The effects of resveratrol on cell cycle, apoptosis, and NF-κB activation

Master's Thesis by Ulrik Stervbo
Department of Life Sciences and Chemistry
Roskilde University, Roskilde, Denmark
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Supervised by

Ole Vang
Department of Life Sciences
and Chemistry
Roskilde University
Roskilde, Denmark

Christine Bonnesen
Department of Virology
and Molecular Toxicology
Novo Nordisk A/S
Måløv, Denmark

Abstract

Purpose: To study the effect of resveratrol, a putative chemopreventive phytoalexin, on DNA synthesis, cell cycle, apoptosis, and the possible involvement of the transcription factor NF-κB.

Methods: HL-60, a suspension human promyelocytic leukemia cell line, and HepG2, an epithelial human hepatoma-derived cell line were chosen as models. Incorporation of ^{3}H-labelled thymidine and cell cycle distribution, assessed by binding of Propidium Iodine and flow cytometry, were used to evaluate the effect on cell proliferation. The effect on apoptosis was studied by the use of several assays. The nuclei were stained with DAPI and subsequently visualised with fluorescence microscopy. Bound Annexin-V and activity of Caspase-8 were evaluated by flow cytometry. Cleavage of Caspase-3 was assessed by Western blot. The effect of resveratrol on NF-κB transactivating activity was studied by a reporter gene assay. Phosphorylation of the RelA/p65 NF-κB subunit was evaluated by Western blot. Levels of resveratrol were evaluated by light-absorption, and size of isolated cell nuclei was assessed by flow cytometry.

Results: Resveratrol induces a complex dose and time dependent reduction in incorporation of ^{3}H-labelled thymidine. The IC_{50} value increased with increase in incubation time and short term treatment had an inhibitory effect on the incorporation of thymidine. These effects are previously unreported for the used cell lines. It was observed for the first time that when in FCS containing solution, levels of resveratrol decreased. It was found that resveratrol induced a dose dependent accumulation in the S and G_1 phases of the cell cycle in both cell lines. In addition, an enlargement of the HL-60 but not HepG2 nuclei was observed. The nuclear enlarging effect is previously unreported. Resveratrol was found to stimulate apoptosis in a dose dependent manner in HL-60 cells. Caspase-3 and Caspase-8 were furthermore found to be activated. Basal and TNF-α stimulated NF-κB activity was found to be inhibited by resveratrol in a dose dependent manner. HL-60 cells were furthermore found not to be suitable for studying phosphorylation of RelA/p65.

Conclusion: Resveratrol modulates the cell cycle in a complex fashion. Apoptosis is stimulated in a dose dependent manner, with activation of the extrinsic and the intrinsic pathways. The involvement of NF-κB in the observed cellular effects remains unclear.

Resumé

Formål: At undersøge effekterne af resveratrol, et formodet chemopræventivt phytoalexin, med hensyn til DNA syntese, cellecyclus og apoptose. Transskriptionsfaktoren NF-κB blev også undersøgt, med henblik på en mulig rolle i observerede cellulære effekter.

Metoder: HL-60, en human promyelocytisk leukæmi suspensionscellelinie og HepG2, en human epitel hepatoma-afledt cellelinie blev valgt som modeller. Inkorporeringen af ^{3}H-mærket thymidin og cellecyklusfordeling, undersøgt ved binding af Propidium Iodid og flowcytometri, blev anvendt til at studere effekten på celleproliferation. Apoptosestimulerende effekter blev undersøgt ved flere assays: Farvning af kerner med DAPI og efterfølgende visualisering ved fluorescensmikroskopi. Mængden af bundet Annexin-V og Caspase-8 aktivitet blev undersøgt ved flowcytometri, mens kløvning af Caspase-3 blev undersøgt ved Western blot. Effekter på transaktivering af NF-κB blev undersøgt ved et reportergenassay, mens fosforylering af NF-κB subuniten RelA/p65 blev undersøgt ved Western blot. Mængden af resveratrol blev undersøgt ved lysabsorption, og størrelsen af isolerede cellekerner blev bestemt ved flowcytometri.

Resultater: Resveratrol inducerede en kompliceret dosis- og tidsafhængig reduktion i inkorporeringen af ^{3}H-mærket thymidin. Det blev observeret, at IC_{50}-værdien steg med inkubationstiden, og at korttidsbehandling havde en hæmmende effekt på inkorporeringen af thymidin. Disse effekter er ikke tidligere observeres i de anvendte cellelinier. For første gang blev det observeret, at niveauet af resveratrol i en FCS-holdig opløsning, mindskedes. Både HL-60 og HepG2 celler såes ophobet i S eller G_1 fasen af cellecyklus, afhængig af den anvendte resveratrol koncentration. Derudover blev størrelsen af HL-60 cellekernerne fundet forøget, en effekt, der ikke tidligere er rapporteret. Resveratrol stimulerede dosisafhængig apoptose i HL-60 celler. Ydermere blev Caspase-3 og Caspase-8 aktiveret. En dosisafhængig reduktion i basal og TNF-α stimuleret NF-κB aktivitet lod sig observere. Slutteligt vurderedes HL-60 at være mindre velegnede til studier af fosforylering af RelA/p65.

Konklusion: Resveratrol modulerer cellecyklus på kompliceret vis. Apoptose stimuleres dosisafhængigt og inkluderer aktivering af den receptormedierede og den mitokondrielle pathway. Involveringen af NF-κB i de cellulære effekter er uafklaret.

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Introduction

Cancer is one of the leading causes of death in the Western world. It is known that the risk of developing the disease can be greatly reduced by a change of lifestyle and, importantly, a change of dietary habits. Many plant-based foods with putative anti-cancer effects, such as garlic, soy beans, ginger, onions, tomatoes, and the cruciferous greens (e.g. broccoli and cabbage) have been identified. In these fruits and vegetables more than 400 compounds are currently under investigation for their health promoting properties.

Resveratrol One of these many compounds is resveratrol, a phytoalexin found in grapes, red wine, and peanuts. The interest in the health promoting properties of resveratrol was sparked when it was originally discovered in red wine, and thus identified as a possible key player in so called "French Paradox". This phrase was coined to explain the lower number of cases of coronary heart disease in various parts of France, compared to other industrialised countries, despite a high consumption of dietary fats.

A discussion of the "French Paradox" is beyond the scope of this report. For a comprehensive review see Goldberg et al. (1995a). The health promoting properties of wine over other alcoholic beverages is not yet consolidated, and was recently discussed in Grønbæk (2004); Li and Mukamal (2004).

Since the discovery of resveratrol as an inhibitor of tumorigenesis in 1997 (Jang et al., 1997), the compound has experienced an increasing attention from the scientific community. Resveratrol has been shown not only to inhibit cancer initiation, but also reverse development of growths in several different types of cancers. However, the mechanism by which resveratrol exerts the health promoting effects is not yet fully elucidated.

Mechanisms regulating cell division and cell death may be affected by phytochemicals. The transcription factor NF-κB is central in controlling the expression of a range og genes, whose products are involved in cell cycle and apoptosis. In the present work, the effects of resveratrol on DNA synthesis and cell cycle, as well as apoptosis in HL-60 ans HepG2 cells will be discussed. Furthermore, the role of NF-κB as a possible mediator will be evaluated.

Nomenclature The nomenclature used in this report is based on the suggestions for gene nomenclature set forth by the Human Gene Nomenclature Committee (Wain et al., 2002, and later post-publication on-line updates[1]) and the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology[2] where applicable. This attempt to adhere to standards will result in rather uncommon names for most of the factors described. In order to alleviate the transition from the somewhat anarchistic nomenclature used in most of the literature to the more standardised nomenclature, the "old name" will be mentioned together with the recommended abbreviation.

[1] `http://www.gene.ucl.ac.uk/nomenclature/guidelines.html`
[2] `http://www.chem.qmul.ac.uk/iupac/jcbn/`

Abbreviations used

BaP	Benzo[a]pyrene
BSA	Bovine serum albumin
Caspase	Cysteine-aspartic-acid-protease – all variants
Cdk	Cyclin-dependent kinase – all variants
CdkN	Cyclin-dependent kinase inhibitor – all variants
DAPI	4',6-Diamidino-2-phenylindole – a DNA binding fluorophor
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
EGFP	Enhanced green fluorescent protein – a fluorophor
EMSA	Electric mobility shift assay
FAM	Carboxyfluorescein – a fluorophor
FCS	Fetal calf serum
FL1-H	Fluorescence intensity height, channel 1
FL2-H	Fluorescence intensity height, channel 2
FL3-H	Fluorescence intensity height, channel 3
FSC-H	Forward scatter height – particle size
HPLC	High performance liquid chromatography
IKBK/IKK	NF-κB inhibitor kinase – all variants
IL-1β	Interleukin 1, beta
LPS	Lipopolysaccharide
MTT	Tetrazolium salt – 3(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NF-κB	Nuclear factor κB – all variants
NF-κBi/IκB	NF-κB inhibitor – all variants
NR	Neutral Red

ODC	Ornithine Decarboxylase
PBS	Phosphate buffered saline
PI	Propidium Iodine – a DNA/RNA binding fluorophor
PS	Phosphatidylserine
RB1	Retinoblastoma protein
RHD	Rel homology domain
RLU	Relative luminescence unit
SD	Standard deviation
SSC-H	Side scatter height – particle granularity
TNF-α	Tumor necrosis factor
TNFRSF	Tumor necrosis factor receptor superfamily
TPA	12-O-tetradecanoylphorbol-13-acetate – also known as Phorbol-12-myristate-13-acetate (PMA)
UV	Ultraviolet

Chapter 1

Background

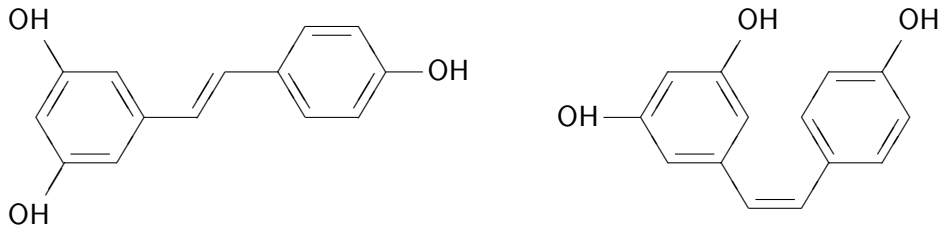
This chapter reviews the current status of research on selected cellular effects of resveratrol. The studies presented in this report comprise the effect of resveratrol on cell growth, cell cycle progression, apoptosis, and the possible involvement of NF-κB. The purpose of this review is to give the reader the required background to interpret the presented data and follow the discussion in the succeeding two chapters.

The chapter starts with a general introduction to resveratrol and the putative beneficial effects of the compound. After a brief introduction to the elements of the eukaryotic cell cycle, the cell cycle modulating effects of resveratrol is reviewed. Mechanisms of apoptosis and the specific effects of resveratrol on apoptosis are subsequently discussed. Finally, current knowledge on the NF-κB signal transduction system and the effect of resveratrol on NF-κB activity is described.

1.1 Resveratrol

Resveratrol is a phytochemical present in a variety of plant species, some of which are used for human consumption, e.g. peanuts, eucalyptus, blueberries, cranberries, and grapes. It is also found in the roots of Japanese knotweed (*Polygonum cuspidatum*), which has been used in traditional Asian herb medicine to reduce inflammation. While the levels of resveratrol are highest in the latter, grapes are probably the most important source of resveratrol for humans, since the compound is also found in one of end product of grapes i.e wine. (reviewed in Aggarwal et al., 2004; Bhat and Pezzuto, 2002; Sobolev and Cole, 1999; Soleas et al., 1997)

Following it's discovery as a phytoalexin in grapes in 1976 by Langcake and Pryce and later in wine (Siemann and Creasy, 1992), resveratrol has enjoyed a growing scientific interest over the years. This interest is mainly due to multiple lines of evidence from *in vitro* and *in vivo* studies suggesting cancer preventive properties (Aggarwal et al., 2004; Jang et al., 1997), as well as epi-



(a) *Trans*-resveratrol (b) *Cis*-resveratrol

Figure 1.1: Chemical structures of resveratrol.

demiological studies indicating an inverse relationship between moderate red wine consumption and coronary heart disease, the so called "French Paradox" (reviewed in Kundu and Surh, 2004; Sun et al., 2002).

The mechanisms behind these effects of resveratrol are not fully elucidated, but the compound has been demonstrated to include 1) anti-oxidant activity, by scavenging and/or regulation of synthesis of scavenging proteins 2) inhibition of carcinogen activation 3) increase carcinogen detoxification 4) inhibition of inflammation 5) modulation of cell cycle progression 6) stimulation of apoptosis (Cao et al., 2003; Dong, 2003; Greten et al., 2004; Kundu and Surh, 2004; Surh, 2003).

However, resveratrol may also have a cancer initiating effect, as genotoxicity has been reported (Ahmad et al., 2000; Matsuoka et al., 2001, 2002).

1.1.1 Physical properties of resveratrol

Structure Resveratrol (3,5,4'-Trihydroxystilbene) is a polyphenol composed of two phenol rings connected by a double bond (figure 1.1). It exists in two isoforms; *trans*-resveratrol and *cis*-resveratrol where the *trans*-isomer is the more stable form (Trela and Waterhouse, 1996). The *trans* to *cis* isomerisation is facilitated by ultraviolet light and high pH, while *cis* to *trans* isomerisation is facilitated by visible light, high temperature, or low pH (Goldberg et al., 1995b; Trela and Waterhouse, 1996). However, when kept in the dark at room temperature *cis*-resveratrol is stable for at least a month (Trela and Waterhouse, 1996).

Physical properties Pure resveratrol is an off-white powder with a molecular mass of 228.24 g/mol. It is hydrophobic but dissolves in ethanol and Dimethylsulfoxide (DMSO). The absorption maxima is approximately 286 nm for *cis*-resveratrol, and 308 nm for *trans*-resveratrol. The different absorption maxima makes high performance liquid chromatography (HPLC) with ultraviolet (UV) detection possible (Deak and Falk, 2002; Goldberg et al., 1995b; Lamuela-Raventos et al., 1995; Trela and Waterhouse, 1996). Other

used means of detecting *cis*- and *trans*-resveratrol are liquid chromatography and gas chromatography (Soleas et al., 1997; Viñas et al., 2000).

Throughout the present report resveratrol refers to *trans*-resveratrol unless otherwise specified.

Biosynthesis In grapes, the phytoalexin resveratrol is synthesized almost entirely in the skin, and the synthethetic potential of grapes is highest just before they reach maturity. The terminal enzyme in the biosynthesis of resveratrol is stilbene synthase, which is activated in response to exogenous stress factors, such as injury, UV irradiation and chemical signals from pathogen fungi. The levels of resveratrol are highest almost immediately after stress exposure, and decline after 42-72 h as result of activation of stilbene oxidase. (reviewed in Pervaiz, 2003; Soleas et al., 1997). A recent report suggested that postharvest UV irradiation can be used to produce grapes with a very high content of resveratrol (Cantos et al., 2001).

Levels of resveratrol in red wine The average levels of *trans*-resveratrol in red wine is often cited as being 5 mg/l. However, the average levels varies greatly from country to country (table 1.1). Since resveratrol is produced by the grape in response to exogenous stress factors, a regional difference is to be expected (Goldberg et al., 1995b, 1996), as well as a difference from vintage to vintage (Goldberg et al., 1996; Martínez-Ortega et al., 2000). The average red wine is estimated to contain approximately 2 mg/l resveratrol. The lowest reported level of resveratrol was barely detectable in a Portuguese red wine made from the Cabernet Sauvignon variety from 1988 (Martínez-Ortega et al., 2000). A Bordeaux, 1991 red wine (variety unknown) has the highest reported levels of *trans*-resveratrol of 7.6 mg/l (Goldberg et al., 1995b). Using the wine making technique of double maceration, levels as high as 18.9 mg/l has been reported in Portuguese red wine (Alonso et al., 2002, vintage not presented).

1.2 Bioavailability and toxicity of resveratrol

Animal studies Using an isolated rat small intestine perfused with resveratrol, it was found that approximately 20 % of availible resveratrol was absorbed. The phytoalexin was mainly absorbed as resveratrol glucuronide and only low levels of unconjugated resveratrol and resveratrol sulfate could be found (Andlauer et al., 2000). In a similar set-up, 6 % of available resveratrol was absorbed in *jejunum* (middle part of small intestine), and only as resveratrol glucuronide. The level of absorption in the *ileum* (lower part of small intestine) was too low to quantify (Kuhnle et al., 2000). By determining the levels of radioactivity in stool fractions (colon plus colon content plus feces) and urine from rats collected over 24 h, as much as 50 to 80 % or radioactive labelled reaveratrol is absorbed (Soleas et al., 2001). Taken together these

Table 1.1: Average *trans*-resveratrol concentration in red wine. The mean values are calculated on basis of levels of *trans*-resveratrol given by the cited references. n is the number of wines studied. An average red wine is estimated to contain 2 mg/l resveratrol

	trans-resveratrol (mg/l)					
Country	Mean	High	Low	Vintage	n	Reference
Australia	2.4	2.6	2.3	1991-1993	2	Goldberg et al. (1996)
Canada	3.5	5.6	1.2	1991-1992	2	Goldberg et al. (1995b)
	2.3	–	–	1995-1996	8	Faustino et al. (2003)
Chile	1.2	1.6	0.8	1990-1992	2	Goldberg et al. (1995b)
	2.3	–	–	1995-1998	10	Faustino et al. (2003)
France	1.6	2.9	0.6	–	5	Careri et al. (2003)
	2.2	4.7	0.3	1988-1993	10	Goldberg et al. (1996)
	3.6	7.6	1.4	1990-1993	6	Goldberg et al. (1995b)
	3	6.8	0.6	1996-1997	7	de Lima et al. (1999)
Greece	1.1	2.5	0.5	1997	29	Kallithraka et al. (2001)
Italy	1.2	3.2	0.7	–	7	Wang et al. (2002b)
	3.7	7.2	1.2	1988 - 1991	15	Mattivi (1993)
	1.2	1.3	1.17	1990-1991	2	Goldberg et al. (1996)
Japan	1	2.3	0.1	–	22	Sato et al. (1997)
Portugal	1	5.7	n.d.[a]	1994-1997	34	de Lima et al. (1999)
Spain	1.6	2.1	1.1	1991	2	Goldberg et al. (1995b)
	0.3	1.3	n.d.	–	5	López et al. (2001)
	0.1	0.2	n.d.	1986-1996	14	Martínez-Ortega et al. (2000)
	1.9[b]	5.1	0.1	1995-2002	45	Moreno-Labanda et al. (2004)
Spain/Portugal[c]	3.5	5.9	2	–	8	Alonso et al. (2002)
USA	0.9	2.2	0.3	1990-1991	4	Goldberg et al. (1996)
	0.9	1.7	0.3	1991-1992	4	Goldberg et al. (1995b)
	1	–	–	1994-1998	7	Faustino et al. (2003)

[a]Not detected; [b]Different wine making methods; [c]No distinction made

findings indicates that resveratrol is absorbed in mainly *duodenum* (the upper part of small intestine), and to a lesser extend in *jejunum*, while no absorption takes place in *ileum*. Also the time course of residence of resveratrol in the small intestine may have an influence on the total absorption of resveratrol.

