THE ANTICANCER MECHANISM OF IBUPROFEN OR INDOMETHACIN IN COLORECTAL CANCER CELLS

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Abbreviations

Aberrant crypt foli	ACF
Adenomatous polyposis coli	APC
Adenosine 5'-triphosphate	ATP
American Type Culture Collection	ATCC
Ammonium persulfate	APS
Apoptosis inducing factor	AIF
Apoptosis protease activating factor-1	Apaf-1
Arachidonic acid	AA
Azoxymethane	AOM
Base pair	bp
B-cell lymphoma 2	Bcl-2
Bcl-2 associated X protein	Bax
Bcl-2-associated death promoter homologue	Bad
Beta-mercaptoethanol	β-ME
Bovine serum albumin	BSA
Caspase recruitment domain	CARD
Chinese hamster ovary cells	CHO
c-jun N-terminal kinase	JNK
Colorectal cancer	CRC
COX-2-negative mice	COX-$2^{-/-}$ mice
COX-2-positive mice	COX-$2^{+/+}$ mice
Cyclooxygenase	COX
Cytokeratin-18	CK-18
Cytotoxic T-lymphocytes	CTLs
Death domains	DD
Death effector domain	DED
Death inducing signaling complex	DISC
Death receptor 4 and death receptor 5	DR4 and DR5
Diethylpyrocarbonate	DEPC
Dimethyl sulfoxide	DMSO
Docosahexanoic acid	DHA
Dulbecco's Modified Eagle's Medium	DMEM
Effective concentration that causes 50% of drug response	EC_{50}
Ethidium bromide	EtBr
Ethylenediamine-tetra acetic acid	EDTA
Fas-associated death domain	FADD
Fetal bovine serum	FBS
European Collection of Cell Cultures	ECACC
Glycosylphosphatidylinositol	GPI
Hour	h
Horseradish peroxidase	HRP
in situ end labeling	ISEL
Inhibitors of apoptosis proteins	IAPs
Insulin-like growth factors I and II	IGF I and II
Isopropyl-beta-D-thiogalactopyranoside	IPTG
Kilo Dalton	kDa
Lactate dehydrogenase	LDH

Ligand binding domain	LBD
Molecular weight	MW
N,N,N'N'-Tetra-methylethylenediamine	TEMED
Necrosis factor-kappa B	NF-κB
Non-steroidal anti-inflammatory drugs	NSAIDs
Optical density	OD
Peroxisome proliferator-activated receptors	PPARs
Peroxisome proliferators-activated receptor response elements	PPRE
Peroxisome proliferators-activated receptor-alpha	PPARα
Peroxisome proliferators-activated receptor-delta	PPARδ
Peroxisome proliferators-activated receptor-gamma	PPARγ
Phenylbutyrate	PB
Phenylmethylsulfonyl flouride	PMSF
Phosphate-buffered saline	PBS
Phosphoinositol -3-kinase	PI3K
Poly ADP-ribose polymerase	PARP
Prostaglandins	PGs
Receptor-interacting protein	RIP
Retinoid X receptor	RXR
RIP-associated ICH-1/CED-3 homologous protein with a DD	RAIDD
Roselle's Park Memorial Institute Medium	RPMI
Serine/threonine kinase	Akt
Sodium citrate	SOC
Sodium dodecyl sulphate	SDS
Standard error of the mean	SEM
T-cell factor	TCF
Tdt-mediated dUTP nick end labeling	TUNEL
Thiazolidinediones	TZD
TNF receptor-1	TNF-R1
TNFα-related apoptosis-inducing ligand	TRAIL
TNFalpha	TNFα
TNF-associated factor 2	TRAF-2
TNFR-associated death domain	TRADD
Tris borate EDTA	TBE
Tumor necrosis factor	TNF
Tumor necrosis factor-alpha	TNFα
volume/volume	v/v
weight/volume	w/v
X-galactosidase	X-gal

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The anticancer mechanism of ibuprofen atau indomethacin in colorectal cancer cells

