Development of a Novel Anticancer Therapy by Targeting Apoptotic Signalling Pathways

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ABSTRACT

This PhD project aimed to examine the anticancer potential of a natural compound, cinobufagin (CBF), and the signal transduction pathways involved in CBF-induced cell death, especially pro-apoptotic pathways. CBF is a major component of the traditional Chinese medicine Chansu, which is extracted from parotoid glands of Chinese toads. Chansu has been widely used in the treatment for inflammation in China for centuries. The role of Chansu in anti-neoplasm has recently been revealed and it leads to a new area of research.

In this study, CBF was initially found to have a potent capacity for anti-proliferation in a variety of cancer cell lines. Our result from cytotoxicity assays showed a particular high sensitivity of CBF in human colon cancer cell lines. Thus, HCT116 and HT29 cell lines were selected for the further investigations. To clarify whether the cell death induced by CBF is apoptosis or necrosis, Annexin V staining was carried out and showed a consequence of CBF-induced apoptosis in both cell lines. The subsequent detection of mitochondrial potential changes post CBF exposure was discovered in the two cell lines. However, the apoptotic pathways elicited by CBF were different in HCT116 and HT29 cells. CBF activated Caspase-3 in HCT116 cells, but the activity of Caspase-3 in treated HT29 cells was inhibited. The translocation of mitochondrial apoptosis inducing factor (AIF), a crucial protein for Caspase-3-independent apoptosis, was also detected but only in HCT116 cells. The nuclear import of AIF was untraceable in the process of HT29 cell death during CBF treatment. Altogether, we concluded that CBF-induced apoptosis in HCT116 is through both Caspase-3 dependent and

independent pathways, while the apoptotic signalling pathways in treated HT29 cells are still undiscovered.

The understanding of precise molecular activities induced by CBF would assist us to eventually develop bufadienolide compounds into novel anticancer agents. Hence, multi-pathway arrays were conducted to disclose the specific pathways involved in the cytotoxicity of CBF treatment. Our results indicated that two pathways were affected the most by CBF exposure, which were the pathways of hypoxia and extracellular signal-regulated kinases (ERK). Subsequently, we focused further on two key proteins related to these two pathways. They were hypoxia-inducible factor 1 α (HIF-1α) and Cortactin (CTTN), required for cell survival and cell migration, respectively. To determine the role of CBF in upregulation or downregulation of the two proteins, the analyses on the expression of mRNAs and proteins were carried out. Our finding showed that in spite of the elevation of HIF-1α mRNA, CBF significantly reduced the expression of HIF-1α protein in both cell lines. This suggested that the CBF-induced inhibition of HIF-1α protein was not a direct consequence of the alteration of HIF-1α transcription level. On the other hand, CBF suppressed the CTTN transcription for a short period (about 12 hours) and the inhibition of CTTN protein was only detected in HCT116 cells. Moreover, a preclinical xenograft tumour model was established to examine the inhibitory effects of CBF in mice. The results from tumour tissue samples showed the capability of CBF in the hindrance of HIF-1α nuclear import and in the downregulation of CTTN expression.

In addition to Chansu, the Australian cane toad extract from parotoid glands was also able to induce cell death in human cancer cell lines. The result from HPLC showed that

the cane toad extract contains four types of bufadienolide compounds, including a main component, marinobufagin. The anti-proliferative capacities between the Australian cane toad extract and CBF were similar in different colon and prostate cancer cell lines. Furthermore, the cane toad is an invasive species and has become a threat to the native environment of Australia, especially in Queensland. Therefore, the goal of this study provides a possibility to convert the biohazardous cane toads to a valuable source of anticancer drugs.

Apart from the investigation on inhibitory capability of CBF, this study also contributed to the development of a drug delivery system using liposome-encapsulation. To enhance the efficacy and safety of hydrophobic drugs like bufadienolides, liposome-encapsulated drugs have been widely applied. Our laboratory generated a functional liposomal curcumin and showed potent inhibitory effects in a human lung cancer cell line. In the future, other therapeutic agents such as bufadienolide compounds could be used to replace curcumin in the reproducible method. As such, liposomal bufadienolides will be developed as a novel group of drugs for anticancer treatment.

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Table of Contents

CHAPTER 1.

1.1 **General Introduction** ---1

1.2 **Apoptosis and Cancer Cell Death** ------------------------------------- 1

1.2.1 Introduction -- 1

1.2.2 Apoptotic Mechanisms --- 2

A. Intrinsic Apoptotic Pathway ----------------------------- 2

B. Extrinsic Apoptotic Pathway ----------------------------- 3

C. Execution of Apoptosis ---------------------------------- 6

D. Elimination of Dead Cells ------------------------------- 7

1.2.3 Cancer Cell Death ---8

A. Hypoxia-induced Apoptosis ------------------------------ 8

B. Chemical-induced Apoptosis in Cancer -----------------10

1.2.4 Summary -- 13

1.3 **MicroRNAs and E2F-Dysregulation in Cancer** ----------------------14

1.3.1 Introduction -- 14

1.3.2 MiRNA Biogenesis --- 14

1.3.3 MiRNAs and Variants of Seed-Matching --------------------------------18

1.3.4 OncomiRs --- 18

A. OncomiR-1 ---21

B. MiR-155 --22

C. MiR-21 --- 23

1.3.5 MiRNAs and Cell Cycle Regulation -------------------------------------24

A. Enhancement of Cell Growth ----------------------------- 24

B. Delay of Cell Growth -------------------------------------25

1.3.6 p53-mediated Cell Cycle Network -- 26

1.3.7 E2Fs and c-Myc --- 27

- A. Structure and Function of E2Fs -------------------------- 28
- B. Deregulation of E2Fs ------------------------------------- 31
- C. E2F and Cell Proliferation ------------------------------- 31
- D. E2F and Apoptosis -- 32

1.3.8 Pro-apoptotic E2F Targeted miRNAs ------------------------------------ 33

1.3.9 Potential Therapeutic Roles of miRNAs ---------------------------------- 37

1.3.10 Conclusion -- 39

1.4 **Elimination of Apoptotic Cell Debris** ---------------------------------- 39

1.4.1 Introduction --- 39

1.4.2 The Processes of Elimination --- 40

- A. Migration towards Apoptotic Debris -------------------- 40
- B. Phagocytic Synapse ---------------------------------------41
- C. Internalisation --- 42
- D. Formation of Phagolysosome and Degradation ------- 42

1.4.3 Inflammation from Impaired Apoptosis --------------------------------- 44

- A. Production of autoantigen by apoptosis -----------------45
- B. Deficiency in Apoptosis Pathway ------------------------45
- C. Failure in Removal of Apoptotic Debris ----------------46

1.4.4 Conclusion -- 46

1.5 **Aims and scope of the Project** -- 47

1.5.1 Overall Objective --47

1.5.2 Specific Aims -- 48

CHAPTER 2.

Materials and Methods -- 52

2.1 Human Cell Lines and Culture Conditions ------------------------------- 52

2.2 Cytotoxicity Assays -- 53

2.3 Detection of Annexin V/Propidium Iodide (PI) Apoptosis ------------- 54

2.4 Analysis of Mitochondrial Potential Changes -------------------------- 54

2.5 Protein Extraction -- 55

2.6 Western Blotting --56

2.7 RNA Extraction --56

2.8 Real-time RT-PCR -- 57

2.9 Immunocytochemistry --- 58

2.10 Caspase-3 Activity Assays --- 59

2.11 Multi-pathway Reporter Assays --59

2.12 Subcellular Protein Extraction ---60

2.13 Transient Transfection Assays ---60

2.14 His-tagged Protein Pull-down --- 61

2.15 Co-immunoprecipitation --- 62

2.16 Xenograft Establishment and Data Collection -------------------------- 63

2.17 Paraffin Wax Infiltration --- 63

2.18 Immunohistochemical Staining --- 64

2.19 Hematoxylin and Eosin Staining -- 64

2.20 Statistical Analysis -- 65

CHAPTER 3

CBF inhibits cell growth in different human cancer cell lines and induces differential apoptotic pathways in colon cancer cell lines

CBF inhibits cell growth in different human cancer cell lines and induces differential apoptotic pathways in colon cancer cell lines -------------------------- 66

Introduction -- 66

Results --- 69

CBF exhibits significant cytotoxicity in a variety of cancer cells, but has the most potency in colon cancer cell lines ---------------------------------69

CBF induces potent pro-apoptotic effects in colon cancer cell lines ------71

Apoptotic cell death in HCT116 is through both Caspase-3-dependent and -independent pathways ---75

Apoptotic cell death in HT29 is through Caspase-3-independent pathway only --- 78

Discussion -- 81

CHAPTER 4

Changes of HIF-1α and CTTN expression are involved in CBF-induced apoptosis in the *in vitro* and *in vivo* experiments ------------------------------------- 86

Introduction -- 86

Results --- 89

CBF significantly affects HIF-1α and serum response factor (SRF) -regulated intracellular pathways -- 89

CBF upregulates mRNA of HIF-1α but downregulates HIF-1α protein expression ---91

CBF inhibited CTTN protein expression in HCT116 in the first 12 hours but not in HT29 cells ---96

CBF promotes nuclear translocation of CTTN in HCT116 cells but not in HT29 --99
CBF did not retard the growth rate of tumour ------------------------------ 103
CBF affects HIF-1α and CTTN expression in mouse tumour tissues ---- 105
Discussion -- 109

CHAPTER 5

Australian cane toad extract and liposomal curcumin inhibit cell growth in different human cancer cell lines -- 118
Introduction -- 118
Results -- 122
The Australian cane toad extract displays significant cytotoxicity in different human colon cancer cell lines ------------------------------------- 121
Liposomal curcumin inhibits lung cancer cell growth in cytotoxicity assays, but is not as effective as sole curcumin ----------------------------- 126
Discussion -- 129

CHAPTER 6

Conclusion and Future Directions -- 132

APPENDICES

Appendix I -- 134
Appendix II --- 135
Appendix III -- 136

REFERENCES -- 139

List of Tables

Table 1 OncomiRs --- 20

Table 2 Predicted apoptotic E2F targeted miRNAs ------------------------------ 34

Table 3 Pro- and anti-apoptotic miRNAs discovered in recent studies --------- 36

Table 4 EC_{50} values of CBF in different cancer cell lines ----------------------- 69

List of Figures

Figure 1.1 A scheme of intrinsic and extrinsic apoptotic pathways. --------------- 5

Figure 1.2 The principle of HIF-1α-regulated pathway. ---------------------------- 9

Figure 1.3 Schematic representation of postulated mechanisms related to CGs intracellular actions. ---12

Figure 1.4 A scheme of miRNA maturation. -- 17

Figure 1.5 A model of a feedback loop regulated by miR-17/20a, E2F1-3 and c-Myc. ---27

Figure 1.6 Distinct structures of E2F family. --28

Figure 1.7 A model of E2F regulated transcriptional repression and activation. - 30

Figure 1.8 Apoptotic signalling pathways and the involvement of miRNA regulation. --- 35

Figure 1.9 A model of an apoptotic-cell-containing a phagosome. -----------------44

Figure 3.1 Cytotoxicity of CBF among various cancer cell lines. ----------------- 70

Figure 3.2 Detection of CBF-induced apoptosis in colon cancer cell lines. -------72

Figure 3.3 CBF-induced apoptosis is both Caspase-3 dependent and independent in HCT116 cells. -- 76

Figure 3.4 CBF-induced HT29 apoptosis is neither Caspase-3 nor AIF-dependent. -- 79

Figure 4.1 HIF-1α-mediated pathway is involved in CBF treatment. -------------- 90

Figure 4.2 CBF affects HIF-1α transcription and protein expression. ------------- 93

Figure 4.3 CBF affects CTTN transcription and protein expression. ------------- 97

Figure 4.4 CBF affects the distribution of HIFα and CTTN. ------------------------ 100

Figure 4.5 CBF treatments in nude mouse models injected with HCT116 cells. - 104

Figure 4.6 Analysis of CBF treatment in xenografts. --------------------------------- 106

Figure 4.7 CBF treatments in mouse colon cancer cell line CT26 and the composition of CBF solution. -- 115

Figure 5.1 Structures and classification of CGs. ------------------------------------- 120

Figure 5.2 Analysis of composition and cytotoxicity of the Australian cane toad extract. -- 123

Figure 5.3 The structure and cytotoxicity of liposomal curcumin in human lung cancer cell line Spc-A1. --- 127

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Publications Related to This Project

1. Yao, Q., Cao, S., **Li, C**., Mengesha, A., Low, P., Kong, B., Dai, S., and Wei, M. "Turn a diarrhoea toxin into a receptor-mediated therapy for a plethora of CLDN-4-overexpressing cancers" *Biochem Biophys Res Commun* 2010 **398**(3), 413-419

2. Yao, Q., Cao, S., **Li, C**., Mengesha, A., Kong, B., and Wei, M. "Micro-RNA-21 regulates TGF-beta-induced myofibroblast differentiation by targeting PDCD4 in tumor-stroma interaction" *Int J Cancer* 2010 **128**(8), 1783-1792

3. **Li, C**. Hashimi, S. M., Good, D. A., Cao, S., Duam, W., Plummer, P. N., Mellick, A. S. and Wei, M. Q. "Apoptosis and MicroRNA Aberrations in Cancer" *Clinical and experimental pharmacology & physiology* 2012

4. **Li, C**., Li, K., Yao, Q., Cao, S., Mellick, A. And Wei, M. " Exploiting proapoptotic factors in colon cancer cells for the development of novel therapies" Abstract for Australasian Gene Therapy Society 6th Meeting 29 Apr – 1 May 2009

5. **Li, C**., Hashimi, S. M., Cao, S., Good, D. A. and Wei, M. Q. "Bufadienolide compound, cinobufagin, inhibits the expression of hypoxia-inducible factor 1 alpha subunit and cortactin in colon cancer cell lines" Abstract for the International Congress Natural Anticancer Drugs, Olomouc, Czech Republic, June 30 – July 4, 2012

CHAPTER 1

1.1 General Introduction:

The most effective method of hindering tumourigenesis is to induce the death of immortalised cancer cells. To achieve this anticancer research has focused on inducing two endogenous cellular pathways leading to cell death: apoptosis and necrosis. Of these two mechanisms of cell death in association with chemotherapy apoptosis has shown the most promising results in impairing cancer growth. Various approaches have been developed to activate endogenous apoptotic pathways. However, due to the failure of current available therapies scientists are still seeking novel agents and molecular therapies that could potentially cure cancer. Recently, small non-coding RNAs, namely microRNAs, have been revealed to possess a central role in cell cycle regulation including apoptosis. MicroRNAs are involved in post-transcriptional gene expression and have been implicated in the regulation of cell differentiation and development. Nevertheless, it is their role in regulating the induction of apoptosis that makes miRNA exploitation a potentially potent and novel therapeutic strategy.

Finally, the clearance of internal apoptotic cells after anti-tumour treatment is a further complication confronting the scientific community and provides an important new direction for the anticancer research.

1.2 Apoptosis and Cancer Cell Death

1.2.1 Introduction

Apoptosis, characterised by programmed cell death (1), and its relevant pathways has been an integral part of anti-carcinogenesis in the last decade. Of the various mechanisms, including necrosis, autophagy, mitotic catastrophe and senescence (2) that are available to kill cancer cells, apoptosis is preferred. This is because it acts inside the cell and is less likely to induce an aberrant immune response (3,4). Apoptosis plays a housekeeping role and is an important part of normal cell cycle regulation (5). Under normal homeostatic conditions, apoptosis restrains cells from inappropriate cell growth. Cells may be induced to apoptosis in response to a wide range of signals, including DNA damage, stress and removal of growth factors. As one of a number of cellular failsafe programmes, apoptosis occurs either contemporarily or after the regulations of checkpoints and cell cycle efforts. Because of this, defects in apoptotic pathways are tightly linked to tumourigenesis. This section will focus on the regulations of apoptosis and its contribution to the success of chemotherapies against cancer.

1.2.2 Apoptotic Mechanisms

There are two basic processes for the activation of apoptosis: the intrinsic pathway and the extrinsic pathway (**Figure 1.1**). Cellular execution and elimination of debris are the results of activation of either of these two pathways acting in concert or separately. These apoptotic activities vary but interrelate with one another so as to form an apoptotic network.

A. Intrinsic Apoptotic Pathway

The intrinsic apoptotic pathway is under the strict control of B-Cell Lymphoma 2 (BCL-2) protein family. It mainly takes place at the outer membrane of mitochondria. Once stimulated by various apoptotic signals, BCL-2 Homology 3 (BH3) only proteins, especially BH3-Interacting-domain Death agonist (BID) and BCL-2-Interacting Mediator of cell death (BIM), activate BCL-2-Associated X protein (BAX) and/or

BCL-2-Antagonist/Killer-1 (BAK) (6). Furthermore, cytosolic BAX assemble on the surface of mitochondria, where localised BAK undergo conformational changes (7,8). Subsequently, BAX and BAK proteins homo-oligomerise and induce pore formation in the outer mitochondrial membrane. Via these pores, various mitochondrial pro-apoptotic proteins, including Second Mitochondrial-derived Activator of Caspase (SMAC), Endonuclease G (Endo G), Apoptosis-Inducing Factor (AIF) and Cytochrome *C*, leak out and play crucial roles in apoptosis induction. For example, SMAC induces apoptosis by deactivating Inhibitor of Apoptosis Protein (IAP) that represses a family of Cysteine-aspartic acid proteases (Caspase) (9). Released Cytochrome *C*, together with members of the apoptosome consisting of Apoptotic Protease Activating Factor 1 (Apaf-1), ATP and pro-Caspase-9, activates Caspase-9 which then activates Caspase-3, forwarding apoptosis (10,11).

In addition, there exists another machinery of mitochondrial fragmentation, which also leads to mitochondrial outer membrane permeabilisation, as well as the further release of Cytochrome *C*. A number of mitochondrial fission/fusion proteins, including Human orthologue of Fis1p (hFis1), Dynamin-Related Protein 1 (Drp1), BCL-2 family proteins and intra-mitochondrial Ca^{2+} signalling, are also responsible for mitochondrial membrane permeabilisation as well as the further release of Cytochrome *C*, however the precise mechanism is largely unknown (12-15).

B. Extrinsic Apoptotic Pathway

The extrinsic pathway takes place as a result of a complex network of several signal transduction pathways. Distinct from the intrinsic pathway, there is no specific site associated with extrinsic apoptosis, which follows a prominent Fas-Associated Death

Domain protein (FADD) – Caspase axis, accompanied by a series of protein kinase and phosphatase activities (16). This process is initiated by the targeting of various death ligands, including necrosins, cytotoxins, lymphotoxins and Tumour Necrosis Factors (TNFs), to their corresponding Death Receptors (DRs) on the surface of cell membrane. Most of these receptors are transmembrane proteins and contain a death domain within their cytoplasmic regions. Following activation the death domain triggers the downstream apoptotic cascade by a direct or indirect interaction with FADD. In many cases, TNF-Receptor 1 (TNF-R1) activates FADD via the intermediate membrane protein, TNF Receptor-Associated Death Domain protein (TRADD), whereas the death domain of Fas receptor (a death receptor of TNF receptor superfamily) directly connects to FADD. Subsequently, the Death Effector Domain (DED) of active FADD disassociates and binds to the adjacent DED of Caspase-8, which then releases its enzymatic motif, known as Caspase-3. This in turn leads to proteolysis in other protein substrates to advance the execution of apoptosis. In addition, to the main FADD-Caspase axis, Caspase-2 seems to influence another apoptotic cascade. This involves association with p53-Induced protein with a Death Domain (PIDD) and RIP (Receptor-Interacting Protein) –Associated ICH-1/CED-3 homologous protein with a Death Domain (RAIDD) (17,18).

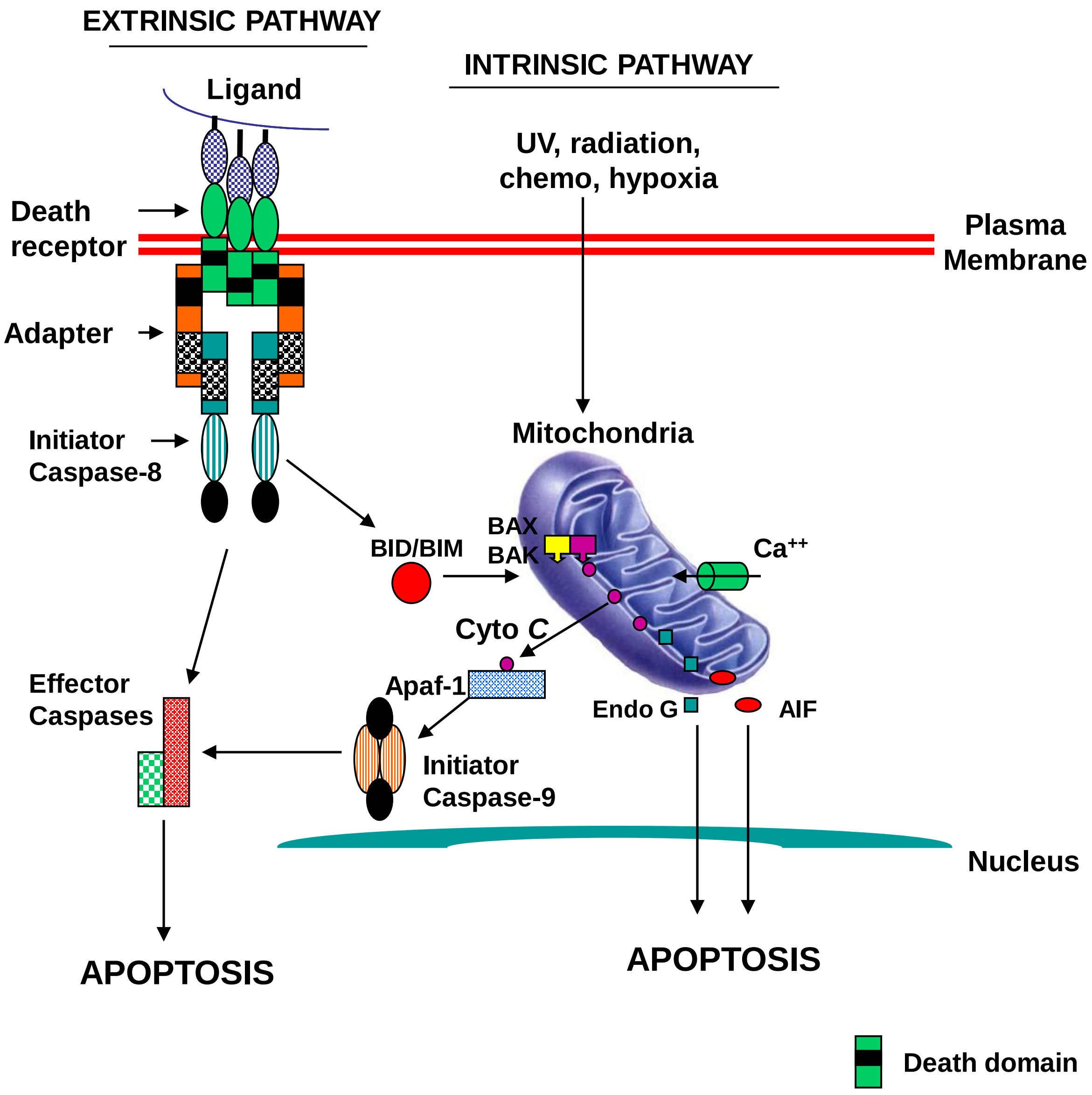

EXTRINSIC PATHWAY
INTRINSIC PATHWAY
Ligand
UV, radiation, chemo, hypoxia
Death receptor
Plasma Membrane
Adapter
Initiator Caspase-8
Mitochondria
BAX
BAK
BID/BIM
Ca++
Cyto C
Apaf-1
Effector Caspases
Endo G
AIF
Initiator Caspase-9
Nucleus
APOPTOSIS
APOPTOSIS
Death domain

Figure 1.1 A scheme of intrinsic and extrinsic apoptotic pathways. The intrinsic pathway mainly occurs in the mitochondria and various mitochondrial permeabilisation proteins play central roles to induce apoptosis. On the other hand, a series of death receptors and intermediate membrane proteins are involved in the extrinsic apoptotic pathway. Original figure from: Gupta, S., Agrawal, A., Agrawal, S., Su, H. & Gollapudi, S. (2006) A paradox of immunodeficiency and inflammation in human aging: lessons learned from apoptosis *Immun Ageing* **3**, 5.

C. Execution of Apoptosis

The procedure of execution of apoptosis is associated with morphological changes, specifically pyknosis (chromatin condensation), karyorrhexis (nuclear fragmentation), cell shrinkage and generation of apoptotic bodies (1). In the face of a complicated apoptotic signalling system, the knowledge of precise mechanisms of execution remains limited. Thus far, two mechanisms have been identified: Caspase-dependent and Caspase-independent cell death.

Caspase dependent execution is a process of irreversible proteolytic activity, which directly or indirectly results in distinct morphologies, depending on the characterisation of cleaved protein substrates. In this case, the “executioners” referring to Caspase-3, -7 and -6 (see above) target a broad spectrum of intracellular proteins including protein kinases, signalling effectors, cytoskeletal proteins and transcription/translation regulators (19). For instance, the cleavage of LaminA-C by Caspases gives rise to the loss of nuclear envelope integrity (20-22). The cleavage of Caspase close to the C-terminal of ROCK I (serine/threonine kinase Rho-associated Kinase I) enables phosphorylation of myosin light chain, resulting in contraction of actin microfilaments and apoptotic blebbing (23,24). Caspase activities also facilitate the attraction of phagocytes for engulfment. In dying cells, Caspase-3 activates iPLA (Independent Phospholipase A), which in turn promotes the release of a chemotactic property, lysophosphatidylcholine (LPC) (17). Such a lipid directs monocytes towards the apoptotic cells.

On the other hand, the apoptogenic factors carrying out non-Caspase dependent cell death normally reside in the mitochondrial intermembrane space, like AIF, Endo G and

High Temperature Requirement protein A 2 (HtrA2) (25-27). These mitochondrial proteins are most likely to undergo a universal process that begins with mitochondrial permeabilisation, resulting in nuclear translocation, followed by DNA degradation or chromatin condensation.

D. Elimination of Dead Cells

The elimination of dying cells is of great importance in the whole apoptotic process. Although the machinery has not been very well characterised, the dogma suggests that the removal is by means of efferocytosis, in tandem with immunological repression (28,29). Initially, the apoptotic bodies and debris release various attraction and electric signals (30) which gather the phagocytes, including macrophages, immature dendritic cells, epithelial cells and fibroblasts (18,31-33). These phagocytes then directly or indirectly interact with the dying cells through phagocytic receptors, specific binding sites and bridge molecules (31,34). Efferocytosis is then triggered by phagosomal Guanosine Triphosphatase (GTPase) Ras-related C3 botulinum toxin substrate 1 (Rac-1) signalling, which causes an assembly of efferocytic actins in close approximation to the apoptotic cells (34,35). The redundant cytoskeletons constitutively extend and cuddle the target (31). Engulfed cells are then degraded through a mechanism mediated by Toll-Like Receptor 4 (TLR-4) signalling (36). The other important details on the removal of apoptotic cells will be presented later.

Along with the clearance, anti-inflammatory substances, including Transforming Growth Factor-β (TGF-β), Prostaglandin E2 and Platelet-activating factors, are also recruited to suppress the local aberrant immune responses (28,29,37,38). Despite this, recent studies have shown that apoptosis is to some extent immunogenic. *In vivo*, the application of anthracyclins, oxaliplatin and irradiation in immunocompetent mice have resulted in a lethal inflammation, indicating that apoptosis stimulated by chemo- and radiotherapies is only provisionally free from inflammation (33,39,40). The immunogenic capability of apoptosis should therefore be taken into account during any study using novel anticancer substances and modalities.

1.2.3 Cancer Cell Death

As a result of the numerous investigation of cell death, induction of apoptosis in cancer has been considered as an ideal way to overcome uncontrolled cell proliferation associated with cancer. An assortment of approaches have been invented and applied both *in vitro* and *in vivo* to initiate apoptosis in immortalised cells, with limited results.

A. Hypoxia-induced Apoptosis

Research has shown that hypoxia can induce apoptosis (41,42). Hypoxia-Induced Apoptosis (HIA) is regulated by pro-apoptotic proteins BAX and BAK (43,44). Under hypoxic conditions, BAX and BAK elicit the alteration in mitochondrial transmembrane potential, resulting in release of Cytochrome *C* and Caspase activation (see above). Hypoxia is a driver of apoptosis in many different cell types. Among several factors involved in HIA, Hypoxia-Inducible Factor (HIF) plays the central role (**Figure 1.2**). HIF is a transcription factor complex composed of -α and Aryl hydrocarbon Receptor Nuclear Translocator (ARNT, aka HIF-1β) subunits (45,46). While ARNT is constitutively expressed, the degradation and expression of HIF-α intimately corresponds to changes in oxygen levels (47,48). During oxygen deprivation, Prolyl Hydroxylase Domain (PHD) responsible for oxygen-dependent degradation of HIF-α is inhibited, permitting HIF-α to enter the nucleus, where HIF-α binds to ARNT and initiates the transcription of hypoxia-responsive genes (49). To date, the mechanisms of HIF-α modulated apoptosis are only partially characterised. The primary focus of most investigations is to understand the relationship between HIF-α and BCL-family proteins, such as BNIP3, a member of BH3 only pro-apoptotic proteins (see above), BAX, BCL-2 and B-Cell Lymphoma-Extra Large (BCL-X_L) (50-53).

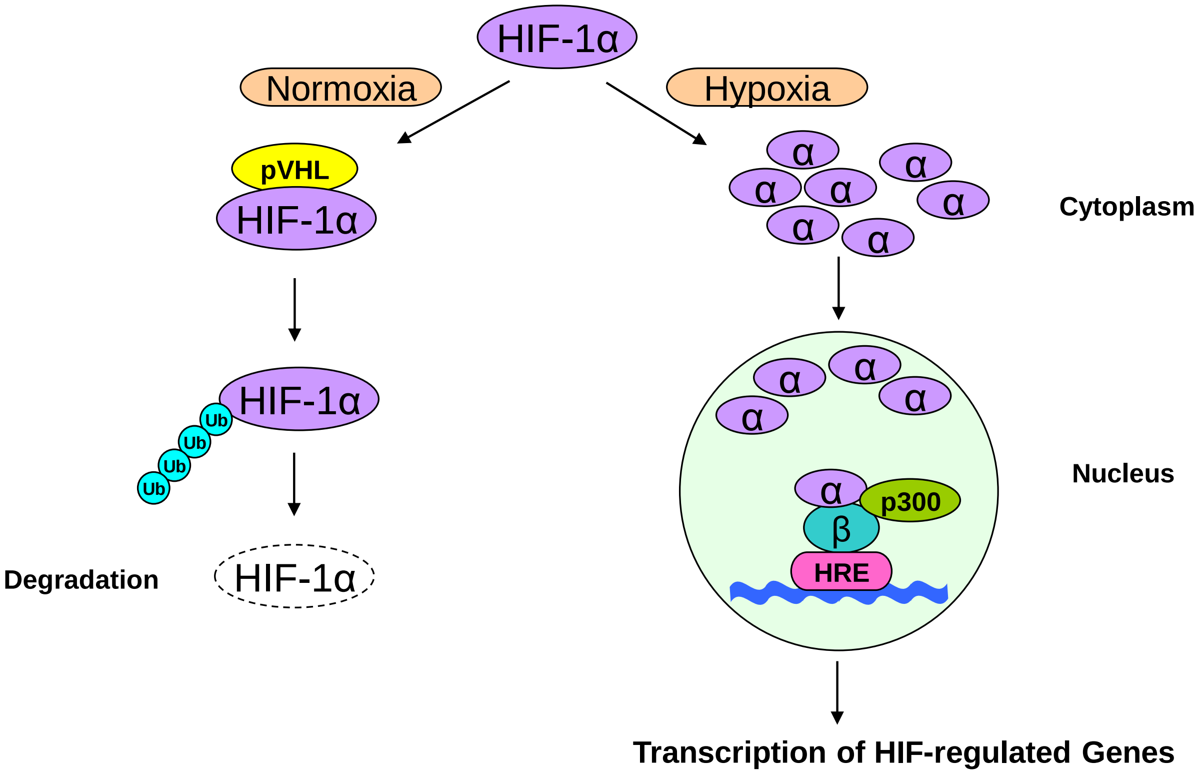

HIF-1α
Normoxia
Hypoxia
pVHL
HIF-1α
α
α
α
α
α
α
α
α
Cytoplasm
HIF-1α
Ub
Ub
Ub
Ub
α
α
α
α
Nucleus
α
p300
β
HRE
Degradation
HIF-1α
Transcription of HIF-regulated Genes

Figure 1.2 The principle of HIF-1α-regulated pathway. In a normal atmosphere, HIF-1α is bound by Von Hippel-Lindau tumour suppressor (pVHL), which causes the subsequent ubiquitination of HIF-1α. As a result, HIF-1α is degraded in the cytoplasm. On the other hand, HIF-1α is translocated into the nucleus in oxygen deprivation. HIF-1α accumulates and forms a complex with ARNT and p300. The complex eventually binds to hypoxia regulated element (HRE), initiating the transcription of hypoxia-responsive genes.

B. Chemical-induced Apoptosis in Cancer

There have been a trend that scientists are hunting for natural inducers of apoptosis in recent years (54). These natural products are typically obtained from the living tissues of higher plants, micro-organisms and animals, and exist in different chemical and biological forms. We will focus on two natural compounds: curcumin and cinobufagin (CBF).

Curcumin (diferuloylmethane) is derived from the plant curcuma longa and is a traditional Indian herb. Curcumin has been found to have profound effects as an anticancer and anti-inflammatory drug (55). Curcumin acts through the intrinsic apoptotic pathway by upregulating p53, as well as a series of p53-mediated pro-apoptotic proteins, like p53 Upregulated Modulator of Apoptosis (PUMA) and Phorbol-12-Myristate-13-Acetate-Induced Protein 1 (Noxa, aka PMAIP1) (56). Curcumin modulates the BCL-2 family proteins by increasing levels of BAK, BAX and BIM, while at the same time inhibiting the expression of anti-apoptotic BCL-2 and BCL-X_L (56,57). The subsequent release of Cytochrome *C* from mitochondria leads to an increasing Caspase-3 activity (58-60), as well as suppression of IAPs (61). Curcumin activates extrinsic pathways by permitting the accumulation of Fas receptors and DRs (62). These receptors are increased after curcumin treatment in both p53-mutant and –wildtype melanoma cell lines. In addition, studies by Takahashi *et al.* have shown that curcumin-induced inhibition of neutrophil activity is the result of reduction of chemotactic cytokine Interleukin-8 (63). Taken together, the wide mechanism of action of curcumin in inducing apoptosis demonstrates its potential as an anticancer drug, though there still remains a dire need for more investigations for its successful translation to the clinic.

CBF is the major component of a traditional Chinese medicine, Chansu (aka Huachansu), which is extracted from toad venom. Chansu has been widely used in the treatment of arrhythmia and inflammation for centuries. Recently, many papers have revealed that Chansu induces apoptosis in cancer cell line studies (64-68). Furthermore, the application of Chansu in clinical trials also gives encouraging results. Chansu combined with gemcitabine-oxaliplatin showed an improvement of life quality of patients with metastatic gallbladder carcinoma (69). Another ongoing Phase II clinical trial is using Chansu and gemcitabine in the treatment for pancreatic cancer by M.D Anderson Cancer Centre (70). In short, Chansu has a potential to be developed as a novel drug for cancer therapy.

To date, the composition of Chansu has been investigated and several main components are commercially available, including CBF, resibufogenin and bufalin (71). In fact, these compounds have a common digoxin-like structure and belong to the family of cardiac glycosides (CGs) or cardiotonic steroids. CGs are a group of compounds which block Na^+/K^+ pumps by binding to the extracellular part of α or β subunit of the ion channel (72). Hence, they have the capacity to increase the intracellular calcium ion concentration and are conventional drugs for congestive heart failure, such as digoxin and digitonin (73). Scientists have been exploring therapeutic roles of CGs in oncology since 1970s (74,75), though mostly focusing on plant-extracted compounds. These numerous studies have presented a complex network related to functions of CGs in apoptosis and tumourigenesis (**Figure 1.3**). The inhibition of Na^+/K^+-ATPases by CGs is direct and results in a high level of Na^+ and Ca^{2+}, which subsequently causes mitochondrial injury and thereby apoptosis (76). Apart from the blockade of Na^+/K^+ pumps, a number of CGs promote the extrinsic apoptotic pathway by upregulating DR4 and DR5 in lung cancer cell lines (77). In the research using breast cancer cell line MDA-MB-435s, ouabain is able to activate Src kinase and secondarily transactivates epidermal growth factor receptor (EGFR), triggering the downstream extracellular signal-regulated kinase (ERK) cascade (78). Moreover, digoxin significantly inhibits the protein expression of HIF-1α but upregulates the mRNA level of HIF-1α in different types of cancer cells and in xenografts (79). Additionally, CGs also induce apoptosis by the suppression of anti-apoptotic proteins, such as nuclear factor-κB (NF-κB), Myc and BCL-2 (80-83).