Organ distribution In mice, following administion of 5 mg/kg radioactive labelled resveratrol through an intra gastric tube (Vitrac et al., 2003), relative high levels of radioactivity was found in *duodenum* after 1.5 h. The levels stayed elevated until 6 h. Levels of radioactivity in the large intestine

peaked after 3 h. In liver and kidney radioactivity was observed after 1.5 h and the levels decreased after 6 h. Radioactivity of lung, spleen, heart, brain, and testis was assessed only after 3 h, and radioactivity was found in all organs. This is in concordance with observations made in gerbils given 30 mg/kg intraperitoneally (Wang et al., 2002a). At 3 h only 27 % of the total radioactivity of liver and kidney could be attributed to labelled *trans*-resveratrol, the remaining 73 % radioactivity originated from conjugates.

In a similar set-up mice were administered 240 mg/kg resveratrol by gastric intubation, the highest amounts of unmetabolised resveratrol was found in plasma, liver, kidney, lung and heart after 15 min, and levels were undetectable 0 after 60 min as determined by HPLC (Sale et al., 2004). The kinetics of unmetabolised resveratrol in plasma was similar to that observed in rats administered 14 mg/kg by gavage (Soleas et al., 2001). In contrast to the observations made by Vitrac et al. (2003) and Wang et al. (2002a), no significant levels of resveratrol were detected in brain, small intestine or colonic mucosa by Sale et al. (2004). The discrepancy may be explained by methodical differences.

Toxicity of resveratrol Juan et al. (2002) studied the harmful effects of resveratrol on male Sprague-Dawley rats. Resveratrol was orally administered as a daily dose of 20 mg/kg for 28 days. No difference in body weight nor food and water intake was observed between the group receiving resveratrol and the control group. This is in full concordance with other observations (Bove et al., 2002; Caltagirone et al., 2000; Carbó et al., 1999; Hecht et al., 1999; Kimura and Okuda, 2001; Tessitore et al., 2000; Ziegler et al., 2004). Hematological and clinical biochemical parameters were not affected by the treatment, with a rise in plasma levels of aspartate aminotransferase, as the only exception. Aspartate aminotransferase is an indicator of liver injury (Borini et al., 2003). Levels of the coronary heart disease indicators low density lipoprotein and high density lipoprotein did not differ, in full agreement with what has been reported by others (Wang et al., 2002c; Wilson et al., 1996). No alterations were observed by a histopathological examination of the body organs, including the liver. The relative weight of major organs did not differ either, except for an increase in the relative weight of the brain and testes in animals receiving resveratrol compared to the control group.

The dose used shows that with an arbitrary safety margin of 100 (human versus rat), a person of 70 kg can consume at least 14 mg resveratrol daily, without any side effects. This is equivalent to drinking 1.8 l Bordeaux, 1991 daily (Goldberg et al., 1995b). Taken together, the study by Juan et al. (2002) indicates that there is a large safety margin regarding resveratrol to repeated moderate wine consumption. However, due to the rise in plasma levels of aspartate aminotransferase, resveratrol induced liver damage cannot be excluded.

Clinical trials In keeping with the findings from animal studies of high absorption of resveratrol, at least 70 % absorption of orally administered resveratrol (25 mg) was detected in a limited clinical trial (Walle et al., 2004). The highest levels of resveratrol in plasma or serum were detected after 0.5 to 1 h, in the range of 2 µM. However, resveratrol was mainly detected as sulfate and glucuronide conjugates in plasma and serum, while levels of free *trans*-resveratrol were negligible, in the range of 35 nM (Goldberg et al., 2003; Walle et al., 2004).

Pharmacokinetics Walle et al. (2004) found that resveratrol and it's conjugates were lost mainly through urine in the course of 72 h with a half-life of approximately 9 h (25 mg resveratrol was orally administered). Interestingly, a rise in the total plasma levels of polyphenols, was observed in men who consumed half a bottle of red wine (375 ml) daily for two weeks (Nigdikar et al., 1998).

Metabolism of resveratrol Resveratrol is glucuronated in the human liver and sulfated in both the liver as well as in the *duodenum* (Santi et al., 2000a,b). The major conjugates are *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4'-*O*-glucuronide, and *trans*-resveratrol-3-*O*-sulfate (Yu et al., 2002). The kinetic data presented indicates glucuronation to prevail over sulfation in the liver, and that the rate of sulfation is the same in the liver and *duodenum*. The metabolic modifications of resveratrol can be inhibited by quercetin, a polyphenol also found in wine (Manach et al., 2004; Santi et al., 2000a,b).

The clinical and *in vivo* studies presented here suggest that free *trans*-resveratrol in plasma is very sparse and short lived. One reason for the short half-life may be conjugation of resveratrol. Another reason may be an accumulation of resveratrol in adipose tissue since due to its hydrophobic properties, albeit this still awaits examination. Resveratrol glucuronides may act as a pool for active *trans*-resveratrol. The existence of human β-glucuronidase (Miller et al., 1990), makes a conversion of resveratrol glucuronide to resveratrol feasible. This implies a steady level of active *trans*-resveratrol within cells.

1.3 Chemopreventive properties of resveratrol

Carcinogenesis is generally viewed as a multistage process in which molecular and cellular changes occur. The process spans several years, and can be simplified to consist of three distinct stages; tumor initiation, promotion, and progression (reviewed in Jakóbisiak et al., 2003). Resveratrol has been shown inhibit initiation as well as promotion (reviewed in Surh, 2003).

Table 1.2: Effects of resveratrol on tumorigenesis. The effects of resveratrol depends on type of tumor, and maybe method of administration. ↓ indicates a great reduction, ↘ indicates a minor reduction, and → indicates no effect at all. When reported, no changes in weight gain, food and water intake, or animal behaviour were observed

Tumor type	Induction	Dosage range	Administration	Study time	Effect	References
Rat						
Hepatoma	Implanted	1 mg/kg	i.p.[a]	7 days	↓ Tumor cell number	Carbó et al. (1999)
Colorectal cancer	Azoxymethane	200 µg/kg	d.w.[b]	100 days	↓ ACF[†]	Tessitore et al. (2000)
					↓ ACF[‡]	
Mouse						
Murine melanoma	Injection	50 mg/kg	i.p.	19 days	↘ Tumor volume	Caltagirone et al. (2000)
Two-stage skin cancer	DMBA/TPA[c]	1 to 25 µM	Topically	18 weeks	↓ Tumor number	Jang et al. (1997)
Two-stage skin cancer	DMBA/TPA	85 nM	Topically	11 weeks	↓ Tumor number	Kapadia et al. (2002)
				21 weeks	↘ Tumor number	
Two-stage skin cancer	DMBA/TPA	5 to 125 µM	Topically	21 weeks	↘ Tumor number	Soleas et al. (2002)
Hepatoma	Implanted	500 to 1500 mg/kg	i.p.	10 days	↓ Tumor size	Liu et al. (2003)
Hepatoma	Implanted	5 to 15 mg/kg	i.p.	10 days	↓ Tumor weight	Yu et al. (2003)
Mac16	Implanted	1 mg/kg	i.p.	3 days	↓ Tumor volume	Wyke et al. (2004)
Gastric cancer	Implanted	500 to 1500 mg/kg	Injection	12 days	↓ Tumor volume	Zhou et al. (2005)
Neuroblastoma	Injection	40 mg/kg	Injection	28 days	↓ Tumor volume	Chen et al. (2004)
Lewis lung carcinoma	Implanted	2.5 to 10 mg/kg	i.p.	21 days	↓ Tumor weight	Kimura and Okuda (2001)
					↓ Angiogenesis	
Lung carcinoma	BaP plus NNK	500 ppm	Food	26 weeks	→ Tumor number	Hecht et al. (1999)
Lung carcinoma	BaP	6 to 8 mg/kg	Food	8 weeks	→ Tumor size	Berge et al. (2004)
					→ Tumor number	
Colorectal cancer	Gene mutation	0.01 %	d.w.	7 weeks	↓ Tumor number	Schneider et al. (2001)
Colorectal cancer	Gene mutation	4 to 90 mg/kg	Food	7 weeks	→ ACF[†]	Ziegler et al. (2004)
					→ ACF[‡]	
Mammary carcinoma	Injection	1 to 5 mg/kg	i.p.	23 days	→ Tumor volume	Bove et al. (2002)
					→ Lung metastases	

[a]Intraperitoneal [b]Drinking water [c]DMBA is a tumor initiator, TPA a promoter

[†]Number of medium and small aberrant crypt foci; [‡]Development of large aberrant crypt foci

***In vivo* effects of resveratrol on tumorigenesis** The first report on chemopreventive effects of resveratrol was by Jang et al. (1997). The authors evaluated the effect of resveratrol on tumorigenesis by a two-stage mouse skin cancer model and found a reduction of skin tumors by as much as 98 %. Since this first study on the inhibitory effect of resveratrol on tumorigenesis, several reports have examined the effect of resveratrol on tumorigenesis in different models.

Resveratrol may have different effects on different types of tumors (table 1.2). Resveratrol even at low doses may inhibit development of implanted hepatoma in different animal models *in vivo*, and may delay development of skin cancers when applied topically. Difference in observations may be due to different times of treatment, but could as easily be explained by differences between animals or induced versus inherited cancer. Taken together, the results summarised in table 1.2 indicates an inhibitory effect by resveratrol on the development of certain tumors *in vivo*. However, there are some discrepancies in this field of research and further studies are needed to fully elucidate the effects of resveratrol.

1.4 Resveratrol modulates the cell cycle

The cell cycle of mammalian cells has several checkpoints to ensure fidelity in the replication of the DNA and progression through the cell cycle (reviewed in Bartek and Lukas, 2001). As uncompromised checkpoints ensure that no cell divides with faulty DNA, nor is able to divide uncontrollably, aberration in the control of the cell cycle is central to the process of carcinogenesis. A cell with uncompromised checkpoints could embark on the apoptotic process of self destruction in the case of irreparable damage. With this reasoning in mind, the effects of resveratrol on the cell cycle and apoptosis is examined.

1.4.1 Cell cycle progression and check points

The cell cycle is a controlled sequence of events from one division of an eukaryotic cell to the next. The cell cycle can be broken down to four phases (figure 1.2), the first growth phase (G_1), the S-phase where the DNA is replicated, a second growth phase (G_2), and finally the M-phase, where the cell actually divides into two daughter cells. Non-dividing cells are said to be in a resting phase, termed G_0.

To control the ordered sequence of events that constitutes the cell cycle, several control points are essential. The checkpoints ensure that each phase of the cell cycle is completed before the next is initiated and some checkpoints ensure that damaged DNA is repaired. The three major checkpoints are at the G_1 to S transition, the G_2 to M transition, and the alignment checkpoint in the M phase (reviewed in Stewart et al., 2003), but also inter S phase

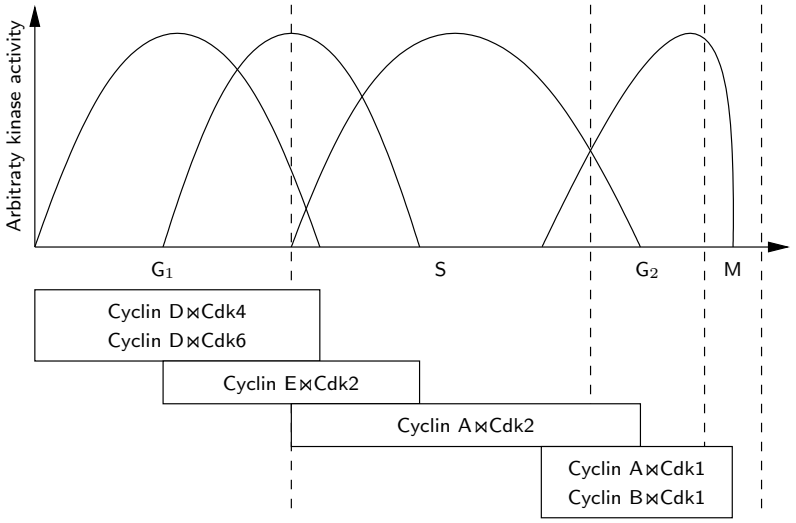


Figure 1.2: The Cyclins and Cdks of the mammalian cell cycle. The activity of Cdks fluctuates throughout the cell cycle as a consequence of fluctuating levels of Cyclins. The cell cycle is initiated by a mitogen. Based on Coqueret (2003); McGowan (2003); Murray (2004); Price et al. (2004)

checkpoints are known. Given the consequence of erroneous replication of the DNA, most checkpoints are concentrated between the G_1 and G_2 phases (reviewed in Bartek et al., 2001) and these are the focus of this report.

If problems are encountered at a checkpoint the cell may embark on the process of self destruction, termed apoptosis, as described in section 1.5. In contrast, malfunctioning cell cycle checkpoints may lead to an accumulation of mutations and genomic instability, which in turn may lead to development of cancer.

Cell cycle progression

The passage of a cell through the cell cycle is controlled by a family of kinases called Cyclin-dependent kinases (Cdks). Binding to specific Cyclins as well as phosphorylation of the Cdk is necessary for full kinase activity. While the level of Cdks stays constant throughout the whole cycle, the levels of Cyclins, and thus the activities of specific Cdks fluctuate with the phase of the cell cycle. The degradation of Cyclins ensures an unidirectional cell cycle (reviewed in McGowan, 2003; Miele, 2004; Murray, 2004). Cyclin D is mainly associated with the G_1 phase (figure 1.2), Cyclin E with the G_1 to S phase transition, Cyclin A with the S phase, and Cyclin A and Cyclin B are associated with the M phase.

Cells in the G_1 phase of the cell cycle usually require extracellular signals, such as growth factors to prepare for entering the S phase. As a response to

growth factors, Cyclin D is synthesised and activates Cdk4 and Cdk6. The active kinases phosphorylate the Retinoblastoma protein (RB1), a repressor of transcription of a number of genes. Inactivation of RB1 results in a sharp increase in levels of Cyclin E, Cyclin E⋈Cdk2 complex, and transcription of S phase genes, through activation of the transcription factor E2F. In a positive feedback loop, Cyclin E⋈Cdk2 phosphorylates RB1 to increase the activity of the Cyclin E⋈Cdk2 complex (reviewed in Price et al., 2004; Stewart et al., 2003).

Shortly after the increase in Cyclin E expression, synthesis of Cyclin A is increased. Where the levels of Cyclin E peaks at the transition from the G_1 to S phase, levels of Cyclin A stay high through the whole S phase, to decline at mid M phase. Towards the end of G_2 phase, Cyclin B accumulates in the cytoplasm. At the beginning of the M phase Cyclin B rapidly translocates to the nucleus, binds to Cdk1/cdc2 and throws the cell into mitosis. During the actual division into two daughter cells Cyclin B is degraded, and thus the divided cells can reenter the G_1 phase (reviewed in McGowan, 2003; Price et al., 2004).

1.4.2 The effect of resveratrol on the cell cycle

When reviewing the literature on the cell cycle modulating effects of resveratrol, a complex picture emerges. The majority of authors study the effect of a single resveratrol concentration at a single treatment time and report an accumulation in the S phase of the cell cycle. The S phase accumulation may be correlated to some interference with the DNA polymerases and/or the ribonucleotide reductase. When varying doses have been analysed, a different pattern appears; at low resveratrol concentrations cells accumulate in the S phase whereas they accumulate and at higher concentrations in the G_1 or G_2/M phase.

Resveratrol modulates the cell cycle of many different cell lines In almost all cell lines studied resveratrol was observed to modulate the progression through the cell cycle (table 1.3). Observations of cell cycle modulating effects are not always subjected to statistical analysis. For this reason the observations presented in table 1.3 should be taken as tendencies which may not necessarily be statistical significant. Extracting a pattern of cell cycle modulating effects of resveratrol is not straight forward. However, resveratrol seems to stimulate an accumulation of cells mainly in the S phase in a dose dependent manner.

Interference with the DNA polymerases Resveratrol has been shown to inhibit replication of the SV40 virus (Sun et al., 1998). The SV40 virus makes extensive use of the host replication system why the result

suggest an interference with the DNA polymerases. Using the calf thymus DNA polymerases Stivala et al. (2001) found that resveratrol in deed did inhibit the DNA polymerases α and δ.The authors found no indication of interference with the assembly of the replication machinery. Taken together, this suggest that resveratrol interferes with the replication machinery through a direct inhibition of the DNA polymerase α and δ.