Abstract

Ibuprofen and indomethacin are among the frequently studied non-steroidal anti-inflammatory drugs (NSAIDs) for their anticancer activities. Besides being non-selective cyclooxygenase-2 (COX-2) inhibitors, both NSAIDs are also direct ligands for peroxisome proliferators-activated receptor-gamma (PPARγ). However, the precise mechanism(s) of action whereby both NSAIDs exert their anticancer effect remain unclear. In this study, we investigated the effects of both NSAIDs in constitutively COX-2-expressing (HCA-7 and HT29) and non-constitutively COX-2-expressing (HCT116) cell lines. Our initial aim of the study was to determine the NSAID growth inhibitory effect as well as the effective concentration to inhibit 50% of cell growth (EC_{50}) of each NSAID in each cell line using lactate dehydrogenase (LDH) release assay. The apoptosis mechanism was then investigated using M30 *CytoDEATH* assay prior to flow cytometry analysis. The apoptotic-related proteins such as caspase-8, -9, -3 and -7 were also investigated using Western blot analysis, whereas the modulation of mRNA expression of relevant molecular targets such as COX-2, *c-myc*, β-catenin, TCF-4 and PPAR subtypes (α, δ, γ1 and γ2) mRNA was quantified using Real-time PCR analysis. Our results demonstrated that both NSAIDs produced remarkable inhibition on the growth of all three cell lines tested. The

inhibitory effect occurred in a concentration- and time-dependent manner, with indomethacin (EC_{50} value >100 μM) being more potent compared to ibuprofen (EC_{50} value >1000 μM). Furthermore, the ability of both NSAIDs in inhibiting the growth of cells is likely not to be associated with COX-2 expression. The evidence from M30 *CytoDEATH* assay suggested that the major mode of cell death caused by both NSAIDs was caspase-dependent apoptosis. This evidence was further supported by Western blot analysis which indicated that the induction occurred *via* caspase-9-dependent pathway, whereas the Real-time PCR analysis showed that both NSAIDs appear to modulate gene expression *via* a variety of different molecular targets in COX-2-dependent and/or independent pathway(s) depending on the colorectal cancer (CRC) cell type. However, alteration of TCF-4 and PPARγ1 mRNA expression are likely essential for both NSAIDs to induce apoptosis. Thus, *Wnt* and PPARγ signaling pathways may be involved in mediating the apoptosis induced by both NSAIDs in CRC cells. In addition, PPARδ is found to be another essential molecular target for indomethacin-induced CRC cell apoptosis. In conclusion, our study may provide additional information and evidence of the various mechanisms and actions of NSAIDs in human CRC cells which may be useful in selecting effective apoptotic drugs against specific CRC types. As we and others have shown, both NSAIDs have anti-CRC activities and are potential anti-CRC agents. Further studies on the effect of both NSAIDs on CRC cells remain important, as they may be developed as chemotherapeutic agents for human CRC.

Antikanser mekanisme ibuprofen atau indomethacin dalam sel kanser kolorektal

Abstrak

Ibuprofen dan indometasin adalah antara drug antiinflamatori bukan steroid (NSAIDs) yang kerap dikaji untuk aktiviti-aktiviti anti kansernya. Selain daripada perencat siklooksigenase-2 (COX-2) bukan pilihan, kedua-dua NSAIDs ini adalah ligan langsung untuk reseptor-gama pengaktif proliferators peroksimase (PPARγ). Walaubagaimanapun, tindakan mekanisme atau mekanisme-mekanisme yang tepat yang mana kedua-dua NSAIDs mengenakan kesan anti kanser masih lagi tidak jelas. Kajian ini menyiasat kesan garis sel pada kedua-dua NSAIDs dalam sebahagian ekspresi COX-2 (HCA-7 dan HT29) dan bukan pada sebahagian ekspresi COX-2. Pada awalnya, kajian ini dijalankan untuk menentukan kesan perencatan pertumbuhan NSAIDs dan juga kepekatan (concentration) yang berkesan untuk merencat 50% daripada sel pertumbuhan (EC_{50}) pada setiap NSAIDs di setiap garis sel menggunakan dehidrogenase laktat (LDH) yang mengeluarkan assay. Mekanisme apoptosis kemudiannya disiasat menggunakan assay M30 *Cyto*DEATH sebelum analisis aliran sitometri dijalankan. Protein-protein berkaitan apoptotic seperti caspase-8, -9, -3 dan -7 juga telah disiasat menggunakan analisis blot Western, manakala modulasi ekspresi mRNA untuk molekul sasaran yang berkaitan seperti COX-2, *c-myc*, β-catenin, TCF-4 dan subjenis-subjenis PPAR mRNA (α,

