 mm^3 for the combination of Ad/AFPtBid with Dox treated group. Although Dox treatment showed significant effect on tumor regression, the mice exhibited serious weight loss. All animals tested survived the experimental period of 8 weeks. As for SK-HEP-1 and DLD-1 tumors, there were no difference in tumor size between Ad/AFPtBid treated

groups and control group (Fig 2.4B). In addition no significant difference in cell viability was observed between Ad/AFPLacZ and Ad/AFPtBid infected cells in DLD-1 and SK-HEP-1 cells *in vitro* (Fig 2.5). These results indicate that Ad/AFPtBid does not kill the non- AFP producing SK-HEP-1 or DLD-1 xenograft, confirming that Ad/AFPtBid specifically attacks AFP-producing tumors only. Taken together, these studies show that Ad/AFPtBid has a specific and significant anti-tumor effect on AFP-producing tumors. Ad/AFPtBid can significantly sensitize the Hep3B xenografts to 5-FU treatment.

To evaluate the potential mechanisms for tumor repression, we performed H&E staining, immunohistochemical staining and *in situ* TUNEL staining on tumor sections. Hep3B tumor tissues of Ad/AFPtBid alone or combination treatment groups showed a decrease in cells positive for cell proliferative marker PCNA (Fig 2.6A-G and Fig H) but an increase in apoptotic cells in TUNEL staining (Fig 2.6A'-G' and Fig I), compared with the tumor treated with Ad/AFPLacZ (Figs 2.6B and 2.6B'). Inflammatory cell infiltration, which may be an indicator of occurrence of apoptosis-related cell damages, was also increased in the tumor tissues of Ad/AFPtBid-treated mice (Fig 2.6c, e, g).

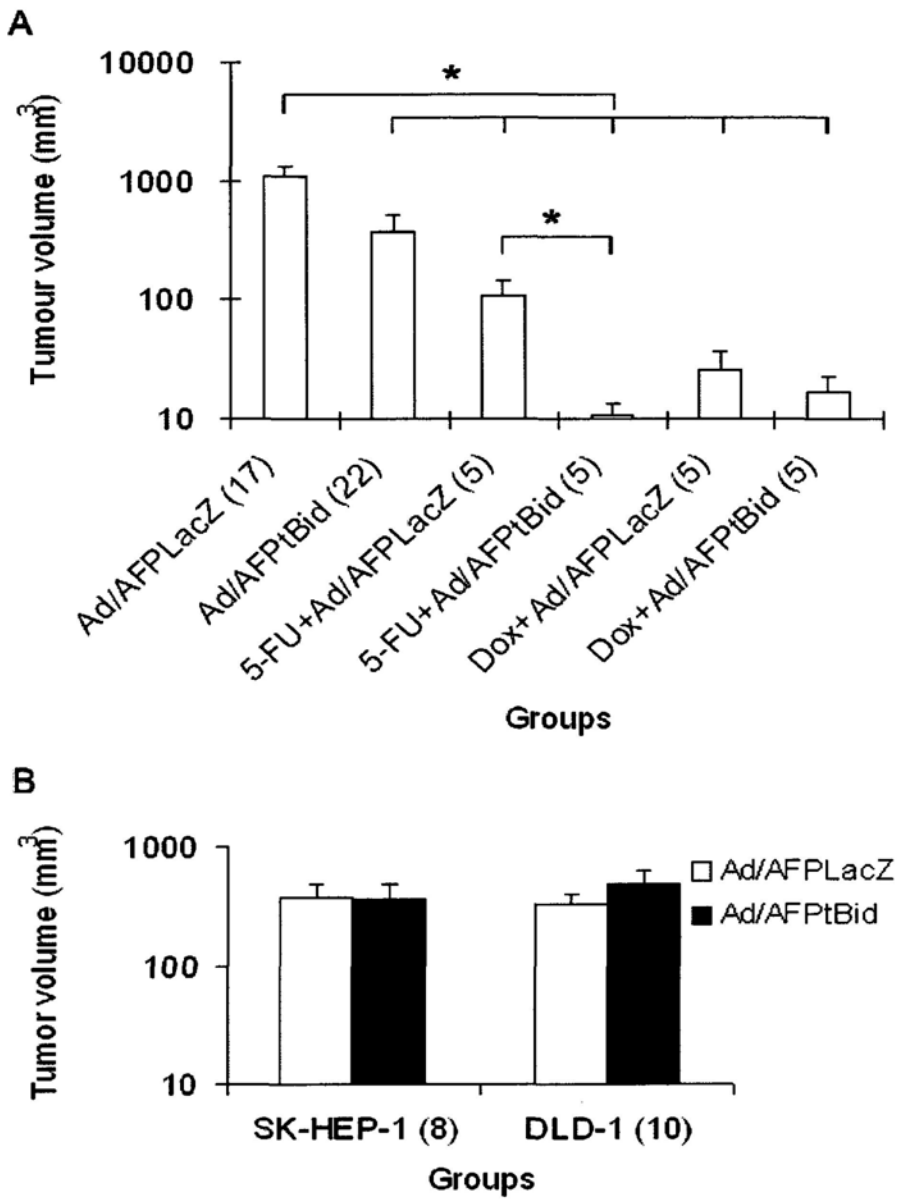


Fig 2.4 Effect of Ad/AFPtBid and 5-FU or Dox in combination on tumors in nude mice.

Number in bracket of all figures indicated mice of each group. **A**, 2×10^6 AFP-producing Hep3B cells were injected into the liver of nude mice. Four weeks after injection, mice were divided into 6 groups and treatments started. Four weeks after the treatment the mice were euthansized and the sizes of tumor were measured. **B**, Two independent experiments were

performed with non-AFP producing human SK-HEP-1 hepatoma cells and DLD-1 colorectal adenocarcinoma cells. Each mouse was inoculated with 2×10^6 of SK-HEP-1 or 3.5×10^6 DLD-1 cells in liver. Ad/AFPtBid treatment was the same as in (A). Values are mean ± SE with * Indicates $P < 0.05$. Error bars, SE (standard error).

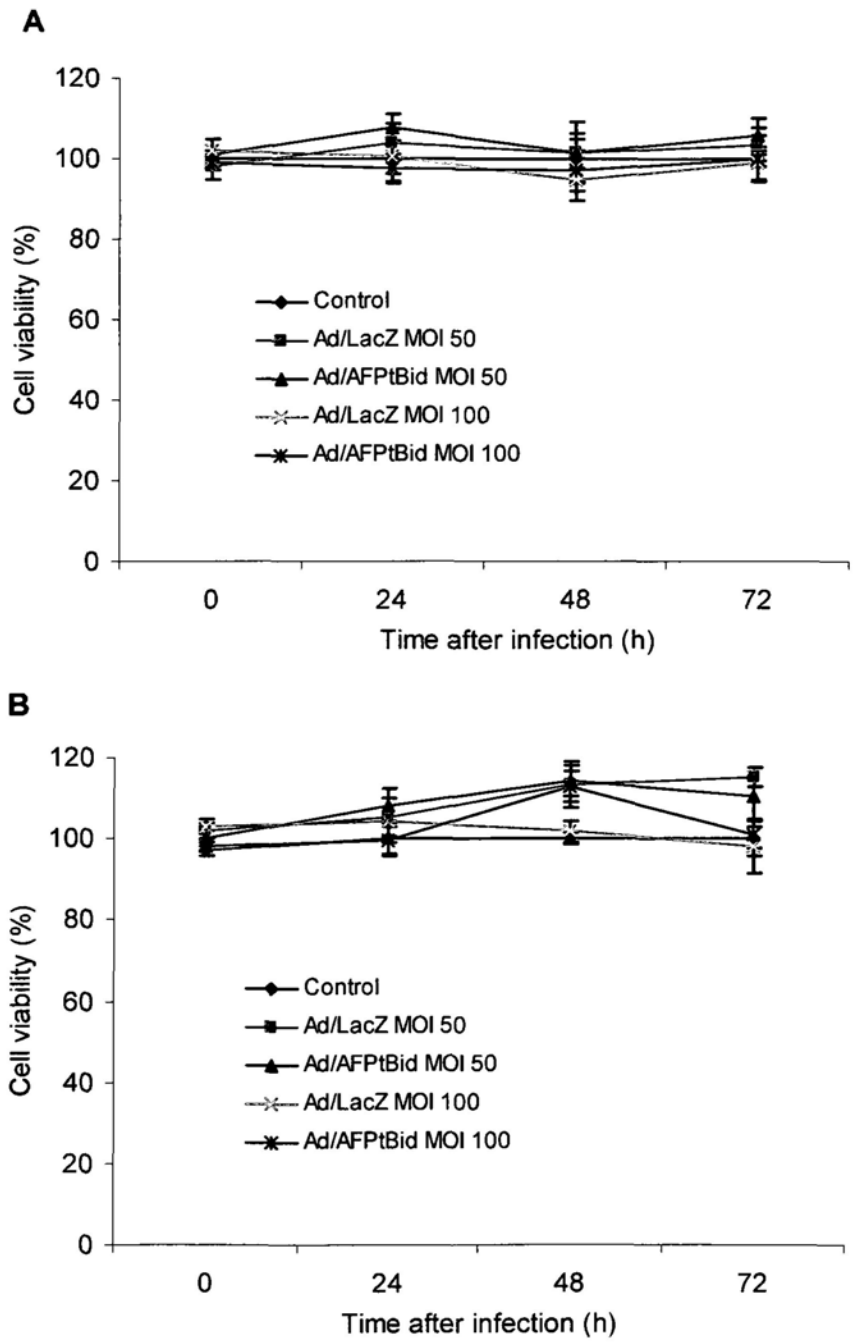
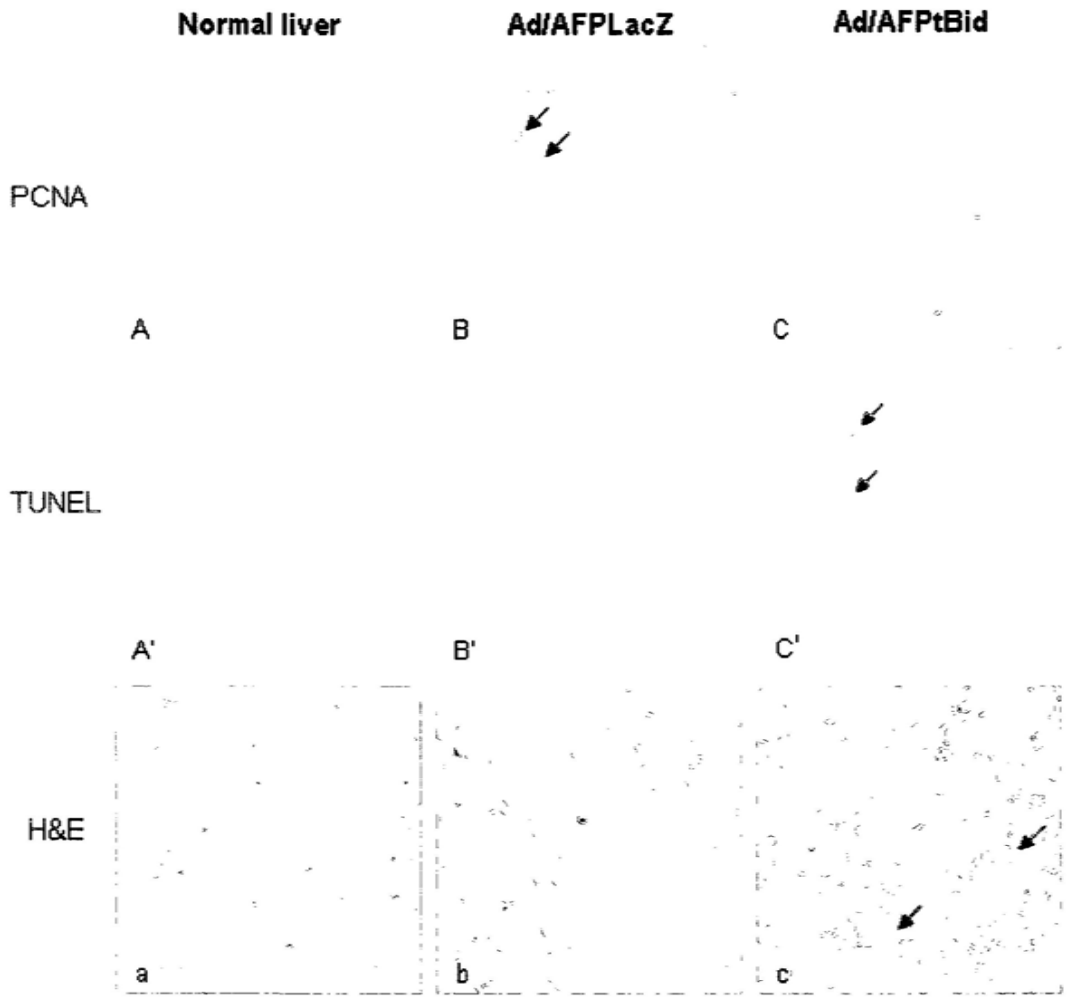
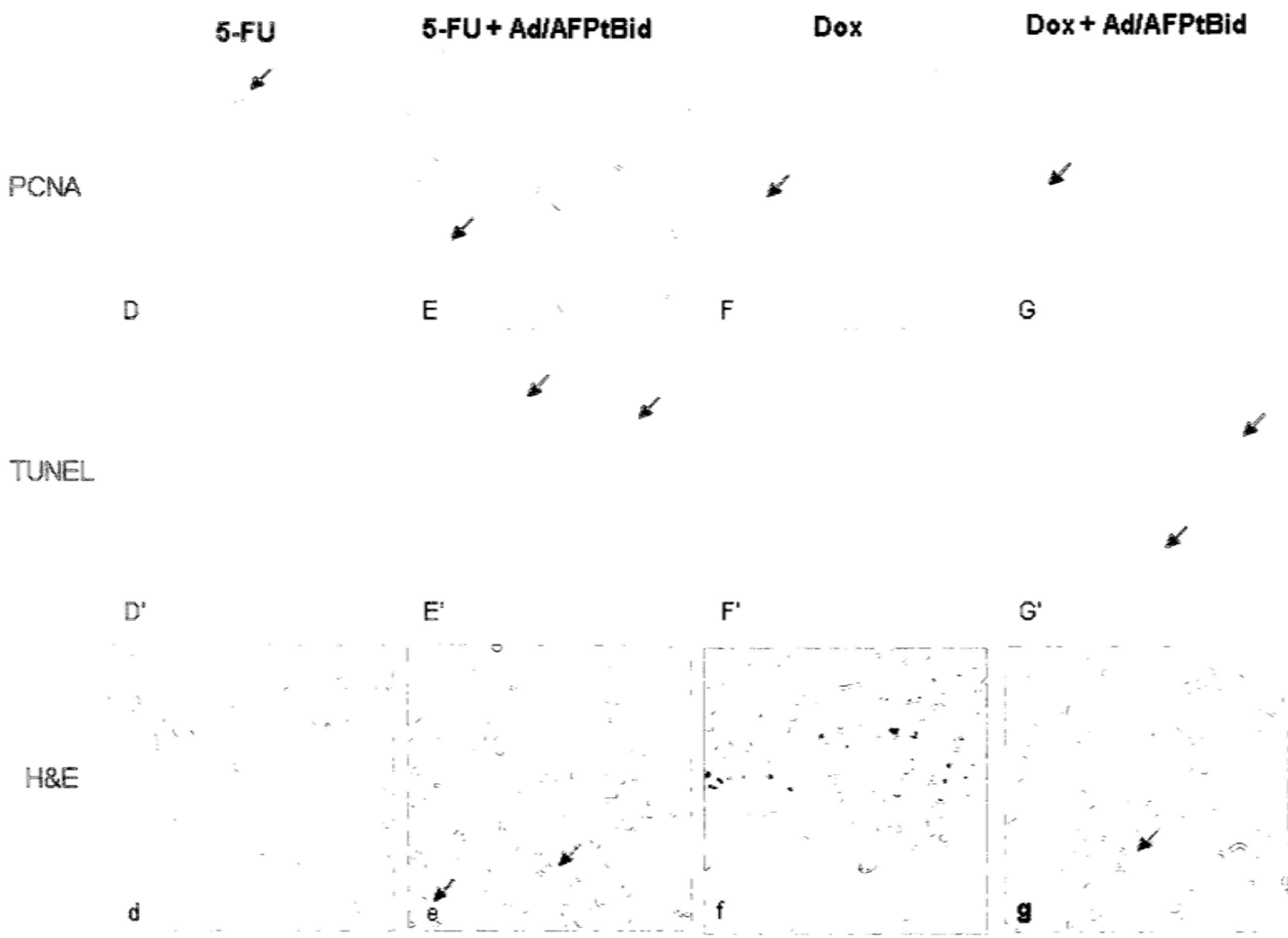


Fig 2.5 Cell viability determined by MTT colorimetric assay after virus infection.