Inhibition of the ribonunleotide reductase An earlier event to trigger S phase delay may be interference with the replication machinery. Resveratrol has been shown to inhibit the ribonucleotide reductase (Fontecave et al., 1998; Pozo-Guisado et al., 2002) which results in depletion of deoxynucleotides (Horvath et al., 2005). The inhibitory effect on the ribonucleotide reductase may be due to the anti-oxidant properties of resveratrol, since resveratrol was found to scavenge the essential tyrosyl radical (Fontecave et al., 1998).

Cell accumulation in different phases depends on the resveratrol concentration In several cell lines, a complex relationship between resveratrol concentration, incubation times, and cell cycle arrest has been reported (table 1.4).

The general emerging picture is that resveratrol stimulates S phase arrest at concentrations below a certain level. Above this level, resveratrol stimulates an accumulation in G_1 or G_2 phase. A very rough estimate of this level would be 50 µM. This estimate should be used with precaution, since it is based on a limited number of observations. Furthermore, the effects of resveratrol on cell cycle arrest appears to depend on incubation time as well as on cell type.

The cell cycle modulating effect of resveratrol is complex The reports summarised in tables 1.3 and 1.4 indicate a pattern of cell cycle modulation caused by resveratrol which is complex and internally contradicting. It appears that resveratrol mainly gives rise to an accumulation of cells in the S phase. However, this may depend on concentration used, and for colon carcinomas the effect may also depend on incubation time. Given the limited number of experiments, these observations may also hold true for other cells lines.

Table 1.3: Resveratrol stimulates cell cycle arrest in many different cell lines. Resveratrol may stimulate S phase accumulation in most cells. Observations presented here are *tendencies*, and may not always have statistical analysis attached

Phase	Model	Dose range	Treatment time	Reference
None				
	LNCap	25 µM	96 h	Hsieh and Wu (1999)
	Seg-1	300 µM	24 h	Joe et al. (2002)
	SK-Mel-28	100 µM	24 and 48 h	Larrosa et al. (2003)
	MDA-MB-231[a]	50 to 150 µM	48 h	Pozo-Guisado et al. (2002)
G_1				
	A431	1 to 50 µM	24 h	Ahmad et al. (2001)
	Bic-1	300 µM	24 h	Joe et al. (2002)
	HCT-116	100 µM	24 h	Fulda and Debatin (2004)
	HL-60	100 µM	6, 16, and 24 h	Kang et al. (2003)
	KATO-III	100 µM	24 h	Atten et al. (2001)
	LNCap	10 µM	24 and 48 h	Narayanan et al. (2003)
	Shep	100 µM	24 h	Fulda and Debatin (2004)
G_1/S				
	HL-60	10 to 50 µM	48 h	Ragione et al. (1998)
	K-562	100 µM	24 h	Ragione et al. (2003)
S				
	A549	12.5 to 50 µM	48 h	Kim et al. (2003)
	Caco2	12.5 to 200 µM	24 h	Wolter et al. (2001)
	DU145	25 µM	96 h	Hsieh and Wu (1999)
	Hce7	300 µM	24 h	Joe et al. (2002)
	HeLa	25 µM	0 to 28 h	Zoberi et al. (2002)
	HepG2	100 µM	48 h	Delmas et al. (2000)
	HL-60	300 µM	24 h	Joe et al. (2002)
	HL-60	4 or 8 µM	20 h	Ahmad et al. (2004)
	JCA-1	25 µM	96 h	Hsieh and Wu (1999)
	K-562	100 µM	48 h	Ferry-Dumazet et al. (2002)
	MCF7	50 µM	6, 12, 24 and 48 h	Banerjee et al. (2002)
	MCF7	300 µM	24 h	Joe et al. (2002)
	Ocim2	10 to 50 µM	6 h	Estrov et al. (2003)
	PC-3	25 µM	96 h	Hsieh and Wu (1999)
	SK-Mel-28	100 µM	72 and 96 h	Larrosa et al. (2003)
	SW480	300 µM	24 h	Joe et al. (2002)
	U-937	10 to 30 µM	24 h	Castello and Tessitore (2005)
S/G_2				
	Kcl22	100 µM	48 h	Ferry-Dumazet et al. (2002)
G_2				
	HT29	100 µM	32 h	Liang et al. (2003)

[a]Synchronised cells

Table 1.4: Resveratrol stimulates cell cycle arrest in several cell lines in a dose dependent manner. Observations presented here are *tendencies*, and may not always have statistical analysis attached

Model	Phase accumulation	Dose range	Treatment time	Reference
Human embryonic fibroblasts				
	S	30 µM	24 h	Stivala et al. (2001)
	G_1/S	90 µM	24 h	
MCF7[a]				
	S	50 µM	36 h	Pozo-Guisado et al. (2002)
	G_0/G_1	100 and 150 µM	36 h	
Cem (subline)				
	S	10, 20 µM	24 h	Bernhard et al. (2000)
	G_1/S	40, 100 µM	24 h	
LNCap				
	S	10 µM	24 h	Kuwajerwala et al. (2002)
	G_1	20, 25 µM	24 h	
A2780				
	S	50 µM	24 h	Opipari et al. (2004)
	G_1	100 µM	24 h	
HCT-116				
	S	50 µM	24 h	Wolter et al. (2001)
	G_2	100 µM	24 h	
	almost none	200 µM	24 h	
SW480				
	S	30, 50, 100 µM	24 and 48 h	Delmas et al. (2002)
	S	30 µM	72 h	
	G_2	50, 100 µM	72 h	
Caco2				
	S	25 µM	16/24 h	Schneider et al. (2000)
	G_2	25 µM	40 h	
	none	25 µM	48 h	
	S	25 µM	64 h	
BPAE				
	S/G_2	50 µM	72 h	Hsieh et al. (1999)
	S	100 µM	72 h	

[a]Synchronised cells

1.5 Resveratrol stimulates apoptosis

1.5.1 Apoptosis

Apoptosis is the term used to describe an evolutionary conserved process which results in distinct morphological features (Samali et al., 1999). The morphological changes *in vitro* include detachment from other cells and the extracellular matrix, resulting in rounding up of the cells. The membrane looses the asymmetry and starts to bulge out, usually referred to as 'blebbing'. The nucleus condenses, and eventually disassembles into fragments of multiples of 180 base pairs in length (reviewed in Hengartner, 2000). The entire cell, including organelles, is organised into vesicles termed 'apoptotic bodies' (reviewed in Häcker, 2000).

Though the term 'programmed cell death' was originally coined to refer specifically to cell death during development (Zhivotovsky, 2004), it is now frequently used as a synonym to 'apoptosis'. To avoid the trap of semantics only the word 'apoptosis' or the more unspecific 'cell death' will be used throughout this report.

Apoptosis is a complex process involving different proteins and families of proteins. The purpose of the following sections are to give an overview of the settings as basis for a discussion of the stimulating effects of resveratrol on apoptosis.

There are two major pathways involved in apoptosis. The extrinsic pathway relies on activation of cell surface receptors by external stimuli. The intrinsic pathway is routed via the mitochondrion as a result of cellular stress and DNA damage. The cysteine-aspartic-acid-proteases (Caspases) are essential in both pathways, thus a short description of these proteins will be given. Activation of the intrinsic pathway converges on a family of proteins called the Bcl2 family. This family of apoptosis regulating proteins is described before a more detailed description of the two pathways leading to apoptosis.

Within the cell at least two more apoptosis pathways exist. One where Caspases are activated by the protease Granzyme B, and one which does not involve Caspases (reviewed in Reed et al., 2004). These two pathways will not be discussed further as they are beyond the scope of this report.

The family of Caspases

The key component of the two major pathways in apoptosis is a family of proteases which recognise and cleave right after aspartic acid (reviewed in Gosslau and Chen, 2004). These aspartic acid specific proteases are collectively called Caspases and at least 14 mammalian Caspases are known (named Caspase-1 through Caspase-14). Members of the Caspase family are involved in apoptosis or inflammation, and those involved in apoptosis are generally subdivided into two groups: the initiator Caspases and effector Caspases (Shi, 2002). Table 1.5

Table 1.5: Overview of the family of Caspases. Subdivision is based on Shi (2002)

Apoptosis		Inflammation
Initiator	Effector	
Caspase-2	Caspase-3	Caspase-1
Caspase-8	Caspase-6	Caspase-4
Caspase-9	Caspase-7	Caspase-5
Caspase-10		Caspase-11
		Caspase-12
		Caspase-13
		Caspase-14

summarises the subdivision of the Caspase family. The Caspases involved in inflammation will not be described further in this report.

Activation of Caspases All Caspases are synthesised as inactive Caspases, called Procaspases, and must be cleaved for activity. Cleavage of Procaspase results in a large (20 kDa) and a small (10 kDa) subunit (figure 1.3), and usually the formation of heterotetramers with two large subunits, two small subunits, and two active sites (Hengartner, 2000; Lawen, 2003). The large subunit of some active Caspases are cleaved once more, though this is unnecessary for catalytic activity (Shi, 2002). While the substrate for initiator Caspases in general are the effector Caspases, the latter are much less selective in their substrate specificity and activated effector Caspases cleave a wide range of proteins – more than 100 proteins are substrates for activated Caspases (reviewed in Thiede and Rudel, 2004).

Selected effects of effector Caspases The proteins flipase (moves phospholipids inward with respect to the cytosol), flopase (moves phospholipids outward) and scramblase (moves phospholipids in both directions) maintain the assymetry of the plasma membrane, and ensure that Phosphatidylserine is in the cytosolic side of the plasma membrane (reviewed in Diaz and Schroit, 1996). Activation of Caspase-3 results in exposure of Phosphatidylserine on the cell surface (figure 1.4), through inhibition of flipase (Hirata et al., 1998; Mandal et al., 2002).

One hallmark of apoptotic cells is the change in nuclear morphology into small condensed fragments (Häcker, 2000). Caspase-6 and Caspase-3 cleave the nuclear mitotic apparatus protein. The cleavage mediates the shrinkage and fragmentation of nuclei as well as DNA fragmentation and chromatin condensation (Hirata et al., 1998).

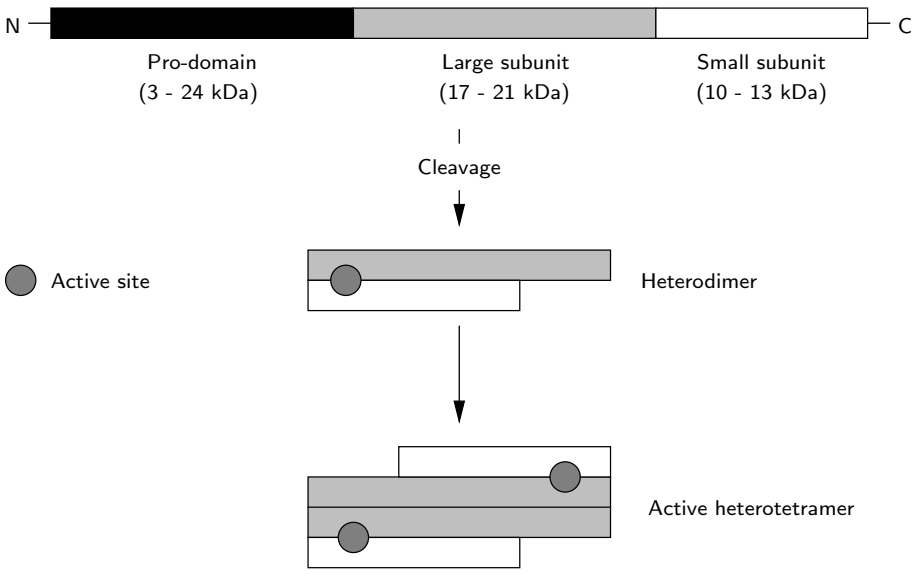
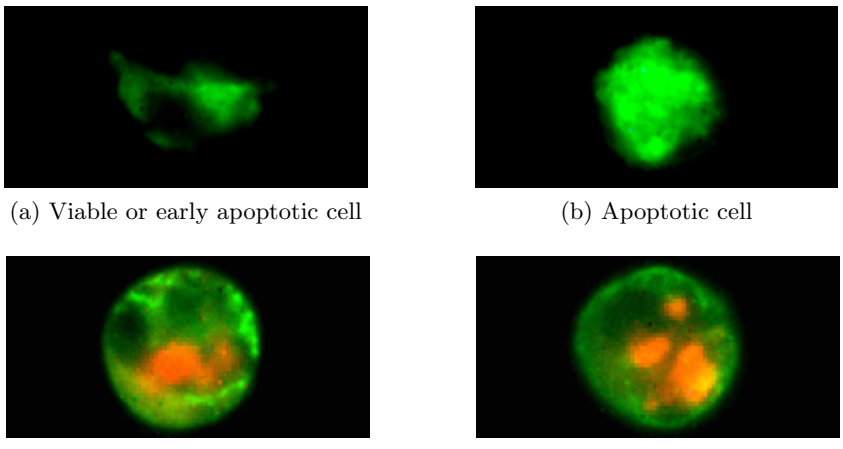


Figure 1.3: Cleavage of Caspases. Procaspase is cleaved to form the large and small subunit. The active Caspase is a heterotetramer with two active sites. Based on Hengartner (2000) and Lawen (2003)



(a) Viable or early apoptotic cell

(b) Apoptotic cell

(c) Dead cell with faint DNA fragments

(d) Dead cell with obvious DNA fragments

Figure 1.4: Double staining of HL-60 cells with Annexin-V-EGFP and PI. Viable or early apoptotic cell (a), apoptotic cell (b), dead cell with faint DNA fragments (c), and dead cell with obvious fragments (d). Personal observations. Cells were treated with 40 µM resveratrol for 18 h, stained with Annexin-V-EGFP (green) and PI (red), and visualised under a fluorescence microscope

Table 1.6: Overview of the human Bcl2 family. Subdivision is based on the presence of the 1 to 4 Bcl2 homology domains (BH). Based on Borner (2003)

Anti-apoptotic		Pro-apoptotic			
Class	Name	Class	Name	Class	Name
Bcl2-like		Bax-like		BH3-only	
	Bcl2		Bax		Bik/Nbk
	Bcl2L1_v1/Bcl-xL		Bak1		Blk
	Bcl2L2		Bok/Bcl2L9		Hrk/Dp5
	Mcl1		Bcl2L1_v2/Bcl-xS		Bnip3
	Bcl2A1/Bfl1				Bcl2L11/Bod
	Bcl2L10/Bcl-B				Bad/Bcl2L8
					Bid
					PmaiP1/Noxa
					Bbc3/Puma
					Bmf

The Bcl2 family of pro- and anti-apoptotic proteins

A central family of regulators of apoptosis is the Bcl2 family of pro- and anti-apoptotic proteins (table 1.6). Some members of the family are anchored in the mitochondrial membrane by a membrane-anchoring domain, while others lack the transmembrane domain (Reed et al., 2004). However, the presence of the transmembrane domain is distributed throughout the whole Bcl2 family with predominance in the Bcl2-like and Bax-like members (reviewed in Borner, 2003; Hengartner, 2000; Reed et al., 2004).

The extrinsic pathway

The extrinsic pathway is activated by extracellular ligands binding to a receptor of the tumor necrosis factor receptor superfamily (TNFRSF) and activation by trimerization of the monomeric receptor (figure 1.5). The Fas/CD95 receptor is the most well-describeed of the extrinsic pathway, but also TNFRSF1A/TNF-R1 is often cited in the literature. The ligand FasLG/CD95L activates the Fas/CD95 receptor which in turn activates Caspase-8. Activated Caspase-8 stimulates cleavage and activation of different effector Caspases as well as the pro-apoptotic protein Bid. by cleavage to tBid. In turn tBid activates the intrinsic pathway (reviewed in Gosslau and Chen, 2004; Lawen, 2003; Muppidi et al., 2004).