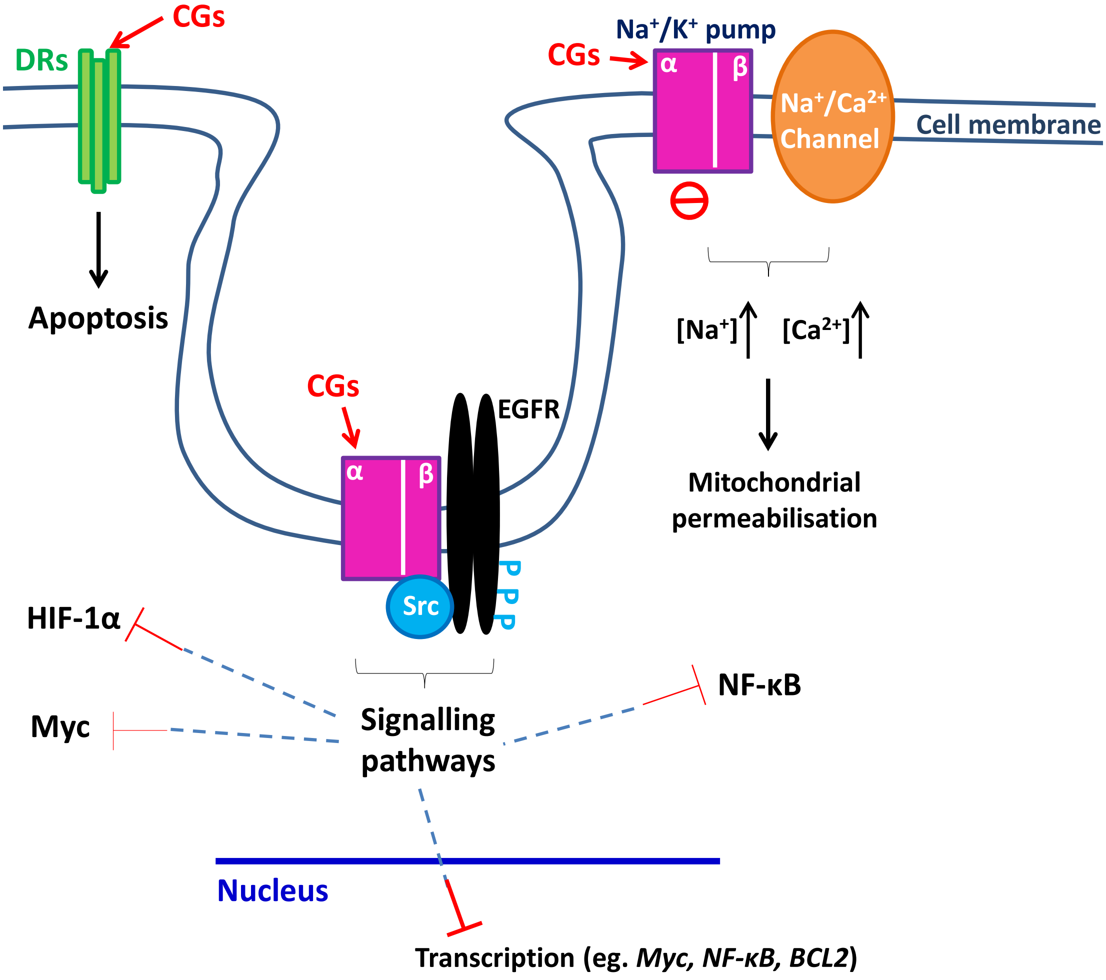

CGs
DRs
Apoptosis
Na$^+$/K$^+$ pump
CGs
α
β
Na$^+$/Ca^{2+} Channel
Cell membrane
⊖
[Na$^+$]↑ [Ca^{2+}]↑
Mitochondrial permeabilisation
CGs
EGFR
α
β
Src
P P P
HIF-1α
Myc
Signalling pathways
NF-κB
Nucleus
Transcription (eg. *Myc, NF-κB, BCL2*)

Figure 1.3 Schematic representation of postulated mechanisms related to CGs intracellular actions. Na$^+$/K$^+$ pumps are located across the cell membrane and in caveolae. The cascade that follows is depicted in the figure and well-characterised key pathway regulators are shown. CG affected apoptotic mechanisms are referred to in the text.

Previous reports have identified CBF as a Na^+/K^+-ATPase inhibitor with treatment resulting in high levels of intracellular calcium (84,85). Lately, CBF has been found to induce apoptosis in various cancer cell lines, such as Hela and LNCaP (86-88). Another advantage of CBF lies in a very low concentration required for the inhibition of cancer cell growth. Compared with that of plant-extracted compounds, the inhibitory concentration of toad-extracted CGs are at least 10 times lower in cancer cells (70). This hypothetically reduces cardiovascular side effects of CBF and supports our laboratory's suggestion that CBF is a potent candidate as a novel anticancer drug. Last but no least, the suppression of immune response or anti-inflammation has been taken into account in cancer therapy (89,90). CBF is commonly used as an anti-inflammatory agent in traditional Chinese medicine, though its mechanism of action remains largely unknown. Thus, CBF could be an ideal solution to treat cancer with low immunogenicity.

1.2.4 Summary

The previous section reviewed the work of apoptotic research with a focus on apoptotic pathways and induction of apoptosis. Previous research has focused on mitochondrial regulation and pro-apoptotic signal transduction pathways. Investigation of death effectors and cascades has shown that the whole apoptotic system appears to be more complicated than scientists once believed. Areas yet to be fully understood include the degradation of engulfed cells and occasional apoptosis linked immunogenicity. Regardless, induction of apoptosis still has a vital role in anticancer therapies.

1.3 MicroRNAs and E2F-Dysregulation in Cancer

1.3.1 Induction

MicroRNAs (miRNAs) are small (20~23mers) non-coding RNA molecules, which act as master regulators of a range of cellular pathways and processes (91). In general, miRNAs post-transcriptionally regulate gene expression by means of binding to their targets in mRNAs and inhibiting subsequent protein translation. A single microRNA can target multiple genes in a given signalling pathway where it acts as regulators of tight gene expression and tissue specific protein synthesis. As such, miRNAs represent key regulators of cell growth and may play important roles in cell death. Recent studies have shown that many miRNAs that mediate the expression of pro- and anti-apoptotic genes/oncogenes have also been implicated in the development of cancer (**Table 1 & 2**). Particularly, a family of miRNAs that are part of the miR-17-92 cluster have been found to regulate the classic adenovirus E2 promoter binding Factor (E2F) / Myelocytomatosis viral oncogene homologue (Myc) regulatory circuit (92). Myc and E2F are involved in signal transduction and are parts of well-established pathways linked to cell cycle progression. This section will seek to assess the current knowledge of E2F targeted miRNAs in apoptosis and the role they play in cancer.

1.3.2 MiRNA Biogenesis

Biogenic pathways of the mature single-stranded microRNAs initiate from primary miRNA transcripts (pri-miRNAs) (93,94) (**Figure 1.4**). Selection of pri-miRNAs to transcribe is determined by certain transcription factors or methylation occurring on miRNA promoter regions (95,96). In the nucleus, due to their typical 5’-methyl G-capped and 3’ polyadenylated structures, pri-miRNAs are mainly recognised and transcribed by RNA polymerase II, less frequently by RNA polymerase III (97-99). Next, a unique step occurs after the transcription, namely microRNA editing (100). During editing, Adenosines (A) in pri-miRNA transcripts are modified into Inosine (I) by Adenosine Deaminases Acting on RNA (ADARs) (101-103). This base-pair-alteration not only enhances or inhibits the subsequent enzymatic cleavage (103,104), but also affects the downstream miRNA-regulated molecular processes (105).

Followed the A to I editing, pri-miRNA transcripts are cleaved into hairpin-like miRNA precursors (pre-miRNAs) by RNase III enzyme Drosha and the DiGeorge Critical Region 8 (Drosha-DGCR8) microprocessor complex (106). The process is a collaboration between DGCR8 that binds to the pri-miRNA and Drosha that cleaves the pri-miRNA (107). This cleavage results in a hairpin structure of pre-miRNA, which contains a double-stranded stem with high fidelity complementary sequence pairing, and a loop region that has less precise sequences for Dicer (a RNase III enzyme) cleavage (108-110). Mutations which cause loss of hairpin structure and impaired recognition by the microprocessor complex can lead to impaired miRNA processing and have been found in human tumours (111).

Pre-miRNAs are then exported to the cytoplasm by Exportin-5 (112). Exportin-5 is supposed to accurately distinguish pre-miRNA short hairpins among other RNA molecules based on their structural characteristics, a short double strand (~33 nucleotides) with a 3’overhang (113-115). In the cytoplasm, RNA-Induced Silencing Complex (RISC) – Loading Complex (RLC), which consists of the RNase III endonuclease, Dicer, Tar RNA Binding Protein (TRBP), Protein Activator of PKR (PACT) and Argonaute1-4 (Ago1-4) recognises hairpin pre-miRNAs (116-119). Dicer then cleaves off the terminal loop of hairpin pre-miRNA to produce a miRNA duplex (~22 base pairs) with 2-nucleotide-overhangs at both 3'ends. In many cases, the cytoplasmic pre-miRNA has firstly to undergo an additional Ago cleavage at the 3' arm of the stem, generating a nicked Ago-cleaved precursor miRNA (ac-pre-miRNA) (120). This nicked strand supports the further dissociation of miRNA duplex and turns out a sense strand for degradation (121). TRBP and PACT act to stabilise Dicer and promote the efficiency of the downstream post-transcriptional regulation of miRNA (117,118). Yet, Dicer is still capable of producing mature miRNAs in the absence of TRBP and PACT (122). In order to produce a single antisense strand (mature miRNA), miRNA duplex is unwound and the sense strand is degraded (123,124). This process is yet to be determined, but Ago is likely to reconstitute with the mature miRNA so as to steady the short molecule during mRNA degradation and translational repression (125,126).

Eventually, the mature miRNA strand is embedded in P bodies that are made up of different mRNA-translational repressed ribonucleoprotein (RNP) complexes (127). Mature miRNAs loaded in P bodies form functional miRNA-induced silencing complexes (miRISCs), which bind to 3' untranslated region (3'UTR) of target mRNAs and lead to translational repression, mediating post-transcriptional silencing (128).

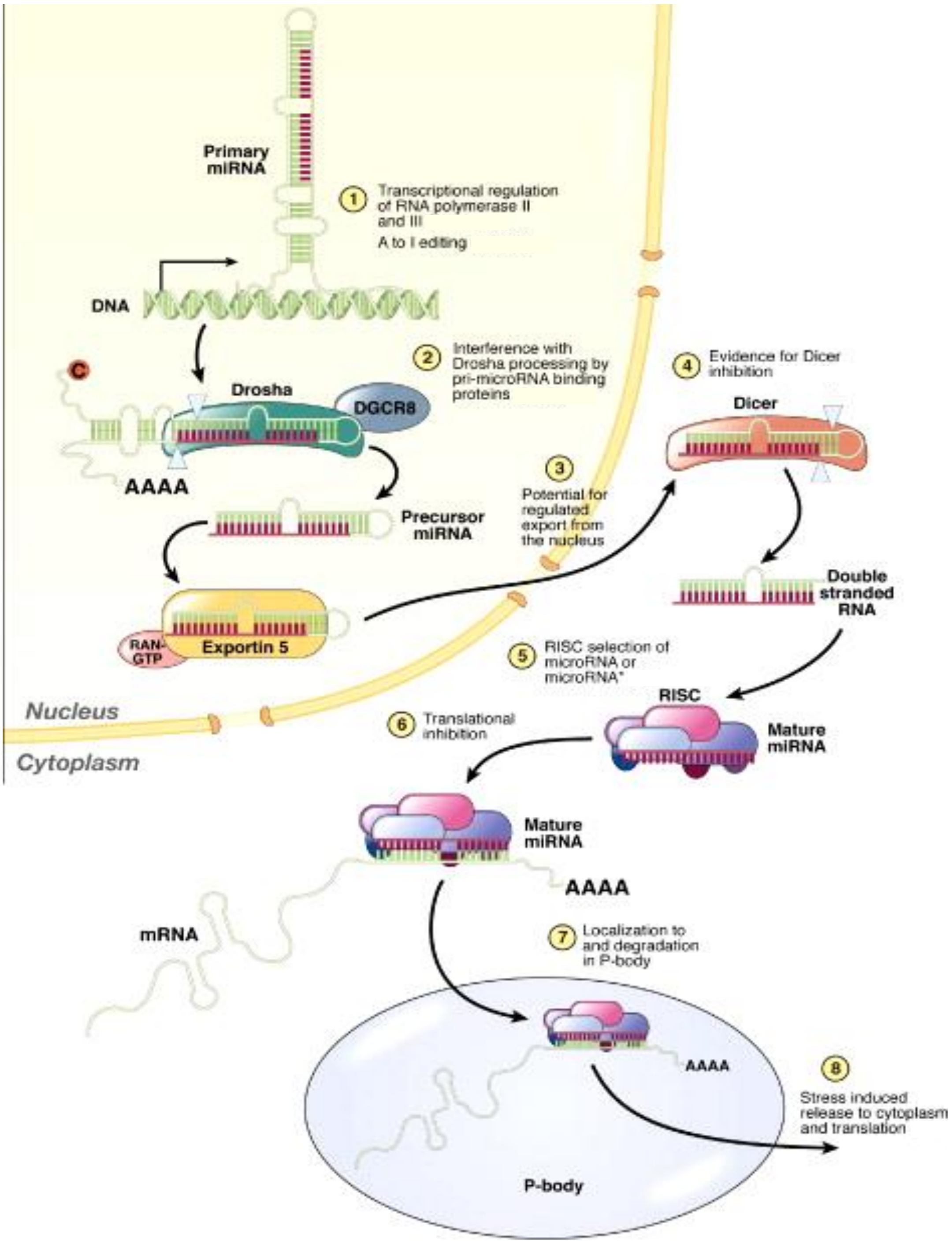

Primary miRNA
1 Transcriptional regulation of RNA polymerase II and III
A to I editing
DNA
C
Drosha
DGCR8
2 Interference with Drosha processing by pri-microRNA binding proteins
AAAA
Precursor miRNA
3 Potential for regulated export from the nucleus
RAN-GTP
Exportin 5
4 Evidence for Dicer inhibition
Dicer
Double stranded RNA
5 RISC selection of microRNA or microRNA*
RISC
Mature miRNA
Nucleus
Cytoplasm
6 Translational inhibition
Mature miRNA
AAAA
mRNA
7 Localization to and degradation in P-body
AAAA
8 Stress induced release to cytoplasm and translation
P-body

Figure 1.4 A scheme of miRNA maturation. MiRNAs are endogenous small non-coding RNAs (20-23 nucleotides) and originally transcribed in the nucleus. Mature miRNAs play a central role in the post-transcriptional regulation of mRNA. As shown above and in the text, a series of protein complexes and enzymatic cleavages are involved in the processing of miRNA maturation. Original figure from: O'Hara, S.P. *et al.* (2009) MicroRNAs: key modulators of posttranscriptional gene expression *Gastroenterology* **136**, 17-25.

1.3.3 MiRNAs and Variants of Seed-Matching

A mature miRNA contains a unique seed region (2-7 nucleotides) within its 5’UTRs (128). For mRNA recognition, the nucleotides in the seed are complementary to the sites within 3’UTR of targeted mRNAs. Due to the shortness of a seed and frequent single nucleotide polymorphism (SNP) within a seed, a single miRNA targets hundreds of mRNAs, and in turn one mRNA is also bound with various miRNAs (129). The perfect matching between a seed and 3’UTR site leads to the degradation of mRNAs. On the other hand, any imperfect pairing with mRNAs might cause loss of functions of mRNAs and alter post-translational regulations. These changes frequently affect either cell death or cell proliferation towards cancer. A very recent study showed that the binding between miR-184 and the tumour necrosis factor α induced protein 2 (TNFAIP2) is closely related to squamous cell carcinoma of the head and neck (SCCHN) (130). Among the SNP frequencies of miR-184, miR-155, miR-105 and miR-550 to the 3’UTR of TNFAIP2, only a base change from T to C within miR-184 seed strongly enhances its binding to TNFAIP2. This leads to a significant reduction of TNFAIP2 expression and elevates the risk of SCCHN onset.

1.3.4 OncomiRs

Aberrations in miRNA expression are closely linked to tumourigenesis. Any miRNA which functions by downregulating a tumour suppressor is referred to as an OncomiR (131). So far several OncomiRs have drawn special attention in the academic arena worldwide (**Table 1**). Nevertheless, an increasing number of studies show that one miRNA targets a variety of mRNAs and plays either anti- or pro-apoptotic roles among different cancers. These characteristics add complexity to function assessment of a single miRNA, and thereby further investigations need to be done with respect to specific cancers to elucidate their roles. Several critical miRNAs have been identified as being important in the regulation of apoptosis during cancer development.

Table 1. OncomiRs			
microRNA	**Putative targets**	**Up/down regulated**	**Types of cancer**
miR-17-92 family	Rbl2 (132) E2F1-3 (133) HIF-1α (134) PTPRO (135) Tsp1 (136) CTGF (137)	Up	Lung (138) Anaplastic thyroid (139) B cell lymphoma (140) Colon (141) Glioblastoma (137)
miR-21	BCL-2 (142) Maspin (143) PTEN (144) PCDC4 (143,145-147) TPM1 (148)	Up	Breast (142,143,148,149) Liver (144) Colorectal (145) Ovarian (150) Cervical (151) Glioblastoma (152)
miR-10b	HOXD10	Up	Breast (153)
miR-27a	ZBTB10 (154,155) Myt-1 (154) Prohibitin (156)	Up	Breast (154) Gastric adenocarcinoma (156)
miR-155	TP53INP1 (157) Pu.1 (158) RhoA (159) Socs (160)	Up	Pancreatic (157) B cell lymphoma (161) Breast (159) Leukaemia (162)
miR-221/222	p27 (163,164) p57 (165) PLZF (166) ERα (167)	Up	Prostate (163) Thyroid papillary (158) Melanoma (166) Breast (164,167) Lung (168) Liver (169) Gastric (170)
miR-372/373	Neo1 Lats2 ESR1	Up	Testis (171)

A. OncomiR-1

The OncomiR-1 (aka miR-17-92) is particularly significant as duplications and deletions have been found in many cancers. The miR-17-92 cluster (locus on chromosome 13q31.3) is composed of seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1 (172). The overexpression of the miR-17-92 cluster has been reported in a variety of cancers, such as B-cell lymphoma (131), lung cancer (138,173), colorectal cancer (174) and thyroid cancer (139). He *et al*. first revealed that a high level of miR-17-92 expression occurs in the *Eμ-myc* transgenic mouse model of lymphoma (131). In this model, miR-17-92 enhances *c-myc*-induced B-cell lymphomagenesis. At the same time, O'Donnell *et al.* demonstrated that cellular-Myc (c-Myc) is also a transcription factor for miR-17-92 (175). Silencing Myc in multiple myeloma cell lines downregulates the expression of several components of the miR-17-92 cluster and induces apoptosis in B lymphocyte cell line P493-6 (176). Also, C-Myc and adenovirus E2 promoter binding Factors (E2Fs) are closely related and recognised as key regulators in advancing cell cycle. In the miR-17-92 cluster, miR-17-5p and miR-20a function as a brake to control the expression of E2F1 through a negative feedback loop (176).

The relationship between the miR-17-92 cluster and pro-apoptotic protein BIM is another point drawing scientists' attention. Kanzaki *et al.* found that BIM was activated in Myc-knockout myeloma cell lines and BIM expression was significantly inhibited when miR-17 and miR-18 were overexpressed in the Myc-knockout cell lines (176). Therefore, the miR-17-92 cluster partially inhibits myeloma cell apoptosis by the suppression of BIM. Similarly, Jason *et al.* reported that glucocorticoid-mediated downregulation of miR-17-92 elevates BIM expression in the murine T-cell lymphoma cell line WEHI7.2 (177). BIM plays a pro-apoptotic role in glucocorticoid-induced apoptosis among different lymphoma models. An overexpressed miR-17-92 cluster partially reduces glucocorticoid-induced BIM levels. In addition, BIM has been found to be a direct target of miR-92a, which is overexpressed in colon cancer tissues (178).

Apart from its OncomiR role, the miR-17-92 cluster is also found to have an anti-proliferation impact. A tight linkage of miR-17-5p and miR-20a with E2Fs reveals a possible pro-apoptotic role for the miR-17-92 family (see below).

B. MiR-155

MiR-155 is largely known as an OncomiR and enhanced expression of miR-155 occurs in a number of B-cell lymphomas. In Hodgkin's lymphoma, miR-155 expression is accompanied with high *BIC* RNA levels (179-181). The *BIC* locus is a common retroviral integration site in avian-leukosis virus-induced lymphomas (182,183), and miR-155 precursor is embedded within its target *BIC* gene (181). Enforced expression of miR-155 in B-cell lineage by using transgenic mice directly shows a preleukemic lymphoproliferation onset, as well as a tendency of B-cell malignancy (184). In solid tumours, Nikiforava *et al.* observed a significant fold change of miR-155 in thyroid cancer (185). This suggested a link between a high miR-155 level and specific papillary linked mutations in isoform B of the serine-threonine kinase Raf (BRAF), RAS and Papillary Thyroid Cancer (RET/PTC) oncogenes. In other words, an elevation in miR-155 expression in thyroid cancer cells could be a sign of the mutations in BRAF, RAS and RET/PTC. Additionally, miR-155 interferes with p53-mediated cellular pathways by repressing tumour protein p53-induced nuclear protein 1 (TP53INP1) (157,186). Thus, overexpression of miR-155 and low levels of TP53INP1 mRNA provide independent clinicopathological and prognostic values in colorectal cancer (187). Besides, another two crucial mediators of apoptosis were reported to be directly inhibited by miR-155 (188). Wang *et al.* suggested that FADD and Caspase-3 are the targets of miR-155, based on their study on the pathogenesis of intervertebral disc degeneration. Mir-155 physically binds to the 3'UTR of FADD and Caspase-3, and downregulation of miR-155 promotes Fas-mediated apoptosis.

On the other hand, miR-155 has recently been revealed to induce apoptosis in dendritic cells (DCs) (189). Upregulation of miR-155 indirectly restrains the degradation of $p27^{kip1}$ and arrests cell cycle progress. MiR-155 initially binds to its putative target KPC1 that is required for polyubiquitilation of $p27^{kip1}$, resulting in stabilised $p27^{kip1}$ and DC apoptosis. In miR-155 knockout mice, the proportion of apoptotic DCs was much lower than that of wildtype mice post 5 days assessment.

C. MiR-21

The oncogene-like miR-21 has been directly implicated in tumour progression and metastasis. MiR-21 is located at 17q23.1 and the mature miRNA is composed of 22 ribonucleotides (miRBaseSequences & Ensembl). The involvement of miR-21 in cancer was firstly described as having a potential anti-apoptotic impact in human glioblastoma cells (152). A target site of miR-21 is in the 3'UTR of the tumour suppressor, tropomyosin 1 (TPM1) and overexpression of miR-21 leads to the downregulation of TPM1 in MCF-7 cells (148,190,191). MiRNA microarray analysis by Meng *et al*. showed a high level of miR-21 expression in human hepatocellular cancer (144,192). In this study, the tumour suppressor Phosphatase and Tensin homologue (PTEN) was identified as a target of miR-21. The level of PTEN, a pro-apoptotic protein, was increased when miR-21 expression was inhibited in malignant hepatocytes, modulating cell migration and invasion. In addition, a novel drug, Difluorinated-Curcumin (CDF), was developed to target and kill pancreatic cancer (PC) stem cells (193). CDF treatment showed that the induction of miR-21 was attenuated and as a consequence, PTEN expression was increased in xenograft models of human PC.

Another validated miR-21 target is programmed cell death protein 4 (Pdcd4) (194-196). In anti-mir-21 treated RKO cells, Pdcd4 expression is upregulated and reduces colon cancer invasion (197). *In vivo*, downregulation of miR-21 or overexpression of Pdcd4 retards the formation and growth of glioblastoma in a murine xenograft model (198). Moreover, elimination of miR-21 increased the proportion of apoptotic cells in skin papillomas in a miR-21-null mouse model (127). The loss of miR-21 notably elevated the expression of PTEN and Pdcd4, and thereby inhibiting the activation of Ras downstream effector pathways.

1.3.5 MiRNAs and Cell Cycle Regulation

As outlined above, miRNAs are closely linked to cell cycle progression. They either enhance or inhibit cell growth, depending on whether they act to inhibit tumour suppressors or oncogenes. MiRNAs have been identified to modulate the levels of several regulators involved in cell cycle. In general terms, the cell cycle progression is under the control of a few key regulators, such as Cyclins, Cyclin-Dependent Kinases (CDKs), E2Fs and p53.

A. Enhancement of Cell Growth

A number of miRNAs advance cell division by reducing the levels of cell cycle inhibitors. In the late G_1 phase, miR-221/222 can promote entry of S phase by suppressing the CDK inhibitors and the members of Cdk-Interacting/Kinase-Inhibiting protein (Cip/Kip) family, p27 and p57 (199,200). MiR-221/222 target sites are in 3'UTR of p27 and p57 mRNA. Under conditions of low serum, premature cells over-expressing miR-221/222 are still driven into S phase, by bypassing the G_1 /S checkpoint(165). *In vivo*, upregulation of p27 in the miR-221/111 knocked-down glioma mouse leads to significant repression in tumourigenesis (201). Similarly, the cell cycle may also be hastened by disrupting Transforming Growth Factor β (TGFβ) - dependent checkpoint. Petrocca *et al*. (2008) elucidated that miR-17-92 and its paralogue miR-106b-25 could blockade TGFβ effector CDKN1A (aka p21) and apoptotic inhibitor BCL2L11 (aka BIM), resulting in the impairment of TGFβ-mediated cell cycle-arrest (202,203).

B. Delay of Cell Growth

Certain miRNAs act to block cell division. The miR-15a/16 clusters, particularly the miR-16, can trigger an accumulation in G_0/G_1 phase in colon cancer cell lines by downregulating the expression of multiple cell cycle-promoting genes, such as Cyclin D1, Cyclin D3, CDK6 and CDC27 (204,205). *In vivo*, enrichment of miR-15a expression suppresses Cell Division Cycle 25 homologue A (CDC25A), reducing hepatic cyst growth in a rat model of polycystic kidney disease (206). A delay in quiescence can also be induced by *lethal-7* miRNA (*let*-7). The study of Johnson *et al.* (2007) in lung and liver cancer cells elucidated that mature let-7 post-transcriptionally inhibits Cyclin D2, CDK6 and CDC25A (207). Furthermore, miR-122, an abundant hepato-specific miRNA, is reduced in human hepatocellular carcinoma (208,209). This observation is predicted to associate with the deregulation of miR-122 target, Cyclin G_1, as well as the Cyclin-related p53 network (208).

1.3.6 p53-mediated Cell Cycle Network

The function of p53 in regulating cell growth has been researched for decades, while the study of miRNAs is relatively new. Members of the miR-34 family (miR-34a, miR-34b & miR-34c) have been implicated in the p53-mediated cell cycle network (210-213). Yet only *pri-miR-34a* transcripts have been directly involved in cell cycle (212). A study by Chang *et al*. in colon cancer showed that induction of miR-34a strongly promotes apoptosis in p53 wildtype HCT116 cells, whereas almost no effects were exhibited in p53-null HCT116 cells (211). This result indicates that miR-34a is a direct target of p53. Likewise, by using wildtype and p53-deficient mouse embryonic fibroblasts, He *et al*. showed that p53 functioned as a promoter to control miR-34b and miR-34c transcription (210). In this study, initial suppression of p53 using shRNA increased the activity of Rat Sarcoma viral oncogene homologue (Ras), resulting in liver carcinoma in mouse models. Conversely, recovery of p53 function caused restoration of all the miR-34 isoforms and spontaneous senescence of liver tumour cells. Furthermore, miR-34 expression can be significantly elevated in a p53-dependent mechanism following irradiation-induced DNA damage (213). MiR-34 has been predicted to target various cell cycle regulators, including CCNE2, CDK4, MET, CCND1, CDK6, MYCN, BCL-2 and SIRT1 (210,214-219). The close relationship between these regulators and p53 strongly implicates miR-34's involvement in p53-regulated network. For example, SIRT1 is a p53 inhibitor and its expression is much higher in chronic lymphocytic leukaemia (CLL) than in normal B lymphocytes. A recent study showed that nicotinamide treatment in CLL effectively elevates miR-34 induction and represses SIRT1 (220). In other words, inhibition of SIRT1 by nicotinamide through the induction of miR-34a activates the p53-mediated apoptotic pathway in CLL cells.

1.3.7 E2Fs and c-Myc

An increasing number of reports have linked miRNAs with the complex regulatory loop surrounding E2Fs and c-Myc regulation of cell cycle, especially the regulatory circuit including miR-17-92 (**Figure 1.5**) (92,133,221,222). Both E2F1-3 and c-Myc are in a positive-feedback loop of transcriptional activation. Initially, c-Myc triggers both expressions of E2F1-3 and miR-17-92 that in turn negatively modulates E2Fs translation. Next, activated E2Fs continuously self-stimulate and stimulate further c-Myc and miR-17-92 expression (133,221). Taken together, the important function of miR-17-92 in the scheme is to restrict excess E2F activity. Intriguingly, miR-17-5p is capable of blockading c-Myc expression so as to strengthen the inhibition of E2F (223). As such miR-17-92 can either be oncogenic (see above) or tumour suppressive in cell growth, depending on the pro- or anti- proliferation role of E2Fs in cancer.

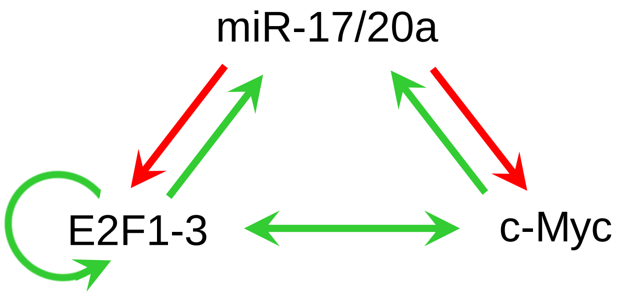


Figure 1.5 A model of a feedback loop regulated by miR-17/20a, E2F1-3 and c-Myc. c-Myc initially activates the expression of E2F1-3 and miR-17/20a. Then active E2F1-3 in turn stimulate their own activities, c-Myc and miR-17/20a. On the other hand, miR-17/20a inhibits E2F1-3 expression and Myc transcription. Therefore, the role of miR-17/20a is like a brake to prevent E2F1-3 overexpression. Red arrow: Inhibition; Green arrow: Activation.

A. Structure and Function of E2Fs

E2F transcription factors are required for various molecular biological functions. Most importantly, E2Fs play critical roles in cell cycle G_1/S phase transition. In mammals, nine E2F family members (E2F1-8) are encoded in eight *E2f* genes (224), of which E2F3a and E2F3b are encoded by *E2f3* but are transcribed by distinct promoters (225). All the E2F subgroups contain at least one DNA binding domain (DB), but only E2F1-5 possess a transactivation domain (224,226) (**Figure 1.6**). This transactivation domain also embeds a pocket protein-binding domain specific for retinoblastoma protein (pRb) and its homologues, p107 and p130.

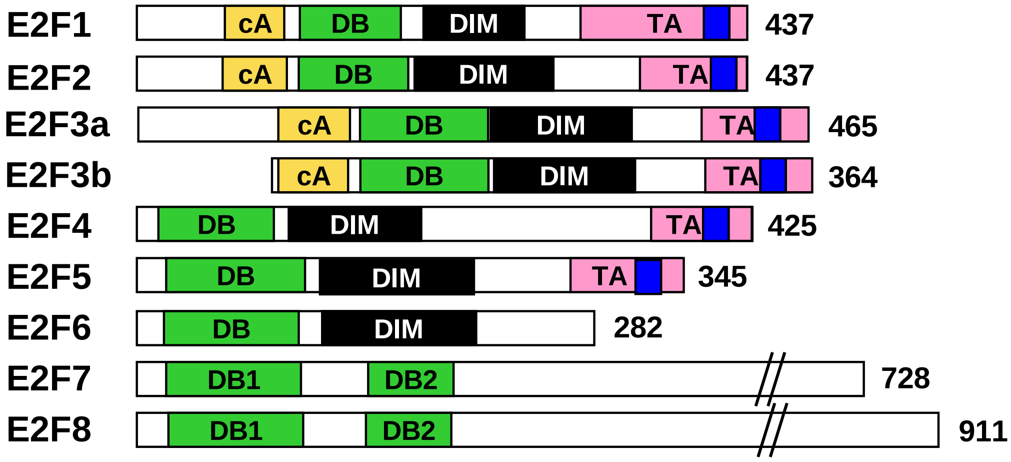

E2F1
cA
DB
DIM
TA
437
E2F2
cA
DB
DIM
TA
437
E2F3a
cA
DB
DIM
TA
465
E2F3b
cA
DB
DIM
TA
364
E2F4
DB
DIM
TA
425
E2F5
DB
DIM
TA
345
E2F6
DB
DIM
282
E2F7
DB1
DB2
728
E2F8
DB1
DB2
911

Figure 1.6 Distinct structures of E2F family. There is at least one DNA binding domain (DB, green) located in all the members of E2F family, whereas only E2F1-6 have a dimerisation domain (DIM, black). By contrast, E2F7-8 contain two DBs which allow them to form dimers with each other. E2F1-5 also contain a transactivation domain (TA, pink), where a domain required for pRb binding (blue) embeds. In addition, E2F1-3 contain a Cyclin A binding site (cA, yellow). The number of amino acids is shown on the right.

Based on their roles in cell cycle, E2Fs can be divided into two groups: Activators (E2F1, E2F2 and E2F3a) and Repressors (E2F4-8) (227). In quiescent cell, the formation of pRb/E2F complex prevents Activators from binding to their E2F promoters, which control the expression of downstream proteins required for DNA replication and cell cycle (228) (**Figure 1.7**). Meanwhile, a repressive complex, composed of E2F4, E2F5, p107/130, Histone Deacetylases (HDACs), Histone Methyltransferases and DNA Methyltransferases, occupies these promoters and represses the expression of Activators. Upon cell cycle entry, the bound pocket proteins are phosphorylated by Cyclin D/CDK4 or /CDK6 complexes, releasing both Activators and Repressors. Free Activators then bind to their promoters and switch on the transcription of genes which regulate cell cycle progression, initiating S phase entry. On the other hand, E2F4 and E2F5 repressors no longer bind to the promoters after G_0/G_1 phase and are exported from the nucleus.

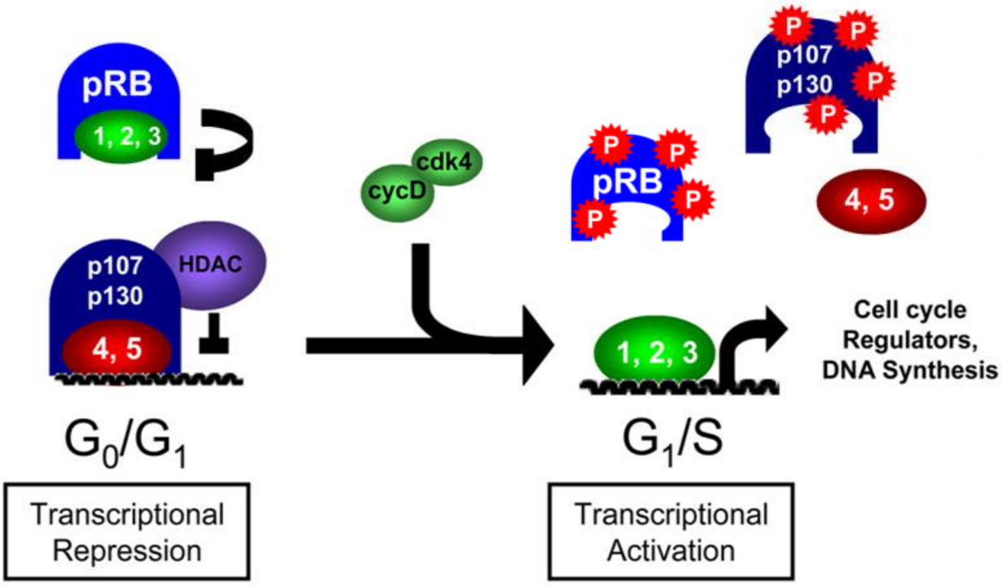

pRB
1, 2, 3
p107
p130
HDAC
4, 5
cdk4
cycD
P
pRB
p107
p130
4, 5
1, 2, 3
Cell cycle
Regulators,
DNA Synthesis
G_0/G_1
G_1/S
Transcriptional Repression
Transcriptional Activation

Figure 1.7 A model of E2F regulated transcriptional repression and activation. The formation of pRb and E2F1-3 complex prevents activated E2Fs from binding to their promoters where a repressive complex (E2F4-5, p107/130 and HDAC) occupies through G0/G1 phase. Once cyclinD/cdk4 complexes phosphorylate pRb and p107/130, free E2F1-3 bind to their promoters and initiate transcription required for DNA replication and cell cycle progression. pRB: pRb; 1,2,3: E2F1-3; 4,5: E2F4-5. Original figure from: Iaquinta, P.J. & Lees, J.A. (2007) Life and death decisions by the E2F transcription factors *Curr Opin Cell Biol* **19**, 649-657.

In the absence of transactivation domain, E2F6-8 function as transcriptional repressors. A hydrophobic heptad repeat domain is embedded in the E2F1-6 structure, facilitating dimerisation with Dimerisation-Partner protein 1 to 4 (DP1-4). The heterodimer between E2F and DP is vital for transcriptional activities of E2F1-6. Alternatively, E2F7 and E2F8 contain an additional DB instead of the dimerisation domain. Because of these two DBs, E27-8 can function via the formation of homodimers (binding to each other) or heterodimers (binding to DNA) (229,230).