δ, γ1 dan γ2) dikuantitikan menggunakan analisis PCR masa-sebenar (REAL-time). Keputusan menunjukkan bahawa kedua-dua NSAIDs menghasilkan perencatan yang luar biasa pada pertumbuhan ketiga-tiga garis sel. Indometasin (nilai EC_{50} > 100 μM) adalah lebih kuat jika dibandingkan dengan ibuprofen (nilai EC_{50} > 1000 μM). Tambahan pula kebolehan kedua-dua NSAIDs dalam merencat pertumbuhan sel-sel mungkin tidak akan dikaitkan dengan ekspresi COX-2. Bukti dari assay M30 *Cyto*DEATH mengusulkan bahawa mod utama kematian sel adalah disebabkan kedua-dua NSAIDs adalah apoptosis yang bergantung pada caspase. Bukti ini seterusnya disokong oleh analisis blot Western yang menunjukkan bahawa cetusan berlaku melalui laluan yang bergantung pada caspase-9, manakala analisis PCR masa-sebenar (REAL-time) menunjukkan bahawa kedua-dua NSAIDs kelihatan memodulat ekspresi gen melalui pelbagai sasaran molekul yang berbeza dalam laluan atau laluan-laluan yang bergantung atau tidak bergantung pada COX-2 dan ini bergantung kepada jenis sel kanser kolorektal (CRC). Walaubagaimanapun perubahan pada TCF-4 dan ekspresi mRNA PPARγ adalah agak penting kepada kedua-dua NSAIDs untuk mencetus apoptosis. Oleh itu laluan-laluan isyarat *Wnt* dan PPARγ mungkin terlibat sebagai perantara apoptosis yang dicetus oleh kedua-dua NSAIDs dalam sel-sel CRC. Sebagai tambahan, PPARδ pula didapati sebagai satu lagi sasaran molekul yang penting untuk apoptosis sel CRC cetusan-indometasin. Kesimpulannya, kajian ini mungkin dapat memberi maklumat tambahan dan bukti pelbagai mekasisme-mekanisme dan tindakan-tindakan NSAIDs dalam sel CRC manusia, yang mungkin

berguna untuk memilih drug apoptotic yang berkesan terhadap jenis CRC yang spesifik. Seperti yang telah kami dan penyelidik-penyelidik lain telah tunjukkan, kedua-dua NSAIDs mempunyai aktiviti-aktiviti anti-CRC dan berpotensi sebagai agen anti-CRC. Kajian-kajian lanjut mengenai kesan kedua-dua NSAIDs pada sel-sel CRC tetap penting kerana mereka boleh dibangunkan sebagai agen kemoterapeutik untuk CRC manusia.

Chapter 1

Introduction

1.1 Introduction of NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) were first introduced in the 1900s, and have subsequently been widely used to treat inflammation, mild-to-moderate pain and fever (Vane and Botting, 1998). Although they have been used for more than two decades and their safety has constantly been reviewed, they have only recently been proposed as anti-cancer agents (Vane and Botting, 1998). NSAIDs work mainly by inhibiting cyclooxygenase (COX) and preventing the formation of prostaglandins (PGs) (Smith and Goh, 1996; Badawi *et al.,* 2000; Han *et al*., 2001). There are two isoforms of COX which are known as COX-1 and COX-2 (Sheng *et al.,* 1997; Badawi *et al.,* 2000). COX-1 is continuously secreted within the stomach and duodenum where it helps maintain healthy stomach lining, normal kidney function and the clotting action of blood platelets (Sheng *et al.,* 1997). In contrast, the COX-2 enzyme is primarily found at sites of inflammation (Sheng *et al.,* 1997). It is induced by inflammatory stimuli such as interleukin-1, and is suppressed by glucocorticoids (Sheng *et al.,* 1997).