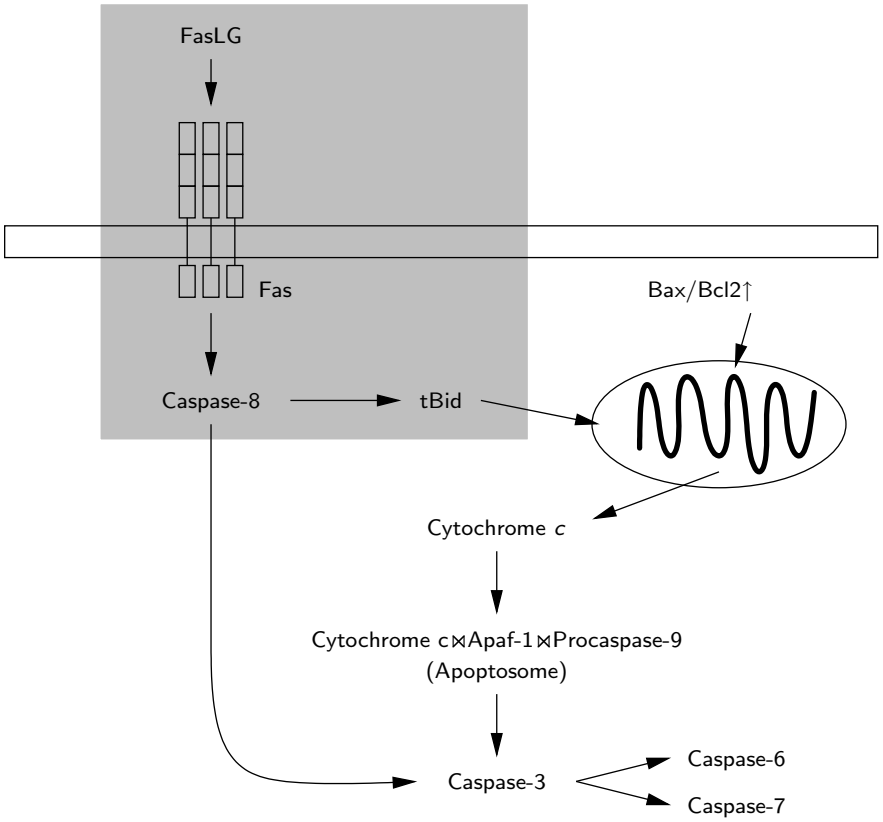


Figure 1.5: The pathways of apoptosis. Simplified illustration to show the Caspase activation pathways. In the extrinsic pathway (gray area), extracellular ligands, such as the Fas/CD95 ligand FasLG/CD95L, bind to membrane bound receptors and result in activation of the initiator Caspase-8. In the intrinsic pathway, intracellular stress signals lead to activation of the intrinsic pathway, resulting in increase in the Bax/Bcl2 ratio, subsequent Cytochrome *c* release from mitochondria and activation of the effector Caspase-3. Caspase-8 may cleave Bid to tBid, which in turn may activate the intrinsic pathway (reviewed in Gosslau and Chen, 2004; Guseva et al., 2004; Lawen, 2003; Muppidi et al., 2004; Shiozaki and Shi, 2004)

The intrinsic pathway

In the intrinsic pathway, apoptosis is activated when Cytochrome *c* is released from the intramembrane space of the mitochondrion into the cytosol (figure 1.5). This release is under tight control by the Bcl2 protein family. The Bax/Bcl2 ratio indicates the relative amounts of pro- and anti-apoptotic proteins of the Bcl2 family (reviewed in Gosslau and Chen, 2004).

The Bax/Bcl2 ratio goes up as response to stimuli such as DNA damage (through p53) and reactive oxygen species. Members of the Bcl2 family stimulates release of Cytochrome *c* and other proteins from the intramembrane

space into the cytosol. Cytosolic Cytochrome *c* binds to apoptotic protease activating factor 1 which stimulates binding of Procaspase-9 to form the mature complex known as the apoptosome. When bound to the apoptosome, Procaspase-9 is activated. Activated Caspase-9 on the apoptosome activates the effector Caspases 3, 6, and 7 by cleavage of their respective Procaspases (reviewed in Lawen, 2003; Shi, 2002).

1.5.2 The effect of resveratrol on apoptosis

There is accumulating evidence that resveratrol stimulates apoptosis in many different tumor cell lines (table 1.7). Few cancer cells are unaffected by resveratrol, and the lack of effect can partly be explained by usage of a sub-effective dose and difference in incubation time.

Caspase dependent stimulation of apoptosis

In reports where the effect on Caspase-3 is studied is the activity of Caspase-3 stimulated by resveratrol. This clearly indicates that resveratrol stimulates a Caspase dependent cell death. Utilising different inhibitors of the active site of Caspases it is possible to study the role of a given Caspase in apoptosis. A selection of Caspase inhibitors are listed in table 1.8

Using z-VAD-fmk, a general Caspase irreversible inhibitor, and z-DEVD-fmk, a specific Caspase-3 irreversible inhibitor, only a reduction in cell death is found in several cell lines (Clement et al., 1998; Estrov et al., 2003; Opipari et al., 2004; Roman et al., 2002). The z-DEVD-fmk and the specific Caspase-8 inhibitor z-IETD-fmk had limited reducing effect on apoptosis stimulated by resveratrol in SW480 cells (Delmas et al., 2003). Only when cells were treated with z-VAD-fmk was apoptosis nearly abolished. In HCT-116, another colon cancer cell line, z-DEVD-cho, a specific Caspase-3 reversible inhibitor, only reduces apoptosis, and z-IETD-cho had no significant effect on apoptosis stimulated by resveratrol (Mahyar-Roemer et al., 2001). Altogether, the results indicate that resveratrol may stimulate Caspase dependent apoptosis, in certain cell lines.

The extrinsic pathway may be involved in resveratrol stimulated apoptosis Resveratrol has been shown to stimulate expression of the Fas/CD95 ligand, FasLG/CD95L, in HL-60 (Clement et al., 1998; Pervaiz, 2001; Su et al., 2005), SNU-1, and KATO-III (Atten et al., 2005) cells. This indicates that the extrinsic pathway may be involved in resveratrol

Table 1.7: Resveratrol stimulates apoptosis in many different cell lines. The difference in stimulatory effect in Caco2 and K-562 cells in different experiments may be due to used resveratrol concentration and/or incubation times

Model	Dose range	Treatment time	Reference
No effect			
Caco2	50 µM	48 h	Schneider et al. (2000)
JCA-1	25 µM	96 h	Hsieh and Wu (1999)
MDA-MB-231	0 to 200 µM	48 h	Pozo-Guisado et al. (2002)
Minor effect			
HL-60	10 to 50 µM	48 h	Ragione et al. (1998)
K-562	100 µM	24 h	Ragione et al. (2003)
Stimulating effect			
Caco2	200 µM	24 and 48 h	Wolter et al. (2001)
K-562	30 and 100 µM	72 h	Ferry-Dumazet et al. (2002)
K-562	100 µM	24 h	Roy et al. (2002)
Eskol and Wsu-Cll	3 to 50 µM	0 to 48 h	Roman et al. (2002)
A2780	50 and 100 µM	24 h	Opipari et al. (2004)
A431	1 to 25 µM	24 h	Ahmad et al. (2001)
A549	12.5 to 50 µM	unknown	Kim et al. (2003)
Cem (subline)	0 to 100 µM	24, 48, and 72 h	Bernhard et al. (2000)
DU145	25 µM	96 h	Hsieh and Wu (1999)
HCT-116	0 to 100 µM	48 and 72 h	Delmas et al. (2003)
HeLa	100 µM	24 h	Roy et al. (2002)
HL-60	32 µM	24 h	Clement et al. (1998)
HL-60	25 µM	24 h	Kang et al. (2003)
HL-60	32 µM	unknown	Pervaiz (2001)
HL-60	100 µM	24 h	Roy et al. (2002)
HL-60	20 µM	24 h	Su et al. (2005)
HL-60	100 µM	6, 24, and 48 h	Surh et al. (1999)
Jurkat	30 µM	72 h	Ferry-Dumazet et al. (2002)
KATO-III	100 µM	24 and 48 h	Atten et al. (2001)
Kcl22	30 µM	72 h	Ferry-Dumazet et al. (2002)
LNCap	25 µM	96 h	Hsieh and Wu (1999)
LNCap	10 µM	24 and 48 h	Narayanan et al. (2003)
MCF7	0 to 150 µM	36 h	Pozo-Guisado et al. (2005)
MCF7	100 µM	24 h	Roy et al. (2002)
MCF7	0 to 200 µM	36 h	Pozo-Guisado et al. (2002)
MiaPaca-2	25 to 100 µM	72 and 24 h	Mouria et al. (2002)
PC-3	25 µM	96 h	Hsieh and Wu (1999)
SNU-1	1 to 100 µM	24 and 48 h	Atten et al. (2005)
SW480	0 to 100 µM	48 and 72 h	Delmas et al. (2003)
Thp-1	100 µM	72 h	Ferry-Dumazet et al. (2002)
U-937	30 µM	72 h	Ferry-Dumazet et al. (2002)
Ocim2	5 to 100 µM	18 h	Estrov et al. (2003)

Table 1.8: Selected Caspase inhibitors.

Inhibitor	Target	Irreversible
z-DEVD-cho	Caspase-3	No
z-DEVD-fmk	Caspase-3	Yes
z-IETD-cho	Caspase-8	No
z-IETD-fmk	Caspase-8	Yes
FAM-LETD-fmk	Caspase-8	Yes
z-VAD-cho	General	No
z-VAD-fmk	General	Yes

stimulated apoptosis. Concomitant treatment of HL-60 and T47D cells with anti-Fas/CD95 and resveratrol inhibited resveratrol induced cell death (Clement et al., 1998). Since anti-Fas/CD95 in an inhibitor of activation of the receptor, this observation is another indication that resveratrol stimulated apoptosis indeed does involve the extrinsic pathway.

Activation of the Fas/CD95 system, and thus the extrinsic pathway may be cell type specific, since no increase in levels of Fas/CD95 or FasLG/CD95L was observed in resveratrol treated Cem (Bernhard et al., 2000) and SW480 cells (Delmas et al., 2003). Interestingly, the authors found that although no interaction is needed between Fas/CD95 and it's ligand, interference with proteins of the death inducing signaling complex inhibits resveratrol stimulated apoptosis. This indicates that resveratrol may stimulate apoptosis through the Fas/CD95 activated extrinsic pathway, independently of FasLG/CD95L but through redistribution of Fas/CD95 in the membrane.

The intrinsic pathway may be activated by resveratrol, but the precise mechanism is unclear An increase in cytosolic Cytochrome *c* following treatment with resveratrol has been reported in several cell lines (Delmas et al., 2003; Opipari et al., 2004; Pervaiz, 2001) indicating that resveratrol activates the intrinsic pathway as well.

Changes in the Bax/Bcl2 ratio are associated with activation of the intrinsic pathway (reviewed in Gosslau and Chen, 2004). The role of the Bcl2 family in resveratrol stimulated apoptosis remains obscure. A decrease in the anti-apoptotic proteins Bcl2, Mcl1, and Bcl2L1_v1/Bcl-xL, and an increase in the pro-apoptotic proteins Bax and Bak1 following resveratrol treatment has been reported in different cell lines (Delmas et al., 2003; Estrov et al., 2003; Pozo-Guisado et al., 2005; Roman et al., 2002; Surh et al., 1999). However, overexpression of Bcl2 and Bcl2L1_v1/Bcl-xL failed to inhibit Cytochrome *c* release and apoptosome formation in the ovarian A2780 cells (Opipari et al.,

2004). In contrast, overexpression of Bcl2, in the human breast cancer cell line MCF7, nearly abolished apoptosis (Pozo-Guisado et al., 2005). This indicates, that at least in some cells, resveratrol may activate the intrinsic pathway independently of the Bcl2 family of anti- and pro-apoptotic proteins.

Caspase independent stimulation of cell death

Resveratrol inhibits the rat liver mitochondrial F0F1-ATPase activity (Zheng and Ramirez, 1999) and rat brain F0F1-ATPase (Zheng and Ramirez, 2000), but has no effect on Na^+/K^+-ATPase from porcine cerebral cortex (Zheng and Ramirez, 2000). Resveratrol inhibits extracellular ATP synthesis in human umbilical vein endothelial cells. No effect was observed on intracellular ATP synthesis (Arakaki et al., 2003, observation presented without data). ATPase inhibitors may stimulate apoptosis in some cell lines (Wolvetang et al., 1994) but suppress and delay apoptosis in others (Shchepina et al., 2002).

1.6 Resveratrol and NF-κB

1.6.1 The NF-κB family of transcription factors

Nuclear factor κB (NF-κB) proteins are transcription factors, which have been shown to be involved in regulation of at least 200 genes (Loop and Pahl, 2003; Pahl, 1999). In addition, more than 100 genes have been predicted to contain recognition sites for the transcription factors by computer-based methods (Shelest et al., 2003). The genes targeted by NF-κBs are involved in a large array of cellular events, such as immune and inflammatory responses, cell proliferation and apoptosis. Dysfunctional NF-κBs has been shown to be involved in a number of diseases, including heart diseases and cancer (Aggarwal, 2004; Dorai and Aggarwal, 2004; Libby, 2002; Robbesyn et al., 2004). A small selection of NF-κB target genes are listed in table 1.9. For an updated and comprehensive (albeit unrefereed) list, consult the website `http://www.nf-kb.org`.

The components of NF-κB controlled gene expression The five members of the mammalian family of NF-κBs are shown in table 1.10. The members are defined by their Rel homology domain (RHD), which is reponsible for DNA binding (reviewed in Chen and Greene, 2004). All members form homo- and heterodimers with each other, all with the capability of stimulating gene transcription. The only exception is RelB which only forms heterodimers with NF-κB1 p105/p50 or NF-κB2 p100/p52 and which acts as a transcriptional activator as well as a repressor (reviewed in Chen and Greene, 2004; Verma, 2004). Furthermore, homodimers of NF-κB1 p105/p50 and NF-κB2 p100/p52 may act as transcription repressors by binding to NF-κB sites on the DNA (Verma, 2004).

Table 1.9: A small selection of genes shown to be controlled by NF-κBs. Based on Loop and Pahl (2003)

Gene	Function
Regulators of apoptosis	
Bax	Pro-apoptotic
Bcl2	Anti-apoptotic
Bcl2L1_v1/Bcl-xL	Anti-apoptotic
Bcl2A1/Bfl1	Anti-apoptotic
Bcl2L10/Bcl-B	Anti-apoptotic
Fas/CD95	Pro-apoptotic
FasLG/CD95L	Pro-apoptotic
CFLAR/FLIP	Anti-apoptotic
Cell cycle	
Cyclin D1	Cell-cycle regulation
Cyclin D2	Cell-cycle regulation
Cyclin D3[a]	Cell-cycle regulation
E2F3	Cell cycle regulator
CdkN1A/p21/Cip1/Waf1[a]	Cyclin-dependent kinase inhibitor
Transcription factors	
Myc/c-Myc	Transcription factor
Rel/c-Rel	Transcription factor
RelB	Transcription factor
NF-κBiA/IκBα	Inhibitor of NF-κB
NF-κBiZ/IκBζ/Mail	Inhibitor of NF-κB
NF-κB1-105kDa	NF-κB1-50kDa precursor
NF-κB2-100kDa	NF-κB2-52kDa precursor
p53	Tumor suppressor
Cytokines	
TNF-α	Tumor necrosis factor
IL-1β	Interleukin 1, beta
Stress response	
Nos2A/iNos	Inducible nitric oxide synthase

[a]Indicates presence of a NF-κB site in the promoter, but not shown to be controlled by NF-κBs

Table 1.10: Overview of the NF-κB family, its inhibitors and the inhibitor kinases. Based on Bonizzi and Karin (2004); Chen and Greene (2004); Hayden and Ghosh (2004); Wong and Chung (2003)

Proteins	Complexes with
NF-κB	
Rel/c-Rel	Rel/c-Rel, RelA/p65, NF-κB1 p105/p50, NF-κB2 p100/p52
RelA/p65	RelA/p65, Rel/c-Rel, NF-κB1 p105/p50, NF-κB2 p100/p52
RelB	no homodimer, NF-κB1 p105/p50, NF-κB2 p100/p52
NF-κB1 p105/p50	NF-κB1 p105/p50, Bcl3/Bcl4, Rel/c-Rel, RelA/p65, RelB
NF-κB2 p100/p52	NF-κB2 p100/p52, Bcl3/Bcl4, Rel/c-Rel, RelA/p65, RelB
NF-κB inhibitors (NF-κBi/IκB)	
NF-κBiA/IκBα	All NF-κB dimers
NF-κBiB/IκBβ	All NF-κB dimers
NF-κBiE/IκBϵ	All NF-κB dimers
NF-κBiZ/IκBζ/Mail	All NF-κB dimers
Bcl3/Bcl4	All NF-κB dimers
NF-κB inhibitor kinases (IKBK/IKK)	
Chuk/IKK-α	Unknown
IKBKB/IKK-β	Unknown
IKBKG/IKK-γ/Nemo	Unknown
IKBKE/IKK-ϵ	Unknown

NF-κB translocates to the nucleus where it exerts the transcriptional activity. By masking the nuclear localisation sequence of the RHD (Karin et al., 2004), NF-κBs are mainly kept inactive in the cytosol. This activity control is carried out by a family of at least five proteins, collectively called NF-κB inhibitor (NF-κBi/IκB) (table 1.10) (reviewed in Wong and Chung, 2003). The family is defined by their ankyrin repeats which makes unprocessed NF-κB1 p105/p50 and NF-κB2 p100/p52 a member of this family as well (Karin et al., 2004).

In order to release and thus activate NF-κBs, the NF-κBi/IκB must be phosphorylated by a NF-κB inhibitor kinase (IKBK/IKK) (reviewed in Hayden and Ghosh, 2004). The known IKBK/IKKs are listed in table 1.10. The most common form of the IKBK/IKK complex consists of the catalytic subunits Chuk/IKK-α and IKBKB/IKK-β and the regulatory subunit IKBKG/IKK-γ/Nemo (Bonizzi and Karin, 2004).