B. Deregulation of E2Fs

Alterations in expression of E2F transcription factors have been shown to lead to either proliferation or cell death. Aberrations in E2F regulation can be induced by DNA damage. Whether cells proliferate or die depends on the cell type, developmental stage and/or specific apoptotic signals. Recent studies have broadened our knowledge of both of these factors involved in E2F-induced apoptosis, as well as E2F targeted miRNAs and their roles in cell cycle.

C. E2F and Cell Proliferation

Regulators of E2Fs overlap during cell cycle progression. Previous work demonstrates that partial loss of function in any of the E2F1-3 subgroups leads to a deficiency in expression of E2F target genes, and blocks S phase entry (231). In the absence of E2F1 or E2F3, cells accumulate in quiescence (232). However, only E2F3 seems to be essential for continued cell growth. Lack of E2F3 allows sustained activity of Alternative Reading Frame (ARF) of CDK inhibitor 2A, which antagonises MDM2 protein and activates p53 (233). Without bound to MDM2, p53 stays active and produces p21 expression. Consequently, the inhibition of CDK to phosphorylate pRb causes cell cycle defect. Hence, the E2F1-3 mediated inappropriate cell growth underlies the ARF-p53 pathway**.** Intriguingly, E2F can also feature an all-or-none control for G_1/S transition (234). In this case, Activators switch on the transcription according to a threshold (a concentration of serum levels), regardless of the later changes in serum. E2F1, E2F2 and E2F3a remain “ON” stage until serum levels drop below the threshold. In addition, E2Fs can contribute to a pro-proliferation role by hastening the transcription of proliferation-related effectors (226). In G_0 phase, E2F1, E2F2 and E2F3a are normally deactivated in bound with phosphorylated pRb.

However, in the presence of abundant activating E2Fs, excessive free E2Fs take over the promoter regions for many anti-apoptotic genes, driving quiescent immortal cells to enter S phase, i.e. proliferation onset.

D. E2F and Apoptosis

Since the overexpression of E2Fs can induce apoptosis, many studies have drawn special attention to E2F-induced apoptosis, particularly E2F1 (235-238). Although E2F1, E2F2 and E2F3a are able to elicit apoptosis in a similar manner, E2F1 appears to dominate cell death (235,238). The reason for this is that E2F2 and E2F3a fail to target many crucial apoptosis related proteins that bind to E2F1. In some cases, E2F2 and E2F3a stimulate apoptosis by propelling E2F1 expression, implying that E2F1 is the only subgroup of the E2F family to individually elicit apoptosis (227). This suggestion is consistent with the observation that overexpression of E2Fs can induce apoptosis in the *E2f1* wildtype mice (239). Furthermore, E2F1 deficiency interferes with the overall level of E2Fs, so that insufficient E2F1 leads to impaired apoptosis (227).

E2F1 expression can also elicit apoptosis in pRb knock-out mice (240) and this response is similar to the scenario of excessive E2F towards the activation of p53 mentioned earlier. E2F1 is able to phosphorylate p53 either on its own or with the aid of Ataxia Telangiectasia Mutated (ATM) and checkpoint kinase 2 (Chk2) (241-244). Paradoxically, E2F-induced apoptosis is also found to occur in a p53-independent manner. In the absence of p53, E2F1 represses cell proliferation by elevating pro-apoptotic regulators, such as Apaf-1, Caspase family and BH3-only proteins (245). Alternatively, E2F1 can promote cell death by blockading certain survival signals from NF-κB or BCL-2 (245).

1.3.8 Pro-apoptotic E2F Targeted miRNAs

An increasing number of E2F miRNAs are found to have pro-apoptotic impact in tumourigenesis, especially the miR-17-92 cluster. Pro-apoptotic E2F miRNAs are summarised in **Table 2** which is based on recent studies and bioinformatic analysis (TargetScan).

Although referred to as an OncomiR complex, miR-17-92 also plays a role in anti-proliferation. The main apoptotic components of the miR-17-92 family are miR-17-5p and miR-20a. It has been shown that miR-17-5p and miR-20a are not only activated by c-Myc, but restrict c-Myc-mediated cellular progression by downregulating the expression of E2F1-3 as well (175,221). MiR-20a was identified to play an anti-apoptotic role by binding to E2F1-3 mRNA in prostate cancer cells (221). Another example is that of miR-17-5p and miR-20a which repress the translation of the oncogene amplified in breast 1 (*AIB1*) in breast cancer (246). AIB1 protein levels were significantly reduced during the treatment of miR-17-5p in MCF7 cells which typically have an abundant level of AIB1. In the same study, however, the siRNA inhibition of miR-17-5p eventually increased AIB1 level in HeLa cells. In addition, the role of miR-17-5p and miR-20a in promoting apoptosis is also supported by the discovery that cell cycle effector Cyclin D1 initially stimulates miR-17-5p and miR-20a expression, which conversely inhibits Cyclin D1 (223).

Table 2. Predicted apoptotic E2F targeted miRNAs

microRNA	Putative targets	Up/down regulated	Types of cancer
miR-34	p53 (210) SIRT1 (219) c-Myc (247) E2F3-5 CDK4 (248)	Down	Lung (216) Prostate (249) Pancreatic (211) Colon (214) Leukaemia (250)
miR-15/16	BCL-2 (251) E2F3,7	Down	Gastric(252) Lymphocytic Leukaemia (253) Prostate (254)
miR206	ERα	Down	Breast (255,256)
miR-17-92	AIB1 E2F1-3	Down	Breast (246)
miR-200	ZEB1-2 (257-260) E2F3	Down	Breast (257) Ovarian (258)
let-7/miR-98	Lin-41 (261) HMGA2 (262,263) TRIM71 (264) Myc (265) Ras (266) Caspase-3 (267) E2F2,5,6 ERα (268)	Down	Ovarian (269) Lung (266,270,271) Colon (272) Lymphoma (265) Breast (273)
miR-143/145	ERK5 (274,275) E2F3 DNMT3A (276)	Down	Colon (277,278) B-cell lymphoma (279) Cervical (151,280) Leukaemia (281) Prostate (282)
miR-181	Tcl1 (283) E2F5,7	Down	Glioma (266) Lymphoma (284)

Other miRNAs discovered in recent studies are listed in **Figure 1.8** and **Table 3**.

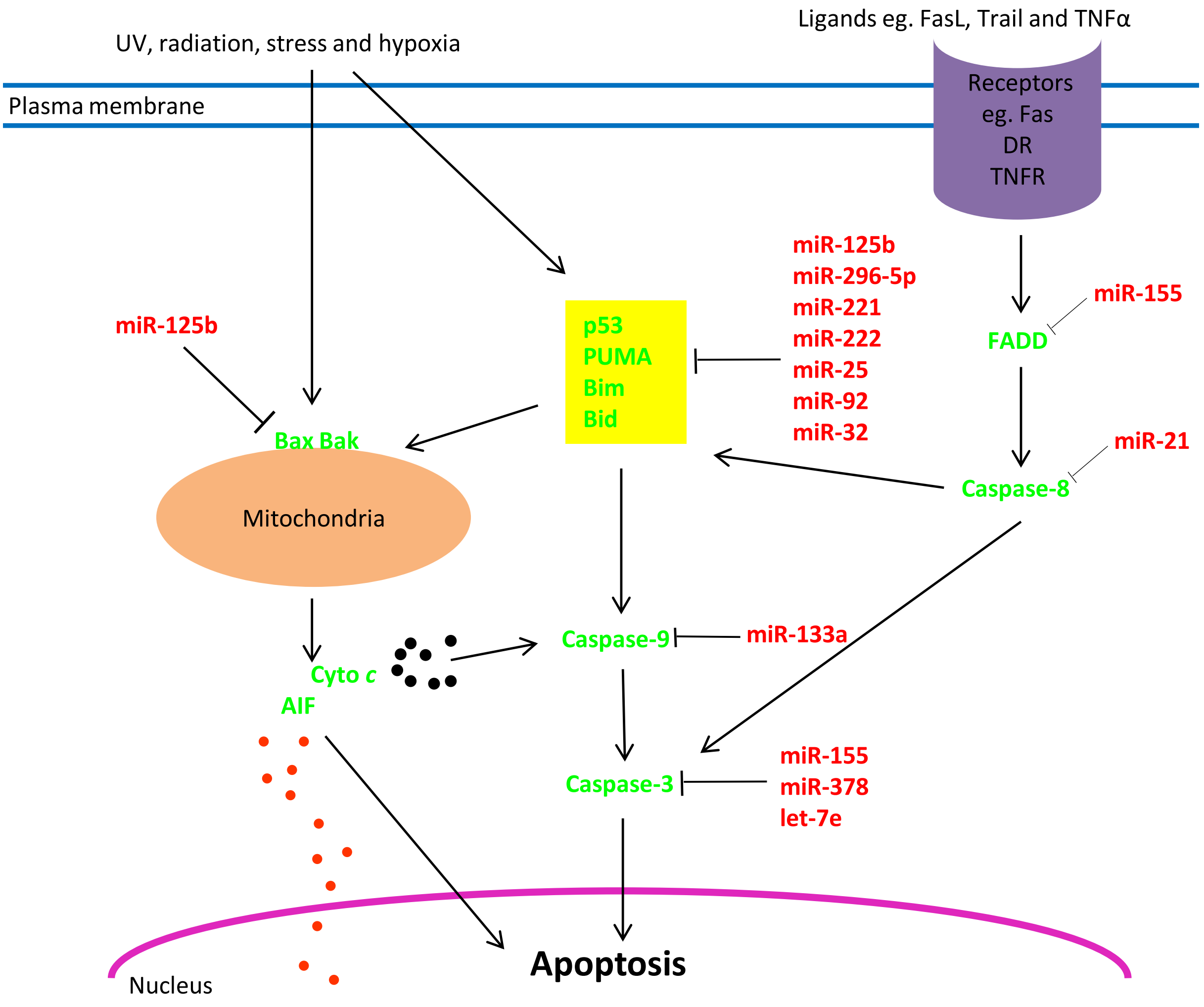

Ligands eg. FasL, Trail and TNFα
UV, radiation, stress and hypoxia
Plasma membrane
Receptors eg. Fas DR TNFR
miR-125b
miR-296-5p
miR-221
miR-222
miR-25
miR-92
miR-32
miR-155
miR-125b
p53
PUMA
Bim
Bid
FADD
Bax Bak
miR-21
Caspase-8
Mitochondria
Caspase-9
miR-133a
Cyto c
AIF
Caspase-3
miR-155
miR-378
let-7e
Nucleus
Apoptosis

Figure 1.8 Apoptotic signalling pathways and the involvement of miRNA regulation. The apoptotic pathway can be either activated by environmental factors or death ligands. The cascade that follows is depicted in the figure and well-characterised miRNAs regulating key genes in the pathway are shown. Apoptotic mechanisms are referred to in the text. Green font: pro-apoptotic proteins; Red font: anti-apoptotic miRNAs.

Table 3. Pro- and anti-apoptotic miRNAs discovered in recent studies.

Pro-apoptotic miR	Targets/Roles	Anti-apoptotic miR	Targets/Roles
miR-1204	↑ p53 (285)	miR-125b	↓ BAK1, PUMA (286,287)
miR-150	↓ DKC1 AKT2 (288)	miR-296-5p	↓ PUMA (289)
miR-10b	↑ BIM (290)	miR-221	↓ PUMA (291,292)
miR-31	↑ BIM (293)	miR-25	↓ BIM (294)
miR-101	↓ EZH2 (295)	miR-92	↓ BIM (178)
miR-203	↑ Bax, Caspase-3 (296)	miR-32	↓ BIM (297)
miR-29b	↓ Mcl-1 (298)	miR-155	↓ FADD, Caspase-3 (188)
miR-143	↑ Caspase-3 (299)	miR-21	↓ Caspase-8 (300)
miR-205	↓ E2F1, E2F5 (301)	miR-133	↓ Caspase-9 (302)
miR-23a	↓ XIAP (303)	miR-378	↓ Caspase-3 (304)
miR-145	↓ BNIP3 (305)	let-7e	↓ Caspase-3 (306)

↑: upregulation induced by a miRNA of a direct target or a regulator in apoptotic pathways.

↓: downregulation induced by a miRNA of a direct target or a regulator in apoptotic pathways.

1.3.9 Potential Therapeutic Roles of miRNAs

Because of the importance of miRNA regulation in tumourigenesis and apoptosis, there is a potential to develop novel therapeutic strategies using miRNAs. Their signatures have been detected in different cancers. Thus, miRNAs can be regarded as good biomarkers for prognosis of cancer. As early as in 2006, Japanese scientists compared the results from real-time RT-PCR of specific *Homo sapiens* miR-155 (hsa-miR-155) between control and cancer patient groups to confirm the diagnosis for lung adenocarcinomas (307). Recently, overexpression of miR-21 and underexpression of miR-34a have been correlated with diagnosis and overall survival of pancreatic ductal adenocarcinoma (308).

Apart from their diagnostic and prognostic functions, several miRNA-involved therapeutic tools have been utilised in cancer research. All of these are based on two principles: induction of anti-proliferation or inhibition of pro-proliferation.

Restoration of expression of an apoptotic miRNA is done by exogenously producing small interference RNA (siRNA) molecules in cancer cells. These synthetic siRNAs induce translational repression like endogenous miRNAs and have showed strong capacity to inhibit target protein expression (309). Alternatively, miRNA precursor sequences are integrated into a viral vector encoding a short hairpin structure. These viral vectors may also encompass reporters and inducible tissue-specific promoters for delivery of the miRNAs (310). Certain viral vectors such as those of the lenti-viral family are also capable of infecting non-proliferating and proliferating cells.

Anti-apoptotic miRNAs (antimiRNAs) can also be blocked by synthetic siRNAs. Designed siRNAs inhibit endogenous miRNA function either by blocking the maturation pathway of targeted miRNA precursors, or are used as antagonists to compete with mature miRNAs. Antisense oligonucleotides usually possess a 2'O-methyl group which enhances stability with no effects on efficiency (311,312). For instance, 2'-MOEs (2'-O-methoxyethyl oligonucleotides) are a type of antimiRs and they may be tagged, or added to a carrier, for cell specific delivery and uptake (313). *In vivo*, Krutzfeldt *et al.* showed that a cholesterol tag prompts uptake of antimiRs by mouse hepatocytes (314). In addition, "miRNA sponges" invented by Ebert *et al*. provides another novel method (315). Several sponges are carried by a vector and expressed in transfected cells. Each sponge contains a specific seed region that targets any endogenous miRNAs, as long as the miRNAs have complementary seed sequences. Therefore, the vectors could block multiple OncomiR functions.

Most recently, Kim *et al.* generated a method that combined the applications of aptamers, miRNAs and nanoparticles, and managed to downregulate miR-221 level by consumption of endogenous miR-221 (316). To deliver nanoparticles carrying miR-221 to astrocytoma cells, nucleolin aptamers were conjugated to the surface of the nanoparticles (317). MiR-221 molecular beacon plus a Cy fluorescent dye (miR-221-Cy) was also conjugated to the nanoparticles by a disulphide bond. After intratumoural injection in mouse xenografts, the aptamers firstly guided the nanoparticles to reach astrocytoma cells where internalisation occurred. The disulphide bond connected miR-221-Cy was then disassociated from the nanoparticles in the cytoplasm. When endogenous oncogene miR-221 bound to free miR-221-Cy, Cy dye was released and gave out fluorescence for imaging. The therapeutic potential of the study is that the attached Cy dye not only enables estimation of nanoparticle delivery, but also could be replaced with other compounds for anticancer treatment.

1.3.10 Conclusion

As we review the roles of miRNAs in cell proliferation and apoptosis, especially the connection between specific miRNAs and E2F transcription factors, there are a number of miRNA referred to as the hallmarks of cancer. Certain miRNAs are closely implicated in classic E2F and Myc signal transduction pathways relevant to cancer. However, the full descriptions of miRNA involved biological mechanisms are still unavailable. Therefore, by exerting more efforts in understanding and modulating the miRNA of interest, we envision the potential of miRNAs in the development of novel therapies against cancer.

1.4 Elimination of Apoptotic Cell Debris

1.4.1 Introduction

Elimination of apoptotic cell debris (efferocytosis) although important has received little attention. The destination of apoptotic bodies is of great importance in biological development, while impaired clearance leads to inflammation and immunogenic effects. Recently, most of studies have focused on *Caenorhabditis elegans* as a model for developmental efferocytosis, with a focus on externalisation of Phosphatidylserine (PS), activation of Ras homologue gene (Rho) family of GTPases and the role of Toll-Like Receptor 4 (TLR-4) in regulating clearance. This section will make a critical overview of current theories on the consequences of efferocytosis, including implications on autoimmunity.

1.4.2 The Processes of Elimination

Phagocytic riddance could be roughly divided into four steps: i) Migration towards Apoptotic Debris; ii) Phagocytic Synapse; iii) Internalisation; iv) Formation of Phagolysosome and Degradation.

A. Migration towards Apoptotic Debris

The localisation of phagocytic cells is the result of signals secreted by dying cells. Lysophosphatidylcholine (LPC) is perhaps the most famous find-me signal (17). LPC was recently revealed to trigger monocytic accumulation by a specific interaction to a G protein-coupled receptor promoting G_2 phase Accumulation (G2A) receptors both in human U937 and mouse J774A.1 cells (318). The knock-down of G2A by RNA-interference and restoration of G2A expression showed a decrease and increase in monocytes migration, respectively. LPC has also been suggested to connect G2A through a bridging protein, rather than physically binding it (319). In some cases, bridging proteins play the role of find-me signals as well. Monocyte chemoattractant protein 1 (MCP-1) is a common macrophage chemoattractant via signalling through its receptors Chemokine C-C motif Receptor 2 (CCR2). CCR2 has been found in both the apoptotic cell and the macrophage membranes (320). Electrical signalling may also play a role in phagocytic attraction and is based on the study of angiogenesis. Notably, the initiation of endothelial sprouts occurs in the direction of apoptotic cells, which contain negatively charged cell membranes (321). Furthermore, an increase of electric potentials in the rat corneal epithelium has been shown to promote the approach of leukocytes (30).

B. Phagocytic Synapse

The phagocytic synapse between dying cells and phagocytes can occur via cell surface rearrangements of various molecules and receptors in the very early stages of apoptosis (322). Most of these communications are facilitated by secondary ligands, and in a few cases via direct interactions (34). The hallmark of this process is phosphatidylserine (PS) exposure to the outer leaflet of the lipid bilayer (323,324). In response to apoptotic stimuli, PS normally in the inner leaflet are preferentially externalised to cell surface by phospholipid scramblase (325,326). Fadok *et al* (2000) utilised monoclonal antibody 217 to identify the specific PS receptor, which distributes on the outer membrane of macrophages, fibroblasts and epithelial cells (327). Furthermore, the induction of the PS receptors in B- and T-cells bestowed the capacity of engulfment for apoptotic cells on the lymphocytes. In addition to PS, the phagocytic synapse is also carried out by the Endoplasmic Reticulum (ER) protein Calreticulin (CRT), whose receptor CD91s [aka LDL-Receptor-related Protein (LRP)] are on the engulfing cell surface (328,329). In apoptotic cells, the assembly of CRTs on the outer plasma membrane resembles the "eat-me" signals and binds CD91 receptors on efferocytes (330). However, this interaction can be disrupted by the "don't eat me" signals, generated by CD47 (an integrin-associated protein) / Signal Regulatory Protein alpha (SIRPα) complexes on the phagocytes (28). This complex usually inhibits engulfment in a viable cell until apoptosis, when the loss of function activates the conjugation. Lastly, there are many poorly-characterised molecules which respond to the recognition between apoptotic cells and efferocytes. However, the exact system has not been established.

C. Internalisation

Prior to the internalisation, the redistribution of efferocytic actins is essential, though the precise initiation of the event is unclear. Based on work with *C. elegans* so far, it seems that two groups of genes modulate ingestion via different pathways (331,332). One group of genes, encodes the proteins Cell Death abnormal-2 [CED-2, aka Chicken tumour virus no. 10 Regulator of Kinase II (CrkII)], CED-5 (aka Dock180, a guanine exchange factor of Rac-1), CED-12 [aka Engulfment and cell Motility protein 1 (ELMO1)] and CED-10 (a homologue to the mammalian Rac-1), and mainly controls the cytoskeleton on efferocyte surface (333). The other group, encoding CED-1 (aka CD91), CED-6 [aka Engulfment adapter protein (GULP)], CED-7 [aka ATP-binding cassette, sub-family A member 1 (ABCA1)] and CED-10, regulates the remodelling of actins (334). Apparently, the presence of CED-10 in both groups implies that the two pathways are not mutually exclusive and GTPase CED-10/Rac-1 appears critical for internalisation (335,336).

Calcium flux can also affect phagocytic ingestion and inhibition of calcium flux actually blocks cytoskeletal reorganisation (337). In this case, the Ca^{2+} Release-Activated Ca^{2+} (CRAC) channels are required for engulfment. Furthermore, the level of calcium ions also affects TGFβ secretion from macrophages.

D. Formation of Phagolysosome and Degradation

After the apoptotic cell is trapped in the engulfment cup, a unique complex functions between internalisation and degradation (**Figure 1.9**). This complex is a Vps34 kinase (a member of PI(3)K), coupled with GTPase DYN-1 (aka dynamin), and localises on the cytosolic side of engulfment cup (338). DYN-1 contains a binding site for Rab5, a

GTPase critical for efferosome maturation, and it also regulates VSP-34. Therefore, the function of the complex assumes that DYN-1 physically binds to Rab5^{GDP}, and then VSP-34 converts Rab5^{GDP} to Rab5^{GTP}. This step is required for the subsequent maturation of apoptotic debris within phagosomes.

There are two unique GTPase proteins, Rab5 and Rab7, which appear to be vital for the maturation of efferosomes (338). Initially, the cytoplasmic engulfed apoptotic cell is coated with DYN-1 and Vps34, namely efferosome. Next, DYN-1 recruits Rab5 to the corpses. Stable binding of Rab5 is due to Vps34-mediated activation of PtdIns(3)P, which Rab5 targets (339,340). The Rab5 coated efferosome then triggers the gathering of the other GTPase Rab7 (338), and afterwards Rab7 replaces Rab5 covering the efferosome. Finally, another regulator of Rab7, Homotypic fusion and vacuole Protein Sorting (HOPS) complex, joins in the progression and is likely to permit the generation of phagolysosome (338). Once the phagolysosome is formed, the degradation of corpses proceeds. To date, the mechanisms involved in digestion are still unclear. In particular, how clearance might result in an aberrant immunogenic response is of current research interest.

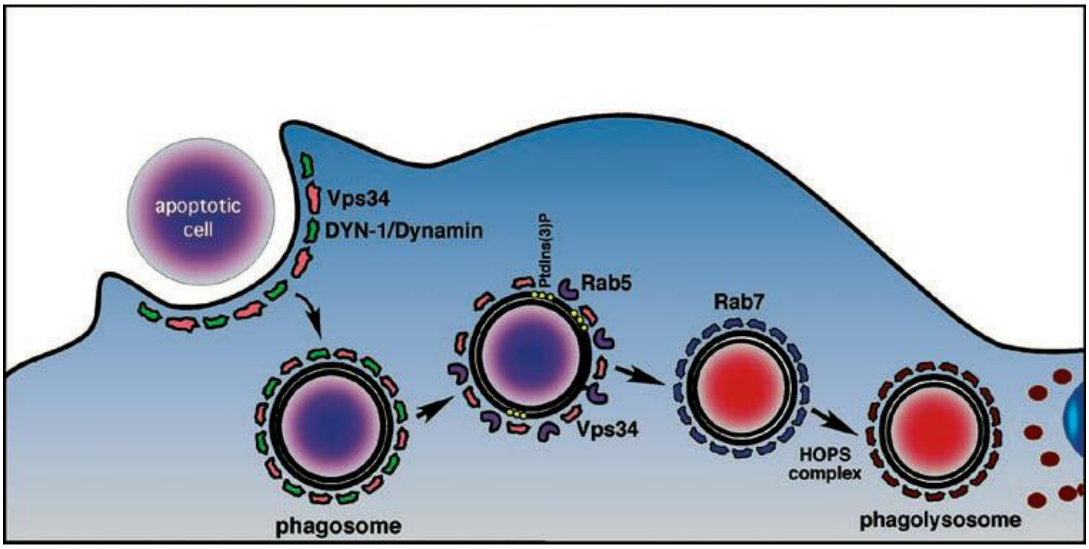


Figure 1.9 A model of an apoptotic-cell-containing a phagosome. After internalisation, the phagosome is coated with Vps-34 and DYN-1. Next, Rab5 binds to the surface of efferosome and initiates recruitment of Rab7. The Rab7-coated phagosome then associates with HOPS complex to form mature phagolysosome, followed by degradation. Figure adapted from: Kinchen, J.M. *et al.* (2008) A pathway for phagosome maturation during engulfment of apoptotic cells *Nat Cell Biol* **10**, 556-566.

1.4.3 Inflammation from Impaired Apoptosis

Completed apoptosis is tolerated by the immune system, whereas impaired removal of apoptotic debris beyond tolerance is immunogenic (341). Substantial papers have implicated a wide variety of autoimmune diseases to the outcomes of damaged apoptosis. Here, three types of apoptotic dysregulation are presented and result from: i) production of autoantigens; ii) deficiency in the apoptotic pathway; and iii) failure in removal of apoptotic debris.

A. Production of autoantigens by apoptosis

During apoptosis, the relocalisation of many subcellular fragments in apoptotic bodies can induce an immune response (342). For this reason molecules that might have once been nuclear or cytoplasmic contents appear on the surface of apoptotic bodies, and resemble antigens, giving rise to inflammation (343). Accordingly, these antigens are referred to as autoantigens, however, efficient apoptosis can avoid this by rapid engulfment of debris before the exposure of immunogenic contents (344). Systemic Lupus Erythematodes (SLE), an autoimmune disease, is an example of how important apoptosis efficiency is in avoiding bad immunological effects (345,346). In this case, inefficient uptake by phagocytes, especially mature dendritic cells, and the appearance of nuclear Histones in apoptotic bodies contributes to disease pathology. Thus, it appears that the autoantigens are Histones and they are responsible for the aberrant T-lymphocytes response.

B. Deficiency in Apoptotic Pathway

The impaired clearance of dying cells results in the presence of redundant molecules, which increase the risk for inflammation (341). Specific deficiency in apoptotic signalling results in impaired immunity. For instance, Autoimmune Lymphoproliferative Syndromes (ALPS) results from an excess of non-functional T-cells, due to Fas signalling deficiency (347). As Fas signalling is required for lymphocyte apoptosis, mutations in Fas constantly yields abnormal T-lymphocytes. The accumulation of impaired lymphocytes eventually outweighs self-tolerance, leading to ALPS onset.

C. Failure in Removal of Apoptotic Debris

Distinct from the examples given in A and B above, hyperactive apoptosis can also be harmful or even fatal. For example, Neonatal Lupus Syndromes (NLS), such as Congenital Heart Block (CHB) is caused by autoantibody-induced aberrant apoptosis. Apoptosis is a homeostatic process and provokes no immune response during the development of foetal heart (348). These apoptotic cells are engulfed by the adjacent healthy cardiocytes in association with Sjögren’s syndrome cytoplasmic antigen/Ro ribonucleoprotein (SSA/Ro) and Sjögren’s syndrome nuclear antigen (SSB/La). Both antigens are localised on cardiocytes and are required for apoptotic engulfment. However, CHB patients passively obtain unique maternal autoantibodies (autoAbs), which inhibit SSA/Ro and SSB/La, blocking cardiocytic mutual uptake (349). Therefore, a redundancy of apoptotic corpses have to be eliminated by macrophages via recognition of Fragment Crystallisable Gamma Receptor ($F_c\gamma R$) and result in secretion of abundant inflammatory cytokines.

1.4.4 Conclusion

The entire process of apoptosis from attraction, communication, internalisation to degradation is poorly-characterised. Comprehensive clarification of these apoptotic mechanisms is needed and will help us to understand the homeostasis and development of multi-cellular organisms and tissues. The exploration of novel therapies against cancer also relies on a well-established apoptotic network. As immunogenicity is due to deficiency of apoptosis, how to successfully eliminate apoptotic cells without inducing immune response will be taken into consideration for future directions of anticancer research.

1.5 Aims and Scope of the Project

1.5.1 Overall Objective

The overall objective of this project is to develop a novel cancer therapy by using natural compounds to target apoptotic signalling pathways.

Cancer is one of the leading diseases causing high death rate every year. Lack of an efficient cure for advanced cancer is probably the most prominent reason for the situation. In fact, current chemotherapy and radiotherapy are often associated with severe side effects. Thus, our laboratory has been looking for compounds which have a high efficacy in cancer treatment along with low therapeutic adverse effects. Moreover, we confine our search to a range of traditional Chinese medicines. Traditional Chinese medicines are generally derived from natural compounds and have been used in China over hundreds of years. Therefore, the efficacy and safety of these drugs have been carefully recorded in many ancient books of medicine. These records are valuable for the novel therapeutic applications of traditional Chinese medicines in the future. In the last decade, an increasing number of traditional Chinese medicines have been revealed to have effects in antitumour and anticancer treatment. Of all, we selected Chansu as our research object to develop a novel anticancer treatment. Chansu is composed of a group of bufadienolide compounds and is well known for its usage in the treatment of heart failure and inflammation. This study set out to identify the anticancer capacity of a major component of Chansu, CBF.

1.5.2 Specific Aims

1. To evaluate the potential of CBF in induction of apoptosis in various cancer cell lines and its respective cytotoxicity (**Chapter 3**).

The initial cytotoxicity assays of CBF in different human cancer cell lines indicated that colon cancer cell line HCT116 and HT29 are the two most sensitive cell lines to CBF. Interestingly, the subsequent examinations about CBF-elicited apoptotic pathways revealed that CBF induces apoptosis through differential pathways in two colon cancer cell lines. A series of apoptosis assays were carried out to identify the exact mechanisms of apoptosis by CBF. Firstly, the Annexin V and PI staining identified that the main proportion of cell death induced by CBF underwent apoptosis, not necrosis. Mitochondrial potential changes were discovered in both colon cancer cell lines and measured by flow cytometry using JC-1 mitochondrial membrane potential dye. After 48 hours CBF treatment, the difference of mitochondrial potential change between treated and untreated cells reached maximum. In addition, the mRNA and protein expressions of Caspase-3, a biomarker of apoptosis, were estimated. Our results indicated that active Caspase-3 activity was significantly increased in HCT116 cell line, but not in HT29 cell line. In spite of a low transcription level of Caspase-3, a strong activated Caspase-3 protein expression in treated HCT116 cells was detected in the immunoblotting, immunocytochemistry and Caspase-3 luciferase assays. Furthermore, the expression of a mitochondrial protein AIF was also tested in the two cell lines. AIF is closely related to Caspase-3-independent apoptosis and its release from mitochondria to the cytoplasm was only detected in HCT116 cells, though mitochondrial permeabilisation was induced in CBF-treated HT29 as well. To this end, we found that

CBF-induced apoptosis in HCT116 cells undergoes both Caspase-3-dependent and –independent pathways. However, CBF inhibition of HT29 is very likely to provoke other cytotoxic signalling pathways.

2. To investigate dysregulation of key proteins in specific signal transduction pathways involved in CBF treatment *in vitro* and *in vivo* (**Chapter 4**).

To further elucidate the molecular mechanisms of CBF in colon cancer cells, 10-pathway reporter assay (comprising of cancer related genes) was used, which identified two important pathways: hypoxia-regulated pathway and mitogen-activated protein kinase (MAPK) /ERK pathway. Hypoxia-regulated pathway involves many transcription factors, especially vascular endothelial growth factor (VEGF) which controls angiogenesis. The key regulator of this pathway is α subunit of hypoxia-inducible factor 1 (HIF-1α), which synthesises in the cytoplasm but translocates to the nucleus under hypoxic conditions. We found that CBF significantly inhibits the protein expression of HIF-1α in HCT116 and HT29 cells, yet irrelevant to the overall increase of HIF-1α mRNA level. Moreover, the protein inhibition of HIF-1α appears to take place by different mechanisms in the two cell lines. By dividing total proteins into four fractions according to their localisation inside of cells, we found a significant suppression of HIF-1α in the fraction of organelle membrane in HCT116 cells, which suggested that CBF interrupts the nuclear import of HIF-1α. Dissimilarly, our analysis in HT29 cells exhibited that CBF represses the cytoplasmic synthesis of HIF-1α and seems to have little inhibitory effect in nuclear translocation and accumulation.

MAPK/ERK pathways mediate phosphorylation of several proteins, which are required for a variety of cellular programmes, including cell cycle, cell migration and metabolism. Among them, our laboratory is interested in a cytoplasmic protein called cortactin (CTTN). CTTN promotes polymerisation of actin and therefore is a key factor for cell migration. CBF significantly suppressed CTTN expression in HCT116 cells and in xenograft nude mouse models injected with HCT116 cells. A nuclear translocation of CTTN was also observed in HCT116 cells. It is the first time that CTTN was found to be distributed in the nucleus. Nevertheless, this nuclear import seems not to be the case in tumour tissue of HCT116 implanted xenograft models.

3. To assess the cytotoxicity of the Australian cane toad extract and liposome curcumin (**Chapter 5**).

This PhD project also covers preliminary studies of the induction of cancer cell death using the Australian cane toad extract that consists of different bufadienolide compounds. The extract sample inhibits the proliferation of human colon and prostate cancer cell lines as effectively as CBF. In addition, to establish a delivery system for water-insoluble natural compounds and to improve our techniques of liposomal drug production, a number of *in vitro* experiments were also performed to compare the efficiency between liposomal curcumin and sole curcumin.

The conclusion and implications of these findings and suggestions for future work are discussed in **Chapter 6**.

One original aim of the study was to address the potential role of miRNAs in the response of cells to CBF given that previous studies had shown an important role for miRNAs in regulation of apoptosis (**Table 3**). However, due to time constraints this aim could not be addressed in this study. The future directions of this project will make more efforts on xenografts, which are expected to include the investigation of miRNA dysregulation in experimental tumour models.

CHAPTER 2

Materials and Methods

2.1 Human Cell Lines and Culture Conditions

Human cancer cell line HCT116 (colon), HT29 (colon), SW620 (colon), A431 (skin), PC3 (prostate), DU145 (prostate), A549 (lung) and MCF-7 (breast) were purchased from American Tissue Culture Collection, VA, USA. Human lung cancer cell line Spc-A_1 was purchased from Cell Bank of Chinese Academy of Sciences, Shanghai, China.

Frozen cells were thawed in a 37°C water bath and were immediately transferred to 25 cm^2 tissue culture flasks (TC25 flasks) containing 5 ml completed culture medium. All the cancer cell lines were cultured in Dulbecco's modified essential medium (Gibco, life technologies, VIC, Australia) supplemented with 10% foetal bovine serum (HyClone, Thermo Fisher Scientific, VIC, Australia), 2 mM GlutaMAX (Gibco), 100 U/ml penicillin (Sigma, Sigma-Aldrich, NSW, Australia), 100 μg/ml streptomycin (Sigma), 110 mg/L sodium pyruvate (Gibco) and 25 mM HEPES (Gibco), in an atmosphere of 5% CO_2 and 95% air at 37°C. The medium was changed two or three times a week depending on the growth rate of the cell lines. When they become confluent in TC25 flasks, cells were subcultured in TC75 or TC175 flasks for subsequent experiments.

For a subculture, adherent cells were detached from the flask bottom by the addition of 0.25% Trypsin+EDTA (Gibco) for 5~15 min at 37°C. Detached cells were centrifuged down (120 x *g*) and resuspended in fresh medium to culture continuously in TC flasks. The leftover cells were stored in liquid nitrogen (approximate 1 million cells per cryovial) and the medium for freezing cells consists of 60% completed culture medium, 30% FBS and 10% dimethyl sulfoxide (DMSO). Prior to immersing in liquid nitrogen, the cryovials containing cells were wrapped in foil and kept at -80°C overnight.

2.2 Cytotoxicity Assays

Cytotoxicity of CBF in all cell lines was studied by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Molecular Probes, life technologies, OR, USA). CBF and NAC were purchased from Sigma and dissolved in 0.05% DMSO (**Appendix I**). Cane toad extract was kindly provided by Ms Jing Jing, University of Queensland. Cells were seeded at densities of 1 ~ 10 x 10^4 cells per well of a 96-well plate, followed by a serum-free culture for 24 hours. After the starvation, cells were exposed to CBF and/or NAC treatment for another 24 or 48 hours, and then MTT reagent was added following the manufacturer's instructions. Briefly, the old medium was replaced with 100 µl of fresh medium plus 10 µl of MTT solution per well. After a further 4 hours incubation at 37°C, SDS solution or DMSO was added to each well to lyse the cells. The final absorbance was read at 570 nm or 540 nm using POLARstar Omega plate reader (BMG Labtech, VIC, Australia) and EC_{50} values were calculated by Prism 5 (GraphPad Software, CA, USA).

For cytotoxicity assays under hypoxic conditions, treated cells were placed in a GasPak pouch (BD, MD, USA) and incubated at 37°C. The indicator within the pouch showed that the percentage of oxygen in the pouch was less or equal to 1%. Alternatively, 100 µM $CoCl_2$ (Sigma) was used to mimic a hypoxic condition in some experiments (**Appendix II**).