Blocking of COX-2 is effective in relieving pain and inflammation, whereas inhibiting of COX-1 often produces unacceptable gastrointestinal side effects such as diarrhea, bloating, heartburn, stomach upset and ulcers (Vane and Botting, 1998). Various conventional NSAIDs such as aspirin, diclofenac, ibuprofen, indomethacin, naprosyn and piroxicam are being used as anti-cancer

agents (Soh and Weinstein, 2003). These NSAIDs inhibit both COX-1 and COX-2 to differing degrees in terms of the relative specificity ratio (IC_{50}; Figure 1.1) (Mitchell *et al.,* 1994; Bishop-Bailey *et al*., 1997; Bishop-Bailey and Warner, 2003).

The lower ratio value reflects the more potent activity of NSAIDs against COX-2 inhibition. Thus, SC58125 (celecoxib derivative) and celecoxib appear to be more selectively involved in COX-2 inhibition activity than naproxen and flurbiprofen (Figure 1.1). Besides indicating the selectivity of NSAIDs in COX-2 inhibition, ratio IC_{50} adequately explains the variations in the side effects of NSAIDs at their anti-inflammatory doses (Vane and Botting, 1998). Selective COX-2 inhibitors such as celecoxib which have higher potency on COX-2 inhibition activity will have potent anti-inflammatory activity with fewer side effects on the stomach and kidney (Henry, 2003; Langman, 2003; Wolfe, 2003), whereas non-selective COX-2 inhibitors such as aspirin, piroxicam, ibuprofen and indomethacin which have a higher potency against COX-1 than against COX-2 inhibition activity, were found to produce high gastrointestinal toxicity and cause the most damage to the stomach at inflammatory doses (Lanza *et al.,* 1989; Rodriguez *et al.,* 1994).

1.2 The non-selective COX inhibitors

The ability of NSAIDs to decrease the COX-1/COX-2 expression has allowed consideration of NSAIDs as potential anti-cancer agents for cancer treatment including human colorectal cancer (CRC) treatment (Vane and Botting, 1998). COX-2 expression has been shown to be up-regulated in human CRC when compared with normal adjacent colonic mucosa (Na and Surh, 2003). Inhibition of the COX-2 expression is important because over expression of COX-2 provides tumor cells with growth and survival advantages including resistance to apoptosis, and increased invasiveness or angiogenesis (Mohammed *et al.,* 1999; Cianchi *et al.,* 2001; Yamada *et al.,* 2001; Na and Surh, 2003).

Thus, targeting the inhibition of COX-2 is regarded as an affective and promising strategy for cancer prevention and treatment (Han *et al*., 2001; Na and Surh, 2003). Indeed, the expression of COX-2 is stimulated by growth factors and tumor promoters, and it is often selectively expressed in tumors cells including CRC cells (Eberhart *et al*., 1994; Sano *et al*., 1995; Han *et al*., 2001). Thus, the expression of COX-2 is believe that plays an important role in cancer, and is often correlated with tumorigenesis. This correlation was supported by the finding that in two mutated adenomatous polyposis coli (*APC*) background mice where the COX-$2^{-/-}$ mice developed fewer intestinal polyps than the COX-$2^{+/+}$ mice (Oshima *et al*., 1996; Han *et al*., 2001).

Many epidemiologic and clinical studies have revealed that regular ingestion of NSAIDs such as aspirin, sulindac and indomethacin can reduce the risk of developing several malignant tumors including CRC (Giardiello *et al.,* 1995; Giovannucci *et al.,* 1995; Boolbol *et al.,* 1996). Based on the evidences above, it is logical to choose a selective COX-2 inhibitor that is as effective as non-selective inhibitors and could prevent PGs formation in inflamed areas without interfering with the activity in stomach and kidney (Vane and Botting, 1998). However, COX-2 appears to have multiple functions and has been shown to be important in ovulation and vasoprotection, and is essential for normal kidney development (Dahl, 1999). Indeed, both COX-1 and COX-2 are expressed in inflamed joints, so it is important that mixed COX inhibition is required for the maximal anti-inflammatory effects (Dahl, 1999).