A, DLD-1 and **B**, SK-HEP-1 cells were cultured in 96 well plates and infected with PBS as control, Ad/AFPLacZ or Ad/AFPtBid at MOI of 50 and 100 for 24h, 48h and 72h until MTT was performed. No significant difference in cell viability was observed between Ad/AFPLacZ and Ad/AFPtBid infected cells in DLD-1 and SK-HEP-1 at indicated time points of both MOI. Values are mean ± SE of four separate experiments. Error bars, SE (standard error).


Normal liver
Ad/AFPLacZ
Ad/AFPtBid
PCNA
A
B
C
TUNEL
A'
B'
C'
H&E
a
b
c


5-FU
5-FU + Ad/AFPtBid
Dox
Dox + Ad/AFPtBid
PCNA
D
E
F
G
TUNEL
D'
E'
F'
G'
H&E
d
e
f
g

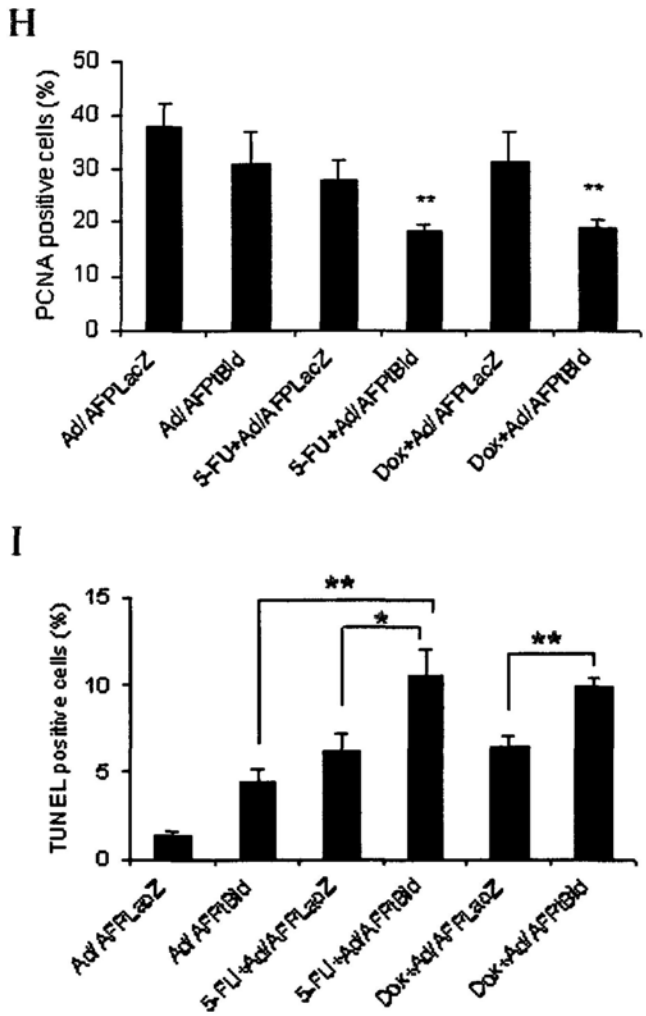


Fig 2.6 Immunohistochemical staining of PCNA, TUNEL staining and H&E staining of Hep3B tumors.

The tumor was harvested 4 weeks after treatments. A-G: Immuno-histochemical staining for PCNA. A'-G': TUNEL staining. a-g: Histology of liver by H&E staining. Original magnification, ×200. H and I: Percentages of cell counts that are either PCNA positive or TUNEL positive. Values are mean ± SE. Error bars, SE (standard error). * indicates $P < 0.05$, ** indicates $P < 0.01$, n=3 per group.

2.3.4 Combination therapy of Ad/AFPtBid with 5-FU or Dox on the survival rate of mice implanted with Hep3B cells

We observed the survival rate of mice receiving various treatments over a six-month period after Hep3B implantation. There were no survivors in the control group and the Ad/AFPtBid in combination with Dox treated group. However, Ad/AFPtBid in combination with 5-FU increased the survival rate compared with the control group (Fig 2.7). On post-mortem of the dead mice in the control group, the sizes of tumors were much bigger than those survived throughout the study period.

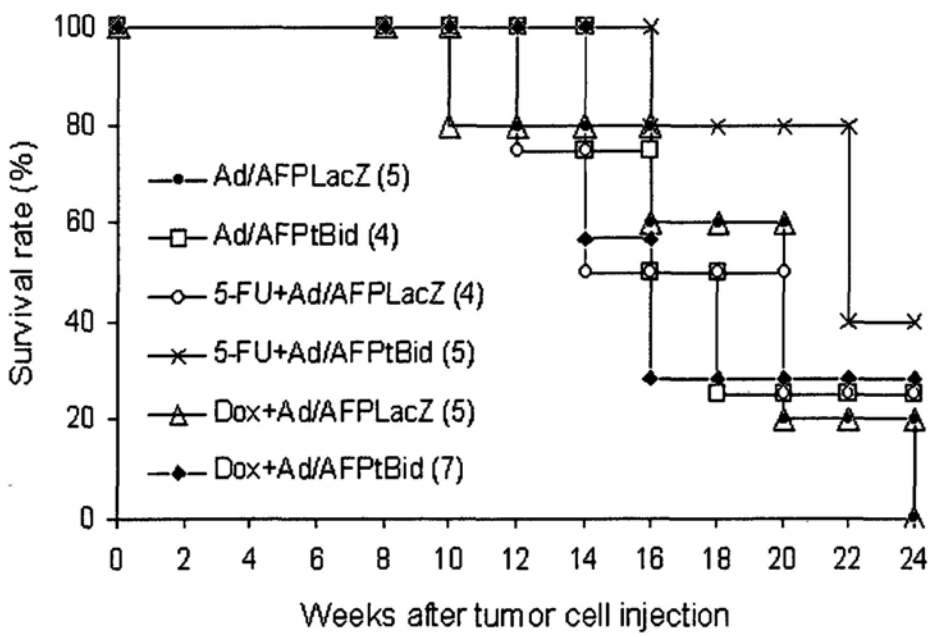


Fig 2.7 Survival rate of mice with orthotopic liver tumor.

Mice with Hep3B implantation were treated with various protocols. Survival rate was calculated six month after Hep3B implantation. Number in bracket of all figures indicated mice of each group. Survival analysis was performed by Kaplain Meier survival analysis.

2.3.5 Assessment of Ad/AFPtBid *in vivo* toxicity

Compared with controls, there were no adenoviral dose-related toxic effects on total white blood cell (WBC) count, WBC differential counts, function of liver (ALT and AST) and kidney (BUN and CRE) (Table 2.3). Differences in mouse daily activity, body weight and microscopic pathology of liver by H&E staining were not found among the groups tested (data not shown).

Table 2.3 Assessment of Ad/AFPtBid *in vivo* toxicity

Parameter	Control	1×10^{10} pfu		1×10^{11} pfu		three doses of 1×10^{10} pfu		three doses of 1×10^{11} pfu	
		4 d mean ± sd	7d mean ± sd	4 d mean ± sd	7d mean ± sd	4 d mean ± sd	7d mean ± sd	4 d mean ± sd	7d mean ± sd
Hemotology									
WBC ($\times10^3$/mm^3)	11.9 ± 2.1	9.6 ± 2.9	11.7 ± 2.6	9.3 ± 1.1	9.7 ± 1.3	14.9 ± 4.0	15.1 ± 4.1	13.4 ± 2.4	15.5 ± 4.8
LYM (%)	67.9 ± 4.6	69.0 ± 7.0	69.0 ± 2.6	63.9 ± 12.1	66.7 ± 4.7	67.6 ± 2.5	64.7 ± 9.7	65.0 ± 7.8	62.5 ± 7.1
NEU (%)	29.4 ± 3.7	28.1 ± 7.4	29.8 ± 2.3	33.6 ± 12.4	30.2 ± 3.4	27.8 ± 2.6	31.2 ± 8.8	30.9 ± 7.7	33.9 ± 5.7
Serum biochemistry									
BUN (mmol/L)	9.92 ± 1.29	8.22 ± 1.39	7.28 ± 1.95	8.98 ± 0.31	8.14 ± 1.29	6.73 ± 1.02	11.2 ± 1.91	7.37 ± 1.07	11.65 ± 0.92
CRE (μmol/L)	7.99 ± 2.66	6.69 ± 5.21	3.92 ± 1.97	15.42 ± 7.27	10.02 ± 7.29	10.51 ± 4.77	19.17 ± 7.45	9.93 ± 7.02	11.05 ± 3.76
ALT (U/L)	11.02 ± 1.57	9.44 ± 9.44	8.81 ± 12.34	7.55 ± 3.21	11.33 ± 6.17	27.7 ± 13.85	18.26 ± 11.51	17.0 ± 4.27	10.7 ± 8.35
AST (U/L)	53.51 ± 9.44	19.94 ± 8.26	17.0 ± 9.46	12.59 ± 5.27	8.18 ± 4.27	22.66 ± 13.41	15.11 ± 2.36	14.48 ± 9.03	19.52 ± 6.42

Note: n = 5 each group. Abbreviation: White blood cell (WBC), lymphocyte (LYM), neutrophil (NEU), blood urea nitrogen (BUN) and creatinine (CRE), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST)

2.4 Discussion

There are several methods to establish HCC tumor in nude mice, all having their advantages and disadvantages. Tumor formed by subcutaneous injection of tumor cells in nude mice is the most common tumor model used. This model is easily created and requires only minimal technical skills. However, this model may not be representative of naturally occurring HCC (Fidler, 1990). Intrahepatic HCC tumors can be established by intrasplenic or intraportal injection of HCC tumors cells. Thus the tumors are formed throughout the liver. However, this model is poorly reproducible due to the technical skills required and the relatively high perioperative mortality (Osada et al., 1996). By injection of tumor cells into the tail vein, HCC tumors can be formed in liver with frequent metastases in the lung as well as in lymph nodes. This model is suit for study of highly metastatic HCC (Ogawa et al., 2001).

In our study, we established orthotopic HCC tumor model in nude mice. The tumor growth is in the same environment milieu as a naturally occurring tumor. Orthotopic xenotransplant model is a useful tool to study liver cancer. It had high fidelity to the actual environment in liver, where a portal system and drug-detoxifying enzymes exist. The organ environment has profound effects on the response of tumor cells to chemotherapy. Studies have shown that response to chemotherapeutic agents varies depending on whether the tumor was ectopic or orthotopic (Wilmanns *et al.*, 1992). The similarity between our *in vivo* mouse model and human HCC is

reflected by the following observations. First, intra-hepatic metastases were found in our model. The intra-hepatic metastasis is an important and frequent event in HCC, which accounts for tumor recurrence and low survival rate (Iizuka *et al.*, 2003, Huang *et al.*, 2005b). Second, our SK-HEP-1 orthotopic tumor is able to metastasize to extra-hepatic organs. Extra-hepatic metastasis also occurs in HCC (Kanda *et al.*, 2008). Third, in our model the tumor growth positively correlates with the level of AFP in serum. AFP is known to be expressed in majority of human HCC but not detected in normal hepatocytes (Peng *et al.*, 1993). It is a valuable marker for not only the diagnosis of HCC but also the monitor of therapeutic response (Trevisani *et al.*, 2001, Marubashi *et al.*, 2007). Therefore, our model highly resembles human HCC and it is a suitable *in vivo* model to study the efficacy of anti-HCC agents.

Ad/AFPtBid is a recombinant replication defective adenovirus, which carries pro-apoptotic tBid gene driven by a hepatoma specific AFP promoter (Miao *et al.*, 2006). Recombinant adenoviruses have gained increasing usage in gene therapy because the vector system combines a high rate of gene transduction that is not cell cycle dependent and the safety of transient expression (Wills *et al.*, 1994). Bid is a pro-apoptotic Bcl-2 family protein, tBid is its activated form. They link the death signals between death receptor pathway and mitochondrial pathway (Yin, 2000). Hepatocytes require a Bid (tBid)-dependent mitochondrial feed-forward amplification loop that releases cytochrome c, oligomerizing Apaf-1 and

caspase 9 to activate sufficient effector caspases to execute apoptosis (Ding *et al.*, 2004, Li *et al.*, 2002, Yin *et al.*, 1999, Zhao *et al.*, 2003). Our previous *in vitro* study demonstrated that the application of Ad/AFPtBid specifically killing Hep3B cells that are known to produce AFP, and significantly sensitized Hep3B cells to 5-FU and Dox (Miao *et al.*, 2006, Miao *et al.*, 2004). In this study we confirmed that Ad/AFPtBid could specifically suppress the AFP-producing HCC but not attack non-AFP-producing tumor in an orthotopic mice model. Application of Ad/AFPtBid can significantly sensitize the effect of other chemotherapeutic agents such as 5-FU, resulting in a synergistic interaction between 5-FU and Ad/AFPtBid in our model. The effectiveness of the combination therapy of 5-FU and interferon-α on advanced HCC has been reported (Sakon *et al.*, 2002, Patt *et al.*, 2003). It is noted that the dose of 5-FU used in this study is 40 mg/kg daily for 3 days, which is much less than the amount of 5-FU used in other cases. Generally, 5-FU is given at 60-66 mg/kg daily in treatment of either human HCC or gastric tumors in animals (Boucher *et al.*, 2002, Ishikawa *et al.*, 1999). Besides the application of 5-FU is often associated with some side effects including myelo-suppression, gastrointestinal and skin toxicity (Macdonald, 1999). However, due to the low dose of 5-FU used, these toxic effects were not found in our mice model. Therefore, the combination of Ad/AFPtBid and 5-FU can achieve potent anti-tumor effect while reducing the possible side effects associated with 5-FU. We also tested the combination effect of Ad/AFPtBid and Dox on HCC. The dose of Dox we used is conventional dose in treatment of

HCC (Li *et al.*, 2001b). The combination of Ad/AFPtBid with Dox or Dox treatment alone showed significant effect on tumor regression. However, the mice in the combination of Ad/AFPtBid with Dox treated group failed to demonstrated better therapeutic response than Dox treatment alone group because the mice that received Dox exhibited serious weight loss in this study. It is known that Dox can result in heavy body weight loss (Klimtova *et al.*, 2002, Nazeyrollas *et al.*, 2003). Further experiments using lower dose of Dox are needed to explore the possibility of its combination with Ad/AFPtBid for treatment of HCC.

Hep3B tumors showed significant number of TUNEL-positive cells after treatment with Ad/AFPtBid, indicating that Ad/AFPtBid induced tumor regression through its pro-apoptotic effect. Our previous study demonstrated that the activities of cytochrome c and subsequent caspase 8, caspase 9, caspase 3 were significantly increased in Ad/AFPtBid-infected Hep3B cells (Miao *et al.*, 2006). Studies have shown that 5-FU executed its therapeutic effect through activation of caspases in colorectal, pancreatic and liver cancer cells including Hep3B HCC cells. 5-FU treatment can also cause a significant increase in the cleavaged Bid in Hep3B cells (Miao *et al.*, 2004, Ikebukuro *et al.*, 2000, Xu *et al.*, 2002b). In addition, this sensitization of HCC cells was accompanied by lymphocyte infiltration. These findings are in accordance with the study by Geoerger *et al* which showed that anti-tumor effects *in vivo* were associated with apoptosis induction and tumor inflammatory cell infiltration (Geoerger *et al.*, 2004).

Usually infection by an adenoviral vector encoding apoptosis-inducing molecules can induce potent inflammatory reactions that contribute to regression of malignancies (Arai *et al.*, 1997, Lamfers *et al.*, 2009).

The other important finding of this study is that Ad/AFPtBid does not produce any obvious side effects in term of the activity of mice, the blood cell number, liver and renal functions. However, data obtained by Choi *et al* showed a dose-dependent toxicity response to systemically administered adenoviral vectors (Choi *et al.*, 2004). Our Ad/AFPtBid was specifically designed to kill AFP-producing tumor cells and it did not attack non-AFP-producing cells. This feature of our therapeutic device can leave normal cells intact and thus may contribute to the lack of obvious toxicity in our study. Therefore, Ad/AFPtBid appears to be a relatively safe anti-HCC agent.

Taken together, our findings show that Ad/AFPtBid can effectively and specifically kill AFP-producing liver caner cells *in vivo* and that Ad/AFPtBid can significantly sensitize liver cancer cells to 5-FU. The combination of Ad/AFPtBid gene therapy with 5-FU results in tumor regression, apoptosis, inflammatory cell infiltration and better survival rate in our model. Also, the therapeutic effect was observed as lower vector doses administered with no detectable toxicity. This combination approach may improve the therapeutic benefits of some chemotherapeutic agents such as 5-FU, rendering Ad/AFPtBid a promising alternative tool in

combination with chemotherapeutic agents for treatment of AFP-producing HCC.

Chapter Three

Susceptibility of Hep3B cells in different phases of cell cycle to tBid-mediated apoptosis

3.1 Introduction

It is well established that many widely used apoptosis inducers act in a cell cycle-specific fashion (Pommier, 2004, Huang *et al.*, 2005a). For example, studies have found that Doxorubicin predominantly induced G2/M arrest (Siu *et al.*, 1999, Potter *et al.*, 2002, Supiot *et al.*, 2005), while 5-FU induced G1, S phase arrest depending on the types of tumor cells (Yoshikawa *et al.*, 2001, Lee *et al.*, 2005, Li *et al.*, 2004, Zalatnai, 2005, Lewin *et al.*, 1987).