2.3 Detection of Annexin V/Propidium Iodide (PI) Apoptosis

HCT116 and HT29 cells were treated with 1 μM CBF and 0 μM of CBF as a control for 24 hours. The cells were harvested and stained with an Alexa Fluor 488 Annexin V/Dead cell apoptosis kit (Molecular Probes). Experiments were performed according to the manufacturer's instructions. Briefly, detached cells were washed in PBS and resuspended in 1X binding buffer. The cell density was adjusted to 2 x 10 ^{6} cells/ml in a test tube, where Annexin V FITC and/or PI were added to cell suspension, followed by a 15 min incubation in the dark. Fluorescence intensity was analysed by FACS (BD Biosciences, CA, USA) within one hour. The percentages on the upper left and lower right quadrants indicated the necrotic cells and apoptotic cells, respectively.

2.4 Analysis of Mitochondrial Potential Changes

HCT116 and HT29 cells were seeded at a density of 1 x 10 ^{4} cells per well of a black μClear 96-well plate (Greiner Bio-One, Frickenhausen, Germany). Cells were then treated with 1 or 0 μM of CBF and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocar-bocyanine iodide (JC-1) dye was added to the cells at 3, 6, 9, 12, 15, 18, 24 and 48 hours. JC-1 dye is a major component of a mitochondria staining kit (Sigma-Aldrich, MO, USA). In healthy cells, JC-1 dye presents red colour and aggregates in the mitochondrial matrix. Once the mitochondrial electrochemical potential gradient changes, JC-1 dye will shift from red to green fluorescence. The experiment was carried out following the protocols provided by the manufacturer. Briefly, adherent cells were firstly incubated in staining mixture for 20 min at 37°C and then were washed in culture medium. At different time points, both red and green fluorescence were recorded using POLARstar Omega plater reader. For JC-1 monomers (green fluorescence), the fluorometer was set at 490 nm excitation wavelength and 530 nm emission wavelength. For JC-1 aggregates (red fluorescence), the fluorometer was set at 525 nm excitation wavelength and 590 nm emission wavelength. Stained cells overlayed with medium were also photographed using a fluorescence microscope.

2.5 Protein Extraction

After exposure to CBF for a required period, cells (*in vitro*) were harvested, followed by washing twice in ice cold PBS. For Bax, AIF, Caspase-3 and α-tubulin proteins, cells were firstly scraped in cold lysis buffer, consisting of 25 mM Tris (pH7.4), 125 mM NaCl, 2.5 mM EDTA, 25 mM NaF, 0.5 mM Na_3VO_4, 0.5% Nonidet-P40 and 0.01% NaN_3, supplemented with 1mM PMSF (Sigma) and protease inhibitor cocktail (Sigma). After centrifugation at 500 x *g* for 5 min at 4°C, the cell pellet was resuspended in SDS lysis buffer, consisting of 62.5 mM Tris-HCl (pH6.8), 2% SDS and 10% glycerol, supplemented with 1 mM DTT, 1mM PMSF and protease inhibitor cocktail. Prior to another centrifugation at 15,000 x *g* for 5 min at 4°C, the suspension was heated to 100°C for 5 min and chilled on ice. The final supernatant was collected and the concentration of cell lysates was measured by DC Protein Assay (Bio-Rad, CA, USA).

For HIF-1α, CTTN and α-tubulin proteins, cells were overlayed with ice cold 100% methanol for less than 10 min at -20°C, followed by two rinses with cold PBS. Cells were then scraped in cold PBS and centrifuged down (500 x *g*) to remove methanol. The pellet was resuspended in cold RIPA buffer (Pierce, IL, USA), supplemented with protease inhibitor cocktail tablets (Complete, Roche Applied Science, Mannheim, Germany). After centrifugation at 13,000 x *g* for 10 min, supernatant was collected for further analysis.

For protein extraction from mouse tumour tissues, frozen tissues were ground in a mortar and pestle and then immersed in cold RIPA buffer plus protease inhibitor. Further homogenisation was performed by passing the tissues 5-10 times through a 21-gauge needle. After centrifugation at 13,000 x *g* for 10 min, the supernatant was collected and mixed with 1X SDS sample buffer, followed by a snap freeze in liquid nitrogen.

2.6 Western Blotting

All the primary antibodies used in Western blotting were purchased from Abcam (Sapphire Bioscience, NSW, Australia). Protein samples were mixed with 1X/4X SDS sample buffer plus β-mercaptoethanol and equal amounts of proteins were loaded onto 7% or 12% SDS-PAGE gels, running in Mini Trans-Bolt module (Bio-Rad). After gel electrophoresis, proteins were transferred to PVDF membranes (Millipore, Merck Millipore, MA, USA). The membrane was blocked in 5% skim milk for 45 min followed by incubation with primary antibodies against Bax (1:1000), AIF (1:1000), active Caspase-3 (1:1000), HIF1-α (1:500), CTTN (1:2000) or α-Tubulin loading control (1:5000) overnight at 4°C. After washing and incubating with horseradish peroxidise-conjugated goat anti-mouse or anti-rabbit (1:10000; Bio-Rad) antibodies, SuperSignal chemiluminescent substrate (Pierce) was added and the chemiluminescence was visualised using a VersaDoc MP 4000 system (Bio-Rad).

2.7 RNA Extraction

Total RNA was isolated from HCT116 and HT29 cells after 12 and 24 hours CBF-treatment using a PureLink RNA mini kit (Ambion, life technologies, CA, USA), according to the manufacturer’s instructions.

For RNA extraction from mouse tissues, fresh tissues (< 125 mm^3) were firstly submerged in RNA*later* solution (Ambion) and stored at 4°C overnight. The subsequent RNA extraction was carried out by using TRIzol reagent (Ambion). Tissues were ground in a mortar and pestle in TRIzol and incubated for 5 min at room temperature. For further homogenisation, chloroform was added to each sample that was shaken vigorously for 15 seconds. After a 3 minutes' precipitation, the samples were centrifuged at 12,000 x *g* for 15 min at 4°C and the aqueous phase of the samples was collected, followed by the addition of 100% isopropanol. After another 10 minutes' incubation, the samples were centrifuged at 12,000 x *g* for 10 min at 4°C. The RNA pellet was washed in 75% ethanol and resuspended in RNase-free water. RNA aliquots are stored at -80°C.

2.8 Real-time RT-PCR

The concentration of RNA samples was measured by NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific, DE, USA). To synthesize first-strand cDNA, 13 μl of reaction volume was set up, by mixing 1 μg of total RNA, 1 μl of oligo $(dT)_{20}$ primer (Invitrogen, life technologies, VIC, Australia), 1 μl of 10 mM dNTP mix (Invitrogen) and ddH_2O. The mixtures were heated at 65°C for 5 min, and then chilled on ice for 1 min. Then 7 μl of master mix, containing 4 μl of 5X First-Strand buffer (Invitrogen), 1 μl of 0.1 M DTT (Invitrogen), 1 μl of ddH_2O and 1 μl (200 units) of SuperScript III Reverse Transcriptase (Invitrogen), was added to the 13 μl reaction volume. Reverse transcription was performed at 50°C for 60 min and following heat deactivation at 70°C for 15 min. Real-time PCR was carried out in 20 μl of reaction solution, consisting of 0.4 μM primers (Sigma), 10 μl of Express SYBR GreenER qPCR SuperMixes (Invitrogen) and ddH_2O. Real-time PCR was performed in iQ5 multicolour real-time

PCR detection system (Bio-Rad). The reaction conditions were 50°C for 2 min and 95°C for another 2 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min. Melting curves were monitored by heat-denaturing amplicons over a 35°C temperature gradient at 0.5°C/sec from 60 to 95°C. No genomic DNA contamination or pseudogenes were detected. Primers used in real-time PCR were: Human AIF (Forward: 5’ - CTG AAA GAC GGC AGG AAG GTA G - 3’, Reverse: 5’ - CTC CAG CCA ATC TTC CAC TCA C - 3’). Human Caspase-3 (Forward: 5’ - GTT TGT GTG CTT CTG AGC CAT G - 3’, Reverse: 5’ - CCA CTG TCT GTC TCA ATG CCA C - 3’). Human HIF-1α (Forward: 5’ - AAG GTA TTG CAC TGC ACA GGC - 3’, Reverse: 5’ - CAG CAC CAA GCA GGT CAT AGG - 3’). Human CTTN (Forward: 5’ - AGG TGT CCT CTG CCT ACC AGA A - 3’, Reverse: 5’ - CCT GCT CTT TCT CCT TAG CGA G -3’). Human GAPDH (Forward: 5’ - GTC TCC TCT GAC TTC AAC AGC G - 3’, Reverse: 5’ - ACC ACC CTG TTG CTG TAG CCA A - 3’).

2.9 Immunocytochemistry

For immunocytochemistry, sterile coverslips were placed in a 24-well plate and cancer cells were seeded at a density of 1.5 x 10^{5} cells per well. After overnight incubation at 37°C, cells were adherent to the coverslips. These cells were then exposed to 1 or 0 μM CBF for another 24 hours. The old medium was then removed and the samples were washed in ice cold phosphate buffered saline (PBS). Fixation was done by the addition of 100% methanol to cells left at -20°C for 10 min, followed by washing in ice cold PBS twice, shaking gently. Usage of 0.2% Triton X-100 (Sigma) in PBS to permeabilise samples was for no more than 10 min, followed by 3 times of wash in PBST, PBS plus 0.5% Tween 20 (Calbiochem, Merck Millipore, Darmstadt, Germany). Prior to staining of primary antibodies, cells were blocked in block buffer (3% normal goat serum and 0.5% BSA in 0.01M PBS) for 30 min. Cells were sequentially incubated with corresponding primary antibodies against active Caspase-3 (1:300; Abcam), AIF (1:100; Cell Signaling, MA, USA), HIF-1α (1:100; Novus, CO, USA) or CTTN (1:300; Abcam) overnight at 4°C. After 3 washes in PBST, a secondary antibody, goat anti-rabbit FITC or Texas Red (1:1500; Abcam), was added to the samples and incubated in dark for 1 hour, followed by washing 3 times in PBST. After one minute of DAPI staining (Molecular Probes), coverslips were drained and sealed onto microscope slides using ProLong Gold antifade reagent (Molecular Probes). Fluorescence images were visualised using confocal microscope FV1000 (Olympus, QLD, Australia) after 24 hours.

2.10 Caspase-3 Activity Assays

Cancer cell lines were seeded at a density of 1 x 10^4 cells per well of white μClear 96-well plates (Greiner Bio-One) and exposed to CBF for 24 hours. The activity of Caspase-3 was evaluated using Caspase-Glo 3/7 assay systems (Promega, WI, USA) according to the manufacturer’s instructions. Luciferase intensity was measured by POLARstar Omega at 4, 8, 12 and 24 hours post CBF addition.

2.11 Multi-pathway Reporter Assays

This multi-pathway activity assay was performed using Cignal Finder Toxicity 10-Pathway Reporter Arrays (SABiosciences, Qiagen, VIC, Australia) according to the manufacturer’s instructions. Briefly, reporter and control DNA constructs of 10 selected transcription factors were transfected into HCT116 cells (3 x 10^4 cells/well) using Lipofectamine LTX reagent (Invitrogen). Twenty-four hours later, cells were treated with 1 μM CBF for 9 hours. The luciferase assays were then carried out by using Dual-

Glo luciferase assay systems (Promega). Both luciferase intensities of firefly and renilla were measured by POLARstar Omega. The ratio of the two types of luciferase readings was normalised. Then the fold changes between treatment and non-treatment was obtained by dividing the normalised luciferase reading of each treated pathway reporter by the normalised reading of control reporters. These changes of CBF-involved signalling pathways were assessed by taking logarithm of 10 ratios and were plotted using Prism 5.

2.12 Subcellular Protein Extraction

3-5 x 10 ^{6} treated HCT116 and HT29 cells were detached, centrifuged down to form a cell pellet and snap frozen in liquid nitrogen. The subcellular protein extraction followed the manufacturer's instructions of ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem). Briefly, frozen cell pellets were washed twice in wash buffer and exposed to Extraction Buffer I plus protease inhibitor cocktail. After centrifugation, the supernatant was collected as the cytosolic protein fraction and the pellet was resuspended Extraction Buffer II to isolate the fraction of membrane/organelle proteins. After a series of centrifugation and usage of specific extraction buffer III~IV, the nuclear and the cytoskeletal matrix protein fractions were separated eventually.

2.13 Transient Transfection Assays

Vectors encoding full-length of human HIF-1α and pEGFP-C1 vector (Clontech, CA, USA) were kindly provided by Dr. Zhou Wang and Associate Prof. Steve Ralph, respectively. The sequences of full-length HIF-1α were amplified by PCR and inserted in a pEGFP-C1 vector, generating pGFP-HIF-1α (**Appendix III**). PCR primers for amplification of intact HIF-1α genes are: Forward 5’ - CACTCTCGGCATGGACGAGC - 3’; Reverse 5’ - TTAGATATCGAGCCACCAGTGTCC - 3’. The forward primer binds to upstream of the restriction site of SacI (NEB, MA, USA) and the underlined sequences (in reverse primer) are the restriction site of EcoRV (NEB). The inserts and SacI/SmaI (NEB) digested pEGFP-C1 vectors were then ligated, as EcoRV and SmaI both produce blunt ends. The fidelity of recombinant plasmid was checked by SacI and BamHI (NEB) digest and the sequencing at Australian Genome Research Facility (AGRF, QLD, Australia). Plasmid pGFP-HIF-1α was transfected into HCT116 and HT29 cells by Lipofectamine LTX with PLUS reagent (Invitrogen). After CBF treatment in transfected cells, the expression level of GFP was measured by POLARstar Omega.

2.14 His-tagged Protein Pull-down

To generate a plasmid contains only HIF-1α sequences and a His-tag, GFP region was successfully removed by AfeI (NEB) and BamHI restriction digest. HIF-1α tagged with six histidines were subsequently cloned into the digested vector, forming pHIF-1α-His (**Appendix III**). PCR primers for amplification of hexa histidine-tagged HIF-1α genes are: Forward 5’ - ATCACTCTCGGCATGGACGA - 3’; Reverse 5’ - GCGGATCCTCAATGATGATGATGATGATGGTTAACTTGATCCAAAGC - 3’. Newly synthetic pHIF-1α-His plasmid was transfected into HCT116 cells by Lipofectamine LTX with PLUS reagent. After CBF treatment, 2 x 10^{7} HCT116 cells were rinsed in cold PBS and scraped in lysis buffer (pH8.0), consisting of 50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole and 0.05% Tween 20. The cell suspension was further sonicated on ice, using six 15 sec bursts at 75 W with a 10 sec cooling period between each burst. Prior to flowing through a Ni-NTA Spin column (Qiagen, Hilden, Germany), the lysate was centrifuged at 10,000 x *g* for 10 min at 4°C. The supernatant was gently added to the column at 4°C, followed by two washes in lysis buffer, collecting each flow-through. The concentration of imidazole for the elution buffer was increased to 250 mM and the eluted protein was further concentrated by using an Amicon ultra-15 centrifugal filter unit (Merck Millipore, Darmstadt, Germany) that assisted to remove any protein size below 15 kDa.

2.15 Co-immunoprecipitation

1 x 10 ^{7} treated HCT116 cells were lysed in non-denaturing lysis buffer, consisting of 20 mM Tris-HCl (pH8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA and fresh protease inhibitors. The lysate was transferred to a microfuge tube and maintained constant agitation for 30 min at 4°C, followed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was then mixed with 1µg of rabbit normal serum and 100 µl of Protein G Plus/Protein A-Agarose bead slurry (Calbiochem) for pre-clearing. To prevent non-specific precipitation, the mixture was incubated for 30 min at 4°C with gentle agitation. The supernatant was collected after another centrifugation at 12,000 rpm at 4°C for 10 min. Subsequently, 2 µg of CTTN antibody (Abcam) was added to 500 µg of the pre-cleared protein supernatant and incubated for 2 hours at 4°C with gentle mixing. To form a lysate-bead mixture, 50 µl of bead slurry was added to the sample and kept at 4°C under rotary agitation overnight. After incubation, the mixture was centrifuged at 25,000 rpm for 15 min at 4°C, followed by 3 washes in lysis buffer and the supernatant from each wash was saved. Target proteins were eluted by heating the samples in 2X SDS sample buffer at 100°C for 5 min. The heating denatured the protein and released the protein from the protein-A/G beads.

2.16 Xenograft Establishment and Data Collection

To establish mouse models, 5 x 10 ^{6} HCT116 cells in PBS (200 µl) were subcutaneously injected into each mouse using 30 - G needles. After tumour growth for 2 weeks, 21 female BALB/c nude mice (aged 8 weeks and weighing 16 - 18 g) were equally divided into three groups (7 mice per group) : intratumoural (i.t.) injection, intraperitoneal (i.p.) injection and control group, with average tumour size of each group about 320 mm^3. To prepare 50 ml injection solution, 10 mg of CBF was dissolved in 4 ml of absolute ethanol and diluted in 10% propylene glycol solution to reach 0.2 mg/ml. The daily dose given to i.t. and i.p. groups was 1.5 mg/kg, while an equal amount of injection solution without CBF was given as a control. Volume of the tumours, body weight, appetite and behaviour of the mice were recorded every day. When the tumour grew to 1 cm^3, the mouse was sacrificed. Tumour tissue specimens were taken for subsequent RNA, protein and immunohistochemistry analysis. All experiments involving animals were approved by Griffith University (AEC No. MSC/01/08).

2.17 Paraffin Wax Infiltration

Before immersed in 10% formalin, mouse tumour tissues were cut to less than 0.5 cm in any single dimension. After soaking overnight, the tissue blocks were rinsed in water for 30 min, followed by a series of immersions for dehydration. The samples were sequentially submerged in 80% ethanol for 1 hour, 95% ethanol for 1 hour, 95% ethanol for another 1 hour and left in 100% ethanol overnight. Prior to paraffin wax infiltration, xylene was used for clearing further the tissue samples, which were submerged in xylene for 3 x 20 min. After fixation, the tissue blocks were embedded in paraffin (70°C) for 3 hours, and were then sectioned in a microtome to 5 µm thick and affixed onto positively-charged slides. The slides were left at room temperature overnight to remove any water.

2.18 Immunohistochemical Staining

The dried sections were deparaffinised and rehydrated before proceeding with the immunohistochemical staining. The slides placed in a rack were sequentially washed in the following solutions: xylene (2 x 3 min), xylene 1:1 with 100% ethanol (3 min), 100% ethanol (2 x 3 min), 95% ethanol (3 min), 70% ethanol (3 min), 50% ethanol (3 min) and running cold tap water. The slides were kept rinsing in tap water and sodium citrate buffer (pH 6.0) for heat-induced antigen retrieval was prepared, consisting of 10 mM sodium citrate, 0.05% Tween 20 and distilled water. The slides were immersed in sodium citrate buffer and heated to boil by microwave. After boiling for 20 min, the slides were rinsed under running cold tap water for 10 min. Subsequently, the slides were washed in TBS plus 0.025% Triton X-100 (TBST) twice for 5 min and blocked in 10% goat normal serum with 1% BSA in TBS for 2 hours, followed by primary antibody (HIF-1α, 1:100; CTTN, 1:100) incubation overnight at 4°C. For secondary antibody incubation, goat anti-rabbit FITC and Texas Red (1:1000) were added to the samples and incubated in the dark for 1 hour, followed by 3 washes in TBST. After one minute of DAPI staining, 20 µl of ProLong Gold antifade reagent was added to the sample, where a clean coverslip was mounted. The slides were kept in the dark and ready for visualisation using confocal microscope after 24 hours.

2.19 Hematoxylin and Eosin Staining

After deparaffinised and rehydrated, the sections were immersed in hematoxylin (Sigma-Aldrich) for 1 min, followed by rinsing in distilled water. The samples were decolourised further in acid alcohol and washed in tap water. The sections were next stained in eosin (Sigma-Aldrich) for 30 sec and transferred into 95% ethanol (15 sec), 100% ethanol (15 sec) and xylene (45 sec) for dehydration. Then a clean coverslip was mounted onto the sample with Permount mounting medium (Fisher Chemicals, Thermo Fisher Scientific, VIC, Australia) and the tissue sample was ready for observation.

2.20 Statistical Analysis

All results are presented as means ± S.E. A student's *t* test computation was used to analyse the data with unequal variance between each group. A $P<0.05$ was considered significant.

CHAPTER 3

CBF inhibits cell growth in different human cancer cell lines and induces differential apoptotic pathways in colon cancer cell lines

Introduction

Cancer is one of the leading causes of human death worldwide due to the lack of effective and curative management strategies. Surgical ablation is only effective for patients who are at an early stage of the disease. For those who suffer from late stage carcinoma, conventional therapies such as radiotherapy and chemotherapy remain as the limited choices (350-353). In fact, these treatments are not only ineffective, but most of them are associated with significant side effects. In the last decade, considerable research has focused on improving chemotherapy by examining the ability of natural compounds to induce apoptosis or programmed cell death, and to activate relevant apoptotic pathways in cancer cells, making them sensitive to cell death (354-357).

Cancer cells can be induced to undergo cell death by one of three mechanisms: necrosis, apoptosis and autophagy. The apoptotic process can be either Caspase-dependent or Caspase-independent (358). Caspases are a family of cysteine-aspartic proteases, the activity of which exhibits a sign of early apoptosis. In Caspase-dependent apoptosis, different initiator Caspase proteins produced in both extrinsic and intrinsic pathways have to be cleaved to form active enzymatic motifs, such as Caspase-3. These active Caspase proteins then lead to proteolysis of their substrates to advance the apoptotic process. On the other hand, Caspase-independent apoptosis requires permeabilisation of mitochondria. Initially, Bax and Bak induce pore formation in the outer membrane of mitochondria. A variety of pro-apoptotic proteins, including Endo G and AIF, are subsequently released from the mitochondria into the cytosol. The proteins regulating Caspase-3-independent apoptosis often cause DNA degradation, although the mechanism for this is not yet established.

As an important pro-apoptotic protein, AIF plays a critical role in Caspase-3-independent apoptosis (359). The precursor of AIF is 67 kDa and localised in the inner membrane of mitochondria in healthy cells. Once apoptosis-induced mitochondrial permeabilisation occurs, AIF is cleaved to a 57 kDa protein that is released into the cytosol (360). The nuclear localisation signal within AIF directs it to the nucleus, where AIF causes chromatin condensation and DNA fragmentation (360). In addition, the inhibition of AIF nuclear import perturbs cell death (361). Several studies have shown that the inhibition of AIF nuclear translocation by the antioxidant N-Acetyl-L-cysteine (NAC) is able to reduce the level of AIF-dependent apoptosis (362-364).

Usage of a therapeutic agent to induce apoptosis in cancer cells is a typical way to treat cancer. Our laboratory is interested in applications of traditional Chinese medicines for cancer treatment. Traditional Chinese medicine Chansu is a dried product of the Asiatic toad (*Bufo gargarizan*) secretion. It contains a group of bufadienolide compounds, including bufalin, resibufogenin and CBF. Chansu has been used for centuries in various prescribed recipes as well as ready-available remedies for anti-arrhythmia, anaesthesia and anti-inflammation purposes in China, Japan and other Asian countries. Furthermore, several previous papers which showed a likely role of Chansu in antiproliferation caught many scientists’ attention (68,87,365). Although Chansu is widely used for numerous medical indications, as with many other natural therapies, the precise mechanism of action is not understood. Thus the claims of effectiveness have not been explained, nor tested.

CBF, as a major component of Chansu (3% ~ 7% of Chansu contents) has been isolated, purified (71,366) and commercially available from Sigma-Aldrich. CBF is a type of bufadienolides and inhibits Na^+/K^+-ATPases (85,367). A number of studies have demonstrated that CBF has an inhibitory effect on cancer cell proliferation, including liver, prostate and cervical cancer cell lines (86-88,368). A pilot work by Kamano *et al.* reported that CBF has a potent inhibitory capacity against primary liver carcinoma cells, to which the half maximal effective concentration (EC_{50}) of natural CBF was 1.672 μM (369). Recently, CBF has been shown to emerge as a novel agent in prevention of cancer proliferation and able to cause cell death in human tumour cells without serious side effects (69).

In this study, we set out to determine the capacity of CBF in inhibiting cancer cell proliferation, and to understand the molecular mechanisms of such an effect. To this end, we found that CBF could efficiently induce apoptosis in different cancer cell lines, including lung, skin, prostate, breast and colon cancer cell lines. However, CBF activated apoptotic signalling cascades varied from cell line to cell line. In colon cancer cells HCT116, CBF induced both Caspase-3–dependent and –independent apoptosis, while in another colon cancer cell line HT29, CBF inhibited Caspase-3 activity. Furthermore, CBF-induced apoptosis in HT29 was AIF-independent but with elevated mitochondrial potential changes. Taken together, our finding indicates that CBF has a potent inhibitory effect on cell growth in different cancer cell lines and CBF-induced apoptotic pathway is cell type dependent.

Results

CBF exhibits significant cytotoxicity in a variety of cancer cells, but has the most potency in colon cancer cell lines.

In preliminary MTT assays, colon (HCT116 and HT29), lung (Spc-A_1 and A549), skin (A431), prostate (PC3) and breast (MCF7) cancer cell lines were treated with five doses of CBF for 24 hours (**Figure 3.1**). Significant decreased cell viability was observed in all the cancer cell lines in a CBF dose dependent manner. Interestingly, the EC_{50} values varied from cell line to cell line, suggesting CBF's differential cytotoxicity (**Table 4**). When compared with the others, colon cancer cell line HCT116 and HT29 were more sensitive to CBF. It is interesting to note that HCT116 cells are p53 wide-type, but HT29 cells are p53 mutants. Therefore, two colon cancer cell line HCT116 and HT29 were chosen for further investigation to ascertain CBF's mechanisms of action.

Table 4. **EC_{50} values of CBF in different cancer cell lines**

Cell Line	Tissue	EC_{50} (nM)
HCT116	Colon	86
HT29	Colon	88
Spc-A_1	Lung	305
A549	Lung	959
MCF-7	Breast	438
PC3	Prostate	92
A431	Skin	5956

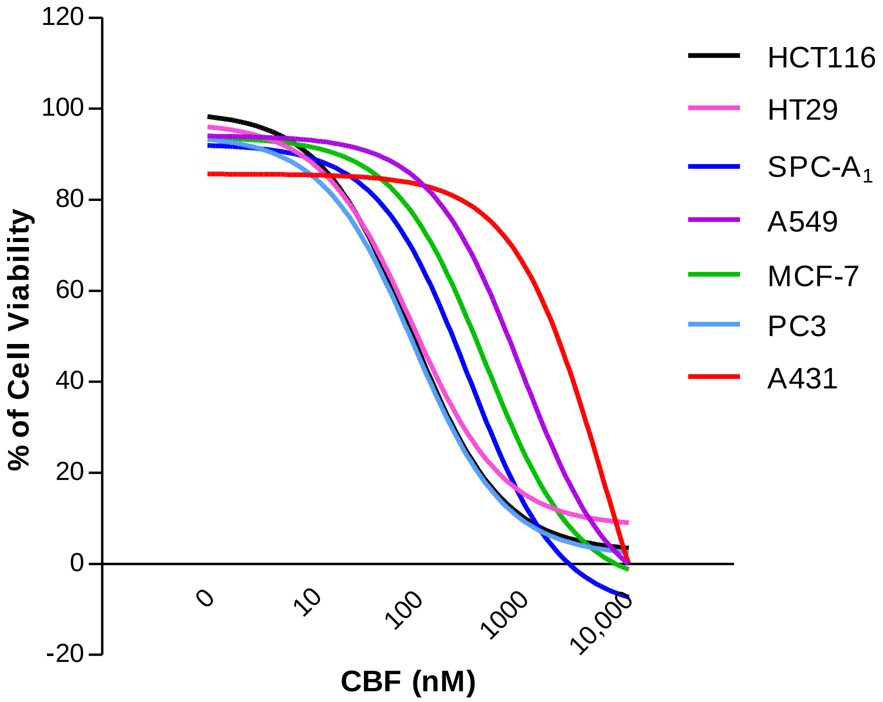
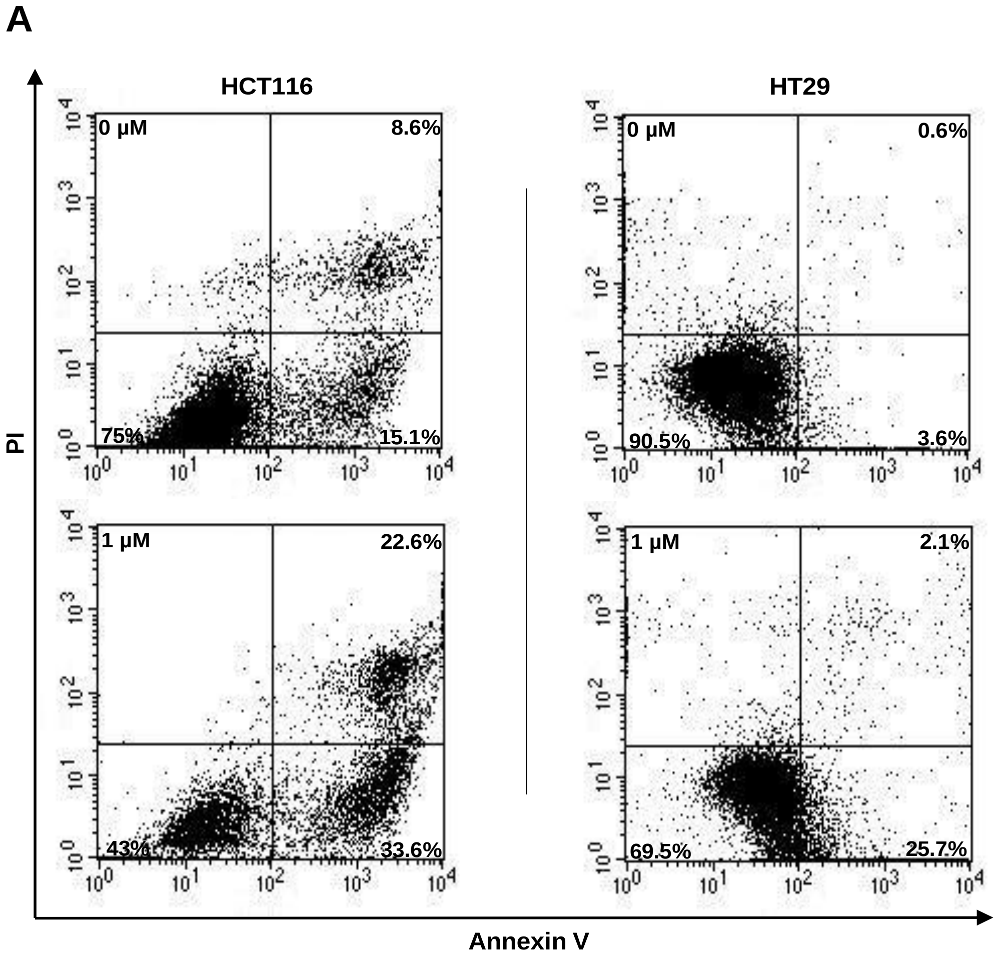
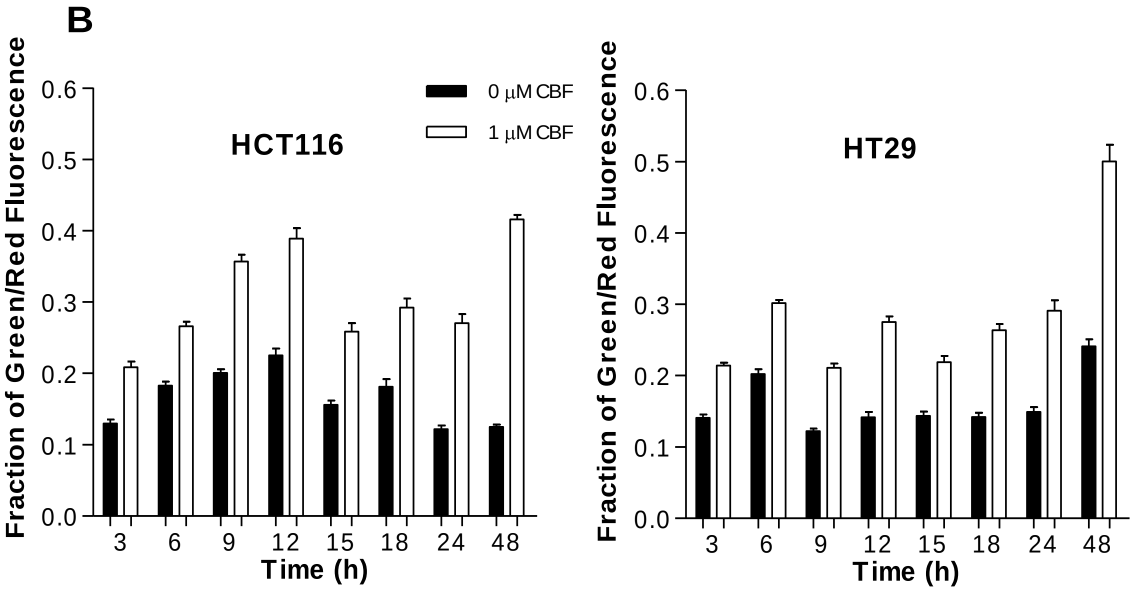


Figure 3.1 Cytotoxicity of CBF among various cancer cell lines. HCT116 and HT29 are human colon cancer cell lines; Spc-A1 and A549 are human lung cancer cell lines; MCF-7 is a human breast cancer cell line; PC3 is a human prostate cancer cell line; A431 is human skin cancer cell line. Preliminary MTT cell viability assays. All cancer cell lines were exposed to CBF for 24 hours and exhibited a reduction in viable cell numbers by using MTT assays. Results are means with standard errors from nine replicates.

CBF induces potent pro-apoptotic effects in colon cancer cell lines.

The decrease of cell viability induced by CBF could result from either apoptosis or necrosis. To clarify the mechanism of cell death, we treated colon cancer cell lines HCT116 and HT29 with 0 or 1 μM CBF for 24 hours, followed by Annexin V and PI staining assays. Annexin V binds to the surface of apoptotic cells, while cell membrane-impermeant PI only binds to DNA in dead cells. Our results showed that both proportions of apoptotic and dying cells were significantly increased after CBF exposure (**Figure 3.2A**). Meanwhile, only a very small percentage of dead cells were stained with PI. Hence, most treated-cells were undergoing apoptosis rather than necrosis. To further verify that the cells died via apoptosis, we measured the change of mitochondrial transmembrane potential using a JC-1 dye. The JC-1 dye aggregates in the interior of mitochondria and gives out red fluorescence in healthy cells, but it turns to green when mitochondrial electrochemical potential alters. Therefore, a strong green signal indicates more cells are undergoing apoptosis. As shown in **Figure 3.2B**, mitochondrial transmembrane potential changed in both cell lines upon CBF-treatment. The fractions of green over red fluorescence were elevated because of an increasing number of cells undergoing apoptosis after the addition of CBF. The images from fluorescent microscope directly exhibited a trend that there were an increasing number of HCT116 cells undergoing mitochondrial membrane potential change (**Figure 3.2C**). Taken together, these results strongly suggest that CBF induces apoptosis in colon cancer cells. Apart from the staining, an unexpected result showed that the expression of the important pro-apoptotic protein Bax localising in the outer membrane of mitochondria was diminished by CBF (**Figure 3.2D**).