1.3 Evaluation of NSAIDs as PPARγ ligands

Non-selective COX inhibitors are regarded as effective and promising anti-cancer agents for cancer prevention or treatment. However, the fact that NSAIDs retain the anti-proliferative activity in a variety of cells that do not express COX-2 would argue against the relevance of COX-2 inhibition as a target for NSAIDs in cancer cells (Hanif *et al.,* 1996; Zhang *et al.,* 1999; Chapple *et al.,* 2000; Smith *et al.,* 2000; Han *et al*., 2001; Hull *et al*., 2003). Indeed, the concentration of NSAIDs required to induce cell death was several orders of magnitude

higher than that required to inhibit the activity of COX (Mitchell *et al*., 1993; Han *et al*., 2001). For example, the IC_{50} ratio of indomethacin to inhibit COX-1 and COX-2 in intact cells were reported to be 28 nM and 1.7 μM, respectively (Mitchell *et al*., 1993), whereas the concentration of indomethacin used to induce cell death in the same cells was 500 μM (Zhang *et al.,* 1999). Therefore, the anti-proliferative activity of NSAIDs would seem to occur *via* COX-independent mechanisms (Hull *et al.,* 2003; Soh and Weinstein, 2003).

One possible mechanism was suggested to be *via* the peroxisome proliferators-activated receptor-gamma (PPARγ), a ligand-activated transcription factor pathway (Smith *et al.,* 2000; Hull *et al.,* 2003; Soh and Weinstein, 2003). According to Lehmann *et al.* (1997), several NSAIDs including ibuprofen and indomethacin, can interact directly and specifically bind with PPARγ ligand binding domain (LBD). Upon ligand binding, PPARγ becomes activated and heterodimerises with retinoid X receptor (RXR) (Figure 1.2) (Boitier *et al.,* 2003). The co-repressors will then dissociate from the complex which leads to an active PPARγ/RXR complex that binds to the gene regulatory sites on DNA termed PPAR response elements (PPRE). There is also evidence of the involvement of co-activator proteins that enhance the activity of the PPARγ/RXR complex. Binding of the PPARγ/RXR complex to PPRE increases the transcription of numerous genes including genes involve in tumorigenesis (Boitier *et al.,* 2003). The ability of NSAIDs to bind to PPARγ has been previously assessed in a competition-binding assay

using [^{3}H] BRL49653 and bacterially expressed PPARγ LBD (Lehmann *et al.,* 1997). Though not all NSAIDs activate PPARγ through direct interactions with the receptor, the fact that NSAIDs bind and thereby activate PPARγ activity, subsequently define them as the PPARγ ligands (Lehmann *et al.,* 1997).

Interestingly, ibuprofen and indomethacin used in this study are non-selective COX inhibitors and direct PPARγ ligands. Activation of PPARγ by ibuprofen and indomethacin was the strongest of any of the NSAIDs that were evaluated in CRC (Nixon *et al.,* 2003). Furthermore, both NSAIDs may activate more than one PPAR subtypes. For example, ibuprofen activates PPARdelta (PPARδ) and PPARγ (Kojo *et al.,* 2003), whereas indomethacin shows agonistic activity for PPARalpha (PPARα) and PPARγ (Lehmann *et al.,* 1997) or PPARγ alone based on two different studies (Kojo *et al.,* 2003). As dual ligands have been proven to be more effective at preventing colorectal carcinogenesis, ibuprofen and indomethacin are therefore potential anti-cancer agents for human CRC.

1.4 The role of PPARγ ligands in cancer

The mechanisms of anti-cancer effect by the PPARγ ligands have, however, not been fully elucidated. In general, activation of PPARγ by its ligand is associated with cancer prevention (Brockman *et al.,* 1998; Sarraf *et al.,* 1998; Kitamura *et al.,* 1999). The PPARγ ligands have anti-

proliferative and pro-differentiation effects in slowing the proliferation of tumor cells including breast (Elstner *et al.,* 1998; Rubin *et al.,* 2000), colon (Sarraf *et al.,* 1998), prostate (Kubota *et al.,* 1998; Hisatake *et al.,* 2000; Shappell *et al.,* 2001), gastric (Takahashi *et al.,* 1999) and pancreatic (Motomura *et al.,* 2000) cancer cells.