Bid, the BH3-only pro-apoptotic Bcl-2 family member, is predominantly defined by its pro-death activity. Its activated form caspase-truncated Bid (tBid) activates Bax and Bak, which are required for the release of cytochrome c from mitochondria and the downstream apoptotic program. Bid/tBid connected the death receptor apoptosis pathway to the mitochondrial apoptosis pathway (Ding *et al.*, 2004), and their expressions were closely associated with the sensitivity of HCC cells to chemotherapeutic drugs (Miao *et al.*, 2004, Miao *et al.*, 2006).

Bcl-2 family members have various roles in DNA damage and cell cycle control (Zinkel *et al.*, 2006). Studies have outlined that Bid is involved in the maintenance of genomic stability, and integrating the control of apoptosis, cell cycle arrest and DNA repair (Zinkel *et al.*, 2003, Zinkel *et al.*, 2005, Kastan, 2005). Bid is phosphorylated in the nucleus and plays a role in cell cycle checkpoint control. Bid-deficient mice are known to be

resistant to Fas-induced hepatocellular apoptosis (Yin *et al.*, 1999). Following treatment, $Bid^{(+/+)}$ but not $Bid^{(-/-)}$ myeloid precursor cells (MPCs) arrested and accumulated in S phase (Zinkel *et al.*, 2005). A recent study in HCC cells showed that Bid exhibited dual functions of S phase arrest and sensitization to apoptosis in response to different degrees of etoposide-induced DNA damage (Song *et al.*, 2008). However, a conflicting result showed that Bid had no role in DNA damage- or replicative stress-induced apoptosis or cell cycle arrest in Bid-deficient mice (Kaufmann *et al.*, 2007).

Our previous study demonstrated that Ad/AFPtBid could specifically target and effectively suppress the AFP-producing orthotopic Hep3B liver tumor in mice. The combination of Ad/AFPtBid with 5-FU was more effective in reducing the tumor and increased survival rate of mice than those treated by either agent alone. To elucidate the mechanism responsible for the effect of combination of Ad/AFPtBid with 5-FU on Hep3B cancer cells, we studied susceptibility of Hep3B cells in defined phases of cell cycle to tBid-mediated apoptosis. In this study, we expressed recombinant human tBid protein, synchronized Hep3B cells at defined cell phases, and tested the susceptibility of Hep3B cells at G0/G1, S or G2/M phases to tBid-mediated apoptosis. The results demonstrated that in contrast to 5-FU that arrested Hep3B cells in the G0/G1 phase and significantly reduced cells in G2/M phase, tBid significantly reduced Hep3B cells in G0/G1 phase, and caused cells arrest at G2/M phase. The elucidation of phase specific effect of tBid

points to a possible therapeutic option that combines different phase specific agents to treat HCC.

3.2 Materials and Methods

3.2.1 Cloning of the tBid gene into the expression vector

Human tBid cDNA (Fig 3.1), which was excised from tBid-pLVCT-tTR-KRAB (804-2, constructed in our own lab) by BamHI/SmaI restriction enzyme, isolated by prep gel, was cloned into the multiple cloning site (MCS) of pGEX-6P-3 (Fig 3.2) with the same restriction enzyme digestion to generate pGEX-6P-3_tBid, which allows the expression of p15 tBid as a fusion protein with an N-terminal glutathione-S-transferase (GST) tag. The ligation mixture was then used to transform MJ109. Positive transformants were identified by polymerase chain reaction (PCR). After PCR verification, colonies of MJ109 cells bearing the recombinant expression vector were selected for mini-prep of plasmid. The purified samples were sequenced. After confirmation of correct sequence, the tested plasmid was then transformed into the *E. coli* BL21 expression cells.

```
301 acgatgagct gcagactgat ggccaacccaa gcagccactc ccgcttggga agaatagagg
361 cagattctga aagtcaagaa gacatcatcc ggaatattgc caggcacctc gcccaggtcg
421 gggacagcat ggaccgtagc atccctccgg gcctggtgaa cggcctggcc ctgcagctca
481 ggaacaccag ccggtcggag gaggaccgga acagggacct ggccactgcc ctggagcagc
541 tgctgcaggc ctaccctaga gacatggaga aggagaagac catgctggtg ctggccctgc
601 tgctggccaa gaaggtggcc agtcacacgc cgtccttgct ccgtgatgtc tttcacacaa
661 cagtgaattt tattaaccag aacctacgca cctacgtgag gagcttagcc agaaatggga
721 tggcactgaac ggacagttcc agaagtgtga ctggctaaag cttgatgtgg tcacagctgt
```

Fig 3.1 tBid gene sequence.

3.2.1.1 Restriction enzyme digestion

The tBid-pLVCT-tTR-KRAB (804-2) and the vector pGEX-6P-3 were digested with restriction enzymes BamHI and SmaI (Promega, Madison, WI). The MCS was shown in Fig 3.3. The reaction was carried out in a total volume of 25 μl in the following condition: 8 μl pGEX-6P-3_tBid or vector, 2.5 μl 10-fold diluted bovine serum albumin (BSA) (Promega, Madison, WI), 2.5 μl 10× buffer B (Promega, Madison, WI), 0.8 μl BamHI, 0.8 μl SmaI and desired volume of double distilled water (ddH_2O). The reaction mixture was incubated in 37°C for 3.5h. The digested products were loaded to a 1% agarose gel in TBE for electrophoresis. The size was checked to confirm expression of respective fragments before purified by agarose gel extraction.

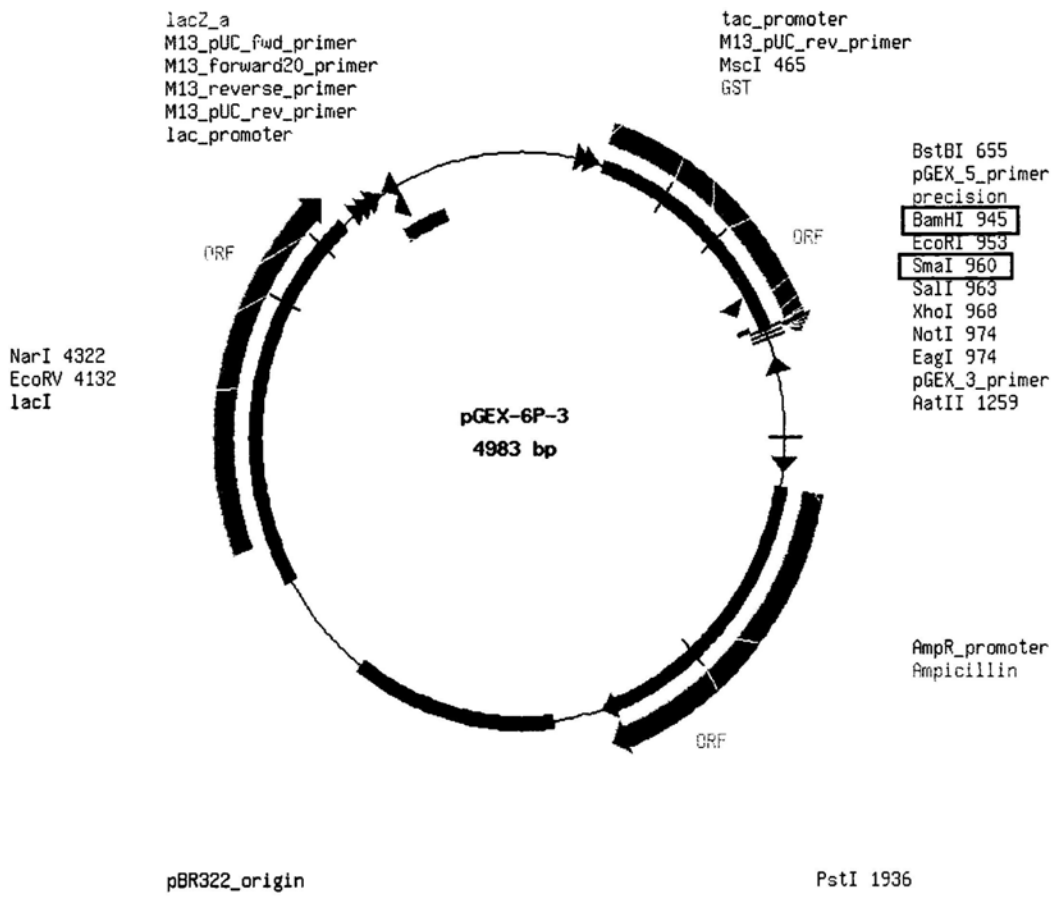


Fig 3.2 Schematic representation of pGEX-6P-3 expression vector

(Adopted from the web site

"https://www.lablife.org/ct?f=c&a=showvecinfo&vectorid=12").

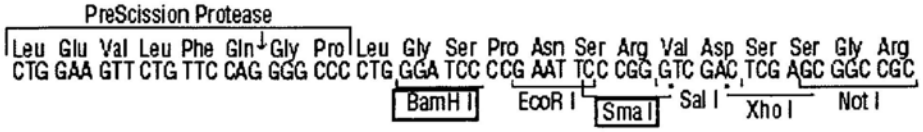


Fig 3.3 Multiple cloning sites of pGEX-6P-3. The restriction enzyme sites used are shown in red boxes.

3.2.1.2 Agarose gel extraction

Electrophoresis of the digested products were done on a 1% (for tBid) and 0.8% (for vector) agarose gel containing ethidium bromide in 1× TBE buffer. QIAquick® Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) was used to purify the product. The DNA fragments were excised from the agarose gel with a clean blade and the slices were weighed in a 1.5 ml eppendorf. Three volumes of Buffer QG was then added to 1 volume of gel and was incubated at 50°C for 10 min with constant vortex every 2-3 min during the incubation. One gel volume of 100% isopropanol was added to the samples after the gel was completely dissolved and mixed. The samples were then applied to the QIAquick columns in 2 ml collection tubes and were centrifuged for 1 min. The flow-through was discarded, 0.5 ml of Buffer QG was added to the same columns and was centrifuged for 1 min. 0.75 ml of Buffer PE with ethanol added before used was added to the columns and centrifuged for 1 min. The flow-through was discarded and the columns were centrifuged for an additional 1 min to remove residual ethanol. The columns were placed into a clean 1.5 ml micro-centrifuge tubes, and finally the DNA was eluted with 30 μl ddH_2O to the center of the QIAquick membrane and the columns were centrifuged at full speed for 1 min.

3.2.1.3 Ligation of vectors and gene of interest

The digested tBid-pLVCT-tTR-KRAB (804-2) and the linearized pGEX-6P-3 were ligated in a mol ratio of 3:1 (insert: vector). A reaction in total volume of 10 μl was done in the following condition: 3 μl digested tBid-pLVCT-tTR-KRAB (804-2), 1 μl pGEX-6P-3, 2 μl 5× ligation buffer, and 1 μl T4 DNA ligase (New England BioLabs Inc., Ipswich, MA). The mixture was incubated at 4°C overnight. pGEX-6P-3_tBid was obtained.

3.2.1.4 Preparation of competent cells for transformation

E.coli strain JM109 (Genotype: *end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′ *tra*D36, *pro*AB, *laq*I^qZΔM15]) (Promega, Madison, MI) was used for transformation of the plasmid. The cells were grown in 100 ml Luria Bertani (LB) medium (10 g Tryptone, 10 g NaCl, and 5 g yeast extract per liter) at 37°C shaker until the stage when OD reached 0.6 at 600 nm. Then, they were separated into 2 tubes and kept at 4°C for 15 min. Cell pellet was obtained by centrifuging the cultures at 3,000 rpm for 5 min. After removing the supernatant, 10 ml ice cold $CaCl_2$ (50 mM, pH 7) were added to each tube and the cells were left on ice for 15 min. Before pelleting, the tubes were poured together and the mixture was centrifuging at 3,000 rpm for 5 min. The pellet was resuspended in $CaCl_2$ (50 mM, pH 7) with 15% glycerol. The mixture was aliquoted into pre-chilled micro-centrifuge tubes and stored at -80°C.

3.2.1.5 Transformation of plasmid into competent cell

The pGEX-6P-3_tBid was transformed into the *E. coli* strain JM109 as described above. Plasmids were introduced into JM109 by $CaCl_2$ mediated transformation. The plasmids were incubated with the competent cells on ice for 20 min. It was then followed by heat shock at 42°C for 5 sec and the DNA-competent cells mixture was put back to ice immediately for 2 min. 900 µl LB medium was added to the cells and incubated at 37°C for 1h. The cells were then centrifuged at 13,000 rpm for 10 sec and were resuspended in 100 µl LB medium with 100 µg/ml ampicillin. They were spread onto ampicillin containing agar plates and incubated at 37°C overnight until single colonies could be isolated.

3.2.1.6 PCR identification of inserted tBid gene

Individual clone was identified by PCR technique, using specific primers: pGEX-5'/pGEX-3' with PCR product ~ 408 bp + 180 bp = 588 bp. The sequences of the primers for amplifying inserted tBid gene were listed as follows:

pGEX-5': GGG CTG GCA AGC CAC GTT TGG TG;

pGEX-3': CCG GGA GCT GCA TGT GTC AGA GG

The amplification was done by using the Expand High Fidelity^{PLUS} PCR System (Roche Diagnostic GmbH, Mannheim, Germany). In a 20 µl PCR reaction, the mixture contained 0.4 µl of primer pairs (0.2 µl from each primer of 100 nM) (Invitrogen, Carlsbad, CA), 6 µl of DNA template (boiled bacterial), 2 µl 10× reaction buffer contains 1.5 mM $MgCl_2$ (Roche

Diagnostic GmbH, Mannheim, Germany), 2 μl 2mM dNTPs and 0.1 μl (5U/μl) of enzyme blend which combined Taq DNA Polymerase and a novel protein that mediated proofreading activity (Roche, Diagnostic GmbH, Mannheim, Germany), and 9.5 μl ddH_2O. PCR program was set as follows:

Steps	**PCR conditions**	**Time**
1	95°C	3 min
2	94°C	15 sec
3	56°C	30 sec
4	72°C	30 sec
5	Repeat step 2 to 4 for 30 cycles	/
6	72°C	10 min

After the PCR, products were loaded to a 0.8% agarose gel containing ethidium bromide in TBE for electrophoresis. The size was checked to confirm expression of interest fragments (Fig 3.4).

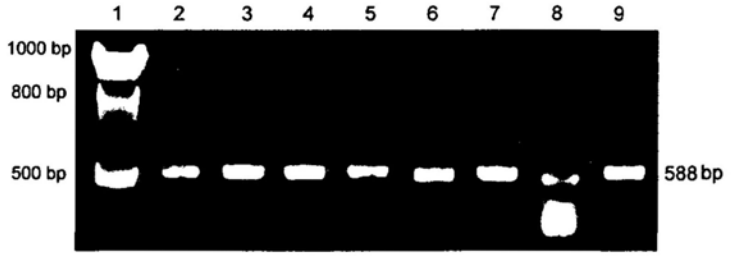


Fig 3.4 PCR product of inserted tBid.

Lane 1: DNA marker; Lane 2-9: PCR of pGEX-6P-3_tBid clones.

3.2.1.7 Plasmid extraction by mini-prep

The plasmids were extracted following the protocol from QIAprep® Miniprep (QIAGEN GmbH, Hilden, Germany). A single colony was picked from each freshly streaked LB ampicillin (LBA) plate and was inoculated in a culture of 4 ml LB medium containing 100 μg/ml ampicillin. They were then incubated for overnight at 37°C with vigorous shaking at 250 rpm. The bacterial cells were harvested by centrifugation at 6,000 rpm in a conventional, table-top micro-centrifuge for 3 min at room temperature. The pelleted bacterial cells were then resuspended in 250 μl Buffer P1 with RNase A solution added and were transferred to a 1.5 ml micro-centrifuge tube. 250 μl Buffer P2 was added to the suspension and was mixed thoroughly by inverting the tubes 4-6 times. And 350 μl Buffer N3 was then added to the lysates and was mixed immediately by inverting the tube 4–6 times. After that, they were centrifuged for 10 min at 13,000 rpm. The supernatants were applied to the QIAprep spin column by pipetting and were centrifuged for 1 min. The flow-through was discarded. Each of the QIAprep spin column was washed by adding 0.5 ml Buffer PB and was centrifuged for 1 min. Then, they were washed by adding 0.75 ml Buffer PE with absolute ethanol added before used, and were centrifuged for 1 min. The residual wash buffer was completely removed by centrifuging for an additional 1 min. The QIAprep column was placed in a clean 1.5 ml micro-centrifuge tube and the plasmid DNA was eluted with 30 μl elution buffer (10 mM Tris·Cl, pH 8.5) added to the center of each QIAprep spin column. The column were allowed to stand for 1 min and centrifuged for 1 min.