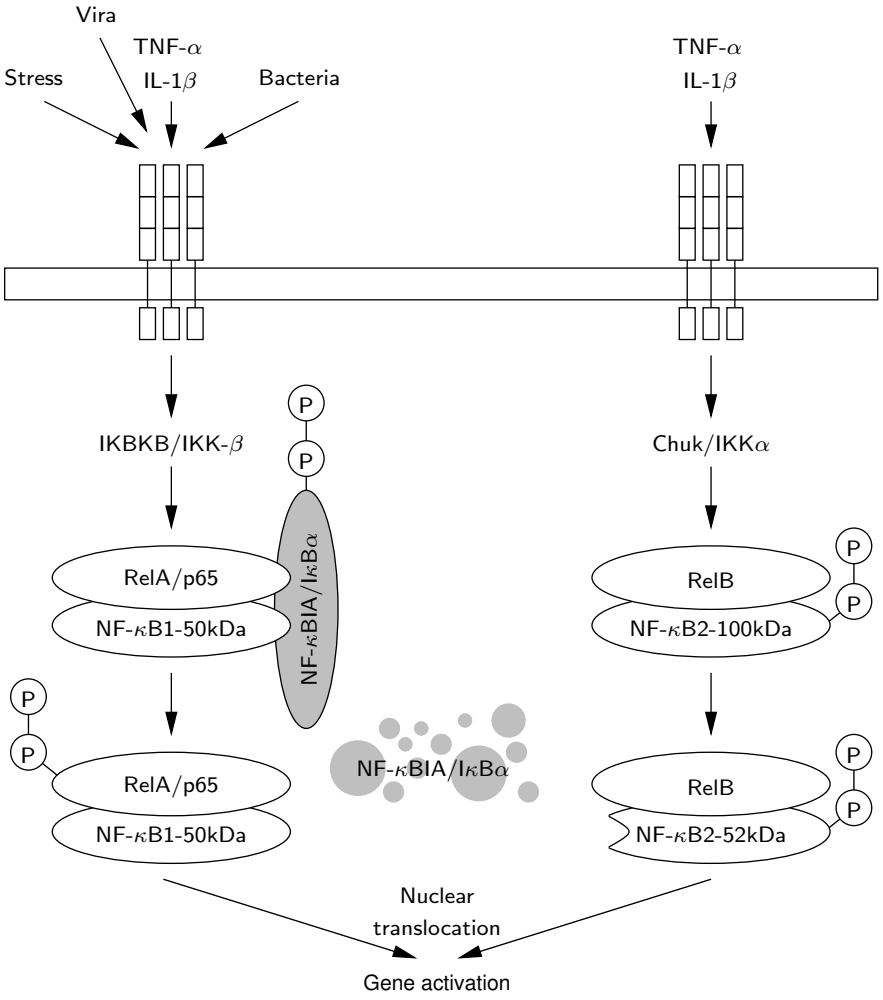


Figure 1.6: The NF-κB activation pathways. Simplified illustration to show the NF-κB activation pathways. In the classical pathway (left), NF-κBiA/IκBα is marked for degradation by phosphorylation (Ⓟ) by IKBKB/IKK-β. The uninhibited complex is phosphorylated before entry into the nucleus. In the alternative pathway (right), NF-κB2 p100/p52 is marked for proteolytic processing by phosphorylation be Chuk/IKK-α. The processed complex then translocated to the nucleus. Based on Bonizzi and Karin (2004); Chen and Greene (2004); Karin et al. (2004)

The NF-κB activation pathways There are in general two pathways leading to NF-κB mediated enhanced transcription of a gene: the classical pathway and the alternative pathway, recently reviewed in Bonizzi and Karin (2004). Both NF-κB activation pathways are activated by pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin 1, beta (IL-1β) (Bonizzi and Karin, 2004; Chen and Greene, 2004). In addition to cytokines, the classical pathway is also activated by stimuli such as stress, bacteria via the Toll-like

receptor, and viral infection (reviewed in Bonizzi and Karin, 2004). Depending on the pathway activated, different genes are expressed (Chen and Greene, 2004)

The classical NF-κB activation pathway RelA/p65⋈NF-κB1-50kDa and RelA/p65⋈NF-κB2-52kDa complexes are kept in the cytosol by attachment to NF-κBiA/IκBα, see figure 1.6. The IKBK/IKK complex acts predominantly through IKBKB/IKK-β in a IKBKG/IKK-γ/Nemo controlled fashion. Activated IKBK/IKK catalyses phosphorylation of NF-κBiA/IκBα of the RelA/p65⋈NF-κB1-50kDa⋈NF-κBiA/IκBα complex at serine residues near the N-terminal. The phosphorylation targets NF-κBiA/IκBα for ubiquitination and subsequent degradation by the 26S proteasome. The uninhibited RelA/p65⋈NF-κB1-50kDa complex is phosphorylated (Sakurai et al., 1999, 2003) and rapidly translocates to the nucleus where is helps general transcription factors and the RNA polymerase to assemble on the DNA (reviewed in Hayden and Ghosh, 2004). A negative feedback mechanism is provided by the NF-κB stimulated expression of NF-κBiA/IκBα, but increased expression of cytokines such as TNF-α and IL-1β, may also be the result of activation of the classical NF-κB pathway (Bonizzi and Karin, 2004; Verma, 2004)

The alternative NF-κB activation pathway This pathway is sometimes refered to as the canonical pathway. In contrast to the classical NF-κB activation pathway, the alternative pathway depends strictly on Chuk/IKK-α (figure 1.6) (reviewed in Karin et al., 2004). The inactive RelB⋈NF-κB2 p100/p52nfkb2:100 complex is activated by a Chuk/IKK-α homodimer, by phosphorylation of NF-κB2-100kDa at two C-terminal sites. This phosphorylation is necessary for ubiquitination of NF-κB2-100kDa and subsequent proteolytic processing of NF-κB2-100kDa to NF-κB2-52kDa. The active RelB⋈NF-κB2-52kDa complex translocates into the nucleus, and causes increased gene transcription (reviewed in Bonizzi and Karin, 2004).

1.6.2 The effect of resveratrol on NF-κB

The effect of resveratrol on unstimulated cells The effect of resveratrol on NF-κB activation in unstimulated cells is not consistent (table 1.11). Some results point to a positive effect of resveratrol on the NF-κB activity, while other observations show a negative effect or no effect at all. Some of the variability may be due to the concentrations used and/or incubation times, which could be sub-effective. Another reason for the diverging observations could be due to cell type specific effects of resveratrol.

The effect of resveratrol on stimulated cells In contrast to the variable effects reported of resveratrol on unstimulated cells, the effect on cytokin

Table 1.11: The effect of resveratrol on NF-κB activation in unstimulated cells.
↑ indicates an increase, ↓ indicates a decrease, and → indicates no effect

Model	Dose range	Stimulation time	Effect	Method	Reference
Huvec	0.1 and 1 µM	over night	↑	Western blot[a]	Pellegatta et al. (2003)
Hasmc	1 and 10 µM	12 h	↑	Western blot[a]	Juan et al. (2005)
Hasmc	1 and 10 µM	8 h	↑	EMSA[b]	Juan et al. (2005)
Hasmc	20 and 40 µM	8 h	↓	EMSA	Juan et al. (2005)
CaSki	75 µM	4 h	↓	EMSA	Woo et al. (2004)
MiaPaca-2	100 µM	24 h	↓	EMSA	Mouria et al. (2002)
MCF7	100 and 150 µM	36 h	↓	EMSA	Pozo-Guisado et al. (2005)
A549	12.5 to 50 µM	48 h	↓	Reporter gene	Kim et al. (2003)
Huvec	0.1 and 1 µM	1 h	→	Western blot[a]	Pellegatta et al. (2003)
Thp-1	30 µM	1.5 h	→	Reporter gene	Holmes-McNary and Baldwin (2000)
MCF7	10 to 50 µM	4 h	→	EMSA	Banerjee et al. (2002)
HeLa	5 µM	4 h	→	EMSA	Manna et al. (2000)
H4	5 µM	4 h	→	EMSA	Manna et al. (2000)
U-937	0.1 to 25 µM	4 h	→	EMSA	Manna et al. (2000)
Jurkat	5 µM	4 h	→	EMSA	Manna et al. (2000)
Kbm-5	20 to 100 µM	8 h	→	EMSA	Takada et al. (2004)
Hasmc	20 and 40 µM	12 h	→	Western blot[a]	Juan et al. (2005)
Hasmc	1 to 40 µM	12 h	→	Western blot[a]	Juan et al. (2005)
A293	10 and 30 µM	32 h	→	Reporter gene	Takada et al. (2004)

[a]Nuclear fraction [b]Electric mobility shift assay

Table 1.12: Resveratrol inhibits NF-κB activation in stimulated cells.
↑ indicates an increase, ↓ indicates a decrease, and → indicates no effect

Model	Dose range	Pretreatment time	Stimulating agent	Stimulation time	Effect	Method	Reference
HT29	25 to 100 µM	1 h	LPS[a]	6 h	↑	Reporter gene	Jeong et al. (2004)
Huvec	15 µM	30 min	LPS	1 h	↓	EMSA[b]	Carluccio et al. (2003)
Thp-1	30 µM	30 min	TNF-α	1 h	↓	Reporter gene	Holmes-McNary and Baldwin (2000)
Thp-1	30 µM	30 min	TNF-α or LPS	1 h	↓	EMSA	Holmes-McNary and Baldwin (2000)
CaSki	75 µM	4 h	TPA[c]	4 h	↓	EMSA	Woo et al. (2004)
MCF7	10 to 50 µM	4 h	TNF-α	30 min	↓	EMSA	Banerjee et al. (2002)
Ocim2	50 µM	4 h	IL-1β	1 h	↓	EMSA	Estrov et al. (2003)
Jurkat	5 µM	4 h	TNF-α	30 min	↓	EMSA	Manna et al. (2000)
HeLa	5 µM	4 h	TNF-α	30 min	↓	EMSA	Manna et al. (2000)
U-937	0.1 to 25 µM	4 h	TNF-α	30 min	↓	EMSA	Manna et al. (2000)
H4	5 µM	4 h	TNF-α	30 min	↓	EMSA	Manna et al. (2000)
Kbm-5	20 to 100 µM	8 h	TNF-α	30 min	↓	EMSA	Ashikawa et al. (2002)
Kbm-5	20 to 100 µM	8 h	TNF-α	30 min	↓	EMSA	Takada et al. (2004)
A293	10 and 30 µM	8 h	TNF-α	24 h	↓	Reporter gene	Takada et al. (2004)
Huvec	0.1 and 1 µM	30 min	TNF-α	30 min	→	Western blot[d]	Pellegatta et al. (2003)
Huvec	100 µM	2 h	TNF-α	1 h	→	EMSA	Pendurthi et al. (1999)
Thp-1	100 µM	2 h	LPS	1 h	→	EMSA	Pendurthi et al. (2002)

[a]Lipopolysaccharide [b]Electric mobility shift assay [c]12-O-tetradecanoylphorbol-13-acetate [d]Nuclear fraction

stimulated cells is far more consistent (table 1.12). Resveratrol generally inhibits the activity of NF-κB, and the effect appears to be independent of factors such as pretreatment duration and chemical properties of the stimulatory compound.

The only stimulatory effect on NF-κB by resveratrol has been reported using the colon carcinoma cell line HT29. Resveratrol used in doses of 25, 50 and 100 µM caused an increase in the levels of the *luciferase* gene product in a dose dependent fashion (Jeong et al., 2004). The NF-κB stimulatory effects of resveratrol appeared to peak at 50 µM, as 100 µM yielded less gene product. However, no statistical analysis was performed, so the significance of the observation is left to be determined.

1.7 Purpose

Resveratrol has been shown to exert a variety of cellular effects. 1) The cell cycle is modulated in a complex manner, where the phase affected depends on the resveratrol concentration used, incubation time, and cell type. 2) Apoptosis is stimulated mainly through the intrinsic pathway, though the extrinsic pathway is also activated. 3) Activation of NF-κB is inhibited.

The purpose of this study was to identify the effect of resveratrol on DNA synthesis and cell cycle progression. The apoptosis stimulating properties of resveratrol were also studied, as well as the possible role of NF-κB in cell cycle modulation and apoptosis.

Model of choice A suspension human promyelocytic leukemia cell line (HL-60), and an epithelial human hepatoma-derived cell line (HepG2), were chosen as models. Resveratrol has previously been shown to modulate the cell cycle of HL-60 and HepG2 cells (Ahmad et al., 2004; Delmas et al., 2000; Joe et al., 2002). The apoptosis stimulating effects of resveratrol on HL-60 cells is well established, though activation of the extrinsic pathway is not yet fully elucidated (Clement et al., 1998; Kang et al., 2003; Pervaiz, 2001; Roy et al., 2002; Surh et al., 1999; Su et al., 2005). Furthermore, resveratrol has been shown to be cytotoxic to different leukemia cells without any severe effect on normal periphal blood cells (Dörrie et al., 2001; Gautam et al., 2000; Tsan et al., 2002). The effect of resveratrol on NF-κB activation in HL-60 cells has not yet been reported.

Chapter 2

Materials and methods

2.1 Chemicals and reagents

Resveratrol, Camptothecin, TNF-α, Neutral Red (NR), 4',6-Diamidino-2-phenylindole (DAPI), Actinomycin-D, tetrazolium salt (MTT), bovine serum albumin (BSA), DMSO, nonfat dried milk, and anti-α-Tubulin were all obtained from Sigma. RPMI-1640, L-Glutamine, fetal calf serum (FCS), Glutamax, Hygromycin, LDS 4× Sample Buffer, NuPAGE 10× Sample Reducing Agent, MES-SDS buffer, antioxidant, and Transfer buffer were from Invitrogen. Phosphate buffered saline (PBS), Penicillin-Streptomycin-Glutamine mix (10000 units/ml penicillin, 10000 µg/ml streptomycin and 29.2 mg/ml L-Glutamine), and trypsin-EDTA were purchased from Gibco. Dulbecco's modified Eagle's medium (DMEM) was obtained from Cambrex. NaCl, NaH_2PO_4, ethanol, and methanol were from Merck. Chaps Cell Extract Buffer, DTT, Antibodies against Caspase-3, cleaved Caspase-3, RelA/p65, phosphorylated RelA/p65 (Ser536), and peroxidase-labelled anti-rabbit IgG were purchased from Cell Signaling. β-Actin and GAPDH antibodies were obtained from Abcam. 4 % Paraformaldehyde in phosphate buffer, pH 7.4 was from Bie & Berntsen. Phosphatase Inhibitor Set II and Protease Inhibitor Set III were purchased from Calbiochem. Tween-20 was obtained from ICN, and Restore Western Blot Stripping Buffer was from Pierce.

Resveratrol and Actinomycin-D were diluted in DMSO and kept at -20 °C until used. TNF-α was kept in aliquots at -20 °C and diluted in cultivation medium prior to use. No compound was stored for more than 6 months.

2.2 Cell lines

HL-60, a suspension human promyelocytic leukemia cell line, and HepG2, an epithelial human hepatoma-derived cell line, were obtained from the American Tissue Culture Collection. The epithelial human cervical carcinoma derived cell line, HeLa stably transfected with a luciferase reporter gene under the

transcriptional control of the NF-κB promoter (HeLa-pNF-kB-Luc) was the gracious gift of Claus Bekker Jeppesen, Novo Nordisk A/S.

Cell media The HL-60 cells were cultivated in RPMI-1640 supplemented with 200 mM L-Glutamine and 10 % heat-inactivated FCS.

The HepG2 cells were cultivated in DMEM supplemented with 1 % Penicillin-Streptomycin-Glutamine mix and 10 % heat-inactivated FCS.

The HeLa-pNF-kB-Luc cells were cultivated in DMEM supplemented with 1 % Penicillin-Streptomycin-Glutamine mix, 400 µg/ml Hygromycin, and 10 % heat-inactivated FCS. For the *luciferase* gene expression assays, the HeLa-pNF-kB-Luc cells were assayed in DMEM without phenol red supplemented with 10 mM Hepes, 2 mM Glutamax, and 10 % heat-inactivated FCS.

Cell cultivation All cells were maintained in a humidified 37 °C, 5 % CO_2 incubator. When the cell density reached approximately 80 % confluence, cells were subcultivated. HL-60 cells were subcultivated by diluting cells in fresh cultivation medium to approximatly 2×10^5 cells/ml. HepG2 and HeLa-pNF-kB-Luc were detached by trypsin-EDTA, and reseeded at approximatly 4×10^4 and 2×10^4 cells/cm^2, respectively. No cell line was passaged more than 30 times. All cell culture flasks as well as 96-well microtiter plate and 6-well Multidishes were fron Nunc, unless otherwise specified.

2.3 Thymidine incorporation assay

This assay utilises the incorporation of ^{3}H-labelled thymidine into newly synthesised DNA. Radioactivity is used as an index of cells progressing through the S phase of the cell, and thus an index of cell growth.

2.3.1 Cell treatment – HL-60

Variation 1 – concurrent resveratrol addition Exponentially growing HL-60 cells were diluted in fresh cultivation medium to 3×10^4 cells/ml. The cell suspension was passed in aliquots to centrifugal tubes (VWR International) and resveratrol or vehicle was added to obtain appropriate concentration (0.1 % vehicle). The cell suspensions were transferred to a sterile 96-well microtiter plate to a total volume of 200 µl/well. All outer wells received 200 µl PBS. Cells were left in the incubator for the desired duration. Thymidine was added and cells were harvested as described in 'Cell harvest and scintillation count'.

Variation 2 – asynchronous resveratrol addition Exponentially growing HL-60 cells were diluted in fresh cultivation medium to 6×10^4 cells/ml. The cell suspension was passed to a sterile 96-well microtiter plate to a total

volume of 100 µl/well. Stock solutions of resveratrol or vehicle (0.1 %) in cultivation medium was made and was kept in the dark in a standard incubator. At different time points prior to cell harvest, 100 µl resveratrol stock solution was passed to each well of the 96-well microtiter plate to a final volume of 200 µl/well. All outer wells received 200 µl PBS. The plates were left in the incubator for the desired incubation time. Thymidine was added and cells were harvested as described in 'Cell harvest and scintillation count'.

2.3.2 Cell treatment – HepG2

Exponentially growing HepG2 cells were diluted in fresh cultivation medium to 3×10^4 cells/ml and transferred to a sterile 96-well microtiter plate to a total volume of 200 µl/well. All outer wells received 200 µl PBS. Cells were placed in the incubator and allowed to adhere overnight. At the day of experiment, stock solutions of resveratrol or vehicle (0.1 %) in cultivation medium were made in centrifugal tubes (VWR International) and kept in the dark in the incubator. At different time points prior to cell harvest, medium was removed by gently inverting the plates, 200 µl resveratrol stock solution was added to each well. All outer wells received 200 µl PBS. The plates were left in the incubator for the desired incubation time. Thymidine was added and cells were harvested as described in 'Cell harvest and scintillation count'.