In support of the former findings, the thiazolidinediones (TZD) group of PPARγ ligands has been shown to inhibit proliferation of breast, prostate and colon cells (Elstner *et al.,* 1998; Kubota *et al.,* 1998; Sarraf *et al.,* 1998). In contrast, the ligands promote the development of colon tumors in transgenic mice in which one copy of the gene encoding the *APC* is knocked out, predisposing them to colon cancer (Lefebvre *et al.,* 1998). However, another study demonstrated that treatment of patients with liposarcomas with a PPARγ ligand results in anti-neoplastic pro-differentiation which may decrease the proliferative rate of the cancer cells and slow the progression of the disease (Demetri *et al.,* 1999). There are also observations that PPARγ ligands induce growth arrest and the synthesis of differentiation markers in human CRC cells (Kitamura *et al.,* 1999). Furthermore, PPARγ ligands have been shown to be potent inhibitors of angiogenesis, a process necessary for solid-tumor growth and metastasis (Xin *et al.,* 1999). Although much of the evidence suggests that activation of PPARγ by its ligands could promote the suppression of tumor growth and inhibit carcinogenesis, the precise mechanisms involved remain unclear.

PPARγ ligands have been shown to exert their anti-cancer cell proliferation through the induction of apoptosis (Yang and Frucht, 2001; Clay *et al.,* 2002). Although the precise role of PPARγ in cancer cells is still debatable, accumulating evidence has reported that PPARγ ligands inhibiting cancer cell growth *via* apoptosis (Yang and Frucht, 2001; Clay *et al.,* 2002; Na and Surh, 2003). The apoptosis pathway is currently the key mechanism by which anti-cancer agents kill tumor cells. The ability of PPARγ ligands to decrease COX-2 expression and initiate apoptosis has made them to be considered as potential therapeutic agents for cancer treatment (Yang and Frucht, 2001; Debatin, 2004). Therefore, identification of the molecular pathways induced by PPARγ ligands will be of both basic and clinical importance.

1.5 Apoptosis

Apoptosis, first reported in 1964, is also known as programmed cell death (Gewies, 2003). It is the physiological process by which unwanted cells are eliminated during development and other normal biological processes (Gewies, 2003). Moreover, cytotoxic T-lymphocytes (CTLs) kill virus-infected cells are also *via* the induction of apoptosis (Roberts, 2000). Currently, understanding of the apoptosis plays an important role in the study of physiological processes, particularly those involved in cancer research (Roberts, 2000). For example, the mutation of the *p53* gene produces defective proteins that are often found in

cancer cells which contribute to resistance to apoptosis (Debatin, 2004). Moreover, immunotherapy of tumors requires target cells with an apoptosis-sensitive phenotype (Debatin, 2004).

Apoptosis is of major importance in the pathogenesis of several diseases including cancer, AIDS and neurological disorders such as Alzheimer's and Parkinson's diseases (Roberts, 2000). It is believed that defects in the apoptotic pathways represent hallmarks of tumorigenesis (Okada and Mak, 2004). Recent research on new cancer therapies has therefore focused on devising ways to overcome this resistance and to trigger apoptosis of cancer cells (Okada and Mak, 2004).

1.6 Morphological features of necrosis and apoptosis

In the cell death process, necrosis occurs when cells are exposed to a serious physical or chemical insult (Gewies, 2003). The necrotic mode of cell death, in which cells suffer a major insult, results in a loss of membrane integrity, swelling and cell rupture (Figure 1.3). During necrosis, the cellular contents are released uncontrolled into the environment which results in damage to surrounding cells and a strong inflammatory response in the corresponding tissue (Gewies, 2003).

In contrast to necrosis, apoptosis is characterised by typical morphological and biochemical hallmarks including cell shrinkage,

nuclear DNA fragmentation and membrane blebbing (Debatin, 2004). The morphological changes such as cell shrinkage and deformation results in the cells loss of contact with neighboring cells and chromatin condensation (Figure 1.3). The chromatin is then localised at the nuclear membrane, the plasma membrane undergoes blebbing and finally the cell is fragmented into compact membrane-enclosed structures termed apoptotic bodies. The apoptotic bodies contain cytosol, condensed chromatin and organelles. They are removed from the tissue by phagocytosis or secondary necrosis without causing an inflammatory response.