3.2.1.8 DNA sequencing of the inserted gene

The sequence of tBid was checked by DYEnamic ET Terminator Cycle Sequencing Kit (GE Medical Systems, Little Chalfont, UK). In each reaction, the following reagents were added: 2 μl (200-500 ng) plasmid DNA as template, 4 μl Sequencing reagent premix, 1 μl (10 pmol/μl) primer, and 3 μl ddH_2O to a total volume of 10 μl. The mixture was placed in a thermal cycler GeneAmp PCR System 9700 for 30 cycles: 95°C for 15 sec, 52°C for 30 sec and 60°C for 1 min. The reaction was terminated at 4°C until purifying.

The purification steps were carried out by using spin columns. The gel material in the columns was tapped to the bottom and 0.8 ml of ddH_2O was added. The upper cap was replaced and the columns were inverted several times to mix the water and gel material. The gel was then allowed to hydrate at room temperature for 2h and was allowed to settle after removing air bubbles by inverting and tapping. The columns were drained completely by gravity after removing the upper and lower end caps and were then inserted into the wash tubes. They were centrifuged at 730×g for 2 min to remove the interstitial fluid and were transferred to 1.5 ml micro-centrifuge tubes. The reaction product was loaded to the top of the gel material and the columns were spun at 730×g for 2 min. The purified samples were collected in micro-centrifuge tubes and were dried in a vacuum centrifuge for 15 min. Each sample pellet was resuspended in 12 μl

of Template Suppression reagent. They were vortexed and spun before heating at 95°C for 2 min to denature. The sequences of the fragments were then read by electrophoresis on the ABI PRISM 377 DNA Sequencer.

3.2.2 Expression and purification of recombinant tBid protein

The GST fusion protein of tBid was produced in *E.coli* BL21 and purified from the cell extracts by affinity chromatography on Gultathione Sepharose 4B column (GE Medical Systems, Little Chalfont, UK) according to the GST Gene Fusion System handbook (Amersham Biosciences). Briefly, a single transformed BL21 colony was grown overnight in 15ml LBA medium at 37°C with shaking at 250 rpm for 12-15h. Five-hundred ml of LB medium was inoculated with the overnight culture and allowed to grow for about 3h at 37°C with shaking at 250 rpm in order to reach an OD 600 at 0.8-1.0. IPTG was added to the culture to a final concentration of 0.1 mM to induce protein expression. After 1h, the culture was harvested by centrifuging at 7,700×g for 10 min at 4°C. The bacterial cell pellets was resuspended in PBS and then were lysed on ice by sonication (Torbeo Ultrasonic Processor 36800; Cole Parmer, London, UK) with five sonication cycles, each consisting of 10 sec pulses at 10 sec intervals. The bacterial lysate was centrifuged at 12,000×g for 10 min at 4°C. The GST fusion protein of p15 tBid was purified from the supernatant by affinity chromatography on Gultathione Sepharose 4B column. Separation of the recombinant protein from the GST moiety was accomplished by site specific proteolysis using PreScission™ Protease (GE Medical Systems,

Little Chalfont, UK). Proteins were stored at -20°C in 50 mM Tris-HCl buffer (pH 8.0).

3.2.3 Detection of tBid protein by SDS-PAGE and Western blot

3.2.3.1 Measurement of protein concentration

Protein concentration was determined by Bradford assay with Coomassie Brilliant Blue G-250 dye (Bio-Rad Laboratories, Inc., Hercules, CA) using bovine serum albumin (BSA) as a standard (Bradford, 1976). Dye reagent was formed by adding 1 volume Dye reagent concentrate with 4 volumes ddH_2O. Five protein standards were prepared by serial dilution of 1.4 mg/ml bovine serum albumin standard to construct the standard curve: 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, 500 μg/ml. 10 μl standards or samples were added to a microplate in triplicate. 200 μl diluted dye reagent was added and was mixed by shaking gently. The plate was incubated at room temperature for at least 5 min, but not more than 1h, then absorbance was read at 595 nm in a microplate reader. Protein concentration of respective samples was calculated from the standard curve.

3.2.3.2 Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was done with Mini-PROTEAN 3 Electrophoresis Module (Bio-Rad Laboratories, Inc., Hercules, CA). Protein samples were diluted with PBS to the desired concentration and 5× SDS loading buffer (60 mM

4× Tris-Cl/SDS, 25% glycerol, 2% SDS, β-ME, 0.1% bromophenol blue) was added. The samples were incubated at 100°C for 5 min. 20 μg sample was then loaded to each lane of a 14% resolving polyacrylamide gel (3.5 ml 40% acrylamide/Bis, 2.5 ml 4× Tris-Cl/SDS, pH 8.8, 4 ml ddH_2O, 10% APS, TEMED). 5% stacking gel was also set above the resolving gel (0.625 ml 40% acrylamide/Bis, 1.25 ml 4× Tris-Cl/SDS, pH 6.8, 3 ml ddH_2O, 10% APS, TEMED). 10 μl Full-Range Rainbow Molecular Weight marker (Amersham Biosciences, Buckinghamshire, UK) was also loaded to the gel as a protein size indicator. The protein was resolved with 120V for 2h.

3.2.3.3 Coomassie Brilliant Blue stain

SDS-PAGE staining solution was prepared by dissolving 0.1g Coomassie Brilliant Blue R-250 (Sigma, USA) in 90 ml 5:4 (v/v) methanol: ddH_2O mixture and 10 ml glacial acetic acid. SDS-PAGE de-staining solution was prepared by mixing 100 ml methanol with 100 ml glacial acetic acid and made up to 1L with ddH_2O.

Gels were stained in SDS-PAGE staining solution at room temperature for 30 min with gentle shaking. The gels were then de-stained in de-staining solution at room temperature with gentle shaking until the background was removed. After rinsing with tap water, the photo of gel (Fig 3.7) was taken by Geliance 600 Imaging System from PerkinElmer.

3.2.3.4 Transfer of proteins to the nitrocellulose membrane

After electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 5 min. The Hybond membrane (Amersham Biosciences, Buckinghamshire, UK), filter paper and fiber pads were also presoaked in transfer buffer. Then, the gel and the membrane were placed between two pieces of filter paper and two fiber pads (avoiding air bubbles formed between the gel and membrane), and were put in a tank containing ice-cold transfer buffer with an ice block. The protein was transferred from the gel to the membrane under 100V for 2h. The whole transfer process was done at 4°C.

3.2.3.5 Immunoblotting of protein

The membrane was equilibrated in Tris-buffered saline (200 mM Tris-Cl, 137 mM NaCl, pH 7.5) with 0.1% Tween 20 (TBST) after protein transfer. It was then blocked in 5% non-fat milk for 1h. After that, the membrane was washed with TBST for 3 times, 5 min each. Then it was incubated with anti-Bid rabbit polyclonal primary antibody (Cell signaling Technology, Inc., Boston, MA) (1: 1000 dilution) at 4°C overnight. After incubation, the membrane was washed with TBST for 10 min and then 5 min each for 3 times before incubated with goat anti-rabbit IgG_1-HRP secondary antibody (1: 2000 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1h. The membrane was again washed with TBST for 10 min, and 5 min each for 3 times. It was then transferred to a flat surface for adding 1.5 ml enhanced chemiluminescence (ECL) system solution, formed by mixing

reagent A and B (Amersham Biosciences, Buckinghamshire, UK). After 1 min incubation, the excess ECL solution was drained away and the membrane was wrapped with Saran-wrap. It was fixed in an x-ray film cassette with protein facing the Hyperfilm™ (Amersham Biosciences Buckingham-shire, UK), and was exposed for 1 min. Finally, the film was developed in the film processor (Fig 3.8).

3.2.4 Activity of recombinant tBid

The biological activity of tBid proteins was assayed by the cytochrome c release assay from isolated Hep3B cell mitochondria (Scorrano et al., 2002).

3.2.4.1 Isolation of mitochondria

Mitochondria were isolated at 4°C according to the instruction of the Mitochondria isolation kit for cultured cells (Pierce Biotechnology, Rockford, IL). Briefly, protease inhibitors were added to Reagent A and Reagent C immediately before use. 2×10^7 cells were collected by centrifuging harvested cell suspension in 1.5 ml eppendorf at 850×g for 2 min and the supernatant was removed. 800 µl Mitochondria Isolation Reagent A was added to the cell pellets and the samples were vortexed at medium speed for 5 sec. The tubes were then incubated on ice for exactly 2 min. Dounce homogenize cells by passage through 21G needle with 1 ml syringe for 20 times. After that, 800 µl of Mitochondria Isolation Reagent C was added to the samples and the tubes were inverted several times to mix

the content. The samples were centrifuged at 700×g for 10 min at 4°C. The supernatant was transferred to new eppendorf tubes and was centrifuged at 3,000×g for 15 min at 4°C. The supernatant was discarded and another 500 µl Mitochondria Isolation Reagent C was added to the pellet. The samples were centrifuged at 12,000×g for 5 min and the supernatant was discarded. The pellets obtained were the isolated mitochondria. Finally, they were resuspended by repeated pipetting in 50 µl PBS pH 7.4. Enriched mitochondria were used in the cytochrome c release assay.

3.2.4.2 Cytochrome c release assay

Assay conditions: Assay volumes were 75 µl and were performed in 0.5 ml micro-centrifuge tubes. Assays require Buffer C containing 25 µg/ml leupeptin, 25 µg/ml pepstatin, 3 µg/ml aprotinin, 100 µM PMSF, and the caspase inhibitor 10 µM Boc-Asp-FMK. Protease inhibitors were added immediately prior to using the buffer.

Incubation of mitochondria with added proteins: Add test proteins to 30 µl in Buffer C. To initiate the cytochrome c release assay, 12 µl of mitochondria and an additional 33 µl of Buffer C were added and the tube was capped. Gently mix the assay mixture for 5-10 sec and incubate in a 30°C water bath for 30 min. Centrifuge samples in a micro-centrifuge at 16,000×g for 5 min at 2 - 8°C. Remove a 50 µl aliquot from the supernatant, transfer it to a new tube, and add 5 µl of 5% Triton-X100. Samples were stored at -20°C in a manual defrost freezer before analysis.

Controls: Two control samples were run for each assay to determine the total amount of cytochrome c that can be released from mitochondria and the amount of spontaneously released cytochrome c from untreated mitochondria. To two tubes add 15 µl of protein dilution buffer and 50 µl of Buffer C. Add 12 µl of mitochondria to each tube and incubate in a 30°C water bath for 30 min. Centrifuge one tube at 16,000×g for 5 min at 2-8°C. Remove a 50 µl aliquot from the supernatant, transfer it to a new tube, and add 5 µl of 5% Triton-X100. This is the background control for spontaneous release of cytochrome c. Do not centrifuge the second tube. Remove a 50 µl aliquot from the reaction mix, transfer it to a new tube, and add 5 µl of 5% Triton-X100. This is the total cytochrome c control. Controls with samples were store at -20°C in a manual defrost freezer before analysis.

Preparation of samples for assay by the Cytochrome c ELISA: Samples were thawed immediately prior to quantifying cytochrome c. The half maximal effective concentration (EC_{50}) for cytochrome c releasing was quantified by using human cytochrome c ELISA Module Set (Bender MedSystems, Inc, CA.). Samples were diluted 150-fold with Assay buffer before analysis.

3.2.5 Cell viability determined by MTT assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to quantify cell proliferation based on the cell viability. To investigate the effect of tBid proteins, Hep3B cells (5×10^3 cells in 100 µl per well) were seeded in 96 well plates (Nunc™ Vernon Hills, Illinois) and incubated overnight to allow cells to attach to the plate. Cells were then incubated with different concentrations of tBid (25, 50, 100, 200, 400, 800, 1600 nM) for 24h 48h, and 72h before the addition of MTT as separate experiments. Having added 10 µl MTT solution (5 mg/ml in PBS, Sigma-Aldrich, St. Louis, MO), cells were incubated for 3h at 37°C in a humidified atmosphere, 5% CO_2. The purple formazan product was solubilized in 200 µl dimethylsulphoxide (DMSO, Sigma-Aldrich, St. Louis, MO). The absorbance at 570 nm of each sample was measured using a reference wavelength of 630 nm with a microplate reader from Molecular Devices (Sunnyvale, CA). The percentage of living cells was defined as the absorbance of the treated cells divided by the absorbance of the control cells and multiplied by 100. Cell proliferation rate of treated cells was compared with non-treated cells. The following formula was used for calculation:

Cell proliferation rate (%) = (Absorbance of treated cells /Absorbance of untreated control cells) × 100%

3.2.6 Cell cycle analysis

Cell cycle

The whole cell cycle is subdivided into mitosis (M phase) and interphase (a phase between cell division). During mitosis, chromosomes are condensed and inactive, whereas at interphase chromosomes are decondensed and transcriptionally active. Interphase is also divided into G1 phase, S phase, and G2 phase. G1 phase is from the end of the previous M phase to the beginning of DNA synthesis and marked by synthesis of enzymes that required in DNA replication. Synthetic S phase is characterized by DNA replication. G2 phase, which follows S phase and precedes M phase is mainly for protein synthesis. Besides the four phases of cell cycle, there is a state of quiescence of cells called G0 phase, on which phase cells are not undergoing proliferation but could be induced to enter the cell cycle from G1 phase. Fig 3.5 showed the overview of the cycles and where chemotherapy comes into play. Most chemotherapy regimens combine drugs of various kinds to use the cell cycle and to overcome the cell cycle.

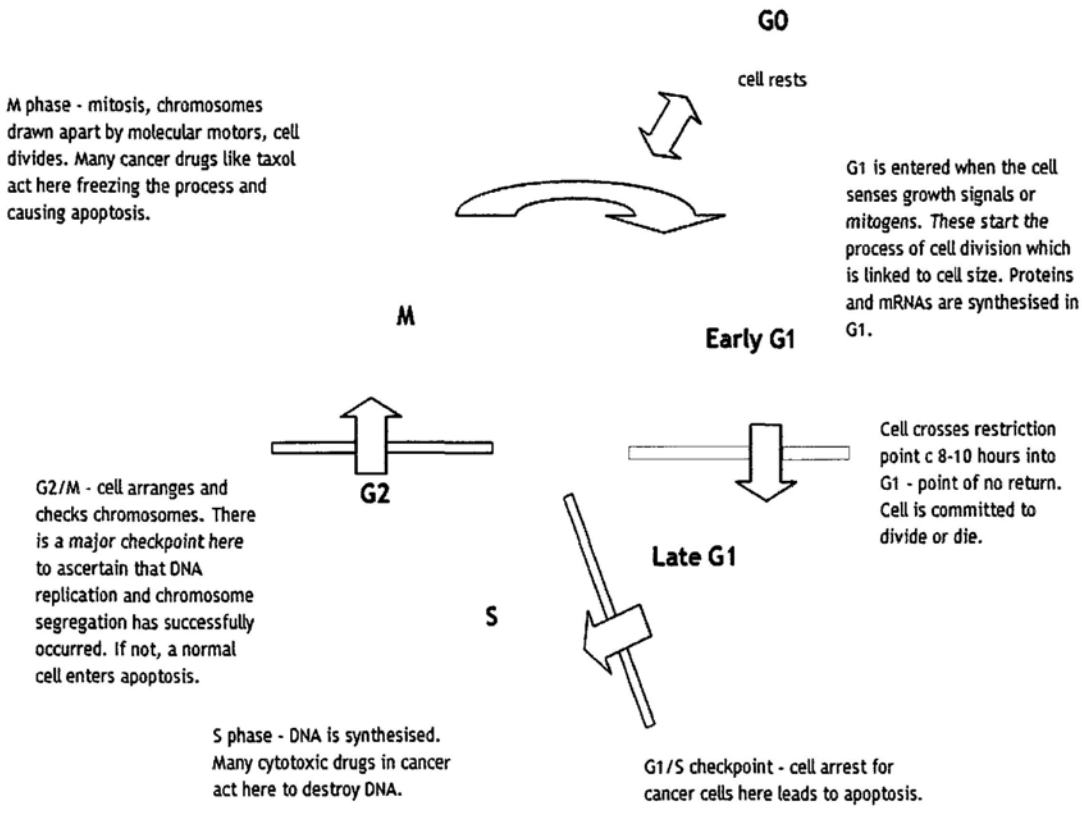


Fig 3.5 Overview of the cycles and where chemotherapy comes into play.

Sample preparation

Cell culture

Hep3B cells (3×10^5 cells in 2 ml per well) were plated in 6-well plates, and after overnight incubation, different concentrations of 5-FU or tBid were added. At the end of incubation period, cells were collected for cell cycle analysis by flow cytometry.

Cell fixation

Medium in each well was collected in a 12×75 mm centrifuge tube. Cells were washed with 1 ml of PBS and the washing was also collected. They

were then detached by 300 µl trypsin and collected to the respective tube. Each tube was centrifuged at 2,000 rpm for 5 min and was washed again with 2 ml PBS. Supernatant was decanted and cell pellets were resuspended in 0.5 ml PBS, pH 7.4. After that, cell suspension was added with 4.5 ml 75% ice-cold ethanol (v/v) slowly with constant vortex. Cells were fixed at -20°C at least overnight before further analysis.