2.3.3 Cell harvest and scintillation count

Two hours before harvest 20 µl 6.25 µCi/ml ^{3}H-Thymidine (Amersham Biosciences) was added to each well, and the plates were left in the incubator. After incubation, cells were transferred to filter plates (PerkinElmer) by the use of a cell harvester (Packard) and left to dry overnight. 30 µl Microscint O scintillation liquid (PerkinElmer) was added to each well of the dried filter plates, and the plates were read in a TopCount NXT scintillation counter (Packard). The TopCount NXT was controlled by the TopCount NXT version 1.06 (Packard) external software on Windows 95.

2.4 Light-absorption scan

The difference in absorption maxima for *cis*- and *trans*-resveratrol was used to qualitatively determine whether *trans*-resveratrol disappears from a solution. PBS with or without 10 % heat inactivated FCS was left with vehicle, 40 or 80 µM resveratrol in centrifugal tubes (VWR International) in a humidified 37 °C, 5 % CO_2 incubator. After 0 to 7 days the solutions were transferred to a UV-transparent 96-well microtiter plate (Costar) to a total volume of 100 µl. The absorption spectra in the range of 200 to 1000 nm were obtained using a SpectraMax Plus 384 (Molecular Devices) microplate spectrophotometer con-

trolled by the SoftMax Pro version 4.7.1 (Molecular Devices) external software on Windows 2000.

2.5 Cell viability estimations

2.5.1 MTT assay

In MTT and other tetrazolium salt based assays insoluble formazan crystals are generated by enzymatic cleavage. The needed enzyme belongs to the respiratory chain of the mitochondria, although one report suggests the cytosol as being the primary residence (Bernas and Dobrucki, 2002). The assay was performed as described by Holian and Walter (2001).

Exponentially growing HepG2 cells were diluted in fresh cultivation medium to 3×10^4 cells/ml and transferred to a sterile 96-well microtiter plate to a total volume of 200 µl/well. All outer wells received 200 µl PBS. Cells were placed in the incubator and allowed to adhere overnight. At the day of treatment, cultivation medium was changed to cultivation medium with resveratrol, TNF-α with Actinomycin-D, TNF-α with vehicle (0.1 %), or vehicle (0.1 %). All outer wells received 200 µl PBS. The plates were left in the incubator for the desired incubation time.

Two hours prior to the end of incubation, 25 µl MTT (5 mg/ml diluted in dH_2O) was added to each well. After incubation, the MTT-medium mixture was discarded by gently inverting the plate. 200 µl DMSO was added to each well, and the plates were left on an orbital shaker for 5 min until all formazan crystals had dissolved. The optical density was read at 550 nm with 650 nm as reference wavelength on a SPECTRA Rainbow Absorbance Microplate Reader (Tecan). The SPECTRA Rainbow was controlled by the Magellan version 2.0 (Tecan) external software on Windows 95.

2.5.2 NR assay

This assay is based on the simple principle of active dye uptake by viable cells. HL-60 cells were stained with Neutral Red (NR) as previously described (Lowik et al., 1993; Rooseboom et al., 2002). Briefly; HL-60 cells were diluted to 3×10^4 cells/ml in cultivation medium. The cell suspension was passed in aliquots to centrifugal tubes (VWR International) and resveratrol or vehicle was added to obtain appropriate concentration (0.1 % vehicle). The cell suspensions were transferred to a sterile 96-well microtiter plate to a total volume of 200 µl/well. All outer wells received 200 µl PBS. Cells were incubated in the incubator for the desired incubation time.

Stock solution of 1 mg/ml NR was made by dissolving the dye in dH_2O, sterilised by filtering through a serum filter with a pore size of 0.2 µm (Nalgene), and shielded from light. A dye buffer was made from one part 1 mg/ml

NR stock solution and one part sterile 1.8 % NaCl. Two hours before destaining, 50 µl dye buffer was added to each well and the plates were left in the incubator. After incubation, cells were pelleted by centrifugation (400 g at 20 °C for 10 min), and medium was discharged by inverting the plate. 100 µl destaining buffer (0.05 M NaH_2PO_4 in 50 % (v/v) ethanol) was added to each well, and the plates were left on an orbital shaker for 15 min. The optical density was read at 550 nm with 650 nm as reference wavelength on a SPECTRA Rainbow Absorbance Microplate Reader (Tecan). The SPECTRA Rainbow was controlled by the Magellan version 2.0 (Tecan) external software on Windows 95.

2.6 Analysis by flow cytometry

2.6.1 Cell cycle analysis by PI staining

The distribution of cells in the cell cycle was analysed by the CycleTEST Plus DNA Reagent Kit (BD Biosciences). The assay is based on the method by Vindeløv et al. (1983) where nuclei are isolated using a mixture of a non-ionic detergent and trypsin. The DNA is subsequently stained with the DNA binding dye Propidium Iodine (PI).

The assay was performed according to manufacturer's protocol. Exponentially growing HepG2 cells were seeded at 1×10^5 cells/ml in 10 ml one day prior to resveratrol treatment and left to settle in a T25 flask. At the day of treatment, cultivation medium was changed to cultivation medium with resveratrol or vehicle (0.1 %). HL-60 cells were diluted to 3.5×10^5 cells/ml in fresh cultivation medium, resveratrol or vehicle (0.1 %) was added, and 3 ml cell suspensions were passed to each well of a sterile 6-well Multidish.

After tratment for 24 h 1×10^6 cells were pelleted by centrifugation (400 g at 4 °C for 5 min), washed twice with PBS, and incubated at room temperature with a buffer containing trypsin and a non ionic detergent. After 10 min trypsin inhibitor in RNase buffer was added, and cells were incubated at room temperature for 10 min. Subsequently, PI was added, and cells were incubated on ice for 10 min. Stained cells were analysed as described in 'Flow cytometric parameters' using the fluorescence intensity height, channel 2 (FL2-H) parameter. The size of nuclei was obtained using the forward scatter height (FSC-H) (particle size) parameter.

The approximate excitation and emission peaks of DNA bound PI are 535 nm and 617 nm, respectively.

2.6.2 Determination of cell size and granularity

To determine the effect on cell size and granularity, intact cells were analysed by the use of the FSC-H (particle size) and side scatter height (SSC-H) (parti-

cle complexity, (i.e. the amount and type of cytoplasmic granules) parameters of the flow cytometer.

HepG2 cells were seeded at 1×10^5 cells/ml in 10 ml one day prior to resveratrol treatment and left to settle in a T25 flask. At the day of treatment, cultivation medium was changed to cultivation medium with resveratrol or vehicle (0.1 %). HL-60 cells were diluted to 3.5×10^5 cells/ml in fresh cultivation medium and resveratrol or vehicle (0.1 %) was added, and 3 ml cell suspensions were passed to each well of a sterile 6-well Multidish. Before analysis by flow cytometry, HepG2 cells were detached from the flask by trypsin-EDTA and resuspended in fresh cultivation medium. HL-60 cells were briefly resuspended to ensure a uniform cell distribution. Cells in suspension were analysed as described in 'Flow cytometric parameters'.

2.6.3 Apoptosis evaluation by Annexin-V binding analysis

Apoptosis was evaluated by the Annexin V-EGFP Apoptosis Detection Kit (Stratech Scientific). The assay is based on the observations that apoptotic cells have lost the membrane asymmetry and exposes Phosphatidylserine (PS). Annexin-V binds to PS, and when conjugated to a fluorophor (enhanced green fluorescent protein (EGFP) in this case), the increase in bound Annexin-V, and thus an increase in apoptosis, can be evaluated by an increase in fluorescence. Using the DNA binding dye PI, cells with compromised membranes can be excluded. HL-60 cells treated with 10 µM Camptothecin for 4 h were used as positive control.

Exponentially growing HL-60 cells were diluted in fresh cultivation medium to 3.5×10^5 cells/ml. The cell suspension was transferred to a sterile 6-well Multidish to a total volume of 3 ml/well and resveratrol or vehicle was added to obtain appropriate concentration (0.1 % vehicle). Cells were incubated in the incubator for 18 h, and treated according to manufacturer's protocol: 1×10^6 cells were pelleted by centrifugation (400 g at 4 °C for 5 min), washed twice with PBS. Cells were resuspended in 500 µl calcium containing Binding Buffer. The cell suspensions were split to aliquots of 250 µl. One cell suspension received 5 µl Annexin-V-EGFP, the other 5 µl PBS. Both recieved 250 µl calcium containing Binding Buffer, and were left to incubate for 15 min in the dark at room temperature.

PI has a tendency to diffuse across the cell membrane, and therefore 5 µl PI was added immediately prior to analysis. Stained cells were analysed as described in 'Flow cytometric parameters' measuring Annexin-V and PI using fluorescence intensity height, channel 1 (FL1-H) and fluorescence intensity height, channel 3 (FL3-H), respectively. The FL3-H channel was to used to minimise overflow from FL1-H.

The approximate excitation and emission peaks of EGFP are 488 nm and 530 nm and of DNA bound PI are 535 nm and 617 nm, respectively.

2.6.4 Caspase-8 activity assessment

Activation of Caspase-8 was studied using the Vybrant FAM Caspase-8 Assay Kit (Molecular Probes). The assay uses the membrane permeable irreversible Caspase-8 inhibitor LETD-fmk. A carboxyfluorescein (FAM) group is linked to the inhibitor peptide as a reporter.

Exponentially growing HL-60 cells were diluted in fresh cultivation medium to 3.5×10^5 cells/ml. The cell suspension was transferred to a sterile 6-well Multidish to a total volume of 3 ml/well and resveratrol or vehicle was added to obtain appropriate concentration (0.1 % vehicle). Cells were incubated in the incubator for 12, 18, or 24 h, and treated according to manufacturer's protocol. Cells were diluted to 1×10^6 cells/ml in fresh cultivation medium. 300 µl cell suspension was mixed with 10 µl 30× FAM-LETD-fmk, and left to incubate in the dark for 60 min. After incubation cells were diluted in 1× wash buffer, and pelleted by centrifugation (400 g at 4 °C for 5 min), and washed twice in 1× wash buffer. Stained cells were analysed as described in 'Flow cytometric parameters' using the FL1-H parameter.

The approximate excitation and emission peaks of the FAM are 488 nm and 530 nm, respectively.

2.6.5 Flow cytometric parameters

All cells were analysed by flow cytometry on a FACSCalibur (Becton Dickinson) equipped with a standard 488 nm laser, and at least 20.000 events were counted. The flow cytometer was controlled by the CellQuestPro version 4.0.2 (BD Biosciences) external software on Mac OS 9.

2.7 DAPI staining

To visualise changes in nuclear morphology, cells were stained with the DNA binding dye DAPI and visualised with fluorescent microscopy. HL-60 cells treated with 10 µM Camptothecin for 4 h were used as positive control.

Exponentially growing HL-60 cells were diluted in fresh cultivation medium to 3.5×10^5 cells/ml. The cell suspension was transferred to a sterile 6-well Multidish to a total volume of 3 ml/well and resveratrol or vehicle was added to obtain appropriate concentration (0.1 % vehicle). Cells were incubated in the incubator for 12 or 18 h.

A stock solution of unit1mg /ml DAPI was made from DAPI dissolved in dH_2O, and diluted to 1 µg/ml in PBS. 500 µl cell suspensions of 2×10^5 cells/ml were spun onto coated glass slides (Thermo Electron) by centrifugation at 1300 rpm at room temperature for 5 min using a Cytospin 4 Cytocentrifuge (Thermo Electron). The slides were left to dry completely for at least 30 min to allow the cells to fully adhere. A hydrophobic barrier

was drawn around the dried cells using a DakoCytomation Pen (DakoCytomation). Cells were fixed with 4 % Paraformaldehyde in phosphate buffer, pH 7.4 at room temperature for 15 min, and washed with cold PBS in a Hellendahl-type staining jar (Bie & Berntsen) for approximately 3 min. Fixed cells were permeabilised with ice cold 0.1 % Triton-X at room temperature for 4 min, and washed twice with cold PBS. Permeabilised cells were stained with 1 µg/ml DAPI in the dark at room temperature for 5 min and washed once with cold PBS. Cover slips were mounted using Vectashield mounting solution (Vector Labs). Stained cells were visualised on a Leica DMR light microscope (Leica). Images of at least 6 random positions on the slide were captured with a Leica DC200 camera (Leica), using the Leica IM50 version 1.20 release 19 (Leica) external software on Windows 2000.

2.8 *Luciferase* gene expression assay

The LucLite Reporter Gene Assay System (PerkinElmer) was used according to manufacturer's protocol.

Exponentially growing HeLa-pNF-kB-Luc cells were diluted in fresh cultivation medium to 1.5×10^5 cells/ml and transferred to sterile white 96-well microtiter plate (PerkinElmer) to a total volume of 100 µl/well. All outer wells received 100 µl PBS. Cells were placed in the incubator and allowed to adhere overnight. At the day of treatment, cultivation medium was removed by carefully inverting the plates, and 100 µl assay medium containing resveratrol with or without 5 ng/ml TNF-α or vehicle (0.1 %) was added to each well. All outer wells received 100 µl PBS. The plates were left in the incubator for 4 h.

After incubation, medium was removed by carefully inverting the plates. Working in subdued light conditions, 100 µl assay medium was added together with 100 µl activated LucLite. The plates were sealed and incubated in the dark at room temperature for 30 min. Luminescence was read in a TopCount NXT luminescence counter (Packard). The TopCount NXT was controlled by the TopCount NXT version 1.06 (Packard) external software on Windows 95.

2.9 Western blot analysis

2.9.1 Buffers and solutions

The following buffers and solutions were used for this analysis:

1× **SDS-PAGE Sample Buffer** 1/4 vol LDS 4× Sample Buffer, 1/10 vol NuPAGE 10× Sample Reducing Agent, 1/100 vol Phosphatase Inhibitor Set II, 1/200 vol Protease Inhibitor Set III, and adjusted with dH_2O

1× **Chaps Cell Extract Buffer** 1/10 vol 10× Chaps Cell Extract Buffer, 1/200 vol DTT, 1/200 vol Protease Inhibitor Set III, and adjusted with dH_2O

5 % BLOTTO 5 g nonfat dried milk diluted in 100 ml PBS with 0.1 % Tween-20

5 % BSA 5 g BSA diluted in 100 ml PBS with 0.1 % Tween-20

2.9.2 Preparation of total cell lysate for immunoblotting anti-Caspase-3 and anti-cleaved Caspase-3

Exponentially growing HL-60 cells were diluted to 4×10^5 cells/ml in cultivation medium. The cell suspension was transferred to a sterile 6-well Multidish to a total volume of 3 ml/well. Resveratrol or vehicle (0.1 %) was added, and the plates were left in the incubator for 18 h.

After incubation, 1×10^6 cells were diluted in as much cold PBS as possible, pelleted by centrifugation (400 g at 4 °C for 5 min), and the supernatant was aspirated. Cells were washed once with cold PBS, transferred to Eppendorf tubes. Cell lysis was performed by adding 100 µl 1× Chaps Cell Extract Buffer. Cells were subjected to 3 cycles of freeze/thawing at -80 °C. Debris was pelleted by centrifugation at 10000 g at 4 °C for 10 min in an Eppendorf centrifuge and discarded. The supernatant was kept at -20 °C until used.

2.9.3 Preparation of total cell lysates for immunoblotting anti-RelA/p65, or anti-phosphorylated RelA/p65 (Ser536)

Treatment and preparation of HeLa-pNF-kB-Luc cells Exponentially growing HeLa-pNF-kB-Luc cells were diluted to 8.3×10^4 cells/ml in cultivation medium. The cell suspension was transferred to sterile 6-well Multidishes to a total volume of 3 ml/well and placed in the incubator and allowed to adhere overnight.

At the day of treatment, medium was removed by gently inverting the plates. 2 ml cultivation medium containing 5 ng/ml TNF-α with or without resveratrol or vehicle (0.1 %) was added. The plates were left in the incubator for the desired incubation time.

After incubation, medium was removed by gently inverting the plates and cells were washed once with 37 °C PBS. Cell lysis was performed by adding 500 µl 2× SDS-PAGE Sample Buffer to each well. Cell lysate was transferred to Eppendorf tubes and subjected to 3 cycles of freeze/thawing at -80 °C. Debris was pelleted by centrifugation at 10000 g at 4 °C for 10 min in an Eppendorf centrifuge and discarded. The supernatant was kept at -20 °C until used.

Treatment and preparation of HL-60 cells Exponentially growing HL-60 cells were diluted to 1×10^6 cells/ml in cultivation medium. The cell suspension was transferred to a sterile 6-well Multidish to a total volume of 2 ml/well. 5 ng/ml TNF-α with or without resveratrol or vehicle (0.1 %) was added. The plates were left in the for the desired incubation time.

After incubation, cells were diluted in as much cold PBS as possible, pelleted by centrifugation (400 g at 4 °C for 5 min) and the supernatant was aspirated. Cell lysis was performed by adding 200 µl 1× SDS-PAGE Sample Buffer. Cell lysates were transferred to Eppendorf tubes and subjected to 3 cycles of freeze/thawing at -80 °C. Debris was pelleted by centrifugation at 10000 g at 4 °C for 10 min in an Eppendorf centrifuge and discarded. The supernatants were kept at -20 °C until used.

2.9.4 Immunoblotting with anti-Caspase-3, anti-cleavedCaspase-3, anti-RelA/p65, or anti-phosphorylated RelA/p65 (Ser536)

Aliquots of 10 or 20 µl cell lysate were loaded and separated on 4-12 % gradient SDS-PAGE gels (Invitrogen). 1× MES-SDS buffer was used as running buffer, and 500 µl antioxidant was added to the top reservoir of the SDS-cell. A Full-Range Rainbow Molecular Weight Markers (Amersham Biosciences) was used as ladder.