1.7 The putative mechanisms of PPARγ ligand-induced apoptosis

As described above, treatment with PPARγ ligands has been widely implicated to reduce the cancer cell growth rate and induce cell terminal differentiation of a number of human cancers including CRC *via* induction of apoptosis (Yang and Frucht, 2001; Boitier *et al.,* 2003). According to Goke *et al.* (2000), the induction of apoptosis by PPARγ ligands is increased by co-stimulation with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family. Furthermore, inhibition of cell growth observed in human breast cancer cells treated *in vitro* with PPARγ ligands is accompanied with a marked increase in apoptosis and a profound decrease of Bcl-2 gene expression (Elstner *et al.,* 1998). In addition, troglitazone-induced apoptosis has

been demonstrated to be associated with caspase-dependent and -independent mechanisms (Yoshizawa *et al.,* 2002). More importantly, Yoshida *et al.* (2003) recently proposed the mechanisms concerning PPARγ ligand-induced apoptosis would be occurring *via* a) perturbation of mitochondrial membrane permeability; b) binding of death receptor to its ligand; and c) phosphatidylinositol-3-kinase (PI3)-serine/threonine kinase (Akt)-apoptosis protease activating factor-1 (Apaf-1) system (down-regulation of survival signaling pathway) (Figure 1.4).

1.7.1 Mitochondrial-dependent apoptosis

PPARγ ligands mainly induce apoptosis *via* perturbation of inner mitochondria membrane permeability (Yoshida *et al.,* 2003). This mitochondrial-dependent apoptosis was termed intrinsic apoptosis where signals from various stimuli disturb the membrane permeability of the mitochondria and subsequently release the apoptogenic factor such as cytochrome *c* into the cytoplasm (Yoshida *et al.,* 2003). During intrinsic apoptosis, anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2) and the pro-apoptotic protein such as Bcl-2 associated X protein (Bax) are induced to oligomerise and migrate from the cytoplasm to the mitochondria escort by various BH3-Only-Protrein (Debatin, 2004). Once the protein is inserted into the outer mitochondria membrane, it induces cytochrome *c* release by the creation or alteration of the mitochondria membrane pores (Debatin, 2004).

In the cytoplasm, cytochrome *c* combines with Apaf-1 and pro-caspase-9 to form a complex termed apoptosome, in the presence of adenosine 5'-triphosphate (ATP) to activate caspase-9 (Figure 1.5) (Roberts, 2000; Gewies, 2003; Debatin, 2004). Caspase-9 subsequently activates downstream executor caspases such as caspase-3, -6 and -7 (Roberts, 2000). Activation of caspase-3 and the subsequent degradative events are probably triggered to initiate the apoptosis (Roberts, 2000; Yoshida *et al.,* 2003; Debatin, 2004). In most PPARγ ligands induced apoptosis cases, caspase-3 is the final molecule where activation of caspase-3 will then causes DNA fragmentation (Yoshida *et al.,* 2003).

However, the mitochondrial pathway must not always associated with caspase activation (Debatin, 2004). The mechanism varies depending on the release of apoptogenic factor such as cytochrome *c*, Smac/Diablo or apoptosis inducing factor (AIF) (Figure 1.5) (Debatin, 2004). The release of cytochrome *c* triggers caspase-9 activation through the formation of cytochrome *c*/Apaf-1/caspase-9 in the presence of ATP to form an active apoptosome complex as described above (Roberts, 2000; Gewies, 2003; Debatin, 2004). Thus, the release of cytochrome *c* from mitochondria always reflects the mitochondrial→caspase-9→executor caspase activation→apoptosis (Cai *et al.,* 2002; Debatin, 2004). The Smac/Diablo promotes caspase activation by neutralising the inhibitory effects of inhibitors of apoptosis proteins (IAPs) and this may also imply the same caspase activation pathway (Debatin, 2004).

However, AIF causes DNA condensation or initiate apoptosis without activating any caspases (Debatin, 2004).

1.7.1.1 Caspase activation in apoptosis

Caspase(s) with the generic name *c* denotes a cysteine protease and *aspase* refers to the aspartate specific cleaving ability of these enzymes (Gewies, 2003). The individual members are then numbered according to their chronological order of publication (Robert, 2000). Caspase activation is recently demonstrated as central importance (hallmark) in the apoptosis signaling network as well as DNA fragmentation, which is activated in most cases of apoptotic cell death (Roberts, 2000; Gewies, 2003; Debatin, 2004). To date, 14 different members of the caspase have been described in mammals with caspase-11 and -12 only identified in mouse (Gewies, 2003). In broad terms, there are two groups of caspases; initiator caspase (such as -2, -8, -9, and -10) and executor caspase (such as -3, -6 and -7) (Table 1.1) (Roberts, 2000; Gewies, 2003; Debatin, 2004).