A
HCT116
HT29
PI
Annexin V
0 μM
8.6%
75%
15.1%
1 μM
22.6%
43%
33.6%
0 μM
0.6%
90.5%
3.6%
1 μM
2.1%
69.5%
25.7%
10^0
10^1
10^2
10^3
10^4


B
HCT116
HT29
0 μM CBF
1 μM CBF
Fraction of Green/Red Fluorescence
0.0
0.1
0.2
0.3
0.4
0.5
0.6
3
6
9
12
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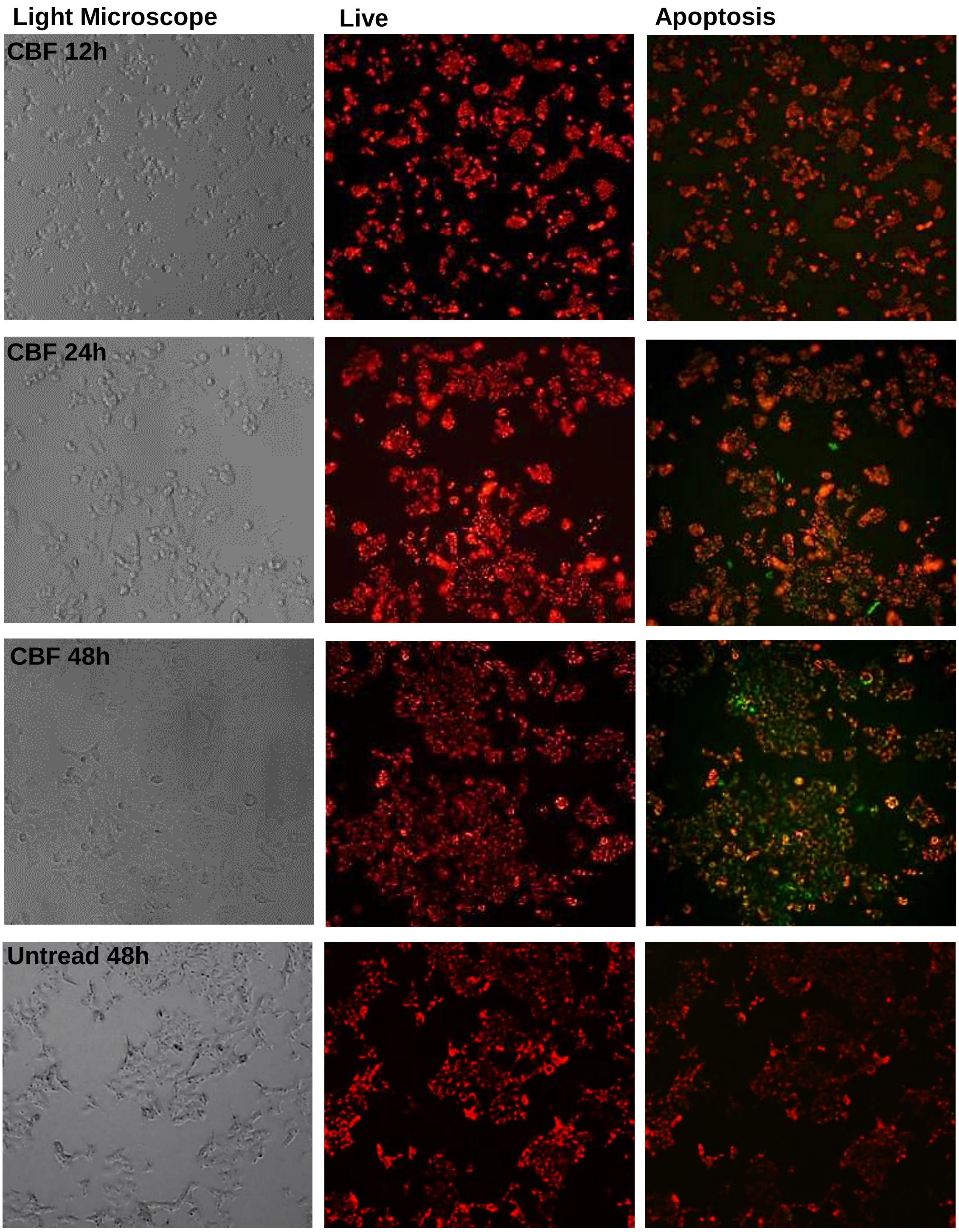
48
Time (h)

C


Light Microscope
Live
Apoptosis
CBF 12h
CBF 24h
CBF 48h
Untread 48h

D

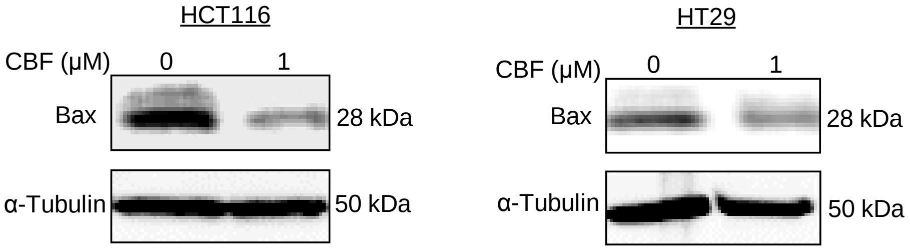

HCT116
CBF (μM)
0
1
Bax
28 kDa
α-Tubulin
50 kDa
HT29
CBF (μM)
0
1
Bax
28 kDa
α-Tubulin
50 kDa

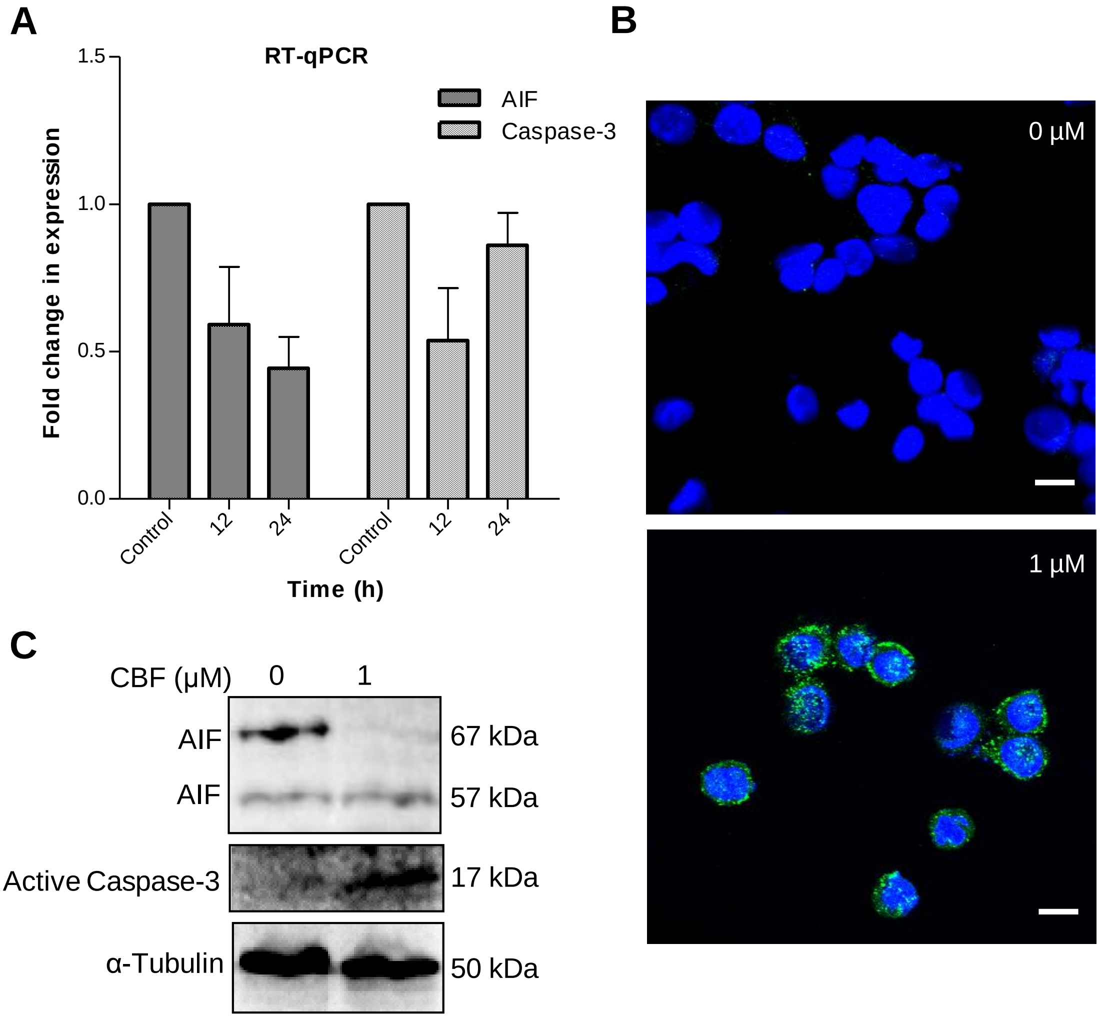
Figure 3.2 Detection of CBF-induced apoptosis in colon cancer cell lines. ***A.*** Annexin V/PI analysis by flow cytometry. HCT116 and HT29 cells were treated with 0 or 1 µM CBF for 24 hours and stained with Annexin V/PI dyes. The lower right percentage indicates the apoptotic cells stained with Annexin V and represents a significant increment after the exposure to CBF, as well as the proportion of dying cells (upper right). Meanwhile, no elevation in PI stained cells (upper left) was observed and a reduction in the living cell number (lower left) after CBF-treatment. ***B.*** The occurrence of mitochondrial permeabilisation in CBF-induced cell death. Untreated and CBF-treated HCT116 and HT29 cells were stained with JC-1 dye, which presents red colour in living cells and green colour in dying cells. The fraction of green over red fluorescence intensity was measured in a time course. The fraction (green/red) between treated and untreated cells indicates the change of mitochondrial potential transition. ***C.*** The change of mitochondrial membrane potential in HCT116 cells. The colour shifting from red (live) to green (apoptosis) indicated that CBF treatment induced mitochondrial membrane potential change. Meanwhile, the green proportion in the untreated cells was very low. ***D.*** Inhibition of Bax by CBF. Immunoblotting showed that the expression of apoptotic mitochondrial protein Bax was inhibited after CBF exposure. Results are means with standard errors from three replicates.

Apoptotic cell death in HCT116 is through both Caspase-3-dependent and -independent pathways.

To further explore the mode of cell death, we studied the activation of a prominent marker for apoptosis, Caspase-3. The total mRNA levels of Caspase-3 in HCT116 cells were initially measured at 12 and 24 hours of CBF treatment. The results showed that mRNA levels decreased at 12 hours and then increased after 24 hours to the level of control groups (**Figure 3.3A**). This indicated that the cytotoxicity of CBF does not directly inhibit the transcription level of Caspase-3. However, the protein expression of Caspase-3 was detected by both confocal microscope and immunoblotting. After 24 hours exposure to CBF, treated and untreated cells were stained with anti-active Caspase-3 antibodies. The bright green fluorescence of active Caspase-3 was only detected in CBF-treated HCT116 (**Figure 3.3B**). Also, the temporal profile of activation Caspase-3 was examined. The level of active Caspase-3 was increased in treated HCT116 cells (**Figure 3.3C**). Moreover, a time course study showed that Caspase-3 levels increased after 12-hours treatment (**Figure 3.3D**). The intensity of active Caspase-3 in treated cells was about three times as much as that of untreated cells after 24 hours.

Since mitochondrial protein AIF can lead to Caspase-3-independent apoptosis, the role of AIF was further investigated in CBF-induced apoptosis. Our results showed that the mRNA level of AIF kept decreasing after CBF exposure (**Figure 3.3A**). This indicated that the transcription of AIF was inhibited, thereby resulting in the low expression level of total AIF (**Figure 3.3C**). The amount of mitochondrial-anchored AIF (67 kDa) was also diminished significantly, leaving the cleaved AIF (57 kDa). Moreover, the addition of antioxidant NAC efficiently enhanced HCT116 resistance to CBF (**Figure 3.3E**).

NAC inhibits the production of reactive oxygen species (ROS) and decreases mitochondrial potential changes, leading to reduction of AIF release. The cell viability assays showed that NAC partially elevated the survival of CBF-treated HCT116. This is most likely due to NAC preventing CBF-treated cells from mitochondrial permeabilisation and subsequent AIF-mediated Caspase-3-independent apoptosis.



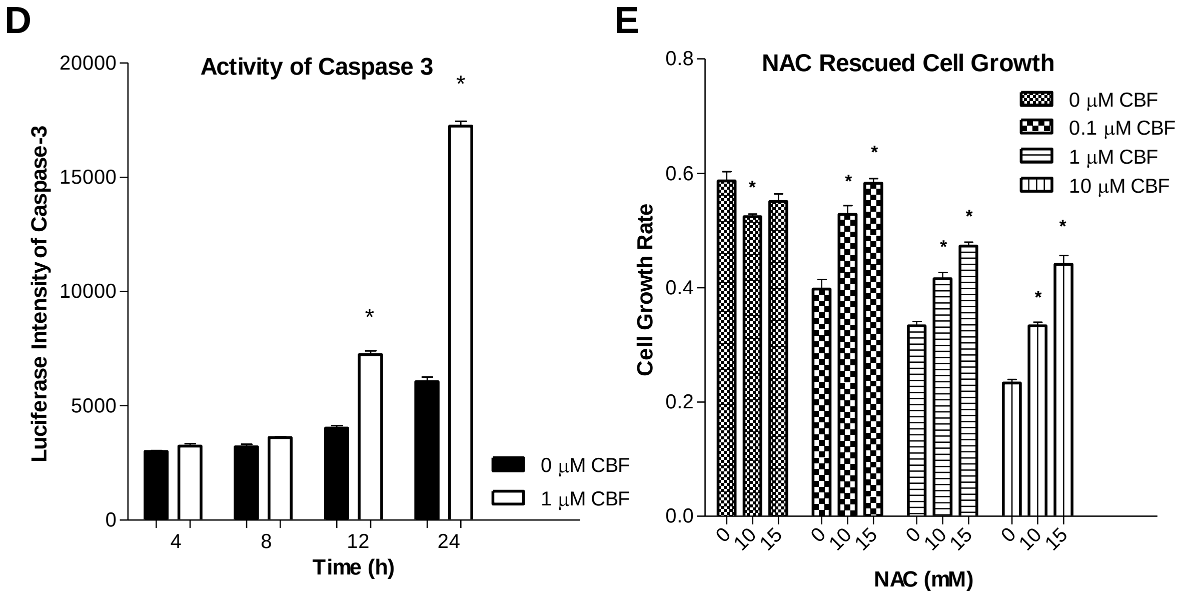
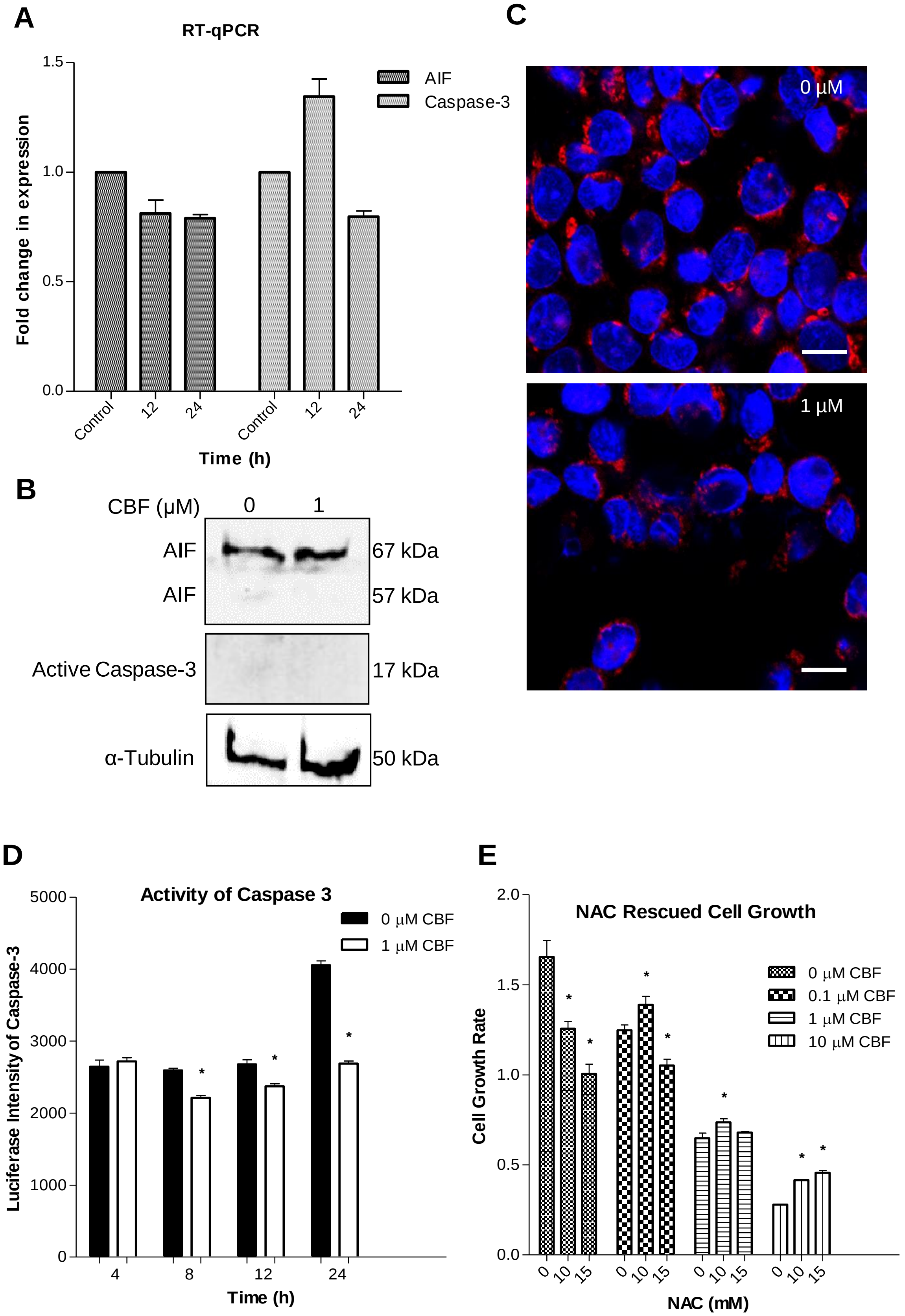


Figure 3.3 CBF-induced apoptosis is both Caspase-3-dependent and -independent in HCT116 cells. *A.* RT-PCR of AIF and Caspase-3. The mRNA level of AIF decreased with time, while Caspase-3 transcript levels decreased after 12 hours but increased at 24hours. ***B.*** Expression of active Caspase-3 detected by confocal microscopy. HCT116 cells were treated with 0 or 1 μM of CBF for 24 hours and analysed by immunofluorescence. The expression of active Caspase-3 (green fluorescence) was only observed in treated HCT116. Cell nuclei were stained with DAPI (blue fluorescence). Scale bars equal 10 μm. ***C.*** Protein expression of active Caspase-3 and cleaved AIF post CBF exposure. After 24-hour-treatment, effective Caspase-3 (17 kDa) and only dissociated AIF (57 kDa) were detected. ***D.*** Detection of Caspase-3 activity via a time course. Treated and untreated cells were lysed and luciferase intensity of Caspase-3 was assessed. A significant increase of Caspase-3 level was found after 24-hour-CBF exposure. (N=9. *P<0.05 compared to the control group). ***E.*** Enhancement of cancer cell survival by NAC. The mixture of different concentrations of CBF and NAC partially inhibited cell death. (N=9. *P<0.05). Results are means with standard errors from three replicates.

Apoptotic cell death in HT29 is through Caspase-3-independent pathway only.

Similar to HCT116 cells, analysis of mRNA level showed no apparent involvement of CBF in transcription of Caspase-3 and AIF (**Figure 3.4A**). The mRNA level of Caspase-3 increased at 12 hours but eventually decreased after 24 hours, while AIF mRNA level was slightly reduced after CBF addition. However, no active Caspase-3 or free AIF proteins (57 kDa) was detected even after 48 hours of CBF treatment (**Figure 3.4B**). Consistently, no significant shift of AIF intracellular distribution between 48-hour-treated and untreated HT29 cells was observed by confocal microscopy (**Figure 3.4C**). The activity of Caspase-3 was reduced in HT29 cells after 24 hours of CBF exposure (**Figure 3.4D**). Furthermore, in contrast to the case of HCT116, NAC was unable to counter the CBF cytotoxicity of HT29 cells (**Figure 3.4E**), indicating that mitochondrial permeabilisation is not a direct cause of the cell death. All these findings and the previous result of inhibitory Bax expression suggest that CBF induced apoptosis in HT29 cells is Caspase-3-independent, but AIF - unrelated.


A
RT-qPCR
Fold change in expression
1.5
1.0
0.5
0.0
AIF
Caspase-3
Control
12
24
Control
12
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Time (h)
B
CBF (µM)
0
1
AIF
67 kDa
AIF
57 kDa
Active Caspase-3
17 kDa
α-Tubulin
50 kDa
C
0 µM
1 µM
D
Activity of Caspase 3
Luciferase Intensity of Caspase-3
5000
4000
3000
2000
1000
0
0 µM CBF
1 µM CBF
4
8
12
24
Time (h)
E
NAC Rescued Cell Growth
Cell Growth Rate
2.0
1.5
1.0
0.5
0.0
0 µM CBF
0.1 µM CBF
1 µM CBF
10 µM CBF
0
10
15
NAC (mM)

Figure 3.4 CBF-induced HT29 apoptosis is neither Caspase-3 nor AIF-dependent. ***A.*** RT-PCR of AIF and Caspase-3. There was no significant change in AIF mRNA level, but Caspase-3 level firstly increased after 12 hours and then decreased at 24 hours. ***B.*** No expression of active Caspase-3 and cleaved AIF. After 48-hour-CBF treatment, only mitochondria-localised AIF (67 kDa) was detected by immunoblotting. ***C.*** No obvious shift in AIF distribution between treated and control groups. After CBF addition of 24 hours, red fluorescence representing AIF expression exhibited no enhancement of AIF nuclear translocation. Cell nuclei were stained with DAPI (blue fluorescence). Scale bars equal 10 μm. ***D.*** The inhibition of Caspase-3 activity in CBF-treated HT29 cells. Treated and untreated cells were lysed and the assessment of Caspase-3 luciferase intensity showed that Caspase-3 level was significantly downregulated after 24-hour-treatment. (N=9. *P<0.05). ***E.*** Failure of cancer cell rescue by NAC. The mixture of CBF and NAC was unable to prevent cells from CBF-induced apoptosis. (N=9. *P<0.05). Results are means with standard errors from three replicates.

Discussion

Since cancer is a leading disease causing death worldwide, the demand for any effective drugs that cure cancer remains urgent. Furthermore, enormous numbers of drugs have been exploited and tested *in vitro* in cancer cells and *in vivo* animal models, as well as humans in clinical trials. However, the development of anticancer treatment has shown almost no breakthrough and taken much longer than what scientists expected. Herein, our laboratory believes that it will be more efficient to put more efforts to ameliorate available traditional medicines, rather than to look for new drugs which require initial time consuming characterisation. Therefore, we focused our research on a traditional Chinese medicine called Chansu. Chansu contains a group of bufadienolides and has been utilised to treat inflammation in China for centuries. Recently, the range of Chansu applications has been widened to the field of anticancer treatment, suggesting a potential for the development of Chansu as a therapeutic agent.

Chansu has been widely used in China, Japan and other Asian countries as an anaesthetic, antibiotic and cardiotonic medication. However, like many of these alternative therapies using natural herbs or compounds, their molecular mechanisms have not been elucidated. As such the claims of effectiveness have not been fully tested. Recently, an increasing number of research papers have revealed the pro-apoptotic function of Chansu and its component in a wide range of cancer cells (64,365,370,371).

CBF is a major bufadienolide compound extracted from Chansu. A variety of molecular mechanisms have been proposed to be involved in CBF related anticancer functions, such as inhibition of NF-κB activation (368) and attenuation of phosphorylation in ERK related pathway (372). According to the classification of bufadienolide, CBF is also a type of CGs. One distinct advantage of CG compounds as anticancer agents is that they have high efficacy and safety profiles (373-375). Further investigation also demonstrated that several CG compounds selectively attack malignant but not normal cell proliferation (376-378). This is consistent with the finding of our laboratory that the human head and neck cancer cell line HN5 was drastically resistant to CBF-induce cell death (EC_{50}>6.5 M, Li and Wei, publication in preparation). Moreover, previous *in vivo* studies and clinical trials that utilised CG alone or in combination with other chemotherapies to treat cancers also gained encouraging outcomes (83,379-381). For example, Qin *et al.* evaluated the synergistic efficacy of gemcitabine-oxaliplatin and CBF in 25 patients suffering from malignant gallbladder cancer (69). The combined chemotherapy clearly improved the patients' quality of life and identified a non-toxic dose of CBF.

CBF caused significant cell death in various cancer cells tested. Interestingly, its inhibitory effects on cancer cell growth were variable, with different EC_{50} values among different cell lines. The lowest EC_{50} exhibited at 86 nM in HCT116 and the most resistance was in A431 at approximately 6 μM of CBF. Cell viability assays showed that colon cancer cell lines exhibited an overall higher cell death efficacy of CBF than that of breast, skin and lung cancer cell lines. This is consistent with the previous finding of Sakai *et al* (2004) who demonstrated that ouabain (a type of CG) has a preferential binding to alpha 3 (α3) subunit of Na^+/K^+-ATPase (382). They showed that there was an elevation of α3 subunit expression in colorectal cancer tissues, as well as in the HT29 cell line. Therefore, it is possible that CBF selectively binds to α3 subunits and give rise to a high inhibitory effect in colon cancer cell lines. As all these findings suggest that CBF has a potential to be used as therapeutic agents in treatment of colon cancer, and therefore our investigations were concentrated on colon cancer HCT116 and HT29 cell lines for this study.

We found that even within two highly sensitive cancer cell lines, HCT116 and HT29 cells underwent very distinct apoptotic processes. Herein, our study focused on elucidating the differential apoptotic pathways induced by CBF. First of all, p53-regulated tumour suppressive pathways may play a prominent role in HCT116 cells after CBF exposure. The classic caspase-3-dependent apoptosis was only detected in CBF-treated HCT116, but not in CBF-treated HT29. HCT116 are p53 wildtype cells, while HT29 cells contain mutant p53 (383). Interestingly, it was found that HCT116 cells are more sensitive to CBF treatment than p53-mutant HT29 cells. However, it is contradictory to a previous publication on prostate cancer, which showed that CBF induced caspase-3 activation in p53-mutant DU145 and p53-null PC3 cells, but not in p53 wildtype LNCaP (87). The reason for the discrepancy is yet to be understood.

Another key feature of the CG-related pro-apoptotic mechanism is mitochondrial injury (384,385). We demonstrated here that the change of mitochondrial potential is involved in CBF-induced cell death and this was linked to AIF-mediated apoptosis. The release of AIF can be due to a sustained increase of intracellular Ca^{2+} level, which causes calpain activation (386). Calpain is a calcium-dependent protease and activated calpain cleaves AIF in mitochondria (387). Yeh *et al.* revealed an elevation of cytosolic Ca^{2+} in prostate cancer cell lines after CBF-treatment (87). This mechanism could also operate in AIF-regulated apoptosis in HCT116 cells. Apart from calpain cleavage, a high intracellular Ca^{2+} concentration also leads to the production of ROS in mitochondria (388). Such enhancement of ROS by CBF is a likely explanation for the disruption of mitochondria in our experiments. Although the expression of dissociated AIF was only observed in CBF-treated HCT116 cells, increased mitochondrial permeabilisation was observed in both cell lines. Moreover, Newman *et al.* (384) found that NAC prevents oleandrin (a type of CG) induced mitochondrial condensation in malignant melanoma by inhibition of ROS production. The addition of NAC to HCT116 cells also reduced CBF-induced apoptosis, to some extent. This further indicated a likely correlation between mitochondrial disruption and CBF cytotoxicity. Intriguingly, pro-apoptotic BAX expression was significantly suppressed after CBF-treatment in both colon cancer cells. The unexpected result conflicts with almost all the available studies about CG induced apoptosis in a variety of cancers (389-391). BAX is a pro-apoptotic protein and responsible for mitochondrial permeabilisation. It has been shown to be upregulated in intrinsic apoptosis and the downregulation of BAX is a feature in cancer cell proliferation in many cases. As this is the first study on effects of CBF in colon cancer, we have limited information to explain what causes the reduction of BAX expression in HCT116 and HT29 cells along with CBF-induced apoptosis. Besides, CBF did elicit apoptosis in HT29, but the apoptotic pathways remain independent of the activity of caspase-3 and AIF. AIF mediated apoptosis has been reported to occur when HT29 cells were treated with a number of agents, such as doxycycline and meclizine (392). However, our findings suggest that CBF seems to activate other apoptotic mechanisms involved in unknown CBF molecular function in HT29 cells.

In conclusion, the discovery that Chansu has anti-proliferation roles in cancer opens a new window to this traditional Chinese medicine. Our results showed that CBF reduced cell growth in different cancer cell lines. Moreover, Caspase-3 activation and AIF translocation were involved in apoptosis in HCT116 cells, but not in HT29 cells, indicating distinct apoptotic pathways activated by CBF. This study is fundamental and useful to the further investigation about the mechanisms involved in CBF-induced apoptosis.

CHAPTER 4

Changes of HIF-1α and CTTN expression are involved in CBF-induced apoptosis in the *in vitro* and *in vivo* experiments.

Introduction

Current chemotherapy has one critical drawback that ought to be overcome, which is drug resistance due to hypoxia. Hypoxic regions are generally located in the areas containing fast and uncontrolled proliferative cells of tumours, which are far from the blood vessels required for oxygen and nutrient supply (393,394). Hypoxia triggers self-survival programmes, including angiogenesis, decrease of metabolism rate and pH homeostasis, preventing cells from death during chemotherapy (395). A tumour microenvironment gradually forms and is characterised by gradients in cell proliferation levels as well as by the size of hypoxic regions (396). This tumour microenvironment diminishes the delivery of therapeutic agents, resulting in inadequate intratumoural drug concentration and distribution (397). In other words, it builds up drug resistance to chemotherapy. So far, scientists have elucidated the basic molecular pathways mediated by hypoxia and found that the major regulator HIF, plays a prominent role in adaptation to hypoxia. HIF consists of two subunits: one is oxygen sensitive α subunit (HIF-1α, -2α and -3α) and the other is a nuclear stabilised β subunit (HIF-1β, aka ARNT) (398). Under normoxic conditions, HIF-1α is hydroxylated in the oxygen-dependent degradation (ODD) domain by PHD, and thereby becomes a target of pVHL (399). HIF-1α is ubiquitinated by pVHL and is rapidly degraded in the cytoplasm. Under hypoxic conditions, HIF-1α is stabilised due to the inhibition of prolyl hydroxylation and rapidly imported to the nucleus (400). In the nucleus, HIF-1α and HIF-1β unite to form a heterodimer that recruits p300/CBP transcriptional co-activators. The activated HIF complex subsequently binds to the hypoxia response elements (HRE), advancing transcription of several genes, including angiogenic growth factors and VEGF (401). Therefore, the key point for the prevention of drug resistance is to inhibit the activity of the HIF-1α regulated hypoxia pathway.

To date, there have been a few types of HIF inhibitors available in the market. These inhibitors are able to interrupt the hypoxia pathway at different points. Small molecule PX-478 (S-2-amino-3-[4'-N,N,-bis(2-chloroethyl)amino]phenyl propionic acid N-oxide dihydrochloride) inhibits the translation of HIF-1α (402). Chetomin blocks the association between HIF and p300 co-activator (403). 17-allylaminogeldanamycin (17-AAG) induces proteasomal degradation of HIF-1α (404). The steroidal HIF inhibitors, especially CGs, are proposed to prompt HIF-1α degradation by producing ROS (405). Our laboratory is interested in using CBF to induce apoptosis in colon cancer cells. CBF belongs to the bufadienolide group of CGs. Our previous results have shown that mitochondrial permeabilisation is involved in CBF induced apoptosis. Furthermore, the ROS inhibitor NAC managed to recover the delayed growth rate of CBF-treated HCT116 cells. Thus, it is expected that CBF will inhibit HIF-1α expression and to hinder the growth rate of human colon cancer cells.

Another consequence of hypoxia induced cell survival is metastasis, a feature of malignant cells. CTTN is one of the crucial regulators for cell migration and metastasis (406). During normal cell migration, CTTN is initially phosphorylated by Src kinase in the C terminal proline-rich domain, while Arp2/3 complex binds to the N terminal of CTTN. The binding of Arp2/3 complex promotes actin polymerisation and facilitate the stabilisation of branched actins (407). Then the formation of cell motility structures like lamellipodia support the movement of certain types of cells, including osteoclasts and macrophages (408). Similarly, phosphorylated CTTN is also able to initiate actin assembly but to form invadopodia in cancer cells, followed by extracellular matrix (ECM) degradation. As a result, detached cells invade surrounding tissues. Therefore, CTTN is used as a marker for detection of invadopodia and overexpression of CTTN indicates the metastatic level of cancer cells (409). Recently, many studies have demonstrated that CTTN is closely related to cell invasion and the inhibition of CTTN efficiently decreases cancer cell motility (410,411). Mutations in Src phosphorylation sites of CTTN weaken cell migration in ECV304 endothelial cells (412). Direct knockdown of CTTN or its partner silent mating type information regulation 2 homolog 1 (SIRT1) by siRNA diminishes cell invasion in human prostate cancer cell line DU145 (413). Moreover, inhibition of the binding of a GTPase effector, AMAP1, to CTTN helps to block the sprouting process of VEGF-induced angiogenesis in HUVEC cells (414). Taken together, CTTN is a master regulator of cell migration and the inhibition of CTTN attenuates cancer metastasis.

In this study, we used CBF to treat human colon cancer cell line HCT116 and HT29. The inhibition of HIF-1α expression was exhibited in the two treated cell lines, whereas overexpression of CTTN was only suppressed in HCT116 cells. Noticeably, this the first report of the distribution of CTTN in the nucleus of HCT116 cells after CBF exposure. However, this observation was not detected in HCT116 implanted xenograft mouse models, though CTTN expression appears to be reduced in tumour tissues.

Results

CBF significantly affects HIF-1α and serum response factor (SRF) - regulated intracellular pathways.

To further explore the signalling pathways underlying CBF-induced apoptosis, ten cellular pathways ranging from stress, cell cycle to cytotoxicity were studied. Due to low reverse transfection efficiency of HT29, only HCT116 cells were examined by transfecting with specific luciferase reporter genes of the 10 pathways. The differences of luciferase expression between treated and control groups indicated the activation level of the respective signalling pathway. A greater fold change in the luciferase activities indicated a higher possibility that a particular pathway is involved. As shown in **Figure 4.1A**, the activity of HIF-1α was downregulated by 2.8 fold between CBF-treated and untreated cells, while serum response factor (SRF/Elk-1) was upregulated by 2.4 fold. Subsequently, it was expected that hypoxia could significantly inhibit the cell growth of CBF-treated HCT116 and HT29 cells, as CBF downregulated the HIF-1α-mediated pathway. HCT116 and HT29 cells were exposed to eight different concentrations of CBF under 1% or 20% oxygen, followed by MTT assays (**Figure 4.1B**). Yet, oxygen deprival rarely affected CBF induced inhibition of cancer cell growth. Compared with cells grown under normal atmospheres, HCT116 and HT29 cells grown under 1% oxygen displayed almost no alteration in the tendency of cell viability.

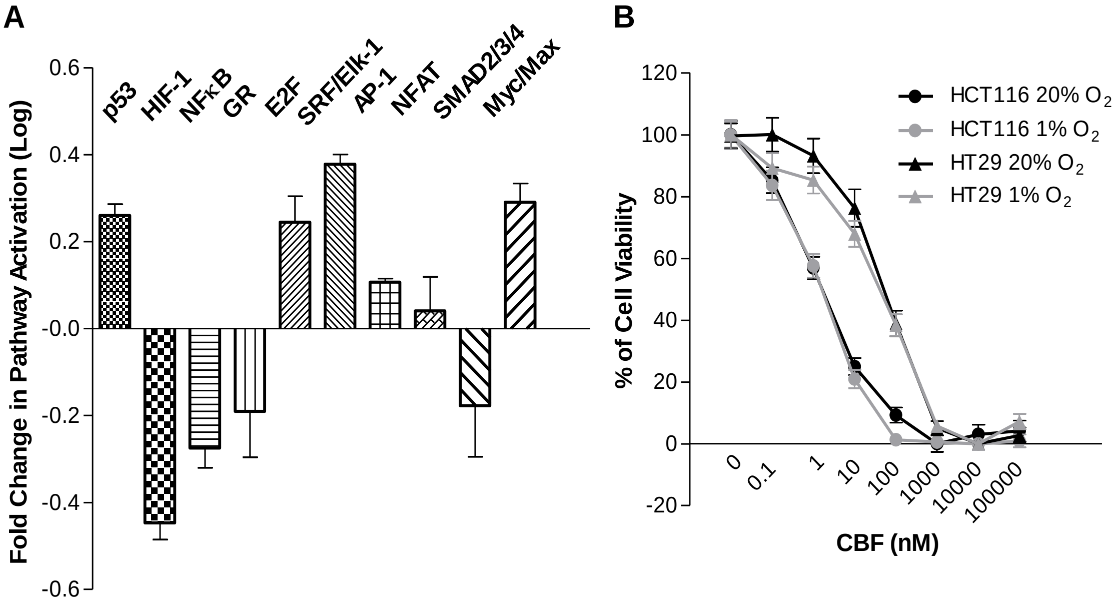


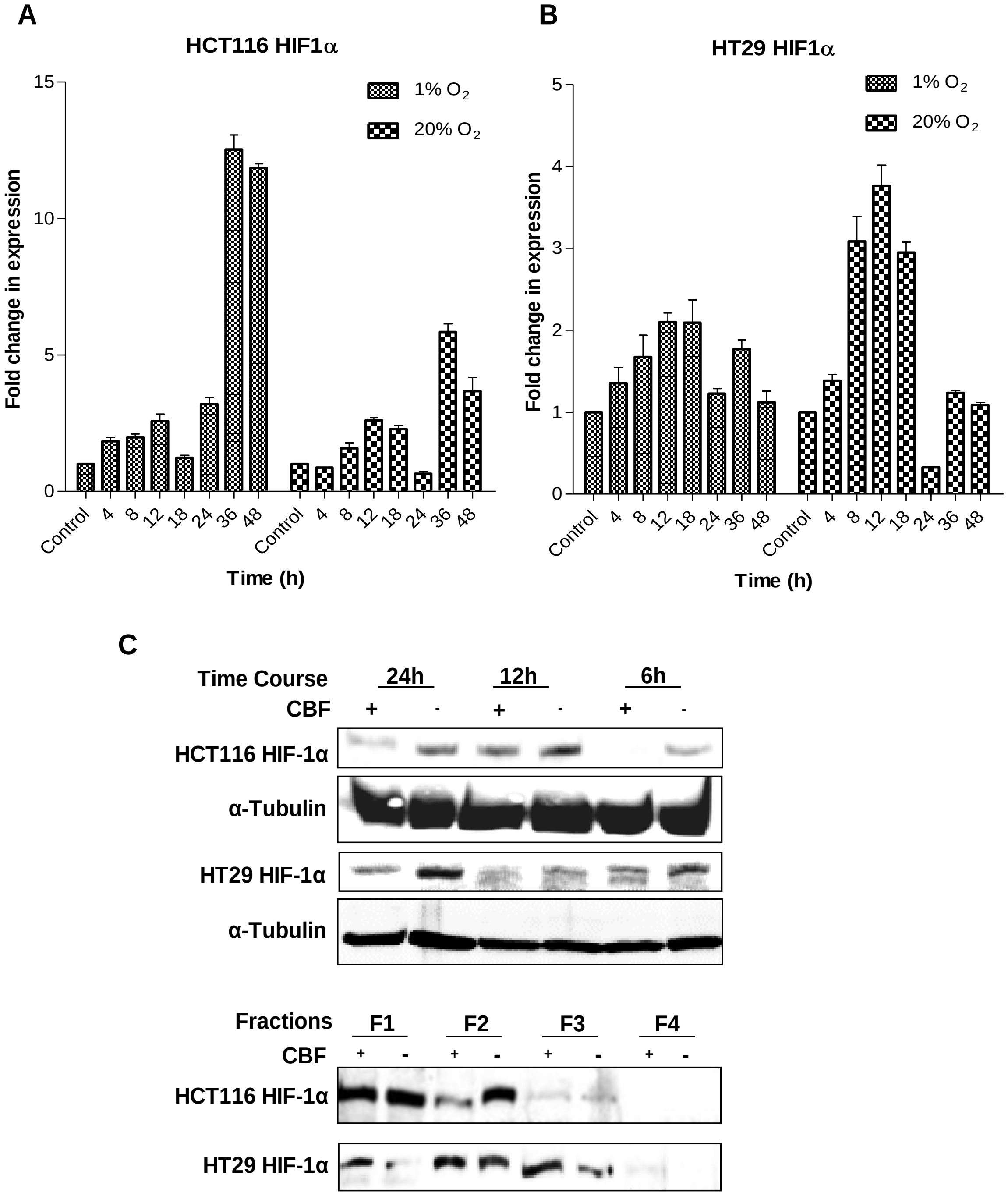
Figure 4.1 HIF-1α-mediated pathway is involved in CBF treatment. ***A.*** Deregulation in multi-pathway array after CBF treatment. HCT116 cells were transfected with ten reporter genes, followed by 9-hour-CBF exposure. These reporters are unique transcription factors representing ten signalling pathways canonically involved in cytotoxicity. The most significant change is in the HIF-1α-regulated pathway, in which HIF-1α level was 2.8 times as low as the untreated HCT116 cells. On the other hand, the most upregulation occurred in the MAPK/ERK pathway, where the expression of SRF/Elk-1 increased by 2.4 times. Results are means with standard errors from 4 replicates. The logarithm of untreated cell luciferase intensity was set to baseline. p53, pathway of p53/DNA damage; HIF-1, pathway of hypoxia; NF-κB, pathway of NF-κB; GR, pathway of glucocorticoid receptor; E2F, pathway of cell cycle/pRb-E2F; SRF/Elk-1, pathway of MAPK/ERK; AP-1, pathway of MAPK/JNK; NFAT, pathway of PKC/Ca^{2+}; SMAD2/SMAD/3/SMAD/4, pathway of TGFβ; Myc/Max, pathway of Myc/Max. ***B.*** Cytotoxicity of CBF in HCT116 and HT29 cells under hypoxic conditions. Two colon cell lines were treated in 8 different concentrations of CBF for 24 hours under 1% of oxygen. The cytotoxicities of CBF are similar between the groups of 1% and 20% oxygen. Results are means with standard errors from four replicates.