Proteins were transferred to a PVDF membrane (Invitrogen) with a pore size of 0.45 µm, using 1× Transfer buffer with 10 % methanol. Membranes were blocked with 5 % BLOTTO for 1 h at room temperature or overnight at 4 °C, and incubated overnight at 4 °C with anti-Caspase-3 ($^1/_{1000}$ dilution in 5 % BLOTTO), anti-cleaved Caspase-3 ($^1/_{1000}$ dilution in 5 % BLOTTO), anti-RelA/p65 ($^1/_{1000}$ dilution in 5 % BSA), or anti-phosphorylated RelA/p65 (Ser536) ($^1/_{1000}$ dilution in 5 % BSA).

A mix of anti-α-Tubulin ($^1/_{6000}$ dilution), anti-β-Actin ($^1/_{250,000}$ dilution), and anti-GAPDH ($^1/_{100,000}$ dilution) all diluted in 5 % BLOTTO was used to check proper loading. Membranes were incubated with the loading control mix for 1 h at room temperature.

Blots were washed 6× for 10 min each with PBS with 0.1 % Tween-20 and incubated with peroxidase-labelled anti-rabbit IgG for 1 h at room temperature. The bands labelled with the antibody were visualised using ECL Advance chemiluminescent reagent (Amersham Biosciences) by exposure to a LAS3000 CCD camera (Fujifilm), using the Image reader LAS3000 version 1.1 (Fujifilm) external software on Windows 2000.

Membranes were stripped for loading control by incubating with Restore Western Blot Stripping Buffer on water bath for 60 to 80 min. Stripped membranes were kept in PBS with 0.1 % Tween-20 until probing with the loading control cocktail.

2.10 Software used

All calculations were performed using Microsoft Excel version 2002 SP-2 (Microsoft) and OpenOffice.org version 1.1.1 (OpenOffice.org). Flow cyto-

metric data were prepared for presentation by Weasel version 2.1, unlicensed (Walter and Eliza Hall Institute). CellQuestPro was utilised for the analysis of cell size, while cell cycle distribution was analysed by ModFitLT version 2.0 (Verity Software House) for Mac OS 9. Band intensities of Western blots were determined using Multi Gauge version 2.3 (Fujifilm) on Windows 2000. Graphs and data fit was done by Grace the 5.1.x series on an appropriate Linux system or Cygwin 1.5.11-1 and images were manipulated by The Gimp version 2.0.x on an appropriate Linux system.

2.11 Calculations

All cells were counted by Brker haemocytometer, depth: 0.1 mm, area of largest square: 0.04 mm^2 (VWR International), and the cell density was calculated by equation (2.1).

$$\text{cells/ml} = \frac{\text{cells counted} \times \text{dilution factor} \times 10^4 \text{ squares/ml}}{\text{number of squares counted}} \tag{2.1}$$

Relative thymidine incorporation, cell viability and relative relative luminescence unit (RLU) was calculated by equation (2.2).

$$\text{relative value} = \frac{\text{mean}_{\text{resveratrol treated}}}{\text{mean}_{\text{vehicle treated}}} \tag{2.2}$$

Relative band intensities was calculated by equation (2.3).

$$\text{relative intensity} = \frac{\text{intensity}_{\text{resveratrol treated}}}{\text{intensity}_{\text{vehicle treated}}} \tag{2.3}$$

Relative RelA/p65 phosphorylation calculated by equation (2.4), the relative intensities was found by equation (2.3).

$$\text{relative phosphorylation} = \frac{\text{relative intensity}_{\text{phosphorylated RelA/p65}}}{\text{relative intensity}_{\text{total RelA/p65}}} \tag{2.4}$$

2.12 Statistical analysis

Statistical analysis were performed using Student's two-tailed paired t-test.

Chapter 3

Results

The purpose of this study was to identify the effects of resveratrol on:

- DNA synthesis – examined by the thymidine incorporation assay
- Cell cycle progression – evaluated by flow cytometric analysis of binding of PI
- Apoptosis – assessed by DAPI staining, to visualise morphologic changes of the cell nucleus, flow cytometric analysis of Annexin-V binding and Caspase-8 activity, and Western blot using antibodies directed against cleaved Caspase-3
- Phosphorylation of NF-κB – investigated by Western blot using antibodies directed against phosphorylated RelA/p65.

Furthermore, the effect of fetal calf serum (FCS) on the levels of resveratrol, as well as that of resveratrol on the size of cell nuclei was also studied.

3.1 DNA synthesis

To study the possible effect of resveratrol on DNA synthesis, the thymidine incorporation assay was utilised, as described in 'Materials and methods', section 2.3.

Resveratrol induces a dose dependent inhibition of thymidine incorporation in HL-60 cells When performing the concurrent resveratrol addition variant of the thymidine incorporation assay, the concentration that reduces the thymidine incorporation by 50 % (IC_{50}) is altered in a time dependent manner. IC_{50} increases from 5 µM resveratrol at 2 h to 20 µM resveratrol at 24 h (figure 3.1). No differences in IC_{50} values was observed between

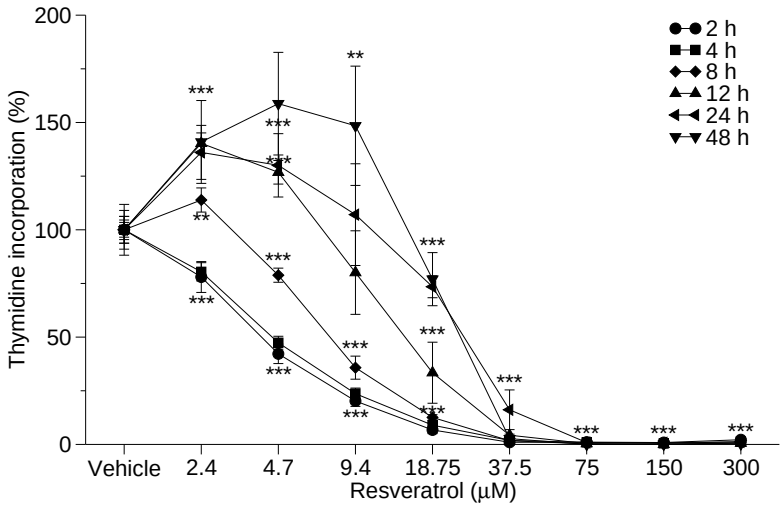


Figure 3.1: Resveratrol induces a time and dose dependent reduction in thymidine incorporation in HL-60 cells. Thymidine incorporation assay was utilised to study the effect of resveratrol on DNA synthesis in HL-60 cells. Resveratrol was added to the cells concurrently, and cells were harvested after the specified treatment times. Data is presented relative to untreated cells. The experiment was performed on 6 occasions and results of a typical experiment are shown, and presented as mean ±SD, $n = 6$. Overlapping data points are annotated with the lowest common p-value (** represents $p \leq 0.01$, *** represents $p \leq 0.001$ compared to vehicle treated cells)

2 and 4 h and between 24 and 48 h. This result indicates that resveratrol inhibits thymidine incorporation in a dose dependent manner, and that the IC_{50} increases as a function of time.

The effect of asynchronous addition of resveratrol to HL-60 cells
To study whether assay medium or cellular activity was the reason for the observed effects on thymidine incorporation, the assay was modified as described in 'Materials and methods', section 2.3.1.

When modifying the assay, no changes in IC_{50} values were observed from 2 to 12 h (figure 3.2), while IC_{50} values increased from 15 µM resveratrol at 12 h to 20 µM resveratrol at 24 and 48 h. The IC_{50} value at 24 and 48 h was not affected by the described modification. This altered result, compared to the result shown in figure 3.1, indicates that a reduction of active *trans*-resveratrol occurs in a non-cellular dependent manner.

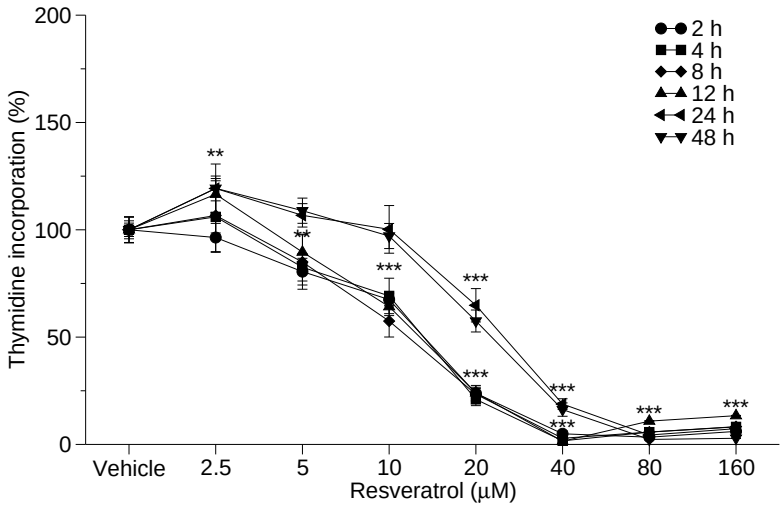
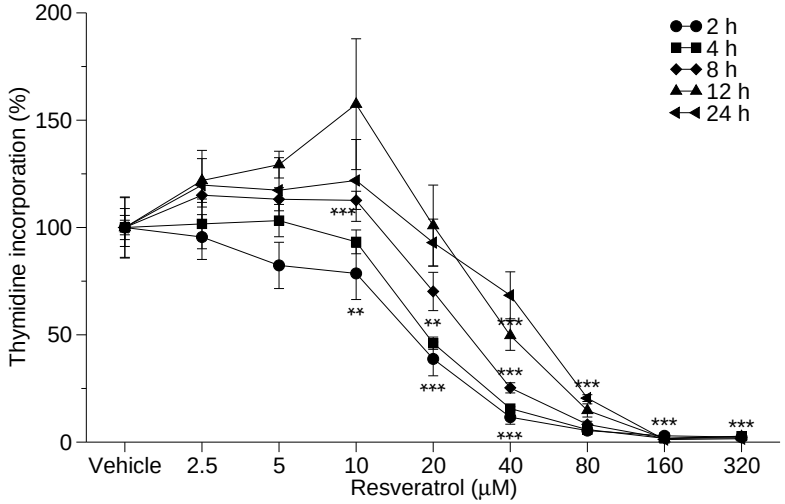


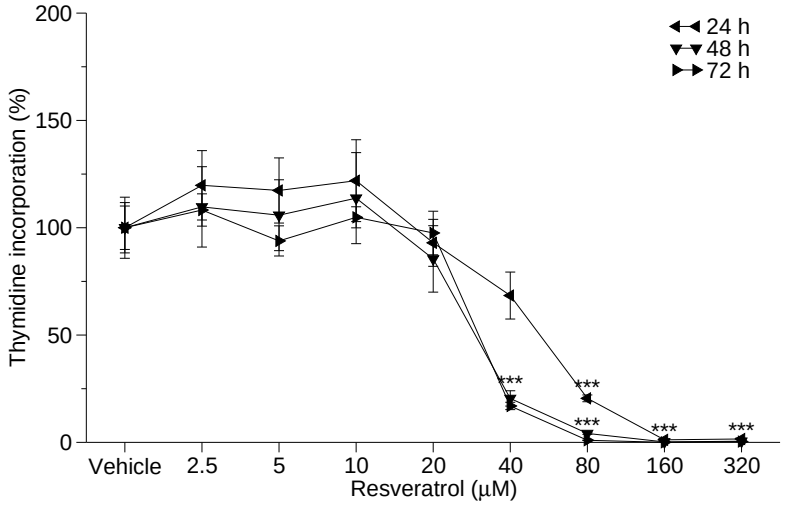
Figure 3.2: Resveratrol induces a time and dose dependent reduction in thymidine incorporation in HL-60 cells. Thymidine incorporation assay was utilised to study the effect of resveratrol on DNA synthesis in HL-60 cells. Resveratrol was added to cells at different time points, and all cells were harvested at the same time after the specified treatment times. Data is presented relative to untreated cells. The experiment was performed on 5 occasions and results of a typical experiment are shown, and presented as mean ±SD, $n = 6$. Overlapping data points are annotated with the lowest common p-value (** represents $p \leq 0.01$, *** represents $p \leq 0.001$ compared to vehicle treated cells)

Taken together, the results presented in figure 3.1 and figure 3.2 indicate, that resveratrol inhibits incorporation of thymidine in HL-60 cells in a dose dependent manner, and that levels of active *trans*-resveratrol may be diminished in cell cultivation medium. The latter is examined in further detail in section 3.2.

Resveratrol induces a time and dose dependent reduction in incorporation of thymidine in HepG2 cells When treated with resveratrol asynchronously, an increase in IC_{50} values of thymidine incorporation in HepG2 cells was observed (figure 3.3). The IC_{50} values ranged from 15 µM at 2 h to 50 µM at 12-24 h (3.3a), and finally decreased to 35 µM at 48 and 72 h (3.3b). This indicates that the modification of the IC_{50} value is a function of resveratrol dose and incubation time, when incubation time is less than 24 h. For incubation times greater than 24 h IC_{50} is modified as a function of dose and an inverse function of time in HepG2 cells.

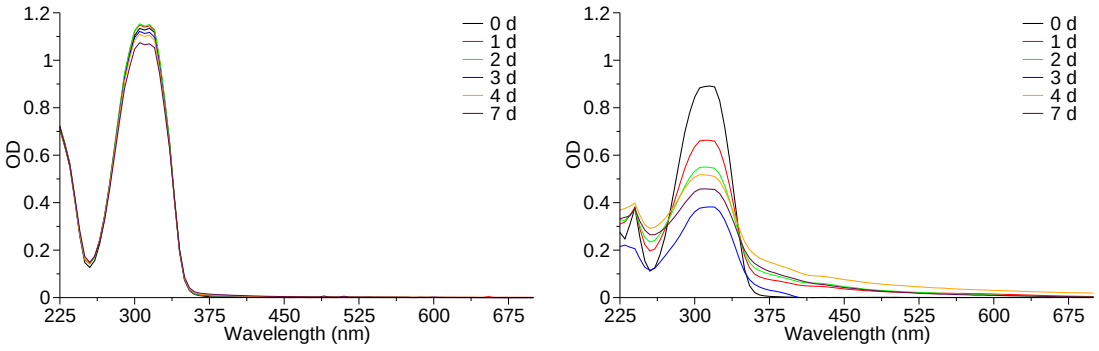


(a) Treatment for 2 to 24 h



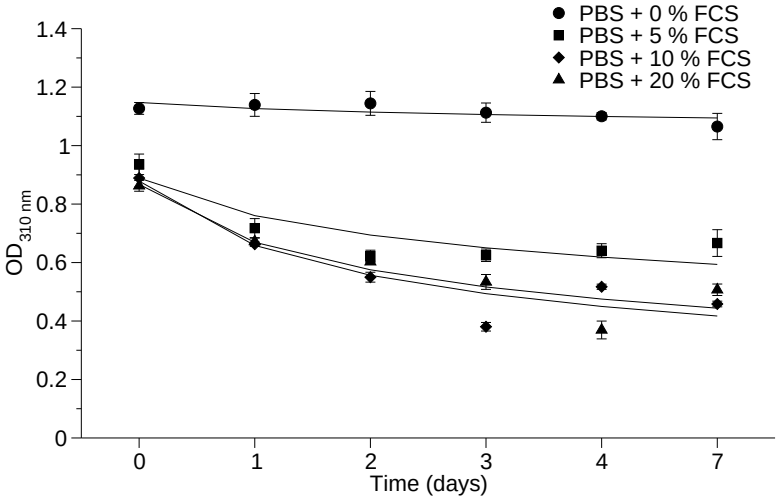
(b) Treatment for 24 to 72 h

Figure 3.3: Resveratrol induces a time and dose dependent reduction in thymidine incorporation in HepG2 cells. Thymidine incorporation assay was utilised to study the effect of resveratrol on DNA synthesis in HepG2 cells. Resveratrol was added to the cells asynchronously, and cells were harvested after the specified treatment times. To clarify the difference in time dependency, the results from the same experiment are split into 2 to 24 h (a) and 24 to 72 h (b). Data is presented relative to untreated cells. The experiment was performed on 3 occasions and results of a typical experiment are shown, and presented as mean ±SD, $n = 6$. Overlapping data points are annotated with the lowest common p-value (** represents $p \leq 0.01$, *** represents $p \leq 0.001$ compared to vehicle treated cells)



(a) 80 µM resveratrol in PBS

(b) 80 µM resveratrol in PBS+FCS



(c) Summary

Figure 3.4: FCS stimulates reduction of *trans*-resveratrol as a function of time. Absorption spectra of 80 µM resveratrol, corrected for background. Resveratrol was diluted in PBS alone (a) or in PBS with 10 % FCS (b) and incubated in a standard incubator. At the specified times aliquots of 100 µL was passed to a UV-transparent 96-well microtiter plate, and an absorption scan was performed. The result of a single experiment is presented as mean, $n = 3$. (c) the absorbance at OD_{310} of 80 µM resveratrol as a function of time and FCS concentration. Data is fitted to $y = A \times x^B$. The result of a single experiment is presented as mean ±SD, $n = 3$

3.2 Light-absorption scan

To further elucidate the role of cell cultivation medium on the apparent reduction in the levels of *trans*-resveratrol, resveratrol was diluted in PBS with or without FCS.

Presence of serum stimulates reduction in the levels of *trans*-resveratrol The absorption spectrum of resveratrol was obtained as described in 'Materials and methods', section 2.4. When diluted in PBS alone, the absorption spectrum of 80 µM was maintained for 7 days (figure 3.4a). The absorption spectrum of resveratrol diluted in PBS with 10 % FCS was clearly reduced as a function of time (figure 3.4b) by almost 33 % after 24 h and 50 % after 48 h. Using standard universal pH indicators, no changes in pH were found (data not shown)

In summary, this indicates that levels of resveratrol is constant when diluted in PBS alone, while the presence of 10 % FCS reduces the level of resveratrol.