In the cell, caspases are synthesised as inactive zymogens. The inactive form of initiator caspase (e.g caspase-8) has longer domain than inactive form of executor caspase (e.g caspase-3) (Figure 1.6 A) (Gewies, 2003). The inactive form of caspase is so-called pro-caspase which at the N-terminus carry a pro-domain followed by a large and a

small subunit. The large and the small subunits are sometimes separated by a linker peptide. Upon maturation, the pro-caspase is proteolytically processed between the large and small subunit where the domain is removed resulting in forming a heterotetramer consisting of two small and two large subunits. The heterotetramer is an active form of caspase. The disappearance of pro-caspase bands in Western blot analysis reflects processing of the zymogen to generate the active form of the specific caspase (Gewies, 2003; Cheah and Azimahtol, 2004). After the initiator pro-caspase is cleaved and activated, it subsequently actives the corresponding downstream executor pro-caspase molecules to become active caspase which in turn initiating the apoptosis (Figure 1.6 B) (Gewies, 2003).

The expression level of individual caspase may have an impact on their overall activity, since deficient expression level may simply impair caspase activation (Debatin, 2004). For example, MCF-7 breast carcinoma cells completely lack caspase-3 expression due to a frame shift mutation within exon 3 of the caspase-3 gene (Janicke *et al.,* 1998). These cells can be sensitised to treatment with cytotoxic drugs by transfection of pro-caspase-3. Alternatively, caspase-8 expression was found to be frequently inactivated by hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumor cells derived from neuroblastoma, malignant brain tumors, Ewing tumors and small-cell lung carcinoma (Teitz *et al.,* 2000; Fulda *et al.,* 2001). Importantly, restoration of caspase-8 expression by gene transfer or

demethylation treatment sensitised resistant tumor cells to death receptor-induced or drug-induced apoptosis (Fulda *et al.,* 2001). In contrast, gene knockout experiments targeting caspase-9 and -3 resulted in mortality as a result of severe defects in brain development (Kuida, 1998). Moreover, caspase-8-deficient embryos died after day 12 (Varfolomeev, 1998). This and the observation indicating that cell lines derived from such knockout experiments are resistant to distinct apoptosis stimuli underlines the importance of caspases as pro-apoptotic mediator (Gewies, 2003). Indeed, it has been recognised that caspases play an important role in the apoptotic signaling machinery (Earnshaw, 1999).

Given the important role of caspases as the final effectors molecule of apoptosis, the ability of anti-cancer agents such as PPARγ ligands to trigger caspase activation appears to be a critical determinant of sensitivity or resistance to cytotoxic therapies (Debatin, 2004). Indeed, caspases are useful biomarkers for apoptosis, as outlined below (Roberts, 2000). As a consequence, determination of caspase activation in various forms of cell death including PPARγ ligand-induced apoptosis may be an important factor in cancer chemotherapies (Debatin, 2004).

1.7.2 Death receptor-ligand apoptosis

The death receptor-ligand system is so called extrinsic apoptosis because the apoptosis signal is initiated by the death receptor at cell surface (Goke *et al.,* 2000; Gewies, 2003; Debatin, 2004). The death receptor belongs to the TNF gene superfamily and generally has several functions other than initiating apoptosis (Debatin, 2004). The best-characterised death receptors are CD95 (or Fas), TNF receptor-1 (TNF-R1) and TRAIL receptor (Goke *et al.,* 2000; Roberts, 2000). The PPARγ ligand-induced death receptor apoptosis is at all times associated with the TNF receptor family (Goke *et al.,* 2000).

1.7.2.1 Signaling by CD95/Fas

According to Ashkenazi and Dixit (1998), there are three main roles of CD95 signaling:

1. mediate killing of cells by T-cells e.g. CTL-mediated killing of virus-infected cells;
2. mediate deletion of activated T-cells at the end of an immune response;
3. mediate destruction of inflammatory and immune cells in immune-privileged sites.