CBF upregulates mRNA of HIF-1α but downregulates HIF-1α protein expression.

Unlike the result from multi-pathways reporter assays, the levels of HIF-1α mRNA exhibited an overall increase in HCT116 cells (**Figure 4.2A**), while no significant difference after 48 hours in HT29 cells (**Figure 4.2B**). HIF-1α mRNA kept increasing during the early 12 hours CBF treatment in both cell lines, and a sharp decrease occurred at 18 or 24 hour time points. After 48 hours treatment, mRNA expression of HIF-1α was significantly elevated in HCT116 but returned to initial level in HT29 cells, regardless of oxygen supply.

On the other hand, the expression of HIF-1α protein was found to be inhibited in CBF treated HCT116 and HT29 cells consistent with the 10-pathway reporter assays (**Figure 4.2C**). The inhibition was most significant after 24 hours treatment under hypoxic (100 µM $CoCl_2$) conditions. Additionally, the expressions of HIF-1α from 4 cellular fractions were also measured. In 24-hour-treated HCT116 cells, a large reduction was found in the fraction of membrane and organelle proteins (F2), while a large difference between treated and non-treated HT29 cells was in the cytosolic fraction (F1). Furthermore, although there is no existing evidence showing the localisation of HIF-1α in the membrane of any organelles or nuclei, our finding, to some extent, indicated that HIF-1α might directly or indirectly interact with the membrane of an organelle or nucleus before nucleic translocation. Thus, CBF is likely to disrupt the binding between HIF-1α and the membrane in HCT116 cells. On the other hand, HIF-1α protein levels were slightly increased in treated HT29 cells in F1, F2 and F3 fractions.

To determine the expression of HIF-1α, it was fused to GFP and expressed in HCT116 and HT29 cells. Intact human HIF-1α sequences was obtained by PCR and inserted into the multiple cloning sites of pEGFP-C1 vector, thereby generating pGFP-HIF-1α for subsequent experiments (**Figure 4.2D&E**). As shown in **Figure 4.2F**, the expression level of GFP in CBF-treated HCT116 and HT29 cells was significantly reduced. Transfected cells were exposed to CBF for 24 hours under 1% of oxygen. GFP expression under 20% of oxygen was also measured and as expected at low levels. Taken together, our findings suggested that HIF-1α accumulation was suppressed by CBF though the mechanisms involved are distinct and unclear in HCT116 and HT29 cells.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20% O₂
Fold change in expression
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Control
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B
HT29 HIF1α
1% O₂
20% O₂
Fold change in expression
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HCT116 HIF-1α
α-Tubulin
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HT29 HIF-1α

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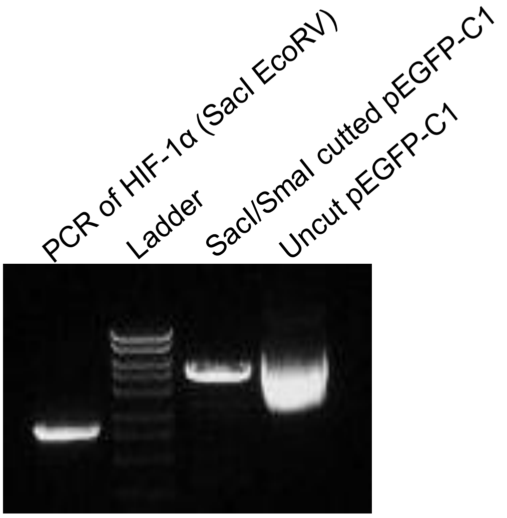
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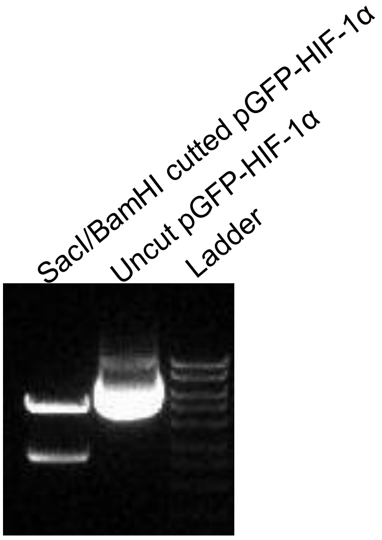
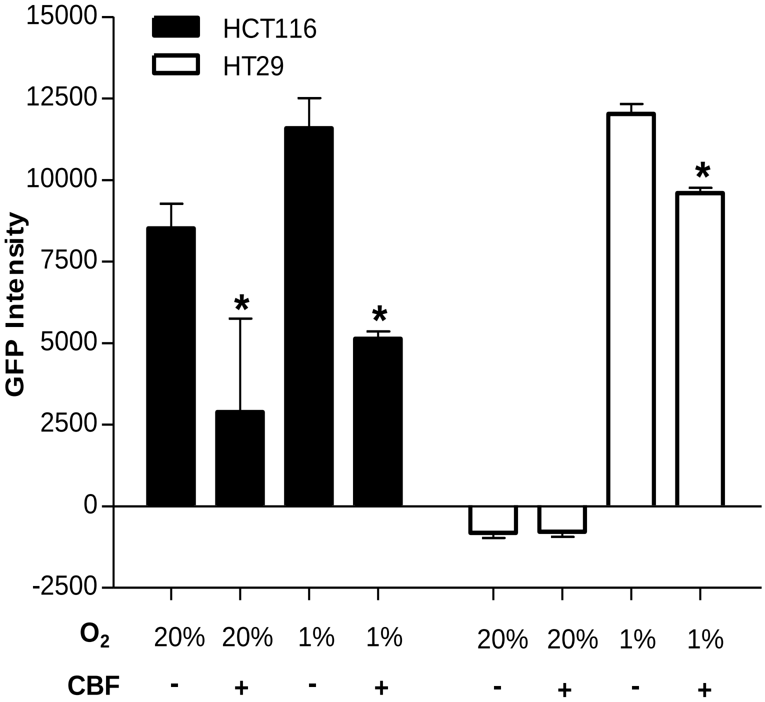
Uncut pEGFP-C1

E


SacI/BamHI cutted pGFP-HIF-1α
Uncut pGFP-HIF-1α
Ladder

F


HCT116
HT29
GFP Intensity
15000
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7500
5000
2500
0
-2500
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20%
20%
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1%
CBF
-
+
-
+
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-
+

Figure 4.2 CBF affects HIF-1α transcription and protein expression. ***A.*** Elevation of HIF-1α transcription level by CBF under hypoxic conditions. Compared with that in a normal atmosphere, the level of HIF-1α mRNA doubled when HCT116 cells were in the lack of oxygen. ***B.*** No overall increase of HIF-1α transcription after 48 hours CBF exposure. The patterns of HIF-1α transcription of HT29 between 1% and 20% were similar. Dramatic upregulations of HIF-1α mRNA were only from 8 to 18 hours' time point, followed by a sharp fall at 24 hours. ***C.*** Inhibition of HIF-1α protein expression. Under hypoxic (100 µM $CoCl_2$) conditions, HIF-1α protein expression was suppressed by CBF at different time points. Moreover, apparent inhibition was detected in membrane/organelle fraction of HCT116 and cytosolic fraction of HT29 cells. F1: cytosolic protein; F2: membrane/organelle protein; F3: nucleic protein; F4: cytoskeletal protein. ***D.*** Restriction digest of HIF-1α and empty pEGFP-C1 vector. PCR amplified HIF-1α inserts were digested by SacI and EcoRV enzymes, and pEGFP-C1 vectors were digested by SacI and SmaI enzymes. ***E.*** Ligation of pGFP-HIF-1α vector. The size of ligated pGFP-HIF-1α was checked by double digest with SacI and BamHI enzymes to produce two fragments. ***F.*** Inhibition of GFP fused HIF-α expression. Plasmids pGFP-HIF-α were transfected into HCT116 and HT29cells, followed by a 24-hour-CBF treatment under 1% or 20% oxygen. Compared to the control groups, 1 µM of CBF exposure effectively inhibited the expression of GFP under hypoxic conditions. Results are means with standard errors from four replicates.

CBF inhibited CTTN protein expression in HCT116 in the first 12 hours but not in HT29 cells.

Based on the results from multi-pathway arrays, transcript factor SRF/Elk-1 related MAPK/ERK pathway was the second most CBF-affected pathway out of 10. Interestingly, CBF has been found to improve the patients' life quality in a clinical trial when combined with the usage of gemcitabine-oxaliplatin to treat gallbladder carcinoma (69). Therefore, the clinical study suggested a role for CBF in the MAPK/ERK signalling pathway and cell migration. As a result, this study focused on CTTN, a key regulator for cell migration, which could be phosphorylated by serine/threonine kinase ERK (415,416). HCT116 and HT29 cells were exposed to CBF under both hypoxic and normoxic conditions and the transcription levels of CTTN mRNA were estimated at different points by RT-PCR (**Figure 4.3A&B**). The results (1% oxygen) showed an inhibition of CTTN transcription from the beginning to the 12 hours' time point, followed by a two-fold increase at 24 hours' time point. When oxygen was sufficient, the highest transcription level of CTTN mRNA in HCT116 cells exhibited at 8 hours' time point, whereas the highest level in HT29 cells was at 24 hours. CTTN protein expression in CBF-treated HCT116 cells was consistent with the exhibition of mRNA transcription under 1% of oxygen (100 μM $CoCl_2$) (**Figure 4.3C**). CTTN protein expression was inhibited by CBF after 6 and 12 hours, but no suppression was observed after 24 hours. By contrast, CBF had no effect on CTTN in HT29 cells. Taken together, these results showed that CBF possesses an inhibitory effect on CTTN transcription and expression in HCT116 cells but is ineffective to HT29 cells. In addition, the protein expression in different cellular fractions was also investigated after 24 hours CBF treatment (**Figure 4.3D**). The outcome showed that

CTTN expression was enhanced in F2 of HCT116 cells and repressed in F4 of HT29 cells by CBF.

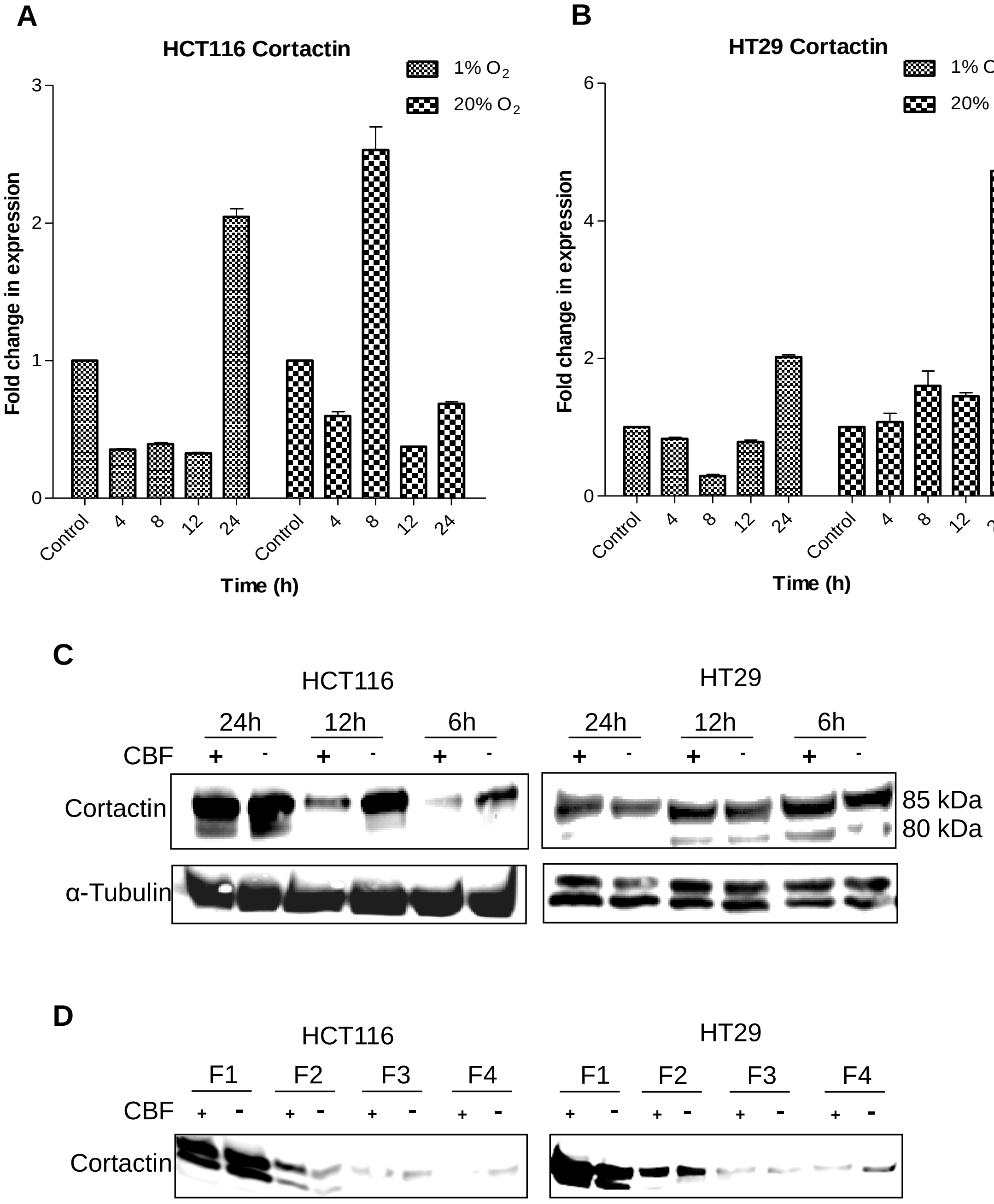
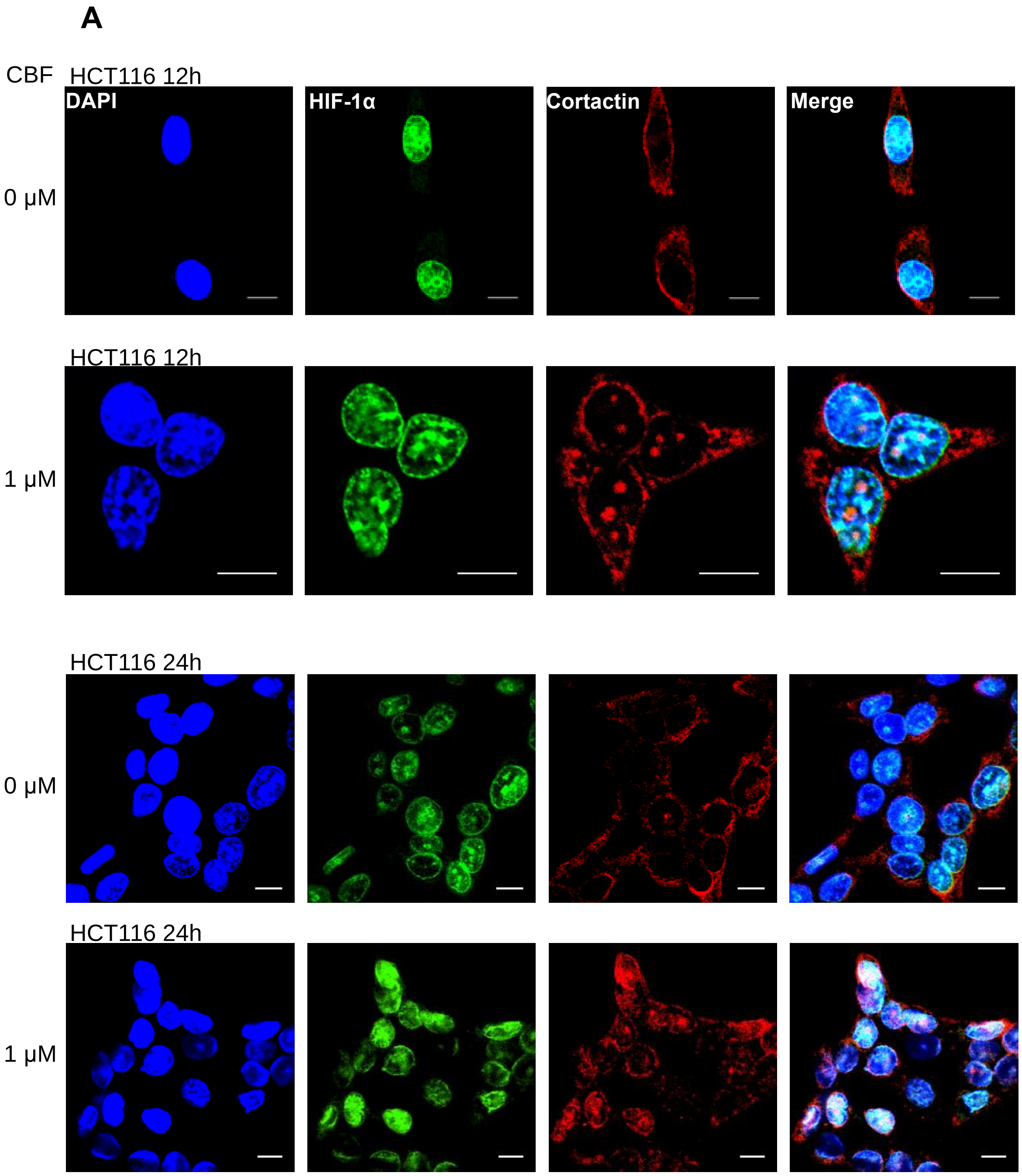


Figure 4.3 CBF affects CTTN transcription and protein expression. ***A.*** The mRNA transcription of CTTN in CBF-treated HCT116 cells. The mRNA level was significantly increased at 24 hours under hypoxic conditions, whereas a dramatic increase was only at 8 hours under normoxic conditions. ***B.*** The mRNA transcription of CTTN in CBF-treated HT29 cells. Transcription level reached the lowest level at 8 hours and increased two-fold at 24 hours under 1% oxygen, while mRNA level was not elevated significantly until 24 hours under 20% oxygen. Results are means with standard errors from 4 replicates. ***C.*** CTTN protein inhibition only in HCT116 (1% oxygen). Expression of CTTN was significantly inhibited at 6 hours' time point in HCT116 and inhibitory capacity of CBF lasted more than 12 hours. In contrast, CTTN protein expression was not affected by CBF in HT29 cells. ***D.*** Increase of CTTN level in F2. The most significant alteration induced by CBF was the upregulation of CTTN expression in membrane/organelle protein fraction in HCT116 cells. However, the effect of CBF in HT29 was hardly detected.

CBF promotes nuclear translocation of CTTN in HCT116 cells but not in HT29.

To further investigate the distribution of HIF-1α and CTTN, both proteins were stained with immunofluorescent antibodies and visualised using confocal microscopy. It was found that CTTN was translocated to the nuclei after CBF treatment for 24 hours in HCT116 cells (**Figure 4.4A**) and the nuclear translocation of HIF-1α was enhanced in HT29 cells (**Figure 4.4B**). The difference in distribution of the two proteins in HCT116 and HT29 cells revealed that there could be distinct mechanisms involved in CBF function in various cancer cells. In other words, the anticancer activity of CBF is cell type dependent. In addition, the nuclear colocalisation of HIF-1α and CTTN observed by immunocytochemistry of HCT116 cells gave rise to the possibility that HIF-1α physically interacted with CTTN. Therefore, to determine this interaction His-tag pull-down and co-immunoprecipitation were carried out. For the application of His-tag pull-down, a new plasmid construct was generated, which contains an intact human HIF-1α and a 6xHis-tag added to the C terminal of HIF-1α (**Figure 4.4C**). Unfortunately, the binding capacity of the newly synthetic His-tagged proteins to a specific His-tag column was weak and most of the His-tagged proteins were washed off in the first washing step. Although the final concentrated eluted protein showed the detection of CTTN in immunoblotting (**Figure 4.4D**), there was no presence of His-tagged HIF-1α proteins. Alternatively, co-immunoprecipitation was performed to examine the interaction between HIF-1α and CTTN post CBF treatment. Treated HCT116 cells were harvested after 24 hours and total proteins were extracted. Specific CTTN antibody and agarose beads were applied to precipitate CTTN and its interacted proteins. The results indicated that there is no direct binding between HIF-1α and CTTN (**Figure 4.4E**).


A
CBF
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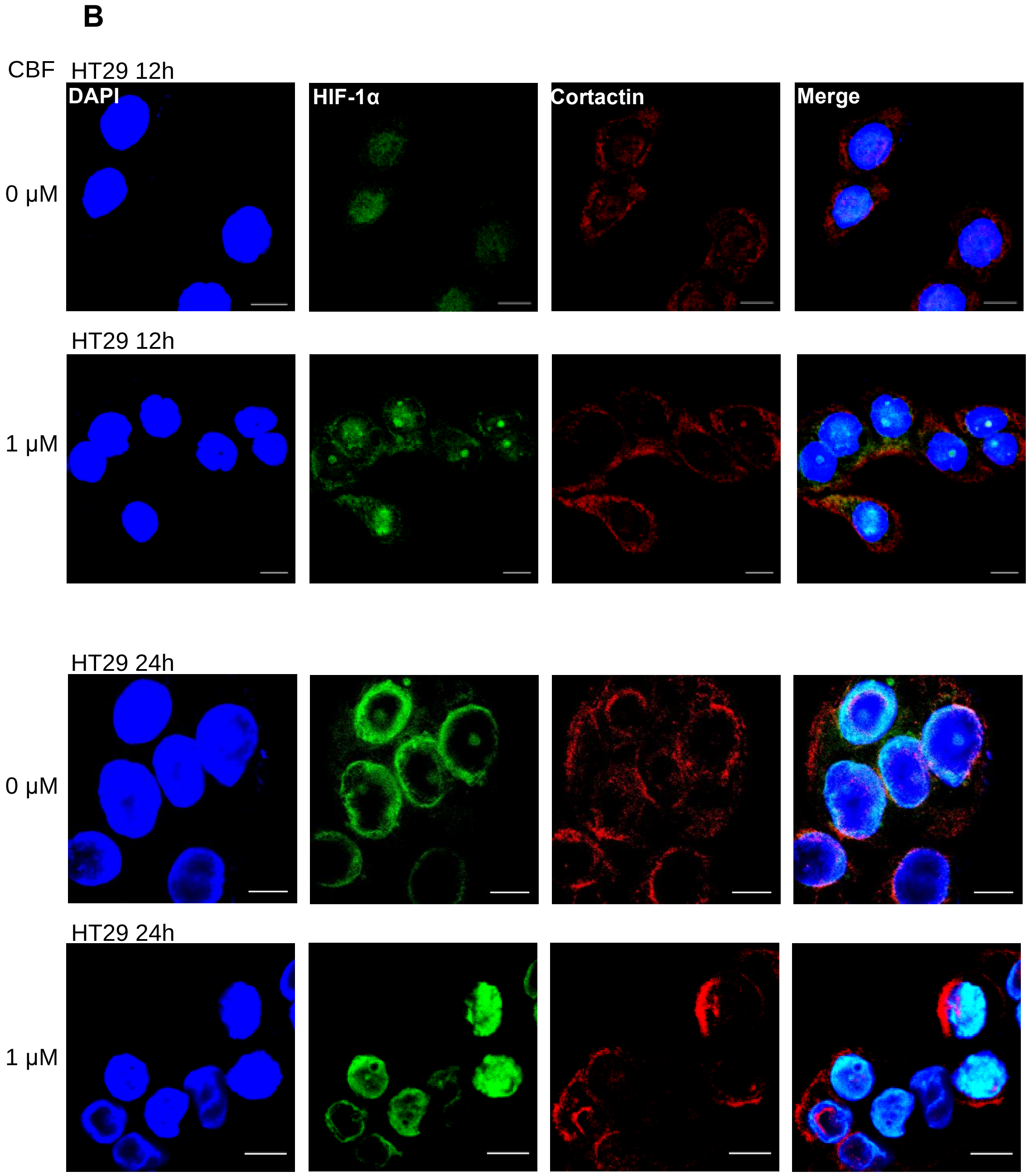
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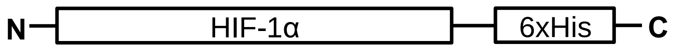
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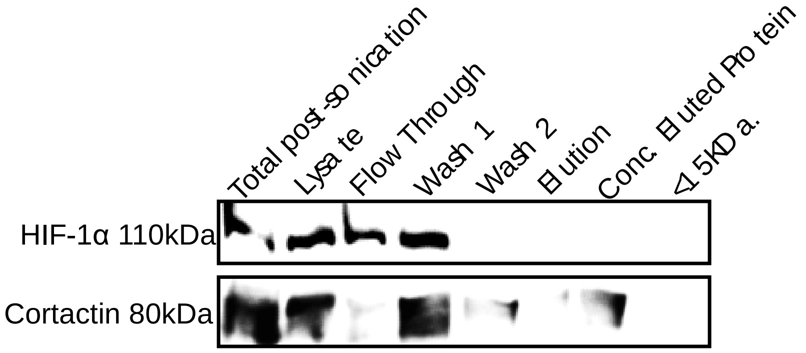
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Cortactin 80kDa

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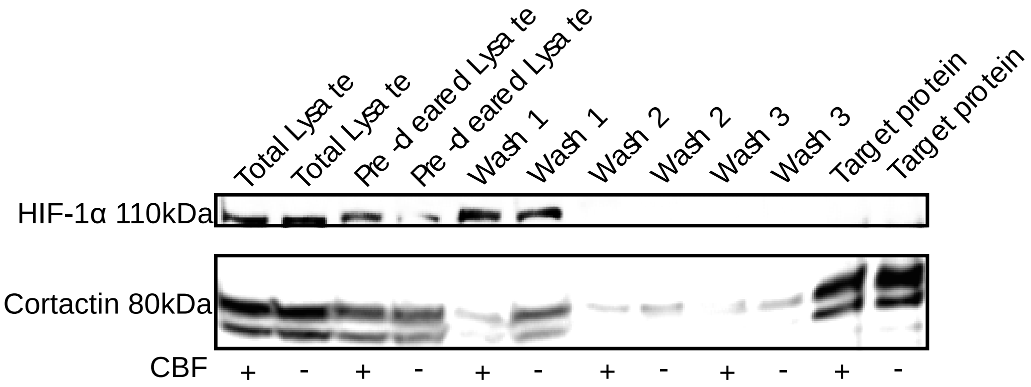
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Wash 3
Wash 3
Target protein
Target protein
HIF-1α 110kDa
Cortactin 80kDa
CBF
+
-
+
-
+
-
+
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Figure 4.4 CBF affects the distribution of HIF-1α and CTTN. ***A.*** Co-localisation of HIF-1α and CTTN in treated HCT116. After 12 hours CBF treatment under hypoxic conditions, the nuclear translocation of CTTN occurred. The subsequent colour overlapping at 24 hours indicated that both HIF-1α and CTTN were localised in the nucleus, compared with no overlap in non-treated HCT116 cells. ***B.*** Enhancement of HIF-1α nuclear translocation in treated HT29 cells. After 24-hour-exposure, CBF accelerated the nuclear translocation of HIF-1α, whereas CTTN proteins appeared to assemble at a point on the surface of cell membrane. Scale bars equal 10 μm. ***C.*** Construct of pHIF-1α-His vector used in His-tag pull-down. Six histidine residues were added to the C terminal of the HIF-1α protein and the plasmids were transfected into HCT116 cells. ***D.*** Sequential steps of His-tagged HIF-1α protein pull-down. The binding capacity of newly synthetic proteins was too weak to bind to the specific His-tag column. Therefore, most of the His-tagged HIF-1α was washed off in the first washing step and was not present in the elution. ***E.*** No direct interaction between HIF-1α and CTTN. After 24 hours CBF treatment, immunoprecipitation using CTTN antibody was performed to extract CTTN from crude protein of HCT116 cells. HIF-1α eluted in the preparation and wash steps, indicating it is not physically bound to CTTN.

CBF did not retard the growth rate of tumour.

In an effort to define the anticancer function of CBF in animal models, we established xenografts by subcutaneous injection of HCT116 cells. The administration of drug started when the average tumour sizes of each group reached 320 mm^3. The longest survival curve was from the group of i.p. injection with 22 days (**Figure 4.5A**). All mice from groups of control and i.t. injection were sacrificed on day 13 and day 15, respectively. Compared with the control group, the tumours of i.t. and i.p. groups showed no significant difference (**Figure 4.5B**). Notably, there was only one mouse leftover in i.p. group after day 16 and this mouse was sacrificed on day 22.

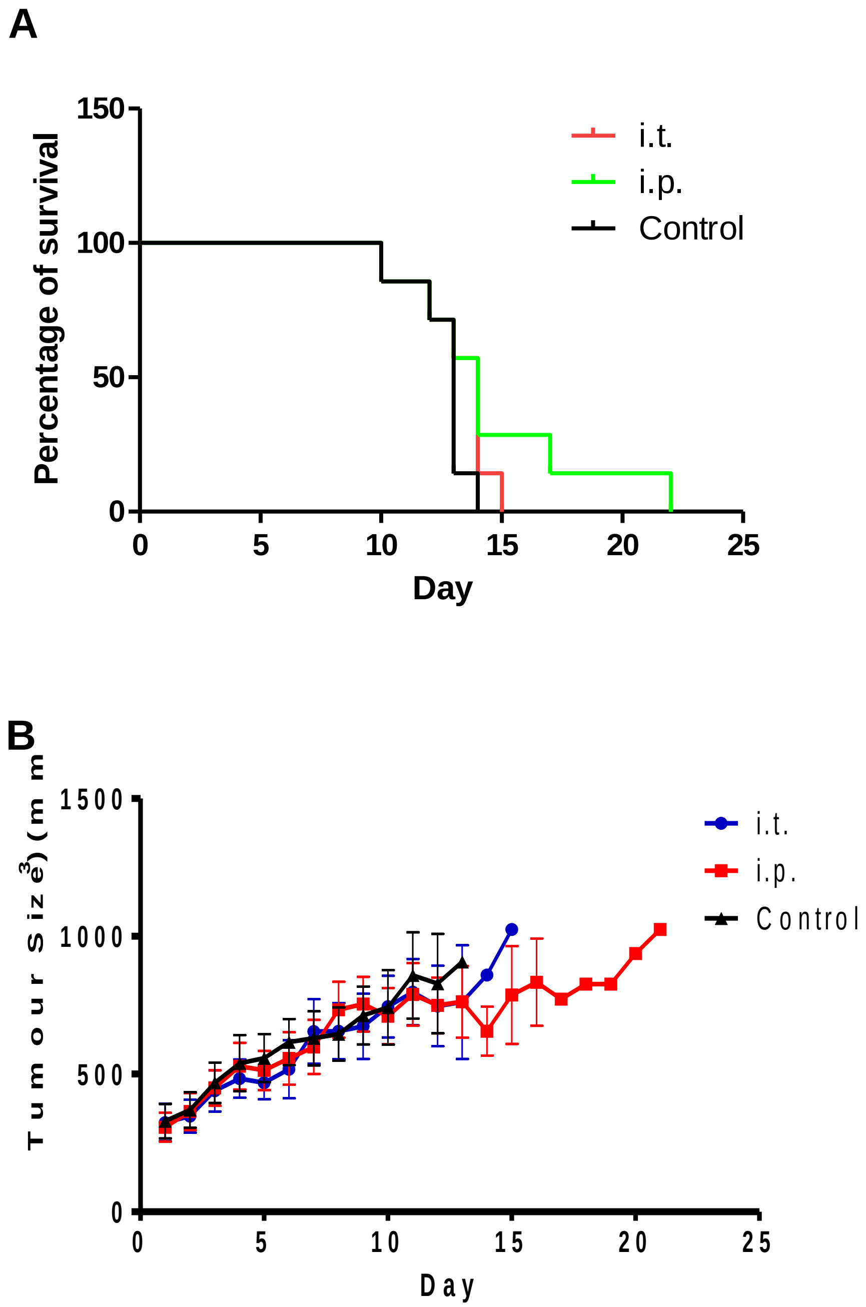
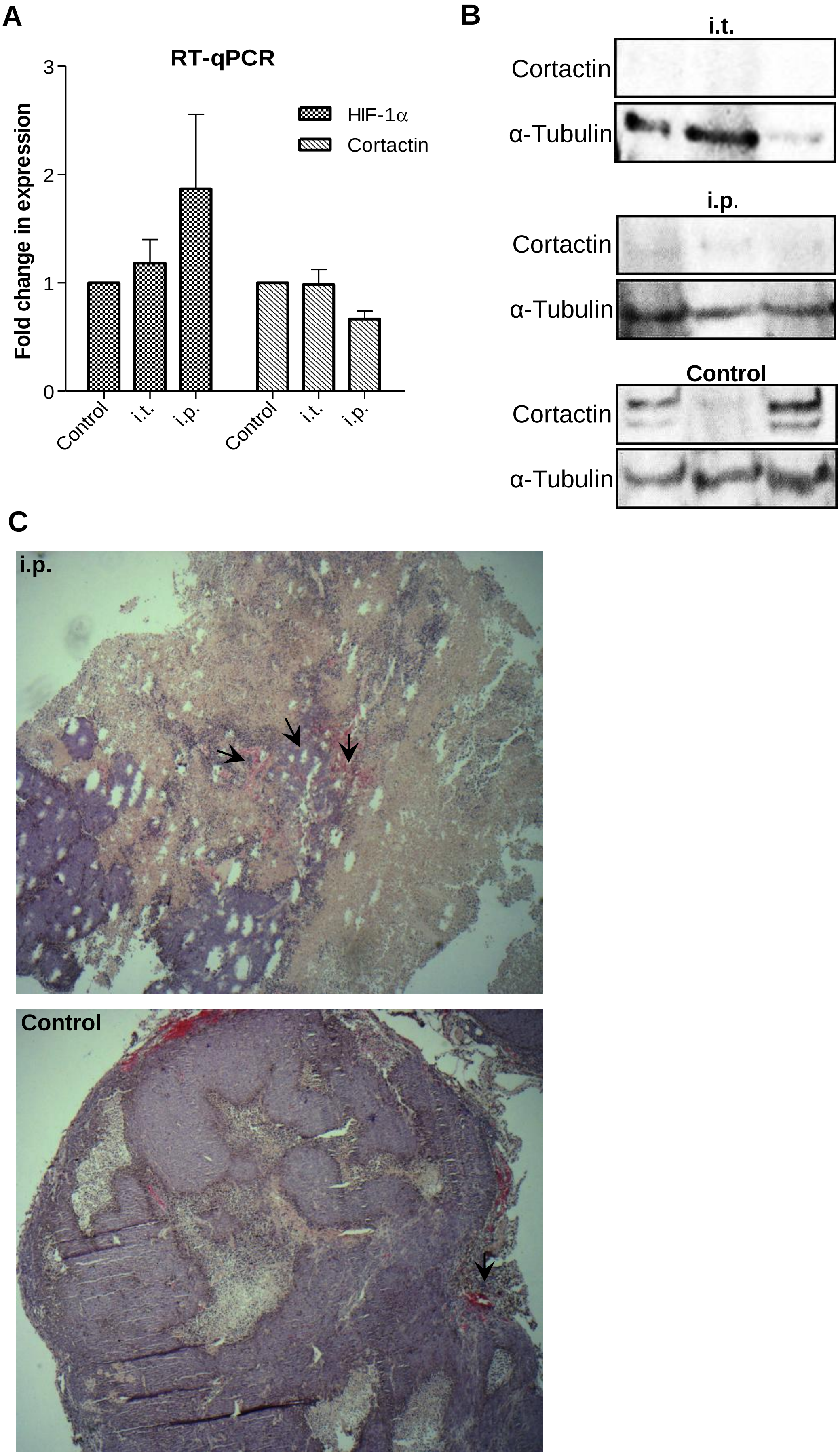


Figure 4.5 CBF treatments in nude mouse models injected with HCT116 cells. *A.* The longest survival is in the i.p. group. After given daily dose (1.5 mg/kg), i.p. group showed the longest survival period with 22 days in the three groups (N=7). The groups of i.t. and control were sacrificed on day 15 and day 13, respectively. ***B.*** Tumour size versus days of CBF administration. There is no significant delay in tumour growth rate during the CBF treatment.