Cell staining

Fixed cells were centrifuged at 2,000 rpm for 5 min and supernatant was decanted. Then cells were washed with 5 ml PBS for two times. After that, cell pellets were resuspended in 0.5 ml of propidium iodide (PI)/RNase A solution and incubated in dark for 30 min at room temperature. Then cells were kept on ice for flow cytometry analysis (Becton Dickinson FACScan). The ratio of cells in the G0/G1, S, and G2/M phases of cell cycle was determined by their DNA content.

Flow cytometry analysis

Cell cycle analysis by flow cytometry is based on the characteristic DNA contents of cells in different growing phases. In the theory, cells in the G0/G1 phases will take up the same amount of DNA-binding fluorochromes and fluoresce in a single channel because they have the same amount and morphology of DNA. Cells in the G2/M phases will take up the double amount of fluorochromes because they have doubled their chromosomes after completing the S phase. Cells in S phase take up

fluorochromes less than those in G2/M phases but more than those in G0/G1 phases and fluoresce in various channels because cells in this phase are undergoing DNA replications and it is impossible for all the cells keep in the same pace of progress. Therefore, there are two peaks showed by flow cytometry respectively inferring two groups of DNA amounts of cells in different cell cycles. Using PI as a DNA binding dye, the following profile should be obtained.

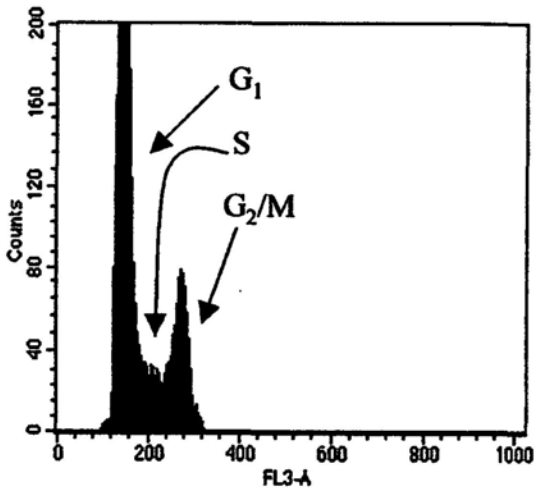


Fig 3.6 Determination of cell cycle distribution by flow cytometric analysis. Histogram representing PI staining (FL3-A).

3.2.7 Western blot analysis of cell cycle-related proteins and caspases

Cells were lysed in RIPA buffer containing protease inhibitors (0.1 mg/ml PMSF, 30-fold diluted Aprotinin and 1 mM sodium orthovanadate). The protein extracts (20 μg) were electrophoresed on 12% SDS-PAGE gels and transferred onto Hybond membranes (Amersham Biosciences, Buckinghamshire, UK) by electroblotting at 100 V for 2h. The membranes

were blocked in 5% nonfat milk and then were incubated with the designated primary antibodies at 4°C overnight, followed by incubation with a HRP-conjugated secondary antibody for another hour. After the membrane was washed with Tris-buffered saline containing Tween-20, the reaction was visualized by means of the ECL reagent kit (Amersham Biosciences). The membrane was exposed to X-Ray films for a period ranging from 30 sec to 10 min. The antibodies against caspase 9, caspase 3, cdc2 p34, p-cdc2 p34 (Tyr 15), cyclin A, cyclin B1 were obtained from Cell signaling (Cell Signaling Technology, Danvers, MA). The antibody against cyclin D1 was obtained from Abcam (Abcam plc, Cambridge, UK). The antibody against cyclin-dependent kinase 2 (Cdk2) was obtained from Chemicon (Millipore, Billerica, MA). The antibodies against p-cdc2 p34 (Thr 161) and actin were obtained from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA).

3.2.8 Assessment of apoptosis by TUNEL assay

APO- BRDU™ Flow cytometry kit for apoptosis (Phoenix Flow Systems, Inc., San Diego, CA) was employed. Cells were fed with the tBid media. At 24h after incubation, cells were harvested by trypsinization. After the cells were washed, resuspended, fixed and stained in staining solution which was a mixture of TdT Reaction Buffer, TdT Enzyme, Br-dUTP, and distilled water for 60 min at 37°C in a temperature controlled bath and were shaken every 15 min. After staining, cells were washed with 1 ml Rinse Buffer for two times. Br-dUTP labeled DNA breaks in apoptotic cells can be detected

by flow cytometry. Apoptosis was measured according to the protocol provided by the kit and the results were presented as percentage of apoptotic cell.

3.2.9 Synchronization of Hep3B cells at defined cell cycles

3.2.9.1 By chemicals

Hep3B cells were synchronized at G0/G1 phase by incubation in serum-free media for 48h. Cells in serum-free medium were synchronized at S phase by two methods. Method one, cells were incubated with 0.2 μg/ml aphidicolin (AP, MP Biomedicals, LLC, Santa Ana, CA) for 24h, washed with PBS for two times, and released in DMEM plus 10% FBS for 1h. Method two, cells were incubated with 0.5 mM hydroxyurea (HY, MP Biomedicals, LLC, Santa Ana, CA) for 12h, washed with PBS for two times, and released in DMEM plus 10% FBS for 6h. Cells in serum-free medium were synchronized at G2/M phase by 0.2 μM etoposide (ET, MP Biomedicals, LLC, Santa Ana, CA) for 24h. Synchronization was confirmed by PI staining and flow cytometry (see above). The concentrations of AP, HY, and ET were found to be without discernible biological effect in Hep3B cells, as cell viability after release from block was routinely over 95%. Synchronized cells were used for experimentation immediately to prevent cells from reentering the cell cycle.

3.2.9.2 G1 phase enrichment by fluorescence-activated cell sorter (FACS)

3.2.9.2.1 Lipofectamine transfection

The human Hep3B cells were transiently transfected with a cDNA encoding Histone H2B and green fluorescent protein (H2B-GFP) (Pharmingen) using the Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). 5×10^5 cells were seeded into 60 mm culture plate with complete medium one day before transfection. For each transfection sample, 4 μg of plasmid DNA was diluted in 100 μl Opi-MEM without serum and mixed. Also, 10 μl Lipofectamine™ 2000 was diluted in 100 μl Opi-MEM and mixed. The diluted DNA and diluted Lipofectamine™ were mixed together and incubated at room temperature for 25 min. The complete medium was removed from the cells, after washing two times by PBS, replaced with 800 μl Opi-MEM. The diluted DNA-Lipofectamine complexes were then added to the wells and mixed gently. The cells were incubated in a CO_2 incubator at 37°C for 4-6h. After the incubation, the transfection medium was aspirated and replaced with fresh, complete medium for growth.

3.2.9.2.2 Construction of H2B-GFP stably expressing Hep3B

Cells were incubated at 37°C with 5% CO_2 for 48h after transfection. They were then trypsinized and transferred to a 100 mm culture dish with selective medium. They were selected in blasticidin (1 μg/ml, MP Biomedicals, LLC, Santa Ana, CA). And the selective medium was

changed twice a week for a total period of about 2 weeks until single colonies could be seen and isolated. The dead cells and debris were removed at the same time. A few colonies from monolayer culture from each plate were isolated with paper discs and were transferred to 24-well plates with selective medium for clonal expansion. H2B-GFP stable Hep3B cell line was obtained.

3.2.9.2.3 FACS-purification

FACS-purification of cells with defined chromatin content was based on their H2B-GFP-dependent fluorescence (FL1, DNA content) and forward scatter characteristics (FSC, size).

3.2.10 Cell phase specific effect of tBid on synchronized Hep3B cells

3.2.10.1 Effect on cell cycle progression and protein expression

Synchronous (at G0/G1, S, and G2/M phase) or asynchronous Hep3B cells were exposed to 200 nM tBid. The working concentration of tBid was calculated based on the typical half maximal effective concentration (EC_{50}) for cytochrome c releasing. Then cell cycle progressions were monitored for 24h. At 0h, 8h, 16h, and 24h aliquots of culture were sampled for flow cytometric analysis and Western blot analysis.

3.2.10.2 Effect on cell viability

Synchronous or asynchronous Hep3B cells were exposed to 200 nM tBid at 37°C for 24h or 48 h. Then cell viability was determined based on the reduction of MTT dye as previously described (Miao *et al.*, 2004). The inhibit effects were expressed as 100% minus the percentage survival of treated cells versus non-treated cells.

3.2.11 Cell phase specific effect of 5-FU

Hep3B cells (3×10^5 cells in 2 ml per well) were plated in 6-well plates and allowed to grow overnight. Then the cells were treated with 20 μg/ml and 50 μg/ml of 5-FU for 12h, 24h, 36h, and 48h at 37°C 5% CO_2 before they were collected for cell cycle analysis by flow cytometry.

3.2.12 Statistical analysis

The differences in the mean of the values between groups were compared for statistical significance using the student's t-test by SPSS 15.0. $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Expression of recombinant tBid protein

Recombinant human tBid (the sequence corresponding to carboxy-terminal fragment of amino acids 61-195 of human Bid) was generated using the

pGEX-6P-3 expression system (GE Medical Systems, Little Chalfont, UK) according to the manufacture's protocol. Then tBid protein was purified from the soluble fraction of disrupted *E. coli*. Purification did not employ detergents. The expression of tBid protein was confirmed by Coomassie Brilliant Blue stain of SDS-PAGE (Fig 3.7) and Western blot (Fig 3.8).

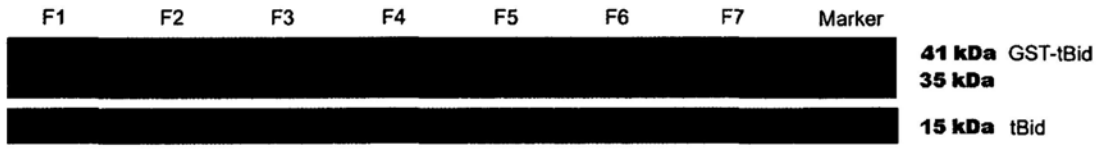


Fig 3.7 SDS-PAGE analysis on the expression of tBid protein.

Upper strip: Fractions collected after supernatant pass Gultathione Sepharose 4B column; Lower strip: Fractions collected after cutting by PreScission™ Protease.

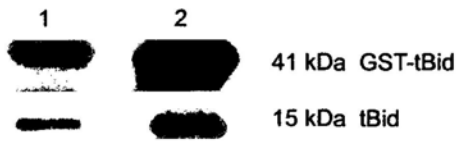


Fig 3.8 Western blot analysis of the expressed tBid protein.

tBid protein in the soluble fraction of the bacterial cell lysate was detected by using Anti-Bid as the first antibody. Upper strip: Fractions collected after supernatant pass Gultathione Sepharose 4B column; Lower strip: Fractions collected after cutting by PreScission™ Protease.

3.3.2 Activity of recombinant tBid

To assess whether purified recombinant tBid proteins were correctly folded and functional, we analyzed the cytochrome c release from isolated Hep3B mitochondria in response to tBid stimulation. The EC_{50} for tBid to release cytochrome c was 200.6 nM (Fig 3.9).

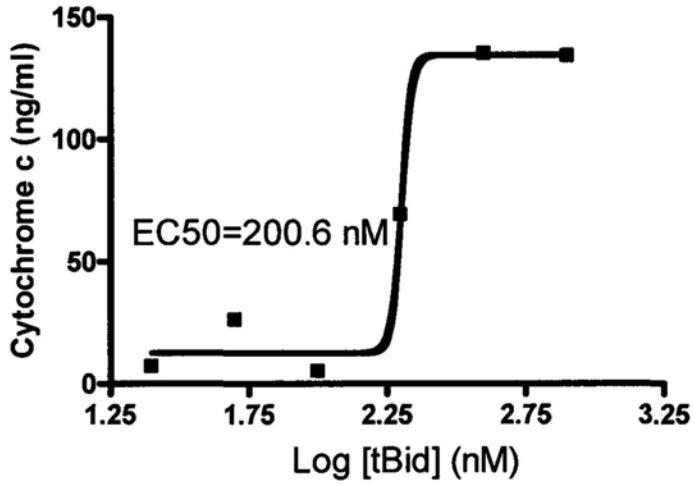


Fig 3.9 Cytochrome c release from the isolated mitochondria of Hep3B cells in response to tBid.

The inhibitory activity of the isolated tBid protein on cell growth was also determined by MTT colorimetric assay. Recombinant tBid inhibited growth of Hep3B cells in concentration- and time-dependent manners (Fig 3.10). Its inhibitory effect on Chang cells was in the same pattern as Hep3B cells but a little weaker (data not shown). This inhibitory effect was in line with other reports showing that the amount of released cytochrome c was proportional to both the incubation time and the concentration of tBid (Luo

et al., 1998, Gross *et al.*, 1999, Madesh *et al.*, 2002). Consistent with the loss of viability, apoptosis determined after 24h tBid treatment was increased in Hep3B cells (Fig 3.11). The level of tBid was increased in Hep3B cells after treatment by different concentrations of tBid for 24h (Fig 3.12).

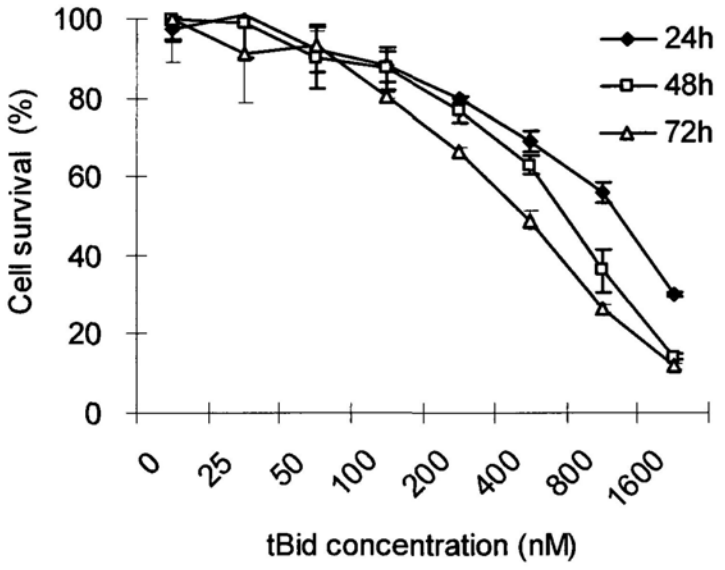


Fig 3.10 Effects of tBid on the viability of Hep3B cells.

Hep3B cells were cultured in 96 well plates and treated with different concentrations of tBid for 24h, 48h and 72h (24h: solid square, 48h: open square, 72h: open triangle) at 37°C until MTT was performed. The viability of cells was expressed as a relative value to that of cells without treatment. Data are the mean ± SE of four separate experiments.

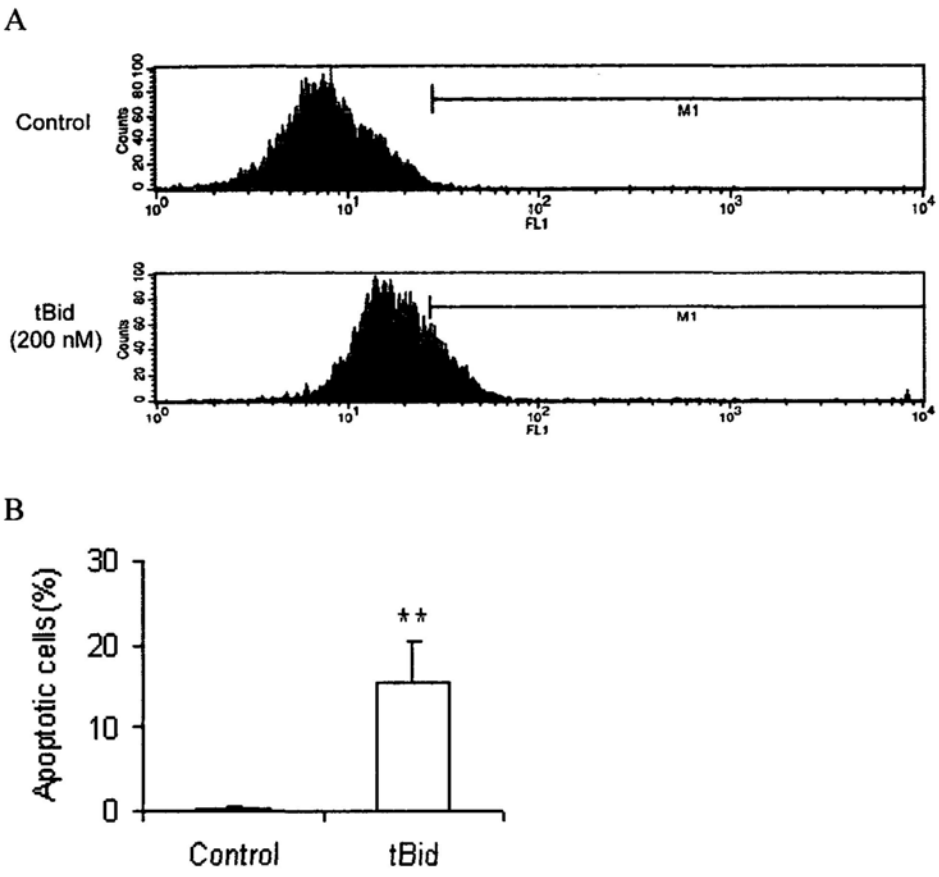


Fig 3.11 Effects of tBid on apoptosis of Hep3B cells.

Cells were treated with 200 nM tBid for 24h. Apoptosis was assayed by TUNEL using Flow cytometry. **A**, TUNEL assay for Hep3B cells after tBid treatment for 24h. **B**, The proportion of the apoptotic cells was shown, non-treatment Hep3B cells as control. Data are the mean ± SE of three separate experiments. ** Indicates $P < 0.01$.

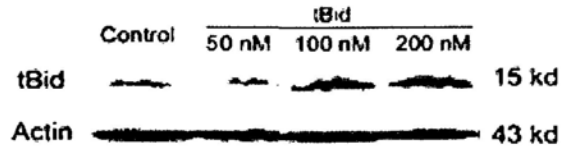


Fig 3.12 Western blot analysis of the levels of tBid.

Cells were treated with 50 nM, 100 nM and 200 nM tBid for 24h. Total protein was isolated after treatment and Western blot was used to detect tBid expression. Actin level was examined and served as the loading control. Experiments were repeated twice.

3.3.3 Cell synchronization

To determine whether tBid specifically or preferably targets a given phase of the cell cycle, Hep3B cells were synchronized at defined cell phases by serum starvation, chemical synchronization, and flow sorting, followed by tBid treatment. The ratio of cells in the G0/G1, S, and G2/M phases of cell cycle was determined by their DNA content.

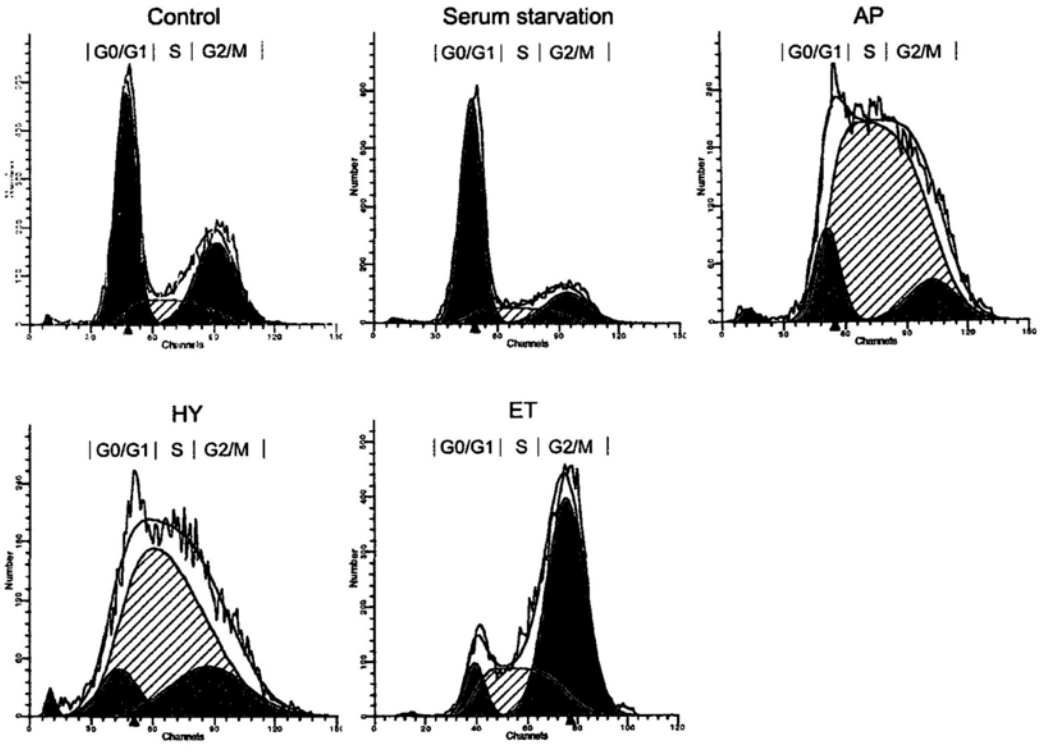
3.3.3.1 By chemicals

Cells were synchronized at G0/G1 phase by 48h serum starvation; S phase either by 0.2 μg/ml AP for 24h with 1h release or by 0.5 mM HY for 12h with 6h release; G2/M phase by 0.2 μM ET for 24h. The cell phases of synchronized Hep3B cells were analyzed by flow cytometry (Fig 3.13A). These selecting methods accumulated more than 60% of cells in G0/G1 phase, 75% (by AP) and 60% (by HY) in S phase, 70% in G2/M phase (Fig 3.13B), yet maintained reasonable viability of the cells.

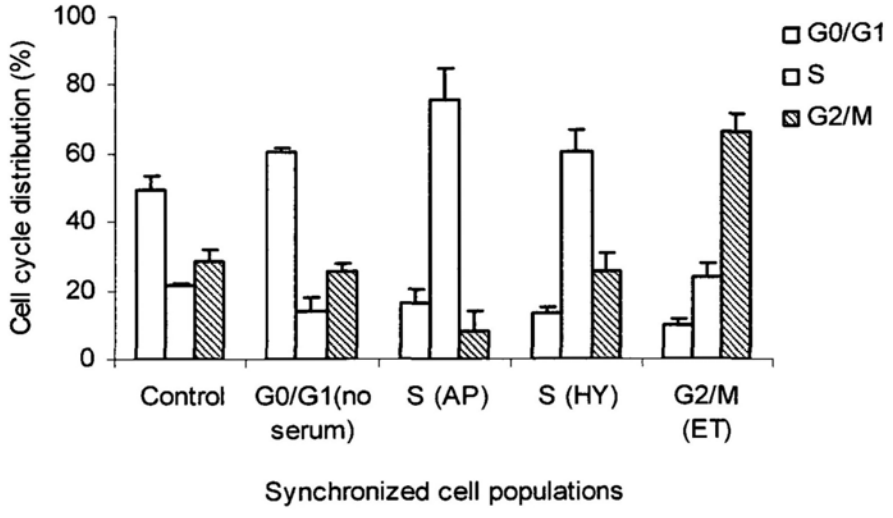
The cytotoxicity of synchronization agents on Hep3B cells was determined by MTT assay. As shown in Fig 3.13C, serum starvation, AP (0.2 μg/ml),

HY (0.5 mM), and ET (0.2 μM), which we used to synchronize Hep3B cell did not reduce cell viability of Hep3B significantly after 24h incubation. When we increased the concentration to two fold and prolonged the incubation time to 48h, there still was not any significant toxicity effect.

A



B



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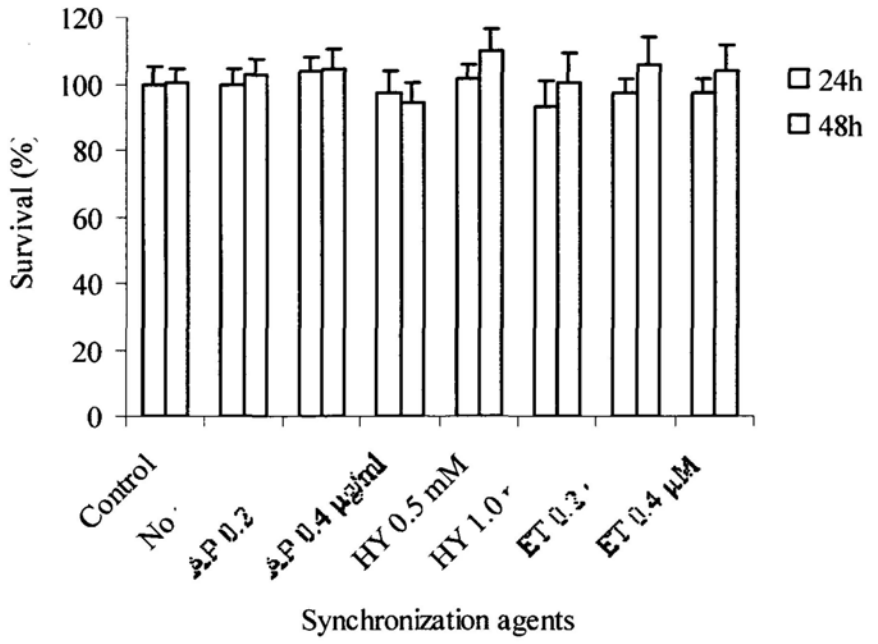


Fig 3.13 Effects of cell phase synchronization agents on Hep3B cells.

A, The DNA histogram of Hep3B cells after synchronization was monitored by flow cytometric analysis. **B**, Cell cycle distribution for Hep3B cells that were subjected to chemical synchronization. The ratio of cells in the G0/G1, G2/M, and S phases of the cell cycle was determined by the DNA content. Data are mean ± SE of three independent experiments. **C**, The cytotoxicity of the synchronization agents to Hep3B cells was determined by MTT assay. Different concentrations of ET, AP, and HY were incubated with Hep3B cells for 24h and 48h. Data are mean ± SE of three independent experiments.

3.3.3.2 G1 phase enrichment by flow sorting

The above methods were the manipulation of the cell cycle by synchronization chemicals that prevent DNA synthesis or that inhibit the formation of mitotic spindles. However, these techniques were reported to have the disadvantage that they may activate cell cycle checkpoint mechanisms and that sometimes they were rather toxic.

To circumvent these problems, we generated Hep3B cells that stably express H2B-GFP fusion proteins as a stoichiometric probe of DNA content, thus by FACS purification we could obtain unmanipulated, non-synchronized G1 populations that were suitable for the study of the impact of cell cycle on apoptosis regulation.

3.3.3.2.1 H2B-GFP stably expressing Hep3B

The Hep3B cell line was cultured as described before. Exponentially growing Hep3B was transfected using Lipofectamine™ 2000 with a cDNA encoding H2B-GFP. After selection by blasticidine, we established H2B-GFP stable expressing Hep3B clones (Fig 3.14).

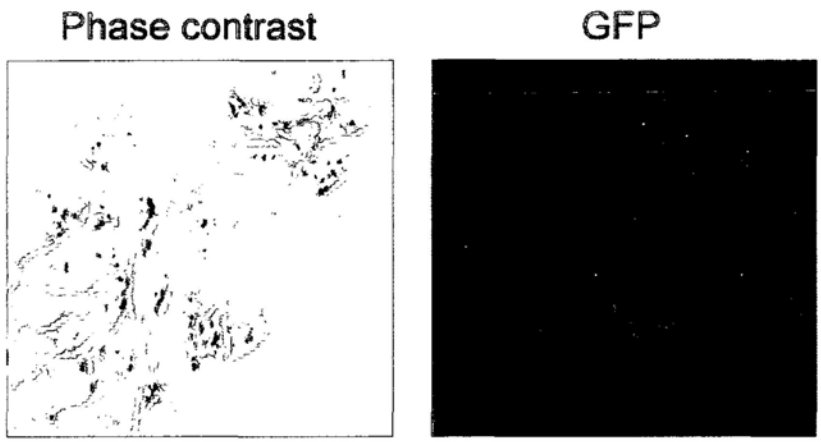


Fig 3.14 Generation of Hep3B clones that stably expressing H2B-GFP. The morphological aspect of the cells was shown, revealing that the GFP fluorescence was spatially restricted to chromatin.

3.3.3.2.2 FACS-purification

FACS-purification of cells was performed as previously described (Coquelle *et al.*, 2006). Briefly, cells sorted in G1 were gated on by defining windows of FL1 and FSC (M1 and M4 for G1 in Fig 3.15A). This procedure led to the enrichment of homogenous populations, as determined by PI staining. The purity at the G1 phase was 88% (Fig 3.15B and C).

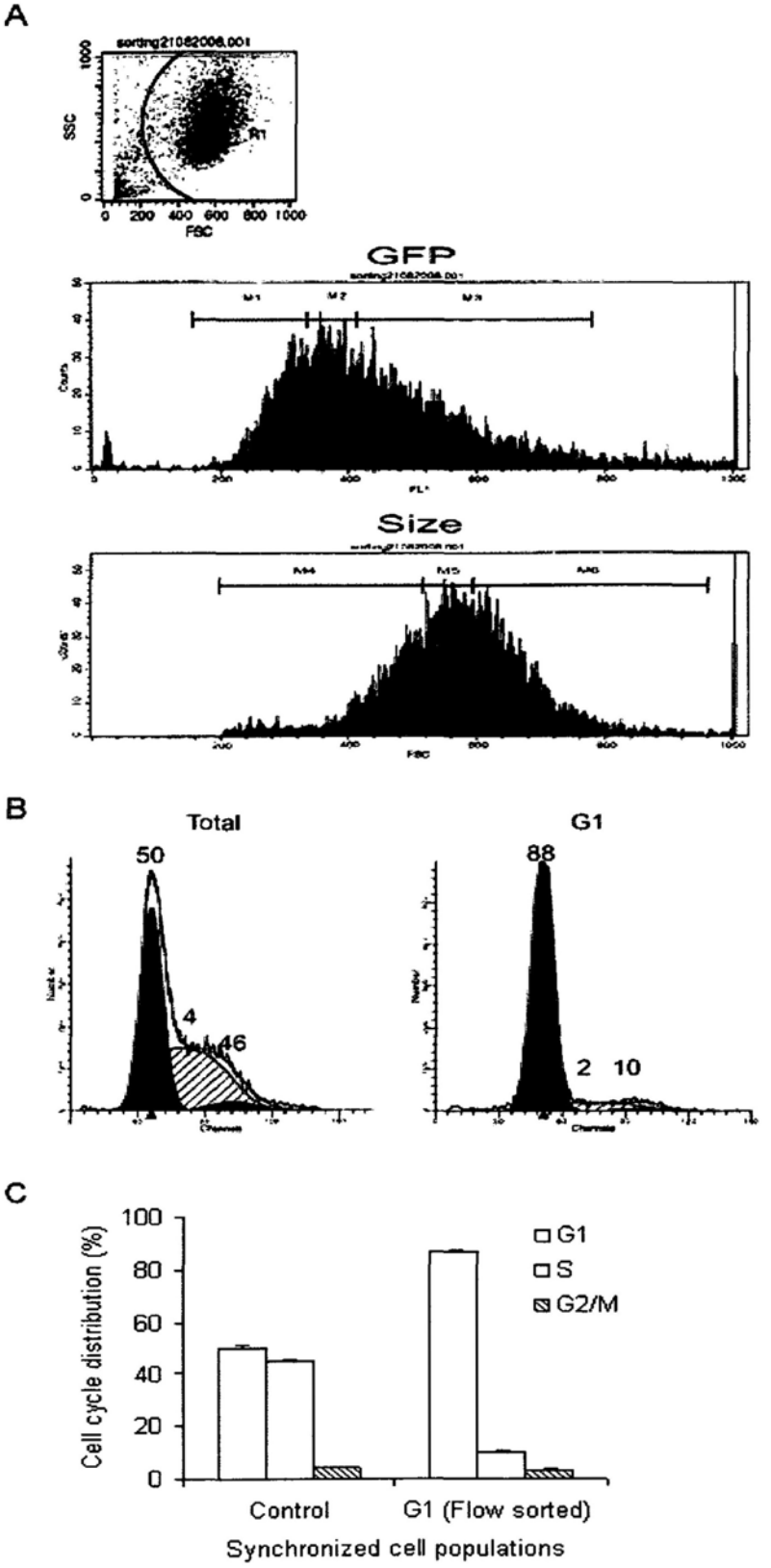

A
SSC
FSC
R1
GFP
Size
B
Total
G1
50
4
46
88
2
10
C
Cell cycle distribution (%)
100
80
60
40
20
0
G1
S
G2/M
Control
G1 (Flow sorted)
Synchronized cell populations

Fig 3.15 Cytofluorometric separation of Hep3B cells expressing H2B-GFP in G1 phase of cell cycle.

A, Cells transfected with H2B-GFP in the logarithmic growth phase were subjected to FACS analysis. When the cells were gated on normal-sized cells (R1 in the FSC vs. SSC plot), as well as their GFP fluorescence (M1, M2, M3) and FSC (M4, M5, M6), they were sorted to bona fide G1 (R1+M1+M4). **B**, The cells were immediately labeled with PI and then subjected to determination of DNA content by flow cytometric analysis. Numbers on each gate indicate the percentage of cells in the G1, S and G2/M phases of the cell cycle. **C**, Cell cycle distributions of flow sorted G1 populations (column chart). Data are mean ± SE of three separate experiments.