CBF affects HIF-1α and CTTN expression in mouse tumour tissues.

Although the overall growth rate of tumour was not reduced, but our analysis still indicated effects of CBF in the molecular biology of collected mouse tumour tissues. The mRNA level of HIF-1α was significantly elevated in i.p. group, whereas the level of CTTN was reduced (**Figure 4.6A**). Furthermore, immunoblotting result showed that CTTN protein expression was only detected in control group (**Figure 4.6B**). Two out of three tissue samples from control group exhibited a clear band of CTTN size. Additionally, in the comparison of tissue samples stained with H&E, the i.p. sections presented more abundant patterns of necrosis than that in the control (**Figure 4.6C**). Intriguingly, the fluorescent images from mouse tissues exhibited a conflicting result in which CBF appeared to delay the nuclear translocation of HIF-1α, whereas HIF-1α import was apparent in the control group (**Figure 4.6D**).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Fold change in expression
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B
i.t.
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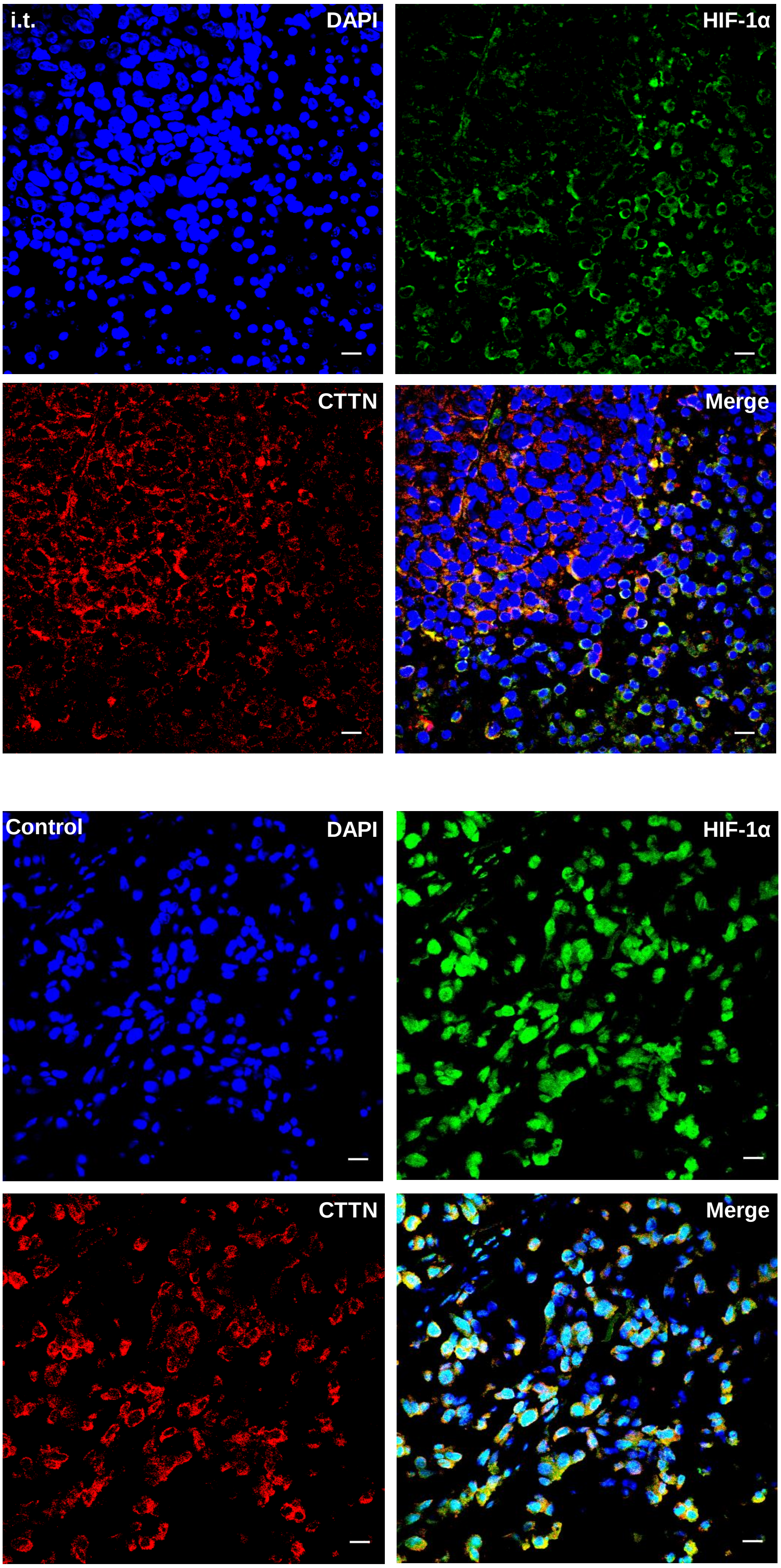
i.t.
DAPI
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CTTN
Merge
Control
DAPI
HIF-1α
CTTN
Merge

Figure 4.6 Analysis of CBF effects in mouse tumour samples. ***A.*** Analysis of HIF-1α and CTTN mRNA level in tumour tissues. Total RNAs were extracted from tissues samples and subsequent PCR result showed an upregulated HIF-1α level as well as a downregulated CTTN level. Results are means with standard errors from 4 replicates. ***B.*** CTTN protein expression only in the control group. Tissue protein crudes were collected and three samples from each group were analysed by immunoblotting. Two out of three samples exhibited the presence of CTTN in the control group. Both i.t. and i.p. groups showed no expression of CTTN. ***C.*** Abundant necrosis patterns in i.p. group (x100). Compared with the control sample, the section of i.p. group contains larger proportions of necrotic cells (black arrows). ***D.*** Inhibition of HIF-1α nuclear translocation in i.t. sample. CBF inhibited HIF-1α nuclear accumulation and the distribution of HIF-1α stained and CTTN stained cells were different. CTTN appeared to express in highly assembled cells, whereas HIF-1α expressed cells were located in loose patterns. Scale bars equal 10 μm.

Discussion

CBF is a major component of traditional Chinese medicine Chansu and standard product of CBF is commercially available. This is the main reason that CBF was used for the subsequent laboratory experiments. The previous cytotoxicity assays also showed a common high sensitivity of CBF in different human colon cancer cell lines. However, the mechanisms involved in CBF-induced apoptosis are still unclear. Our previous results showed that the classic Caspase-3-dependent apoptosis was only detected in CBF-treated HCT116, but not in CBF-treated HT29. Herein, a reporter assay was used to elucidate the differential apoptotic pathways induced by CBF.

The assessment of the 10-pathway reporter arrays suggests a multi-factorial network involved in CBF-mediated apoptosis. Overall, the most significant inhibition was in HIF-1α-regulated pathway. The expression of HIF-1α intimately corresponds to changes in oxygen levels (47). During oxygen deprivation, degradation of HIF-1α is inhibited, permitting HIF-1α to enter the nucleus, where it binds to ARNT and initiates the transcription of hypoxia-responsive genes (49). HIF-1α regulates the transcription of several oncogenic genes, especially VEGF, and thereby promotes cell proliferation (417). In relation to the inhibition of HIF-1α, although there is no direct relationship established between HIF-1α and CBF, several previous studies have shown that digoxin and other CGs inhibit HIF-1α synthesis to retard tumour growth (418). Thus, there is a possibility that the digoxin-like molecule CBF reduces HIF-1α level in colon cancer cell lines as well. Interestingly, our findings revealed a paradox in the CBF inhibition of HIF-1α at the mRNA and protein levels. CBF induces HIF-1α mRNA expression, but inhibits the expression of the protein. This is consistent with another study in which HIF-1α mRNA expression was increased after exposure to digoxin in human hepatoma

Hep3B cell line (79). However, the subsequent protein expression of HIF-1α was significantly inhibited by digoxin. In fact, HIF-1α protein is repressed by p53 during normal cell function (419). The 10-pathway reporter showed that p53 protein is activated by CBF in HCT116 cells, which provides a plausible explanation for the decreased HIF-1α protein. However, the increase in HIF-1α mRNA by CBF is by an unknown mechanism, possibly by repression or activation of a transcription factor. Another result from the estimation of GFP expression also confirmed the inhibition of exogenous HIF-1α protein expression in the presence of CBF.

Additionally, based on the discovery of HIF-1α downregulation, it was hypothesised that the inhibitory effects of CBF would be enhanced in p53-mutant HT29 cells. Furthermore, Blagosklonny *et al.* demonstrated that p53 represses the activity HIF-1α in breast cancer cells (420). Other studies have showed that p53-mutant cancer cells sustain longer periods of proliferation under hypoxic conditions than p53-wildtype cancer cells (421). Our observation also showed that p53-wildtype HCT116 cells are more sensitive to CBF treatment than HT29 cells in a normal atmosphere. On the other hand, it was expected that HT29 cells would show less resistance to CBF during hypoxia, but there was no significant difference of cell growth rate between normoxic and hypoxic HT29 cells. The result from the 10-pathway reporter arrays showed that p53 protein was significantly upregulated in CBF treated HCT116 cells. Hence, the functional status of p53 seems relevant to CBF induced inhibition of HCT116 cells.

Apart from the inhibitory function of CBF, it was discovered that the nuclear translocation of HIF-1α in HT29 occurs earlier than that in HCT116 cells, including a large proportion of HIF-1α bound to the fraction of nuclear membrane. Combined with the results from immunostaining, it is postulated that the addition of CBF accelerates the nuclear translocation of HIF-1α, especially in HT29 cells. This assumption might be due to the activation of the MAPK/ERK pathway, the second most affected pathway shown in 10-pathway reporter assays. A number of previous studies have revealed that stimulation of MAPK pathway not only enhances HIF-1α transcriptional activity (422), but induces the nuclear accumulation of HIF-1α as well (423-425), which may partially explain the nuclear shift of HIF-1α in our case. Therefore, taken together our findings imply that the hypoxia-regulated cellular pathway is involved in CBF driven cell death.

The capacity of CGs to upregulate MAPK pathways have been reported previously, including the studies about ouabain and digoxin (426,427). MAPK activation is initiated by the binding of CG to Na^+/K^+-ATPase, resulting in the release of Src kinase. Src belongs to the tyrosine kinase family and are normally bound to alpha subunit of Na^+/K^+-ATPase (426). Dissociated Src subsequently triggers the Ras-MAPK cascade, followed by a serial of phosphorylation and eventually activation of ERKs which induce many transcription factor activities involved in cell survival (428). CTTN as a substrate for Src and ERK kinases directly regulates actin polymerisation via Src/ERK phosphorylation (429). The activation of MAPK/ERK pathway by CBF might explain the significant increase in transcriptional activity of CTTN (under 1% of oxygen). After the 24 hours treatment, the high level of CTTN mRNA leads to increased CTTN protein expression. The subsequent overexpression of CTTN protein overwhelms the inhibition induced by CBF in HCT116 cells. Furthermore, this is the first report that CTTN is imported to the nucleus, but the mechanism for the translocation remains unclear. Nevertheless, we eliminated the probability that HIF-1α and CTTN physically bind to each other. To investigate the interaction between HIF-1α and CTTN, we initially applied a His-tag pull-down using a plasmid containing intact human HIF-1α sequences tagged with six histidine residues at the C terminal. Unfortunately, the binding capacity of the newly synthetic proteins to a specific His-tag column was too weak to capture His-tagged proteins. The detection of His-tagged HIF-1α proteins with CTTN in the elution therefore failed. Nonetheless, the alternative co-immunoprecipitation suggested that there could be no direct interaction between HIF-1α and CTTN. Here, we cannot formally exclude the possibility that CTTN and HIF-1α do associate, but the association could not be detected if anti-CTTN antibody and HIF-1α binding sites on CTTN overlap. In the future, we might repeat the His-tag pull-down but with a plasmid containing six histidines at the N terminal of HIF-1α. This may enhance the binding capacity of His-tagged proteins to the column. On the other hand, hemagglutinin (HA) peptides could be tagged to the N or C terminal of CTTN protein for co-immunoprecipitation experiments. The use of HA-tag specific antibody helps to extract the tagged CTTN without directly binding to the CTTN native protein, preventing the break of protein-protein interactions. The chromatography of using protein tags associated with both HIF-1α and CTTN would decipher the physical connection between these two proteins.

CBF showed a potent anti-proliferation capacity in cancer cell lines, and therefore the next step of this study was to examine the efficacy of CBF in xenografts. A previous study reported that CBF has no effect in tumour growth in nude mouse models injected with HT29 cells (430). Our finding also showed a lower sensitivity of HT29 than that of HCT116. Hence, we initially established nude mouse models only subcutaneously injected with HCT116 cells. Since several previous clinical trials of CBF or Chansu had showed encouraging results (69), it was expected that the usage of CBF in xenografts would significantly retard the growth rate of colon cancer. However, this is not the case and we summarised a few points to explain the *in vivo* experiment. First of all, almost no information is available for the functions of bufadienolides in murine cells (430,431). A pilot study of Kawazoe *et al.* (1998) pointed out that bufalin induces apoptosis in human colon cancer Colo320DM cells but failed to do so in murine leukaemia P288D1 cells (431). This could be due to the insensitivity of sodium and potassium pumps in murine cells, which were found to have no alteration of plasma membrane potential in the presence of bufalin. On the other hand, bufalin reduces concentrations of potassium ions in human leukaemia and colon cancer cell lines by inhibiting Na^+ K^+ -ATPases. Our result of CBF cytotoxicity assay in mouse colon cancer fibroblast CT26 also showed no effects in CT26 growth after exposure to CBF even for 48 hours (**Figure 4.7A**). Therefore, a possibility concluded is that the dose applied in our mouse study was too low to produce effective CBF activities. We used the concentration of 1.5 mg CBF per kg of mouse body weight for the treatment based on previous experiments using similar Chansu compounds (379,432). To find a proper dose for the administration, we will repeat the experiment with variable concentrations in the future.

Another main reason could be due to the insolubility of CBF occurred in the preparation of the solution. During the experiment, organic compound CBF was initially dissolved in 10% propylene glycol, but failed to be completely dissolved. CBF was then entirely dissolved in 50% ethanol and this solution was injected to nude mice (1.5 mg/kg). Unfortunately, 50% ethanol solvent is too toxic to the mice and caused death of several mice after consecutive injection for 3 days. Eventually, CBF was dissolved in 8% ethanol and the solution diluted to 0.2 mg/ml in 10% propylene glycol. Because of several trials of drug solutions, the interrupted CBF treatment actually started when the average tumour size were over 700 mm^3. The fast growth rate of large tumours apparently could not be retarded by the therapeutic effects of CBF.

In addition, we also tested the composition of the final CBF solution used in nude mouse models. The solution was analysed using high-performance liquid chromatography (HPLC) at the end of *in vivo* experiment. The HPLC gave an unexpected result that there are two peaks in our prepared CBF solution (**Figure 4.7B**). The previous CBF solution for *in vitro* experiments was not tested by HPLC and there was no stock solution remaining in our laboratory. The CBF compound we are currently using is from another vendor. Therefore, we cannot formally exclude the possibility that the CBF solution used for *in vitro* studies might also show two peaks. The HPLC results of CBF solution *in vivo* indicated that CBF may have undergone a chemical modification or have become partially degraded and this contributed to the lack of biological effect we observed *in vivo*. The degradation of CBF could result from the instability of CBF in ethanol and propylene glycol solutions. The other possibility is the contamination of CBF solution or the reagent contained in the original CBF product for compound stability. The real cause and the corresponding solution to the problem need to be worked out in future experiments.

Despite of the failure in the reduction of the tumour size, tumour samples were obtained for subsequent mRNA and protein analysis. The results of the xenograft models were mostly consistent with what was observed in cell experiments. However, surprisingly, the distribution of HIF-1α and CTTN were dissimilar to that under *in vitro* conditions. In the presence of CBF, HIF-1α localised in the cytosol of tumour cells and did not accumulate in the nucleus. As well, the nuclear translocation of CTTN did not occur, either. Although there is little information in hand to elucidate the mechanism involved in CBF function in tumour tissues, an inhibitory role is still revealed in the blockade of HIF-1α import in CBF-treated mouse models. Last but not least, CBF-treated mice showed no significant sickness in behaviour assessment. Compared with the control group, treated goups were more energetic even when the mice carried a large sized tumour (>800 mm^3). This observation was supported by a previous clinical trial showing that CBF combined with gemcitabine-oxaliplatin treatment improves the life quality of patients suffering from advanced gallbladder carcinoma (69).

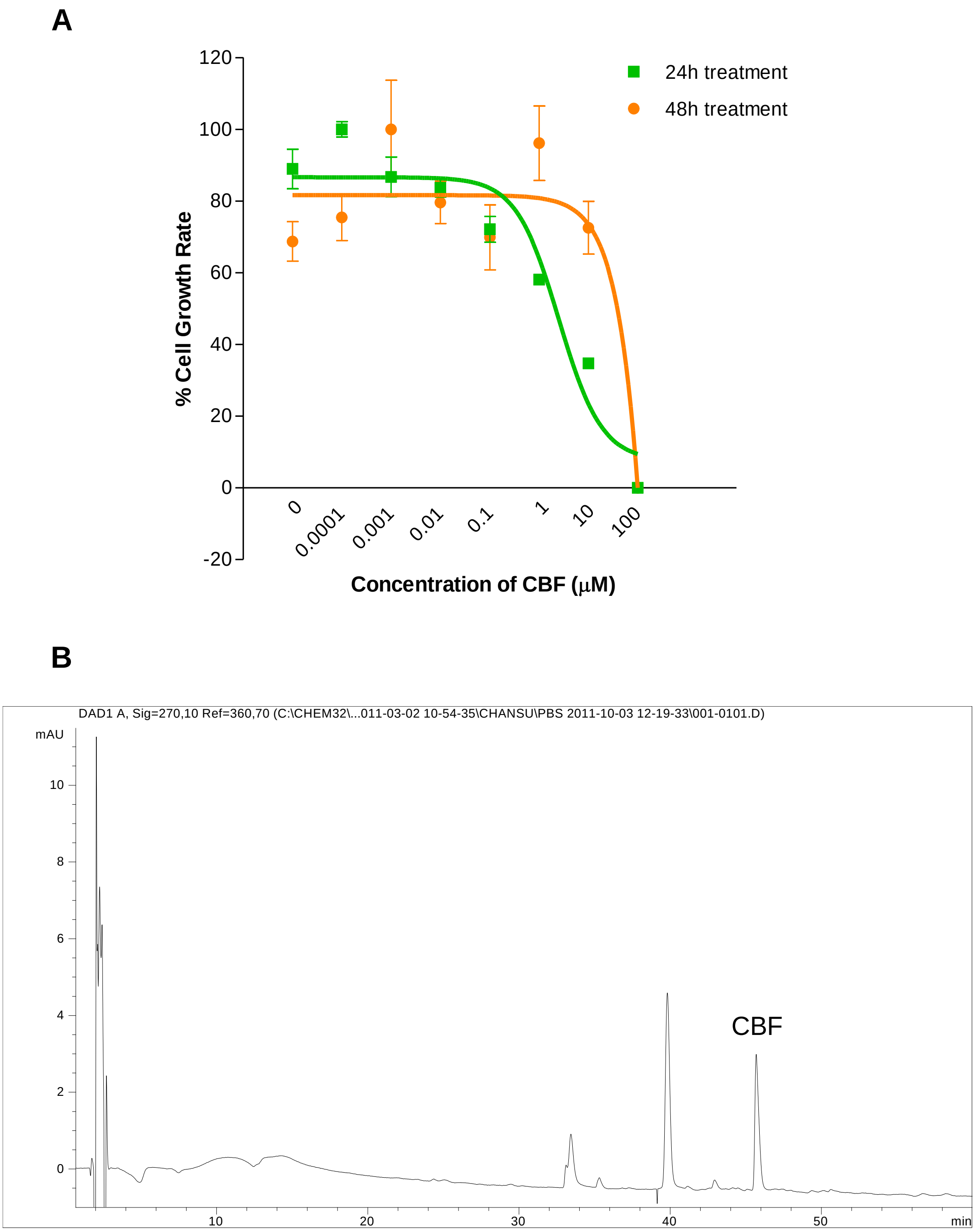


Figure 4.7 CBF treatments in mouse colon cancer cell line CT26 and the composition of CBF solution. *A.* Cytotoxicity analysis of CBF in CT26 cells. After 24 and 48 hours of CBF treatment, CT26 cells show a resistance to CBF killing capacity. ***B.*** HPLC chromatogram of prepared CBF in ethanol and propylene glycol solution. The peak of standard CBF was at a retention time of 46 min, while an unknown constituent showed at retention time of 40 min. Results are means with standard errors from 12 replicates.

In conclusion, CBF induces different inhibition levels of HIF-1α and CTTN proteins between the two cell lines, which confirmed further that the mechanisms for CBF-induced apoptosis are not identical but cell type dependent. Moreover, the knowledge of bufadienolides treatment in xenograft is limited and leads to the difficulties in our first nude mouse experiment. Nevertheless, our findings will be useful to ameliorate the experiment protocols for future *in vivo* investigations of CBF. To this end, our findings showed that CBF is a highly potent inhibitor of colon cancer cell growth, has high efficacy and safety profiles, and has the potential to be an effective anticancer chemotherapeutic agent.

CHAPTER 5

Australian cane toad extract and liposomal curcumin inhibit cell growth in different human cancer cell lines.

Introduction

The induction of apoptosis by chemicals and drugs has been an ideal way to overcome uncontrolled cancer cell proliferation. Of all the available chemicals and drugs, a family of classic drugs CGs have recently expanded their therapeutic applications to the field of anticancer treatment. As Na^+ K^+ -ATPase inhibitors, CGs have a long history in clinical use for the treatment of congestive heart failure and atrial arrhythmia. CGs are steroid-like compounds and are divided further into two groups, cardenolides and bufadienolides, in accordance with their structures (**Figure 5.1**). Common compounds from the cardenolide class are digoxin, digitalis and ouabain. Compounds extracted mainly from toads, such as CBF and marinobufagin, belong to the bufadienolide class. In spite of the unknown precise mechanisms, several types of CGs have been revealed to have antiproliferative capacity in clinical observations, especially several cardenolides (433-437). From a pilot work of Stenkvist *et al.* (1979) who found that digitalis treatment could decrease the growth rate of breast cancer to a recent statistical analysis that digoxin reduced the risk of prostate cancer (438,439), CGs have showed a potential to become new agents for anticancer treatment. Apart from their direct inhibitory roles, CGs are also combined their therapeutic potential with other anticancer medications (69). Therefore, these encouraging discoveries advocate investigating the nature of molecular biological activities of CGs in cancer cells. Recently, scientists have found that multiple signalling pathways are involved in the actions of CG in mammalian cells, including apoptotic, NF-κB-regulated and HIF-1α-regulated pathways (418,440-

442). To activate the apoptotic cascade, CGs initially bind to α subunit of Na^+ K^+ -ATPase and inhibit the influx of potassium ions, followed by an increase in intracellular concentration of sodium and calcium ions (90). The elevated Ca^{2+} concentration may cause mitochondrial permeabilisation and the release of apoptotic proteins, leading to intrinsic apoptosis (443). Alternatively, a high level of Ca^{2+} may enhance the transcript of Fas ligands, which induce extrinsic apoptosis as a consequence (444). Moreover, Frese *et al* (2006) found that CGs upregulate DR4 and DR5, close initiators of extrinsic apoptosis, in lung cancer cells (77). Another important pathway affected by CGs is the HIF-1α-regulated hypoxic pathway. Digoxin, ouabain and proscillaridin A have been found to inhibit the synthesis of HIF-1α protein in liver and prostate cancer cell lines and xenografts (79).

The use of bufadienolides for cancer treatment is a relatively new area. For example, a couple of bufadienolides strengthens the efficacy of drugs acting on TRAIL-induced apoptosis (65). They inhibit the activation of signal transducer and activator of transcription 3 (STAT3) and downregulate anti-apoptotic induced myeloid leukaemia cell differentiation protein (Mcl-1) expression. As a result, the inhibition of Mcl-1 significantly increases the sensitivity of human breast cancer cells to TRAIL triggered cell death. Our laboratory has shown that the main component of Chansu, CBF, has a potential in the induction of apoptosis among a variety of cancer cell lines. Except for Chansu from Chinese toad secretion, the bufadienolides extracted from the toads of other territories have also been pointed out to have killing ability in cancer cells (445). This also provides a possibility that the extract from the Australian cane toads could be modified to become an anticancer medicine. More importantly, Australia is currently facing an environmental problem of cane toad invasion. Thus, the conversion of these troublemakers to a medicine source is of great significance. Herein, we assessed the cytotoxicity of the Australian cane toad extract that contains several bufadienolides. Our findings are encouraging which suggest that the Australian cane toad extract shows a similar killing capacity as CBF does in human colon cancer cell lines.

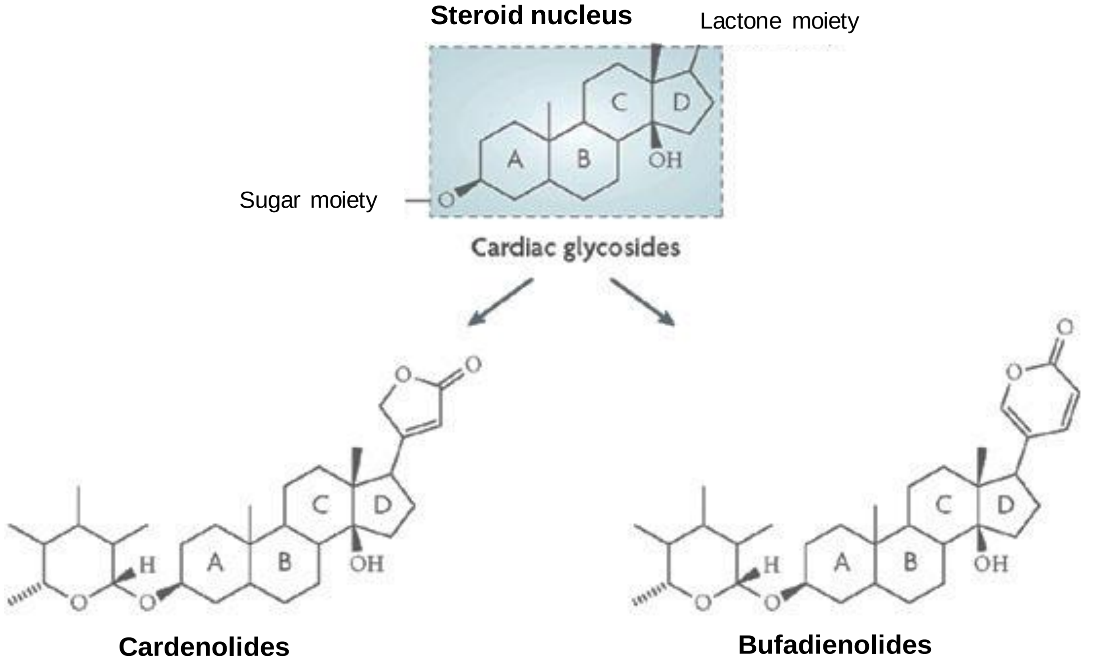


Figure 5.1 Structures and classification of CGs. According to their structure, CGs are divided further to cardenolides and bufadienolides. Original figure from: Prassas, I., and Diamandis, E. P. (2008) Novel Therapeutic applications of cardiac glycosides *Nat Rev Drug Discov* **7**, 926-935.

Apart from inhibitory effects, the efficacy and safety of bufadienolides also need to be taken into consideration for the preclinical *in vivo* studies. Bufadienolides are steroid-like compounds and are hydrophobic. This directly leads to a low bioavailability of bufadienolides in the body. Building up drug resistance might also be a problem and frequently happens to patients during the treatment for recurrence of cancer. Another drawback, which is common in available chemotherapeutics, is that natural bufadienolides are non-tissue specific to neoplasms, and might cause severe side effects. Therefore, to overcome these limitations of conventional bufadienolides, liposomal Chansu or bufadienolides will be generated for anticancer therapy.

With the development of nanotechnology, different forms of nano-vehicles have been invented in association with drug delivery systems. Liposome is a typical example and has been widely used for *in vitro* and *in vivo* studies (446-449). Liposome for drug delivery system generally consists of three parts:

(i) Lipid bilayer: The lipid bilayer not only prevents carried molecules from free traffic in blood vessels, but also enhances the uptake of therapeutic molecules by fusing to the cell membrane (450);

(ii) Substances attached to the lipid bilayer for targeting purpose: These substances could be aptamers, siRNA, peptides and ligands, and are all responsible for guiding the carried compound to aimed tissues (451);

(iii) Therapeutic agents: Drugs and compounds are embedded in the core of lipid bilayer and are supposed to be released after internalisation by the cells of interest.

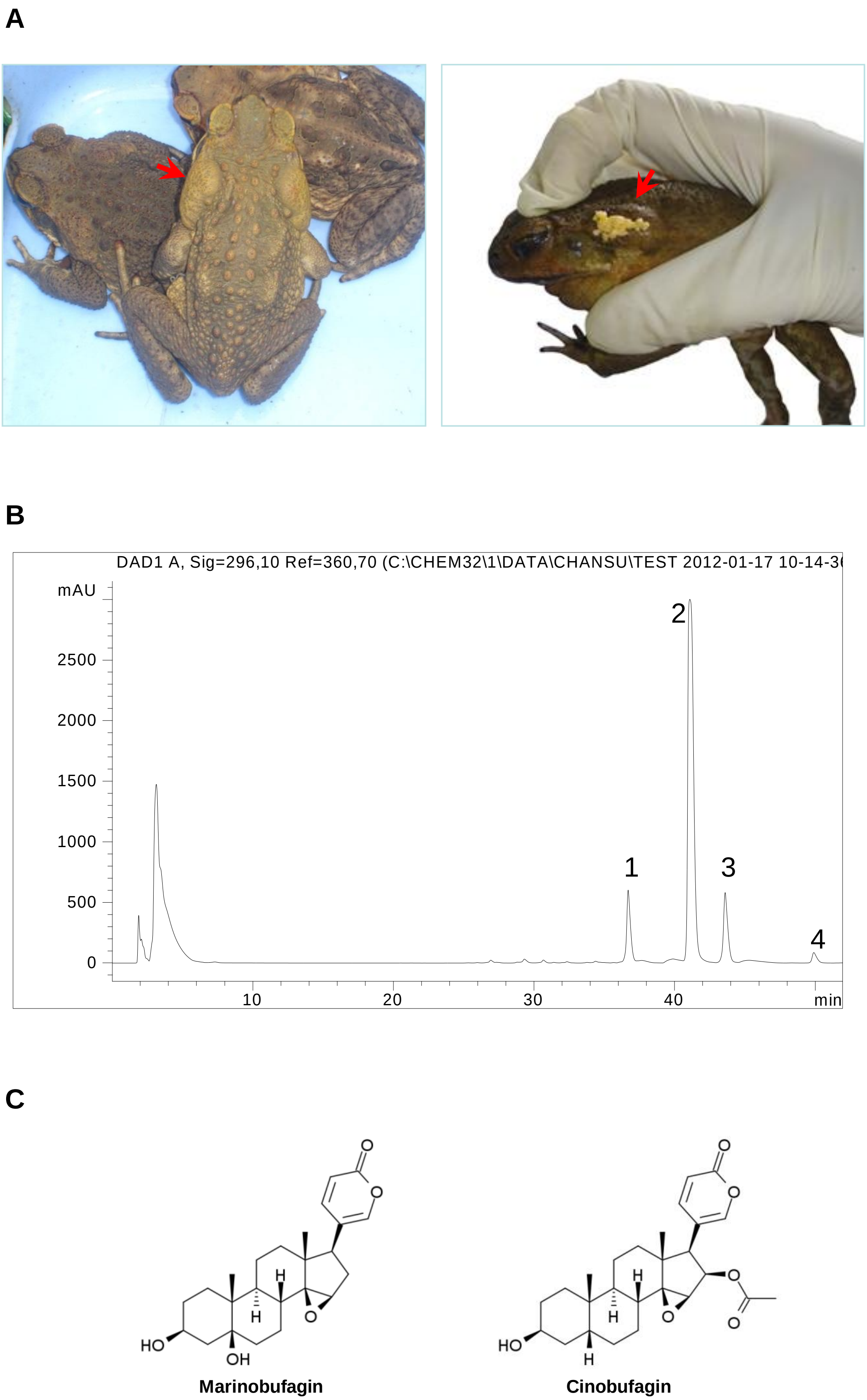
All features of liposome are designed to elevate the efficacy and safety of drugs such as bufadienolides. Therefore, a drug delivery system using liposome-capsulated bufadienolides will be established for preclinical *in vivo* experiments in the near future.

As there were no previous protocols available for the generation of liposomal bufadienolides, many trials needed to be undertaken to develop functional liposomal CBF. Furthermore, the cost of CBF for a large number of preliminary experiments is too high and beyond the budget of this study. Therefore, CBF was replaced with curcumin to develop a reproducible method for the early stages of this study. Curcumin has been reported to have a strong anticancer function in a variety of cancer types (452). It is almost aqueous insoluble but very cheap. In this study, the production of liposomal curcumin was done by our collaborative research group (Dr Parekh's laboratory, the University of Queensland). Our laboratory performed cytotoxicity assays using synthetic liposomal curcumin that showed an inhibitory capacity in a human lung cancer cell line.

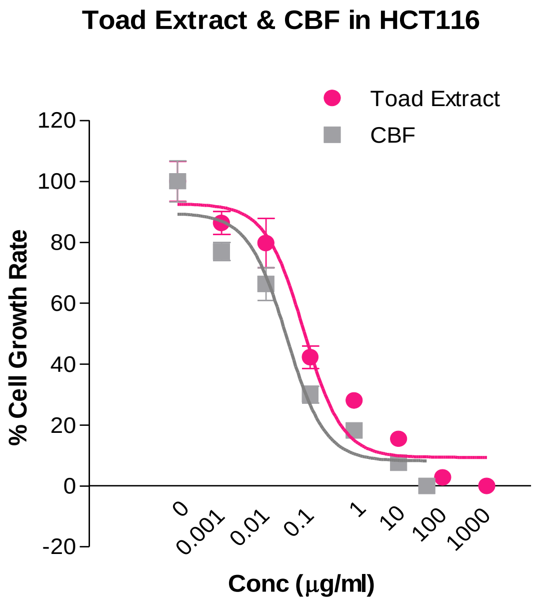
Results

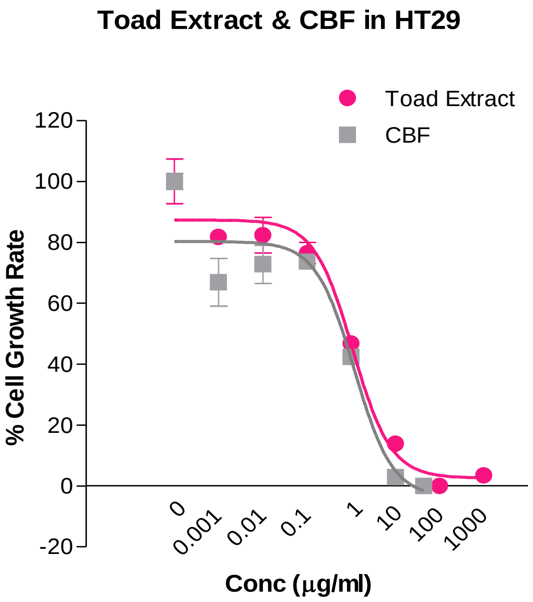
The Australian cane toad extract displays significant cytotoxicity in different human colon cancer cell lines.