3.3.4 Cell phase specific effect of tBid

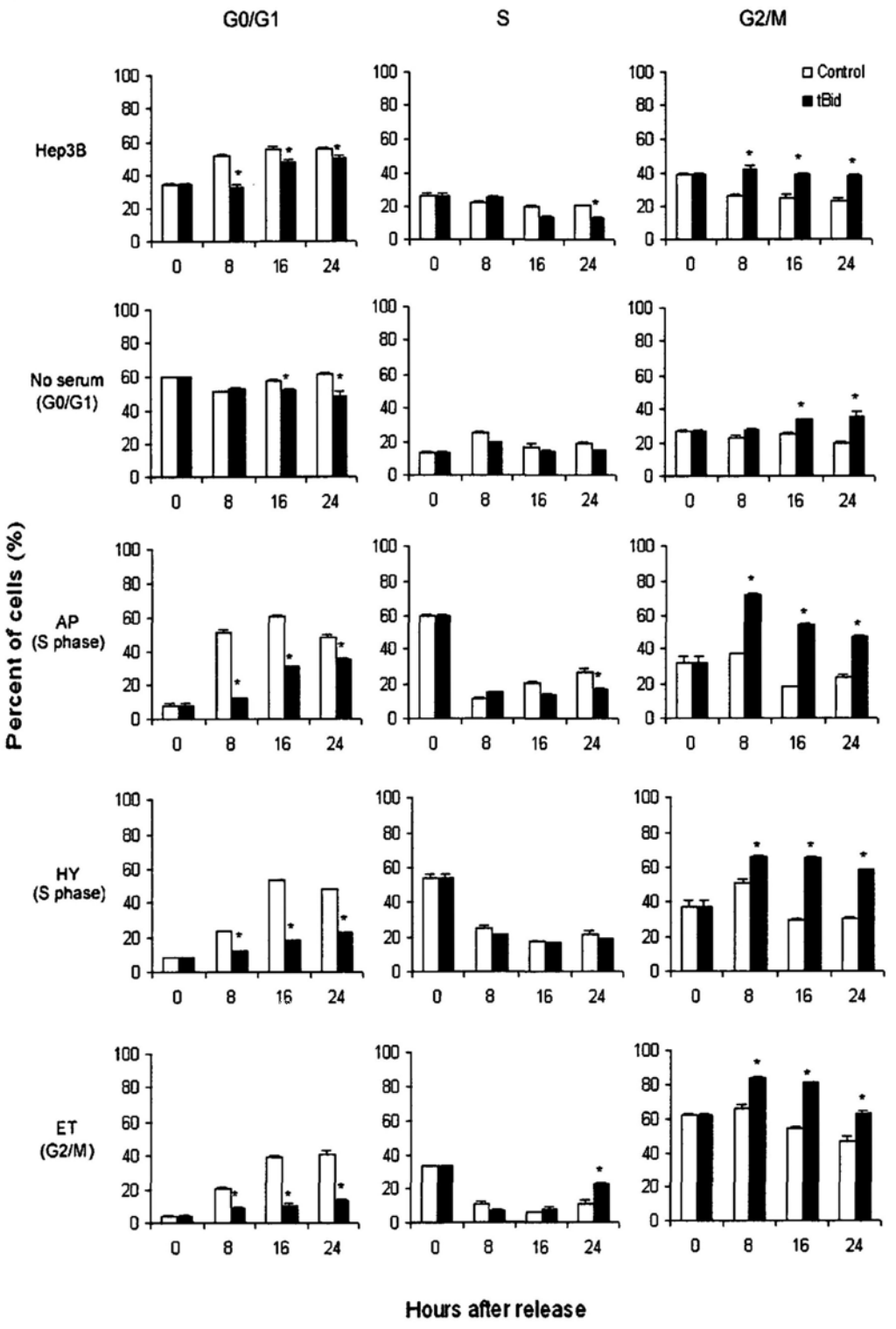
To determine whether tBid specifically or preferably targets a given phase of the cell cycle, Hep3B cells were synchronized at defined cell phases by serum starvation, chemical synchronization, and flow sorting, followed by tBid treatment. Cell cycle progression, protein expression and cell viability were determined.

3.3.4.1 Cell cycle progression, cell cycle-related proteins and caspases expression in synchronized Hep3B exposed to tBid

Hep3B cells were synchronized to define cell phases as descried above. Synchronous (at G0/G1, S and G2/M phase) or asynchronous Hep3B cells were exposed to 200 nM tBid. At the indicated times, aliquots of cultured cells were sampled for flow cytometric analysis and protein were extracted for Western blot analysis. The percentages of phase fractions were shown in Fig 3.16A. Results showed that treatment with tBid arrested the Hep3B cell in G2/M phase of cell cycle accompanied by the reduction of cells in the G0/G1 phase. The ratio of G0/G1 phase fraction in tumor cells treated by tBid was statistically less than the ratio in control non-treated cells ($P < 0.05$), which indicated cells in the G0/G1 phase were particularly sensitive to growth inhibition of tBid. The result could be confirmed when cell proliferation was assayed by MTT assay (Fig 3.17).

In addition, Western blot analysis results showed that the levels of Cdk2 and p-cdc2 p34 (Tyr 15) decreased. The level of p-cdc2 p34 (Thr 161) increased in the tBid-treated cells compared with the control cells, but the levels of cdc2 p34, cyclin A, cyclin D1 and cyclin B1 were not significantly changed. The expression of procaspase 9, cleaved caspase 9 and caspase 3 was significantly increased in cells treated by tBid compared with the control cells (Fig 3.16B).

A


G0/G1
S
G2/M
Control
tBid
Hep3B
No serum (G0/G1)
AP (S phase)
HY (S phase)
ET (G2/M)
Percent of cells (%)
Hours after release

B

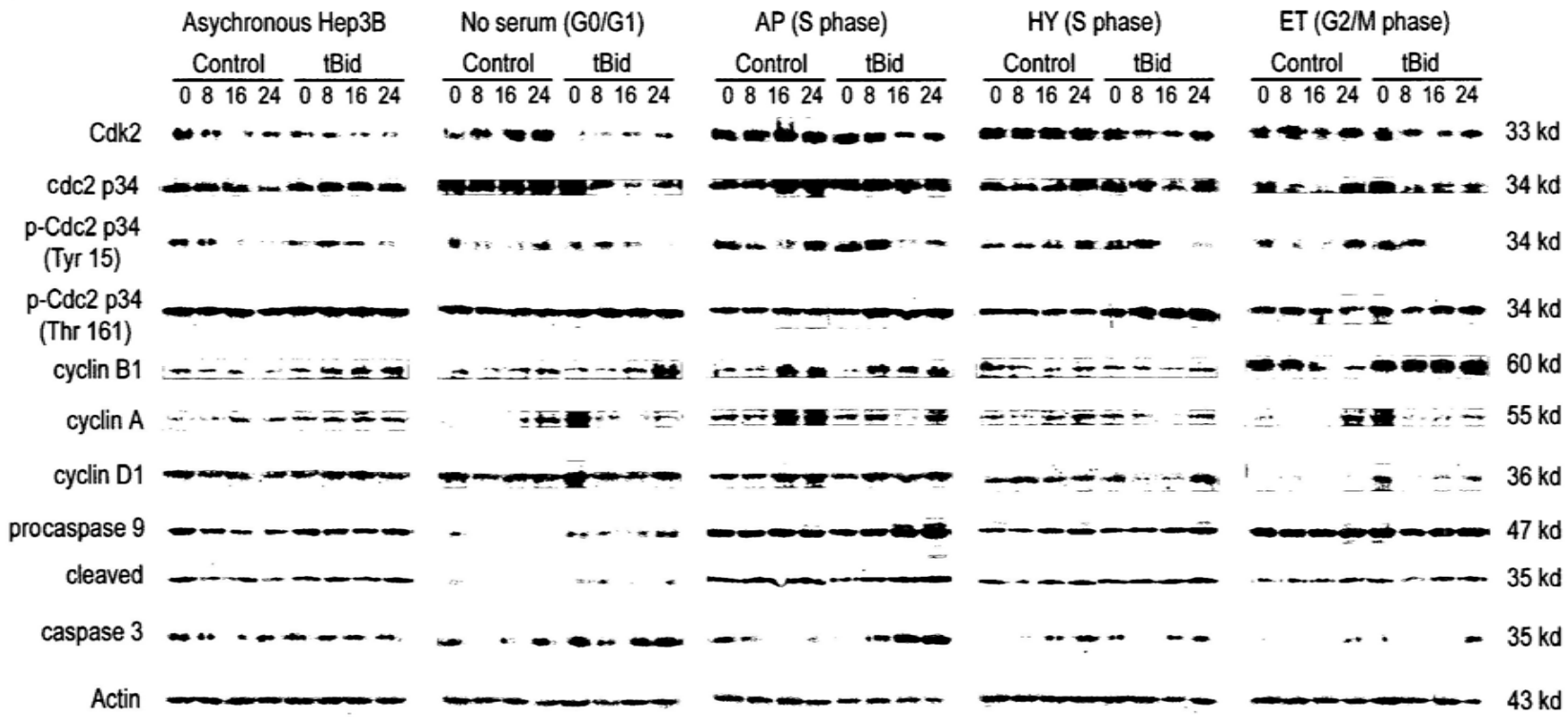


Fig 3.16 Flow cytometric analysis of cell cycle progression and protein expression detected by Western blot of asynchronous and synchronous Hep3B cells exposed to tBid.

Hep3B cells were synchronized at G0/G1 phase by serum starvation, S phase by either AP or HY, at G2/M phase by ET as described in Materials and Methods. Then cells were treated with 200 nM tBid or the same volume of 50 mM Tris-HCl buffer (pH 8.0) as control. **A**, Flow cytometric analysis of cell cycle progression. Data are mean ± SE of three separate experiments. **B**, Cell cycle related proteins, caspase 9 and caspase 3 expressions. At indicated time points, aliquots of cells were sampled, total protein was isolated and the levels of Cdk2, p-Cdc2 p34 (Thr 161), cyclin B1, cyclin A, cyclin D1, caspase 9 and caspase 3 at each time point were analyzed with Western blot analysis. Actin level was examined and served as the loading control. Experiments were repeated at least twice.

3.3.4.2 Cell viability detected by MTT assay

As shown in Fig 3.17A, synchronization with serum starvation at G0/G1 phase permitted tBid exposure to inhibit 31% of cells, compared to 16%-24% in the asynchronous counterparts or cells synchronized at other phases of the cell cycle. The G1-enriched Hep3B-GFP cells were also demonstrated a significantly increased vulnerability to tBid-mediated cell death compared to the asynchronous population (Fig 3.17B). These results indicated that Hep3B cells at G0/G1 phase were relatively more susceptible to tBid-mediated apoptosis.

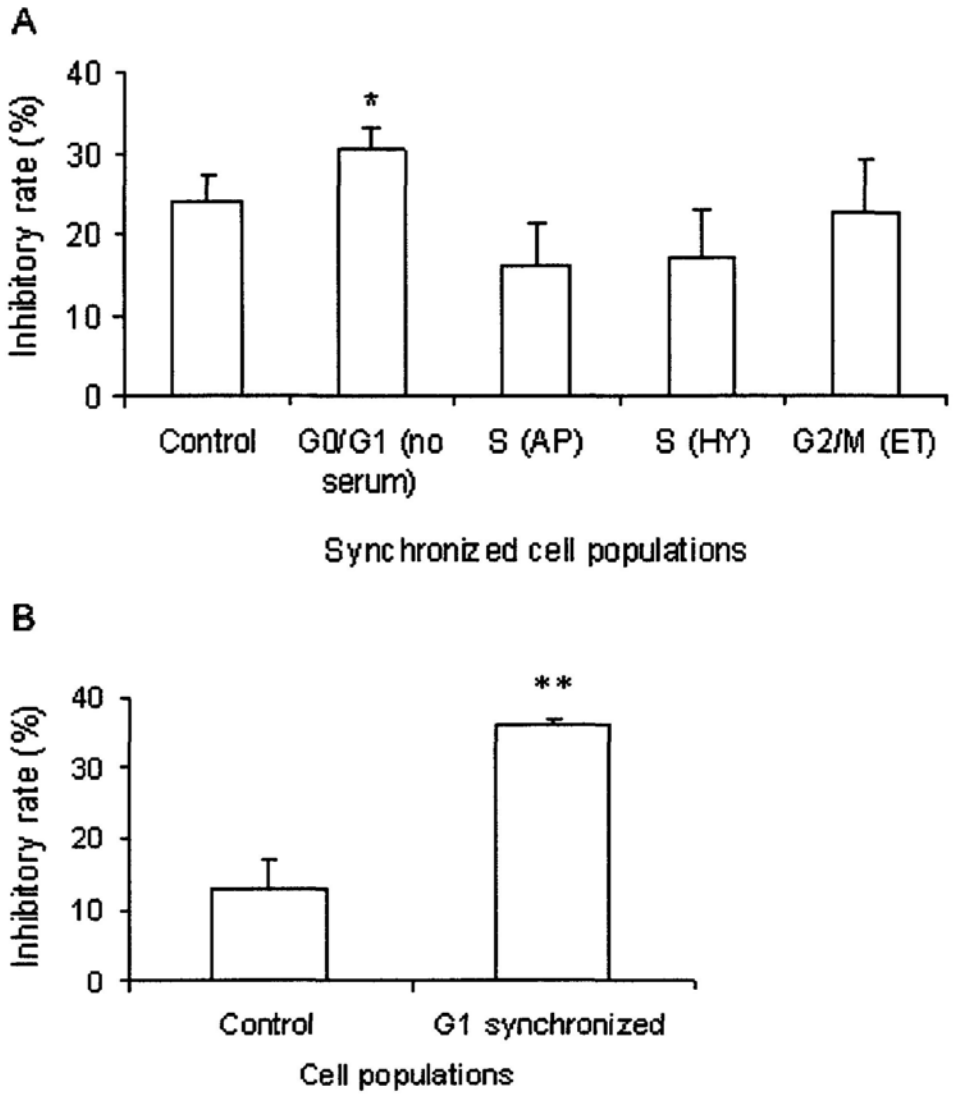


Fig 3.17 Susceptibility of phase-synchronized Hep3B cells to tBid-mediated apoptosis.

Cell viability was determined based on the reduction of MTT dye. The inhibitory effects were expressed as 100% minus the percentage survival of treated cells versus non-treated cells. **A**, Hep3B cells at G0/G1, S and G2/M phases of cell cycle were treated with 200 nM tBid for 48h. Data are mean ± SE of three independent experiments. * Indicates $P < 0.05$, compared with each of the rest 4 columns. **B**, G1 phase Hep3B-GFP cells obtained by flow sorting as described in Fig 3.15 were treated with 200 nM

tBid for 24h. Total Hep3B-GFP cells were use as control. Data are mean ± SE of three independent experiments. ** Indicates $P < 0.01$.

3.3.5 Cell phase specific effect of 5-FU

Most chemotherapeutic agents play roles in anti-growth of cancer cells with variations of cytotoxic effect in the cell cycles. 5-FU may act throughout the cell cycle varying from the types of cells. As shown in Fig 3.18A and B, 20 μg/ml and 50 μg/ml 5-FU arrested Hep3B cells in the G0/G1 phase, significantly reduced cells in the G2/M phase since 12h after treatment.