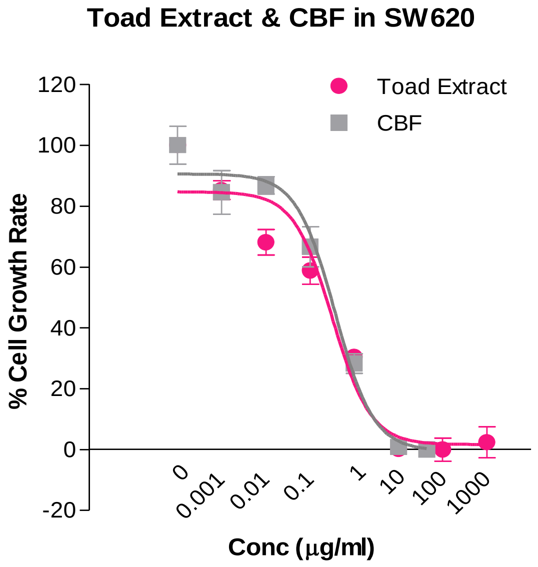
The original compounds were extracted from the parotoid glands of cane toad *Bufo marinus* (**Figure 5.2A**). Hydrophilic constitutes were firstly removed from the toad secretion, and the insoluble part was completely dissolved in methanol to extract hydrophobic compounds. The final dry sample was obtained after rotary evaporation. The most abundant component of cane toad extract is marinobufagin (**Figure 5.2B&C**), which has already showed a potent inhibitory role in various cancer cell lines (445,453). Therefore, the inhibitory capacity of the Australian cane toad extract was tested in human colon and prostate cancer cell lines. SW620 cell line was found to be the most sensitive colon cancer cell line to cane toad extract exposure (0.3~0.4 µg/ml, **Fig. 5.2D**), whereas DU145 prostate cancer cells showed the highest sensitivity (0.1~0.2 µg/ml, data not shown) compared to prostate cancer cell line PC3 and ALVA. Of all six cell lines tested, only HCT116 cells exhibited a slightly higher sensitivity to CBF, compared to cane toad extract.


A
B
DAD1 A, Sig=296,10 Ref=360,70 (C:\CHEM32\1\DATA\CHANSU\TEST 2012-01-17 10-14-3
mAU
2500
2000
1500
1000
500
0
10
20
30
40
min
1
2
3
4
C
O
O
H
H
O
HO
OH
Marinobufagin
O
O
H
H
O
O
O
HO
H
Cinobufagin

D







Human colon cancer cell line	EC_{50} of Cane Toad Extract (μg/ml)	EC_{50} of Cinobufagin (μg/ml)
HCT116	0.722	0.295
HT29	1.072	1.169
SW620	0.318	0.365

Figure 5.2 Analysis of composition and cytotoxicity of the Australian cane toad extract. ***A.*** Photos of Australian cane toads. The secretion from parotoid glands (red arrow) is firstly dissolved in water to remove hydrophilic constitutes. The insoluble component is dissolved in 100% methanol to extract bufadienolides. The photos were taken by Ms Jing Jing. ***B.*** HPLC chromatogram of cane toad extract. Numbered peaks represent four main components of the sample. No. 1: Telocinobufagin; No. 2: Marinobufagin; No. 3: Arenobufagin; No. 4: Resibufogenin. The HPLC analysis was done by Ms Jing Jing in Dr. Parekh's laboratory, the University of Queensland. ***C.*** Structures of two common bufadienolides. Marinobufagin and CBF are the main components of *Bufo marinus* and *Bufo gargarizan* extracts, respectively. ***D.*** Inhibitory effects of cane toad extract and CBF in human colon cancer cell lines (HCT116, HT29 and SW620). Different concentrations of toad extract and CBF were added to three human colon cancer cell lines. SW620 cells showed the most sensitivity to CBF after 24 hours treatment in the two drugs. Results are means with standard errors from six replicates.

Liposomal curcumin inhibits lung cancer cell growth in cytotoxicity assays, but is not as effective as sole curcumin.

To improve further the efficacy of water-insoluble drugs *in vivo*, low aqueous soluble compounds can be fixed in the core of nanoparticles (454), like liposomes. Basically, a liposome is composed of a lipophilic bilayer and the core of the particle, where the DNA fragments or drugs of interest are embedded (**Figure 5.3A**). For targeting purposes, specific peptides or DNA sequences like aptamers are designed to be attached on the surface of the liposomal membrane (316). Since steroid-like bufadienolides have very low solubility in water, liposome-encapsulation could directly enhance the delivery *in vivo*. Our laboratory intends to utilise nanoparticles to carry CBF into the body. To improve the likelihood of producing functional liposomal CBF and reduce the cost, curcumin was selected for preliminary tests instead of CBF. Curcumin is almost aqueous insoluble and very cheap. The *in vitro* study of the liposomal curcumin showed a potent killing capacity in human lung cancer cell line Spc-A1 (**Figure 5.3B**). However, the sole curcumin appears to have a stronger inhibitory ability in Spc-A1 cells. Nevertheless, the preliminary results only revealed that our liposomal curcumin is functioning and able to suppress growth in cancer cells. The accurate efficacy of liposomal curcumin and liposomal CBF will be measured in mouse models where the advantage of liposome for drug delivery is expected to be more dramatic.

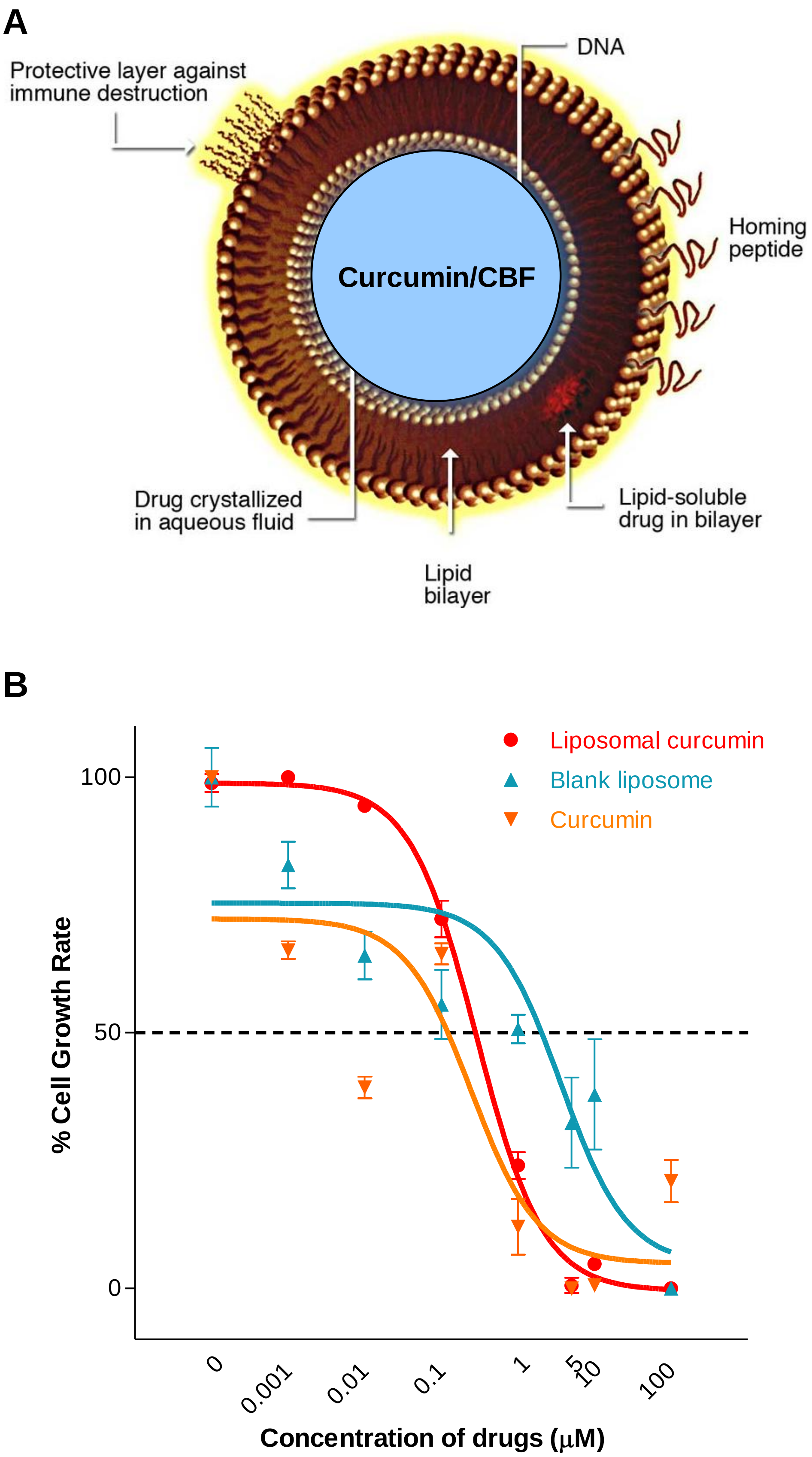

A
Protective layer against immune destruction
DNA
Homing peptide
Curcumin/CBF
Drug crystallized in aqueous fluid
Lipid-soluble drug in bilayer
Lipid bilayer
B
Liposomal curcumin
Blank liposome
Curcumin
% Cell Growth Rate
100
50
0
0
0.001
0.01
0.1
1
5
10
100
Concentration of drugs (μM)

Figure 5.3 The structure and cytotoxicity of liposomal curcumin in human lung cancer cell line Spc-A1. ***A.*** Liposome for drug delivery. A liposome consists of cell membrane-like bilayer and a core containing the drug of interest. After internalisation of liposomes, insoluble drugs can be released directly inside of cells. Original figure from: http://en.wikipedia.org/wiki/File:Liposome.jpg. ***B.*** Cytotoxicity of liposomal curcumin in Spc-A1 cells. The sole curcumin showed the strongest anticancer capability, whereas blank liposome has little effects in inhibition of lung cancer cell growth. Results are means with standard errors from nine replicates. Curcumin was liposome-encapsulased by Dr Shafiur Rahman in Dr. Parekh’s laboratory, the University of Queensland.

Discussion

Traditional CGs have recently been exploited for their therapeutic role as anti-neoplasms. The anticancer capacity of the cardenolide group, such as ouabain, digoxin and oleandrin, has been widely studied and a number of compounds have already been applied in clinical trials (455). On the other hand, the knowledge of the bufadienolide group about anti-proliferation is still very limited. Recently, many research groups have discovered the killing capability of bufadienolides (405), including traditional Chinese medicine, Chansu. Our laboratory is interested in development of novel drugs for cancer treatment. Our findings showed that different bufadienolide compounds have a common ability of inhibiting cancer cell growth. Furthermore, these bufadienolides could be carried in liposomes, which increase the efficacy and safety of the therapeutic agents. Therefore, innovation of the bufadienolides with potent anticancer functions seems to be very worthwhile.

Initial studies showed that CBF has a high potency in colon cancer cells. Furthermore, the prostate cancer cell line PC3 also showed a relative low EC_{50} value among different cell lines. The effects of CBF in prostate cancer cell lines have been previously investigated by Yeh *et al.* in 2003 and Yu *et al.* in 2008 (87,88). Their studies showed a potent anti-proliferation capacity of two bufadienolides, bufalin and CBF, in LNCaP, DU145 and PC3 human prostate cancer cell lines. Thus, to explore further the efficiency of bufadienolides in colon and prostate cancers, we treated colon cancer lines (HCT116, HT29 & SW620) and prostate cancer cell lines (PC3, ALVA & DU145) with the Australian cane toad extract and measured the corresponding EC_{50} values. In the experiments, CBF was used as a control and the results showed similar efficacy of cane toad extract and CBF, with a slightly stronger cytotoxicity of CBF in HCT116 (0.295

µg/ml) and PC3 (0.142 µg/ml) cancer cell lines (data not shown). Despite the knowledge of using the Australian cane toad extract to treat cancer is very limited, marinobufagin as the most abundant constitute of the toad extract has been found to kill HCT-8 human colon cancer cells *in vitro* (445). Taken together our findings suggest that bufadienolides have a potential to be used as therapeutic agents in treatment of colon and prostate cancer.

With the development of nanotechnology, the usage of liposomes and nanoparticles for delivery systems *in vivo* has markedly enhanced the bioavailability of water-insoluble drugs (454,456). These specific nanoparticles are internalised in targeted cells and tissues to directly release the therapeutic agents. To take advantage of this technology, our laboratory has used liposomes to carry insoluble drugs for anticancer purposes. Initial experiments were designed to generate liposomal curcumin and to subsequently apply the skills gained for the future production of liposomal bufadienolides. The results from the cytotoxicity assays revealed that liposomal curcumin retains anti-proliferation capacity and similar doses of blank liposomes are not toxic to cells. However, the lower efficacy of liposomal curcumin, compared to that of sole curcumin, could be attributed to some reasons. Firstly, uptake of liposomal particles might delay the effects of curcumin in the cell culture. The curcumin could be embedded in the liposomes and cannot directly in contact with cells. In other words, the actual function time of curcumin in treated cells was delayed by the process of endocytosis. This process is affected by a number of factors, such as the structural or dynamical characterisation of the particle (457). On the other hand, the effects of sole curcumin are more dramatic to cells with no obstructions. Another point of view is that the advantage of liposomal drugs for delivery lies in the application of *in vivo* delivery. The structure and solvation dynamics of nanoparticles are of great importance in the body. Under sophisticated conditions, structural and dynamical characterisation directly affects the efficacy of therapeutic agents and the comparison of bioavailability between liposomal curcumin and curcumin alone is useful. Therefore, more investigations of liposomal curcumin in mouse models need to be done in the near future.

In conclusion, the discovery that traditional Chinese medicine Chansu has anti-proliferation roles in cancer extends the application of the bufadienolide group of drugs in new areas. Our laboratory and other research groups have shown that bufadienolides commonly have anticancer capacity. Moreover, the development of nanoparticles for drug delivery has broadened the limitation of traditional water-insoluble drugs and provides an optimistic future for these traditional medicines to be used in the field of anticancer treatment.

CHAPTER 6

Conclusion and Future Directions

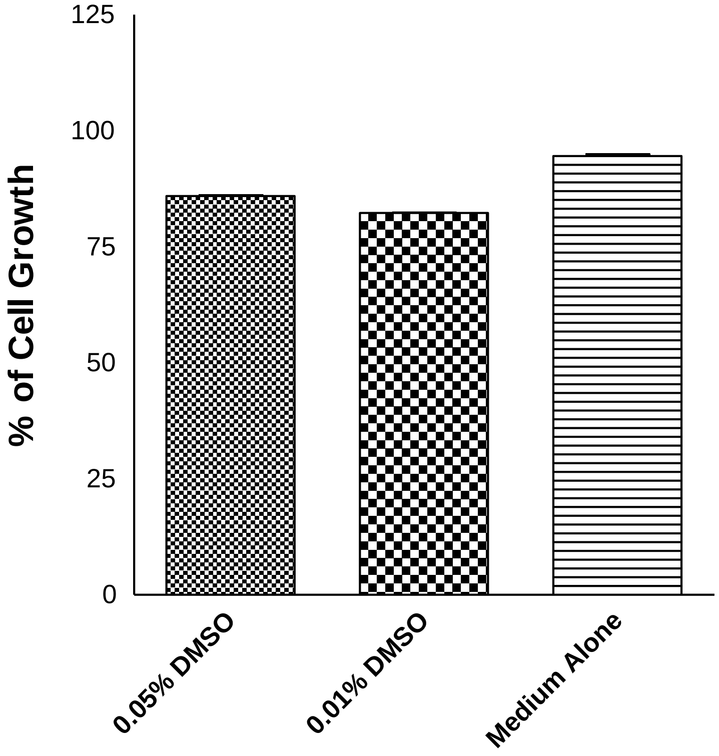
Due to time and fund limitation of this PhD study, it was difficult to take a close look at many perspectives of CBF molecular functions. To this end, our laboratory has revealed that CBF and Australia cane toad extract induce cell death in many cancer cell lines, especially human colon and prostate cancer cell lines. We identified that this kind of cell death is apoptosis but the involved apoptotic pathways varies from cell line to cell line. Moreover, the molecular mechanisms of CBF led to the reduction of HIF-1α expression in HCT116 and HT29 cells. Additionally, CTTN expression was also significantly suppressed in HCT116 cells. The result from HCT116 implanted xenograft mouse models showed clearly that CBF treatment causes the inhibition of HIF-1α nuclear translocation. Our findings have shed light on the molecular mechanisms of CBF inhibition of cancer cells and provide encouragement for future detailed analysis of this potent drug.

Future work on the application of bufadienolides to treat cancer should follow two axes. One is to focus on the efficacy of a bufadienolide drug in xenografts. Initially, the major components from crude Australia cane toad extract need to be isolated, such as marinobufagin and arenobufagin. The most effective bufadienolide compound to colon or prostate cancer should be selected using CBF as a control in cytotoxicity assays. After the examination of EC_{50} values, the selected bufadienolide should be directly used in xenograft models. In a second study bufadienolides should be tested for their ability to repress tumour growth in colon or prostate xenografts. Prior to the *in vivo* experiment, the selected bufadienolide compound and CBF (as a control) have to be completely dissolved in a proper concentration of ethanol and propylene glycol solution. Protocols of the repeat experiment will be modified in accordance with previous experience. Apart from the usage of raw bufadienolides, it might also be appropriate to utilise nanoparticles to carry bufadienolides and test the bioavailability in mouse models. The stability of liposomal drug needs to be amended from the current time of less than one month to about six months as stated by Li *et al.* (458). The stable product of liposomal bufadienolide will then need to be intravenously injected into xenografts, followed by the measurement of tumour volume. According to the theoretical elevation of the uptake of liposomal drugs *in vivo*, it is expected that a higher efficacy will be shown in the nanoparticle injected group.

During the present study, a number of unexpected but interesting results were obtained from the *in vitro* experiments. The understanding of these results will help us to achieve a comprehensive idea about the mechanisms involved in bufadienolide-induced apoptosis. Therefore, the other direction is to continue the exploration of CBF-related apoptotic pathways in cancer cell lines. HIF-1α-regulated hypoxia pathway will be the prominent pathway to look at. HIF-1α tightly controls the transcription of many transcription factors that play important roles in cell survival and migration (459). Inhibition of HIF-1α expression would significantly blocks angiogenesis which is regulated by the downstream protein of HIF-1α, VEGF (417). Further experiment in our laboratory will focus on suppression of HIF-1α expression by CBF combined with specific miRNAs. On the other hand, to confirm that CBF inhibits CTTN expression in HCT116 cells, cell migration assays need to be performed. Furthermore, knockdown of CTTN by siRNA will be a control group for comparison in these experiments.

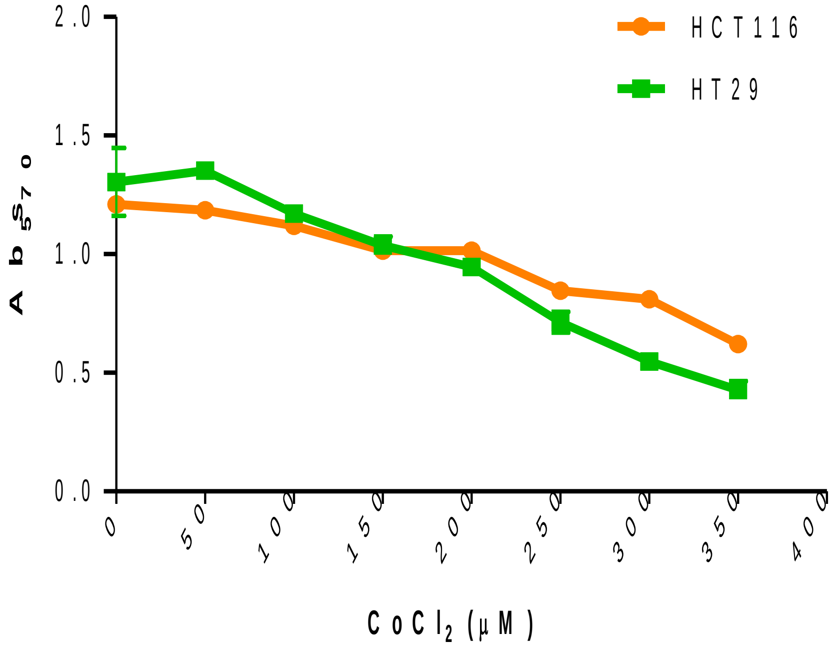
APPENDICES

Appendix I: Cytotoxicity assay of DMSO solvent.



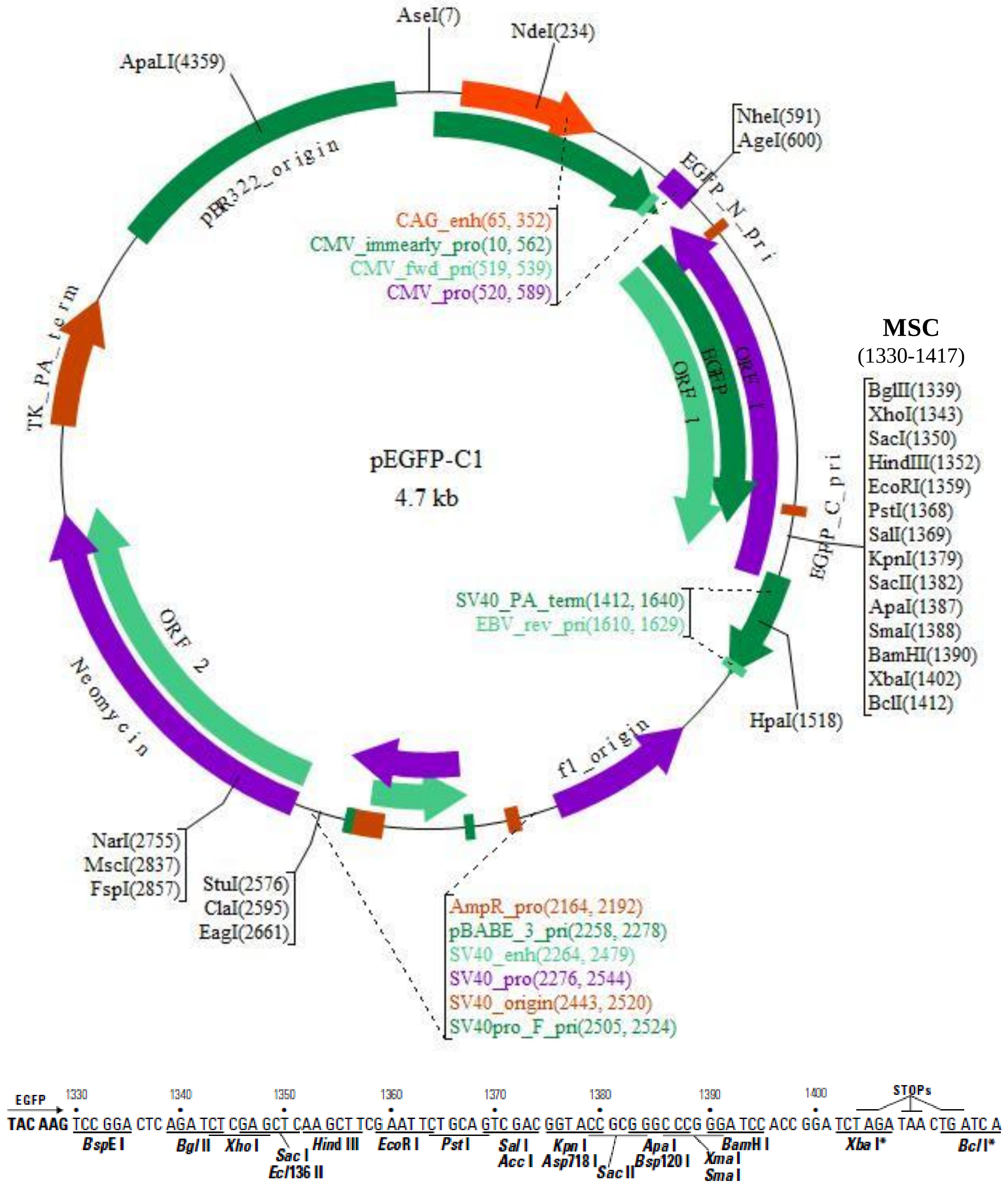
Appendix I. Cytotoxicity assays of DMSO in Spc-A$_1$ cells. DMSO was diluted in complete culture medium to reach different concentrations. Human lung cancer cell line Spc-A$_1$ was grown in 0.05% DMSO, 0.01% DMSO and complete medium alone. Compared with medium alone, the cell growth rates in 0.05% and 0.01% DMSO were not reduced significantly, indicating that 0.05% and 0.01% DMSO are safe to use as a solvent and do not affect the subsequent CBF cytotoxicity assays. Results are means with standard errors from triplicates.

Appendix II: Cytotoxicity assay of $CoCl_2$ in HCT116 and HT29 cells.



Appendix II. Cytotoxicity assays of $CoCl_2$ in HCT116 and HT29 cells. $CoCl_2$ was diluted in complete culture medium to reach the certain concentrations. Human colon cancer cell line HCT116 and HT29 were then grown in different concentrations of $CoCl_2$ for 24 hours. Compared with medium alone, the cell growth rates in 100 µM of $CoCl_2$ were not inhibited significantly. This result indicated that 100 µM of $CoCl_2$ is safe to use for producing a hypoxic condition and does not affect the subsequent CBF cytotoxicity assays. Results are means with standard errors from nine replicates.

Appendix III: Plasmids.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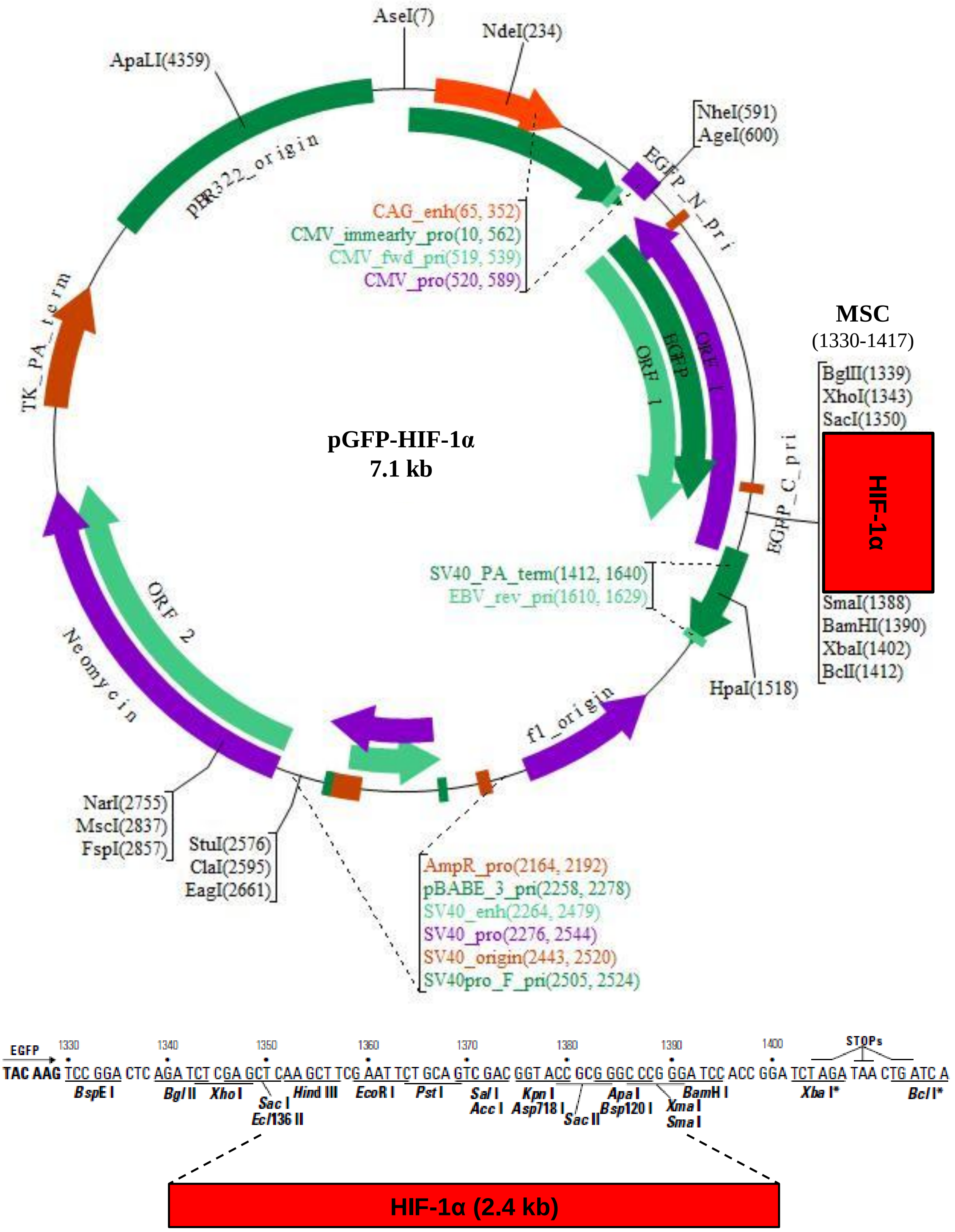
NdeI(234)
ApaLI(4359)
NheI(591)
AgeI(600)
EGFP_N_pri
pBR322_origin
CAG_enh(65, 352)
CMV_immearly_pro(10, 562)
CMV_fwd_pri(519, 539)
CMV_pro(520, 589)
TK_PA_term
ORF 1
EGFP
ORF 1
MSC
(1330-1417)
BglII(1339)
XhoI(1343)
SacI(1350)
HindIII(1352)
EcoRI(1359)
PstI(1368)
SalI(1369)
KpnI(1379)
SacII(1382)
ApaI(1387)
SmaI(1388)
BamHI(1390)
XbaI(1402)
BclI(1412)
EGFP_C_pri
pEGFP-C1
4.7 kb
SV40_PA_term(1412, 1640)
EBV_rev_pri(1610, 1629)
HpaI(1518)
ORF 2
Neomycin
f1_origin
NarI(2755)
MscI(2837)
FspI(2857)
StuI(2576)
ClaI(2595)
EagI(2661)
AmpR_pro(2164, 2192)
pBABE_3_pri(2258, 2278)
SV40_enh(2264, 2479)
SV40_pro(2276, 2544)
SV40_origin(2443, 2520)
SV40pro_F_pri(2505, 2524)
EGFP
1330
1340
1350
1360
1370
1380
1390
1400
STOPs
TAC AAG TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC GGA TCT AGA TAA CTG ATC A
BspE I
Bgl II
Xho I
Sac I
Ecl136 II
Hind III
EcoR I
Pst I
Sal I
Acc I
Kpn I
Asp718 I
Sac II
Apa I
Bsp120 I
Xma I
Sma I
BamH I
Xba I*
Bcl I*

Appendix III.1 Map of plasmid cloning vector pEGFP-C1 and sequence of the multiple cloning sites (MCS). This vector is commercially available from Clontech.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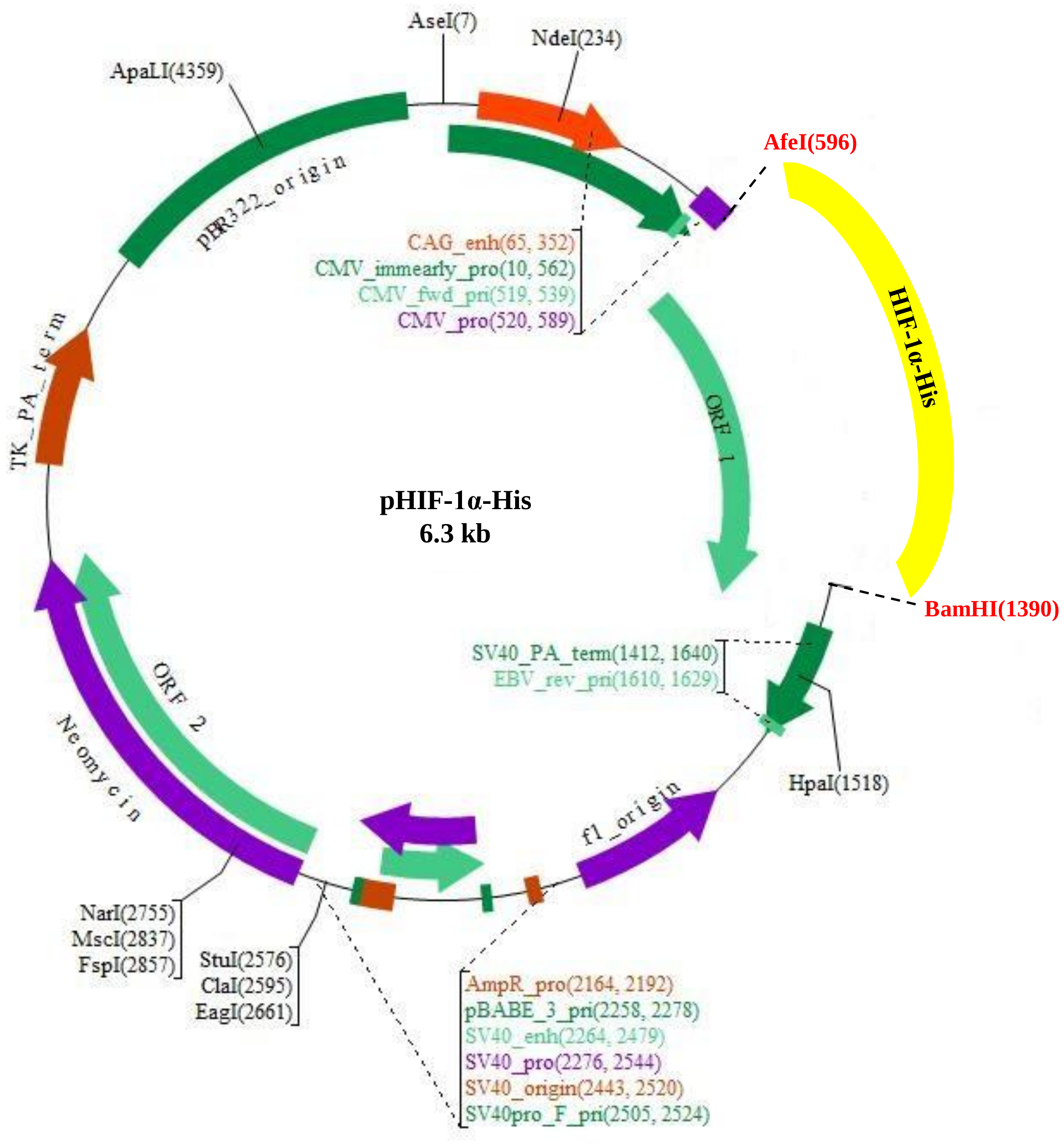
NdeI(234)
ApaLI(4359)
NheI(591)
AgeI(600)
pBR322_origin
EGFP_N_pri
CAG_enh(65, 352)
CMV_immearly_pro(10, 562)
CMV_fwd_pri(519, 539)
CMV_pro(520, 589)
TK_PA_term
ORF 1
EGFP
ORF 1
MSC
(1330-1417)
BglII(1339)
XhoI(1343)
SacI(1350)
HIF-1α
SmaI(1388)
BamHI(1390)
XbaI(1402)
BclI(1412)
EGFP_C_pri
pGFP-HIF-1α
7.1 kb
SV40_PA_term(1412, 1640)
EBV_rev_pri(1610, 1629)
HpaI(1518)
ORF 2
Neomycin
f1_origin
NarI(2755)
MscI(2837)
FspI(2857)
StuI(2576)
ClaI(2595)
EagI(2661)
AmpR_pro(2164, 2192)
pBABE_3_pri(2258, 2278)
SV40_enh(2264, 2479)
SV40_pro(2276, 2544)
SV40_origin(2443, 2520)
SV40pro_F_pri(2505, 2524)
EGFP
1330
1340
1350
1360
1370
1380
1390
1400
STOPs
TAC AAG TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC GGA TCT AGA TAA CTG ATC A
BspE I
Bgl II
Xho I
Sac I
Ecl136 II
Hind III
EcoR I
Pst I
Sal I
Acc I
Kpn I
Asp718 I
Sac II
Apa I
Bsp120 I
Xma I
Sma I
BamH I
Xba I*
Bcl I*
HIF-1α (2.4 kb)

Appendix III.2 Map of recombinant cloning vector pGFP-HIF-1α. Sequence of HIF-1α (the red fragment) was inserted into MSC of pEGFP-C1 at the restriction sites of SacI and SmaI.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NdeI(234)
ApaLI(4359)
AfeI(596)
HIF-1α-His
pBR322_origin
CAG_enh(65, 352)
CMV_immearly_pro(10, 562)
CMV_fwd_pri(519, 539)
CMV_pro(520, 589)
TK_PA_term
ORF 1
pHIF-1α-His
6.3 kb
BamHI(1390)
SV40_PA_term(1412, 1640)
EBV_rev_pri(1610, 1629)
HpaI(1518)
ORF 2
Neomycin
f1_origin
NarI(2755)
MscI(2837)
FspI(2857)
StuI(2576)
ClaI(2595)
EagI(2661)
AmpR_pro(2164, 2192)
pBABE_3_pri(2258, 2278)
SV40_enh(2264, 2479)
SV40_pro(2276, 2544)
SV40_origin(2443, 2520)
SV40pro_F_pri(2505, 2524)

Appendix III.3 Map of recombinant cloning vector pHIF-1α-His. Sequence of HIF-1α-His (the yellow fragment) was inserted into the digested pEGFP-C1 vector at the restriction sites of AfeI and BamHI.

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