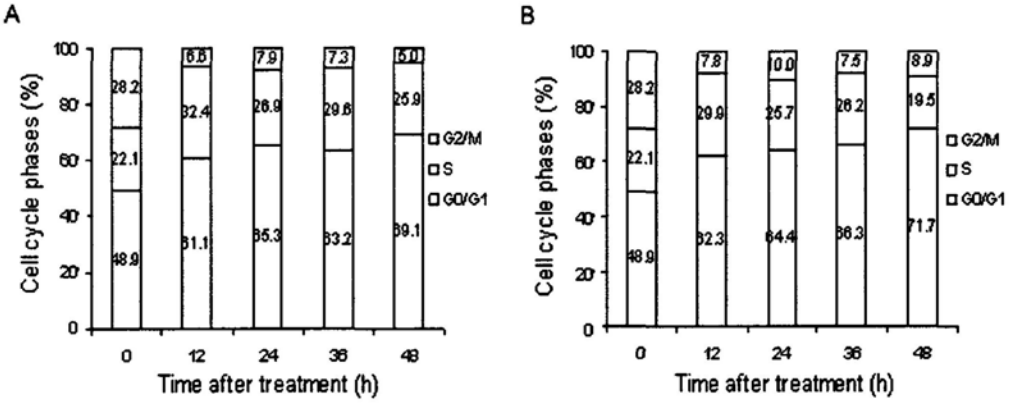


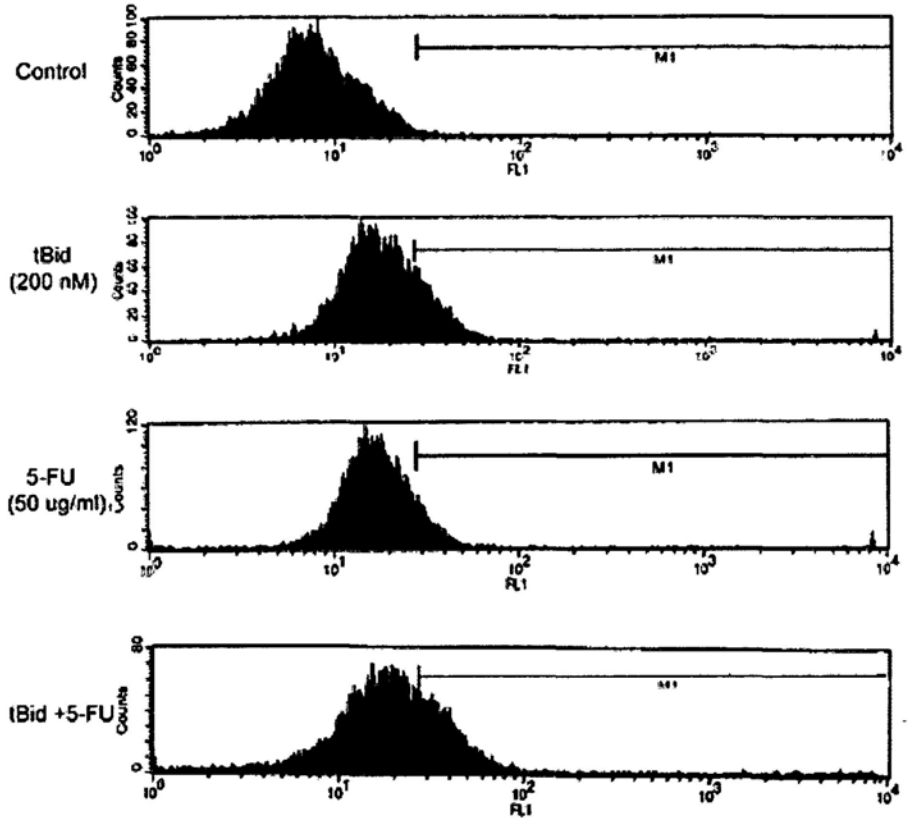
Fig 3.18 Cell cycle distribution for Hep3B cells treated with 5-FU.

A, 5-FU 20 μg/ml. **B**, 5-FU 50 μg/ml. Data are mean of three separate experiments.

3.3.6 Effect of tBid in combination with 5-FU on Hep3B cells

As shown in Fig 3.19A and B, 200 nM tBid in combination with 50 μg/ml 5-FU caused more Hep3B cells to be apoptotic than either agent alone. In line with this, the level of tBid was increased (Fig 3.19C).

A



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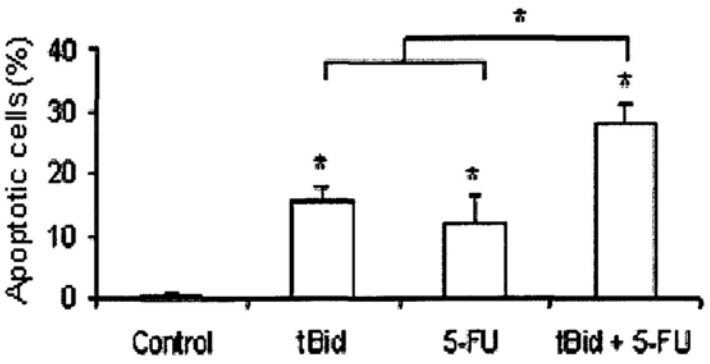

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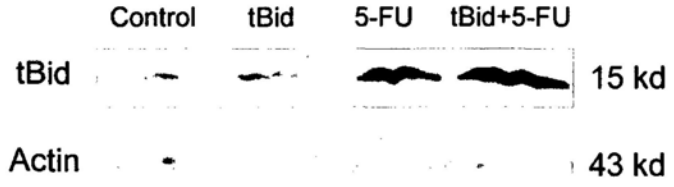

Fig 3.19 Effects of tBid in combination with 5-FU on apoptosis of Hep3B cells.

Cells were treated with 200 nM tBid in combination with 50 μg/ml 5-FU or either agent alone for 24h. Apoptosis was assayed by TUNEL using Flow cytometry. **A**, Representative TUNEL assay for Hep3B cells after treatment. **B**, The proportion of the apoptotic cells was shown, non-treated Hep3B cells as control. Data are the mean ± SE of three separate experiments. * Indicates $P < 0.05$. **C**, Total protein was isolated and Western blot was used to detect tBid expression. Actin level was examined and served as the loading control. Experiments were repeated twice.

3.4 Discussion

Several antitumor agents of different classes have been characterized in terms of the cell cycle phase specificity of induction of apoptosis (Pommier, 2004, Huang *et al.*, 2005a, Halicka *et al.*, 1997). Most chemotherapy regimens combine drugs targeting different phases of the cell cycle to increase the efficiency of treatment. Previous studies indicated that the introduction of Bid or tBid could effectively induce cell death in various kinds of cells including hepatocytes (Yin, 2000, Esposti, 2002). The combination of Ad/AFPtBid with 5-FU was more effective in reducing

Hep3B tumor and increased the survival rate of mice than either agent alone. 5-FU may act throughout all phases of the cell cycle depending on the types of cells (Yoshikawa *et al.*, 2001, Lee *et al.*, 2005, Li *et al.*, 2004). In this study we evaluated susceptibility of Hep3B cells in defined phases of cell cycle to tBid-mediated apoptosis.

We used two different methods to get synchronized fractions of Hep3B. The first one is to use cell cycle synchronization agents. AP is an inhibitor of DNA polymerase-α that has been reported to arrest cells in S phase without adverse effect. It is reported to be low cytotoxic, easy to be removed by washing and it produces the best synchrony for S phase (Fox *et al.*, 1987). HY is an inhibitor of ribonucleotide diphosphate reductase that arrests cells in early S phase (Kumar *et al.*, 2005). ET, an inhibitor of topoisomerase 2, arrests cells at the G2/M boundary. Some synchronization agents have disadvantages as they were rather toxic. In our experiment, pharmacological synchronization procedures of cells in the G0/G1, S or G2/M phases of the cell cycle were non-toxic (Fig 3.13C) and did not induce cell apoptosis detected by TUNLE assay (data not shown). They yield cell cycle fractions with an acceptable degree of purity (more than 60% for G0/G1, 60% and 75% for S, and 70% for G2/M) (Fig 3.13B). Upon re-culture, the cells readily resume the cell cycle. This is in line with previous studies showing that cell cycle arrest induced by serum starvation or treatment with AP had only minor effects on the viability of hematopoietic cell lines, human breast cancer cell lines, and did not

abrogate the subsequent onset of apoptosis (Sangfelt *et al.*, 1997, Blajeski *et al.*, 2001). The doses of ET (0.2 μM) we used were very low but it was enough to arrest cells. It has been reported that 10 μM of ET needs to induce repairable damage while 100 μM is usually used to induce apoptosis (Chen and Hitomi, 1999, Kamer *et al.*, 2005). The second method used is to purify G1 phase cells by flow sorting, which is non-toxic and does not perturb the general apoptosis susceptibility of the cells. As shown in Fig 3.15B, this method yield G1 fractions with a more acceptable degree of purity (88%), compared with the result obtained using cell cycle synchronization agents.

After obtaining synchronized cells, we explored the susceptibility of Hep3B cells to tBid-mediated apoptosis. The changes in the cell cycle of Hep3B after tBid treatment were determined by flow cytometry. Previous studies have shown that cell cycle phase fraction of the tumor tissues from HCC patients were G0/G1 (77.25%), S (6.17%) and G2/M (16.62%), in contrast to cell cycle phase fraction of normal liver G0/G1 (97.1%), S (2.1%) and G2/M (0.8%) (Chao *et al.*, 1998, Paterlini *et al.*, 1995). In this study, the time-course progression revealed that tBid significantly increased Hep3B cell population of the G2/M phase and significantly reduced the percentage of G0/G1 phase (Fig 3.16A). Activation of cdc2 and cyclin B1 are essential to trigger G2/M phase transition (Draetta and Beach, 1988). Cdc2 is positively regulated by the phosphorylation of Thr 161 and dephosphorylation of Tyr 15 (Atherton-Fessler *et al.*, 1993). Although the

levels of cyclin B1 and cdc2 p34 did not show any significant change in our Western blot analysis, the expression of p-cdc2 p34 (Thr 161) was significantly increased and the level of p-cdc2 p34 (Tyr 15) was markedly decreased in the tBid-treated cells compared with the control cells. At the same time, the level of Cdk2 decreased, which is essential for cell cycle G1/S phase transition. The levels of cyclin A and cyclin D1 were not significantly changed. Furthermore, tBid treatment caused activation of caspase 9 and caspase 3 (Fig 3.16B). Our experiment demonstrated that cells in G0/G1 phases were relatively more susceptible to tBid-induced apoptosis. These results indicate that tBid may cause Hep3B cells arrest at the G2/M phase of cell cycle, and primarily induced the cells from the G0/G1 phase to apoptosis. This finding is in agreement with Bid is important for the accumulation of embryonic fibroblasts cells in the S and G2/M phase of the cell cycle following ET induced DNA damage (Kamer *et al.*, 2005). Bid has been known to exhibit S phase checkpoint activation (Song *et al.*, 2008). It may link DNA repair processes and apoptosis, translating the cell damage into either cell cycle arrest (at low levels of damage) or apoptosis (at high levels of damage) (Kamer *et al.*, 2005). Therefore, DNA-repair and cell cycle checkpoint pathways allow cells to deal with damages. The G2/M checkpoint prevents cells from initiating mitosis when they progress into G2/M with some unrepaired damage inflicted during previous S or G0/G1 phases (Xu *et al.*, 2002a, Nyberg *et al.*, 2002). These cellular responses are important for determining responses to current cancer therapies.

It has been reported that G2/M arrest occurs in response to many types of DNA damages. Hep3B cells used in this study were p53 mutant. The fact that tumor cells with mutant p53 tend to selectively accumulate in G2/M after tBid treatment, indicates that p53-independent mechanisms are sufficient to sustain the G2/M arrest. At the same time, this phenomenon has inspired efforts to manipulate or regulate the G2/M checkpoint as a potential strategy to improve the efficiency of cytotoxic drugs and sensitize cancer cells to radiation- or chemotherapeutic agents (Zhou and Bartek, 2004, Links *et al.*, 1998). Fortunately, we found that 5-FU significantly reduced Hep3B cells in G2/M phase, while mainly arrested cells in the G0/G1 phase. The high percentage of tumor cells staying in the G0/G1 phase is known to contribute to resistance to 5-FU (Wang *et al.*, 2004). Our finding indicates that tBid mainly target the tumor cells in the G0/G1 phase. Therefore, tBid and 5-FU in combination may maximize their anti-tumor effect by targeting tumor cells in the different phases of the cell cycle. Such a complementary effect of tBid and 5-FU on different phases of the cell cycle may partly explain the synergetic therapeutic result when Ad/AFPtBid is used in combination with 5-FU to treat HCC cells in our previous studies (Miao *et al.*, 2004, Miao *et al.*, 2006) and in this study (Fig 3.19). Our findings also suggest that in order to achieve the maximal killing effects of 5-FU and tBid, 5-FU should be first used to line up the tumor cells in G1, and then tBid is applied to induce apoptosis, as the cells in G1 is most sensitive to tBid. Our present data thus provide useful information

for the optimization of tBid and other anti-tumor agents in combination to inhibit tumor growth.

In conclusion, this study showed that tBid may play roles in the interplay between apoptosis and cell cycle regulation. The elucidation of phase specific effect of tBid points to a possible therapeutic option that combines different phase specific agents to overcome resistance of HCC and treat HCC more effectively.

Chapter Four

Conclusion and Future Perspectives

4.1 Summary of results

1. Ad/AFPtBid can effectively and specifically kill AFP-producing liver caner cells *in vivo* without obvious toxicity.

2. Ad/AFPtBid can significantly sensitize liver cancer cells to 5-FU. The combination of 5-FU with Ad/AFPtBid was more effective in reducing the tumor and increasing the survival rate than either agent alone.

3. The combination of Ad/AFPtBid gene therapy with 5-FU results in tumor regression, apoptosis, and inflammatory cell infiltration in our model.

4. tBid significantly reduced the percentage of G0/G1 phase Hep3B cells, caused cells arrest at G2/M phase of cell cycle, while 5-FU arrested Hep3B cells in the G0/G1 phase, significantly reduced cells in G2/M phase. The levels of cell cycle-related proteins and caspases were in line with the result of the cell cycle.

4.2 Conclusions

In conclusion, our study has demonstrated that Ad/AFPtBid could effectively and specifically kill AFP-producing liver caner cells *in vivo* and that Ad/AFPtBid could significantly sensitize liver cancer cells to 5-FU. Hep3B tumors showed significant number of TUNEL-positive cells after treatment with Ad/AFPtBid, indicating that Ad/AFPtBid induced tumor regression through its pro-apoptotic effect. In addition, this study showed that tBid may play roles in the interplay between apoptosis and cell cycle regulation. Hep3B cells in G0/G1 phase were more susceptible to tBid-mediated apoptosis, while 5-FU significantly reduced cells in G2/M phase.

Therefore, tBid and 5-FU in combination may maximize their anti-tumor effect by targeting tumor cells in the different phases of the cell cycle. The pro-apoptotic effects of Ad/AFP tBid and 5-FU and the complementary effect tBid and 5-FU on different phases of the cell cycle may explain the synergetic therapeutic results when tBid is used in combination with 5-FU to treat HCC cells in this study. Our findings also suggest that in order to achieve the maximal killing effects of 5-FU and tBid, 5-FU should be first used to line up the tumor cells in G1, and then tBid is applied to induce apoptosis, as the cells in G1 is most sensitive to tBid. Our present data thus provide useful information for the optimization of tBid and other anti-tumor agents in combination to inhibit tumor growth.

Our present study has studied the therapeutic effect of Ad/AFPtBid and chemotherapeutic agents in combination on orthotopic HCC in nude mice, and the susceptibility of Hep3B cells in different phases of cell cycle to tBid-mediated apoptosis. These may provide the novel idea for the therapy of HCC.

This combination approach may improve the therapeutic benefits of some chemotherapeutic agents such as 5-FU, rendering Ad/AFPtBid a promising alternative tool in combination with chemotherapeutic agents for treatment of AFP-producing HCC. Further experiments to determine whether the response of HCC cells to Ad/AFPtBid treatment is AFP-concentration dependent are needed. This will be helpful for the future use of Ad/AFP tBid in HCC patients, because the level of AFP is different in patients with

HCC and AFP is also a valuable marker for the monitor of therapeutic response. The elucidation of phase specific effect of tBid points to a possible therapeutic option that combines different phase specific agents to overcome resistance of HCC and treat HCC more effectively. The different susceptibility of HCC tumor cells in specific cell phases of cell cycle toward treatments also points to the possibility that combine synchronization agents with therapeutic agents to treat patients with HCC. However, more work is needed in order to further understand the molecular basic of cell phase specific effect of tBid.

4.3 Future Perspectives

Currently, viral and nonviral vector systems are used in gene therapy for cancer. Among them adenovirus vector is the most commonly used vector for gene therapy due to its well-understood structure and high rate of gene transfer *in vivo*. Other viruses used as vectors include retroviruses, adeno-associated viruses, lentiviruses, poxviruses, and herpes viruses. These viruses differ in how well they transfer genes to the cells they recognize and are able to infect, and whether they alter the cell's DNA permanently or temporarily. Some promising viral vectors for gene therapy of cancer includes Newcastle disease virus, attenuated vaccine strains of measles virus (Schirrmacher and Fournier, 2009, Russell and Peng, 2009).

Although a variety of virus gene delivery vehicles has been developed, most of them suffer from some limitations, including inadequate tumor

targeting, inefficient gene transfer, limitations in the size of the carried therapeutic genes, potential toxicity, possibly causing harmful mutations to the DNA, the viral vector could cause immune reaction, and that the virus could be transmitted from the patient to other individuals or into the environment (Seow and Wood, 2009).

Strategies to improve delivery and targeting of adenovirus vectors include genetic modification of viral coat proteins, non-genetic modifications including polymer encapsulation approaches and pharmacological interventions (Bachtarzi et al., 2008). For example, a nanoparticle carrying the cytocidal gene may selectively target primary tumors and metastatic foci.

While continuing to improve these vectors, efforts to investigate nonviral vectors remain a viable alternative option (Seow and Wood, 2009). Nonviral vectors include synthetic liposomes, vectors based on the use of cationic lipids or polymers, minicircles, bacteria, bacteriophage, virus-like particles, erythrocyte ghosts, and exosomes. The current challenge to these non-viral vectors is obtain a similarly effective transfection to viral ones.

We now understand better the strengths and weaknesses of various gene transfer vectors; this facilitates the choice of appropriate vectors for individual diseases. Combinations of the different targeting strategies and techniques to deliver genes more efficiently, specifically target on tumor

cells and evade the immune system harbor the future for adenoviral gene therapy in patients with cancer. However, there are still some limitations that need special consideration before adenovirus-targeted cancer gene therapy emerges as a routine treatment in the clinical using.

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