The degree of reduction of *trans*-resveratrol is correlated to the levels of FCS (figure 3.4c). 5 % FCS stimulates reduction of the levels of *trans*-resveratrol to a lesser extent than 10 and 20 % FCS.

The absorption spectra shown in figure 3.4 were obtained for 80 µM resveratrol. Similar results were obtained for 40 µM resveratrol, and the difference in absolute absorption reflects the difference in resveratrol concentration (data not shown). The result obtained at 3 days is considered flawed.

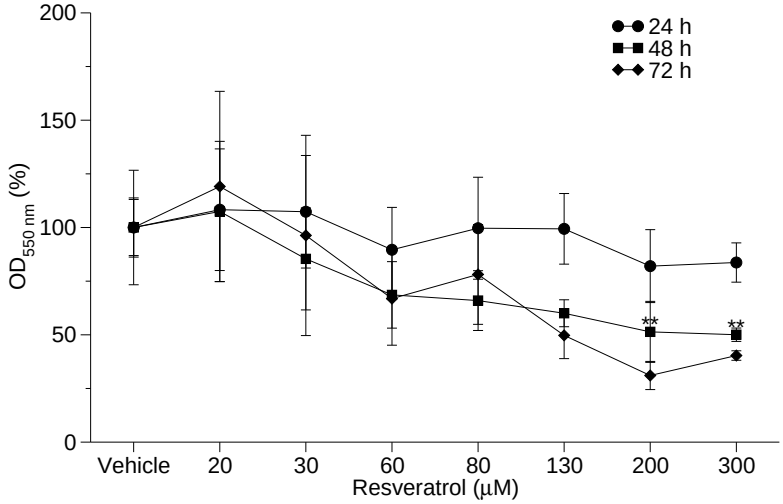
Notation of resveratrol concentrations To avoid confusion between resveratrol concentrations used in this report and values stated by others, all resveratrol levels mentioned are added start concentrations.

3.3 Interference with common cytotoxicity assays

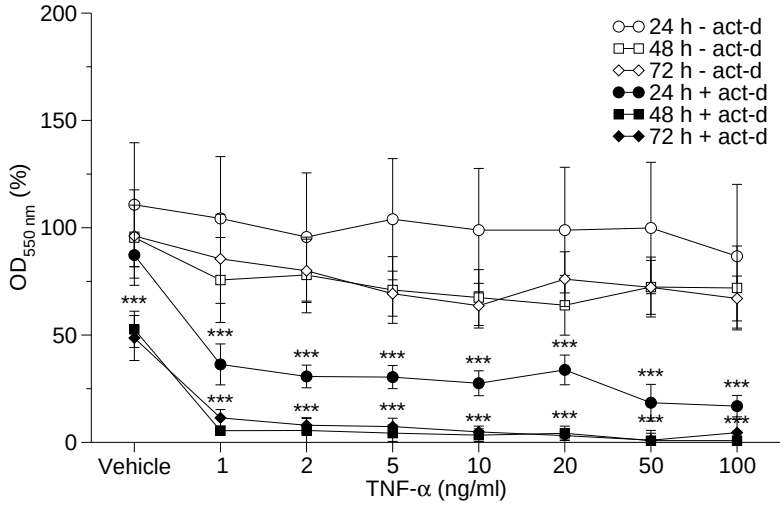
To further study the effects of resveratrol on cell proliferation, we turn to other assays to gain insight into the cytotoxicity of resveratrol on HL-60 and HepG2 cells.

Resveratrol interferes with the MTT assay The MTT assay is commonly used to asses cytoxicity of a compound and was performed as described in 'Materials and methods', section 2.5.1. Based on the data obtained by the thymidine incorporation assay, it was expected that a cytotoxic effect could be measured using the MTT assay. However, when treating HepG2 cells with resveratrol, the assay fails to produce statistically significant data (figure 3.5a).

The assay itself was demonstrated in the present work to be reliable by reproducing the results of Hill et al. (1995) and Yoshikawa et al. (1999) (figure 3.5b): TNF-α together with Actinomycin-D, but not alone stimulated cell death in HepG2 cells. There was no significant increase in cell death, when HepG2 cells were treated with TNF-α alone for up to 72 h. Co-treatment with TNF-α and Actinomycin-D reduced the viability of HepG2 cells after 24 h. Actinomycin-D alone significantly reduced viability after 48 and 72 h, and the reduction was further enhanced by co-treatment with TNF-α.



(a) Resveratrol



(b) TNF-α with and without Actinomycin-D

Figure 3.5: Resveratrol interferes with the result of the MTT assay. HepG2 cells were treated with test compound and viability was determined at the specified times by the MTT assay. Cells were treated with resveratrol (a). The experiment was performed on 6 occasions and results of a typical experiment are shown, and presented as mean ±SD, $n = 6$. Cells were treated with TNF-α with or without 0.5 µg/ml Actinomycin-D (b). The experiment was performed on 3 occasions and results of a typical experiment are shown, and presented as mean ±SD, $n = 6$. Data is presented relative to untreated cells. Overlapping data points are annotated with the lowest common p-value (** represents $p \leq 0.01$, *** represents $p \leq 0.001$ compared to vehicle treated cells)

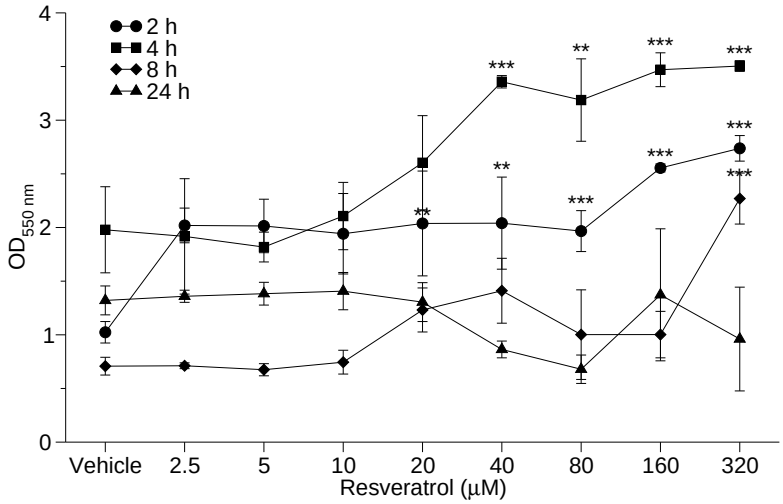


Figure 3.6: Resveratrol interferes with the result of the NR assay. HL-60 cells were treated with various concentrations of resveratrol and viability was determined at the specified times by the NR assay. The result of a single experiment is presented as mean ±SD, $n = 6$ (** represents $p \leq 0.01$, *** represents $p \leq 0.001$ compared to vehicle treated cells)

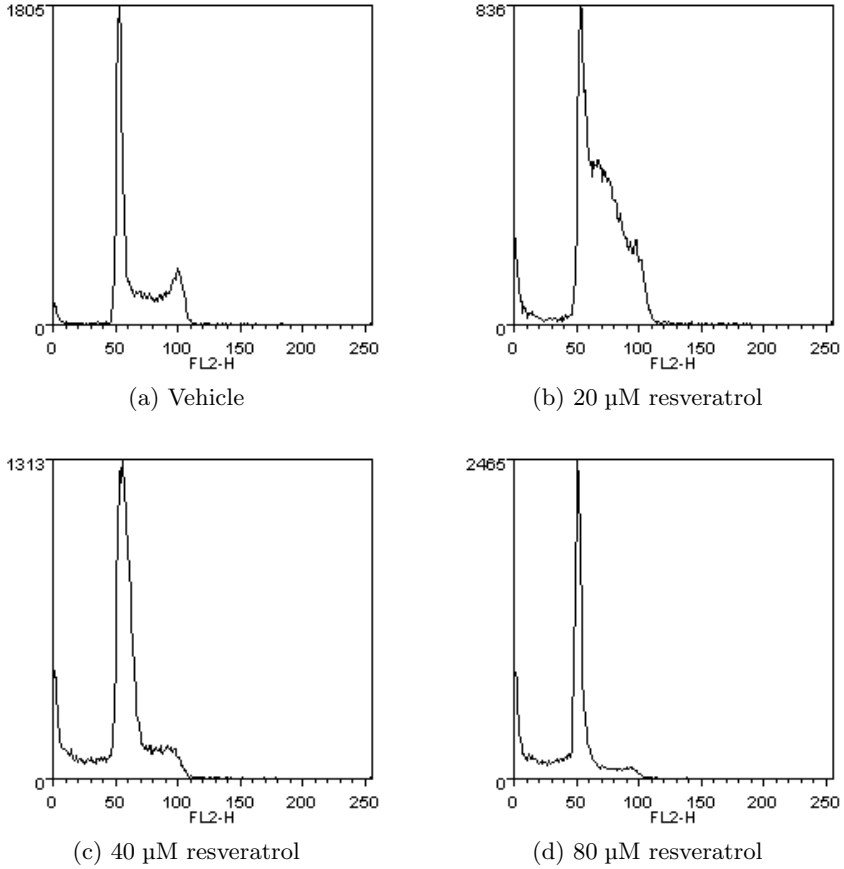
Taken together, these results indicate that in my hands resveratrol may interfere with the result on the MTT assay.

Resveratrol interferes with the NR assay To overcome the problems observed in the MTT assay, a different assay was chosen. The NR assay is based on active dye uptake by viable cells, as described in 'Materials and methods', section 2.5.2. The uptake of NR dye was an unreliable measurement of the viability of resveratrol treated HL-60 cells (figure 3.6), with large standard deviations and only few measurements with significance less than 5 % compared to vehicle treated cells. This demonstrates that resveratrol interferes with the NR assay.

3.4 Cell cycle progression

To further explore the effects of resveratrol induced inhibition of cell growth measured by thymidine incorporation, the cell cycle modulating properties of resveratrol were examined. These experiments were performed as described in 'Materials and methods', section 2.6.1.

Resveratrol induces cell cycle arrest in HL-60 cells After treatment with resveratrol for 24 h, there was a clear dose dependent induction of cell cycle accumulation (figure 3.7). At low doses (20 µM) the number of HL-60 cells



(a) Vehicle (b) 20 µM resveratrol

(c) 40 µM resveratrol (d) 80 µM resveratrol

Figure 3.7: Resveratrol induces cell cycle arrest in HL-60 cells. Cells were treated with vehicle (a), 20 (b), 40 (c), or 80 µM (d) resveratrol for 24 h and the cell membrane was removed. The nuclei were stained with PI and the DNA content was examined by flow cytometry. Data is presented as cell count as a function of DNA content. The results are summarised in figure 3.8.

in the S phase were clearly increased. At increased resveratrol concentrations (40 and 80 µM) the cell cycle was affected in the G_1 phase.

Resveratrol induces cell cycle arrest in HepG2 cells The effect on cell cycle progression in HepG2 cells (figure 3.9), appeared to follow the same pattern as was observed with HL-60 cells. Incubation with 20 µM resveratrol for 24 h had no effect on the cell cycle distribution, while 40 µM increased the number of cells in the S phase. At increased concentrations (80 µM), resveratrol also caused accumulation of cells in the S phase, but to a lesser extent than was observed for 40 µM.

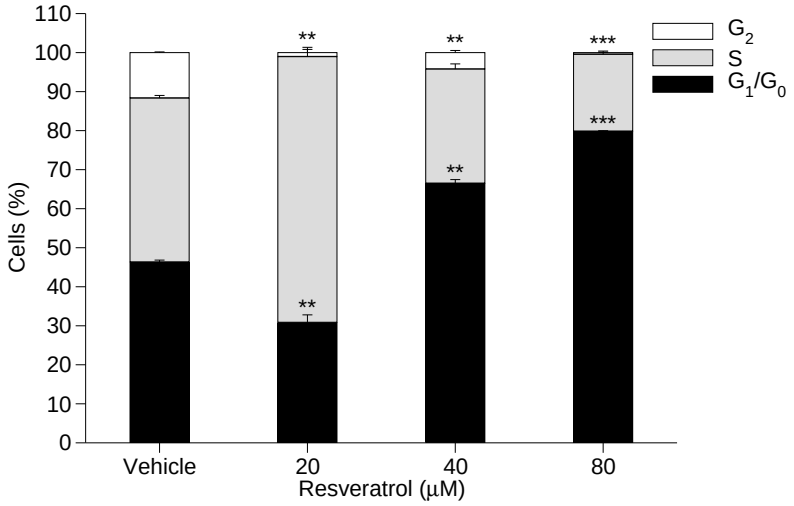


Figure 3.8: Summary of the cell cycle modulating effect of resveratrol in HL-60 cells. The results of figure 3.7 are summarised. Data is presented as relative phase distribution. The experiment was performed on 3 occasions and results of a typical experiment are shown, and presented as mean ±SD, $n = 3$. Overlapping data points are annotated with the lowest common p-value (** represents $p \leq 0.01$, *** represents $p \leq 0.001$ compared to vehicle treated cells)

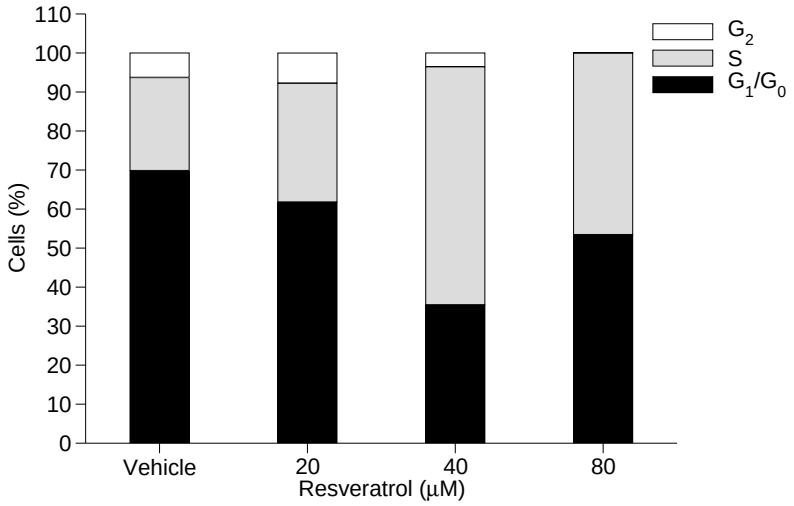
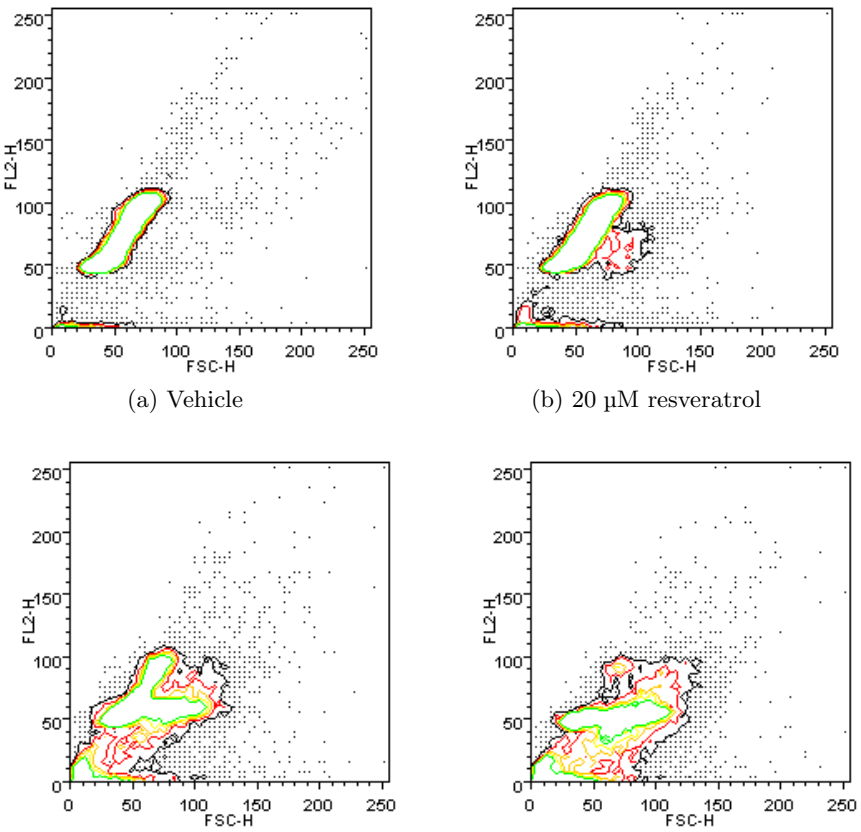


Figure 3.9: Resveratrol induces cell cycle arrest in HepG2 cells. Cells were treated with vehicle or 20, 40, or 80 µM resveratrol for 24 h. The nuclei were stained with PI and the DNA content was examined by flow cytometry. Data is presented as relative phase distribution. The experiment was performed on 3 occasions and results of a typical experiment are shown



(a) Vehicle (b) 20 µM resveratrol

(c) 40 µM resveratrol (d) 80 µM resveratrol

Figure 3.10: Resveratrol stimulates an increase in the nuclear size in HL-60 cells. Cells were treated with vehicle (a), 20 (b), 40 (c), or 80 µM (d) resveratrol for 24 h and the cell membrane was removed. Nuclei were stained with PI and the DNA content examined by flow cytometry. Data is presented as contour plots of DNA content (FL2-H) as a function of nucleus size FSC-H. Cell number is represented as iso-lines. The sub-G_1 population of dead cells is visible in the lower left corner. The experiment was performed on 3 occasions and results of a typical experiment are shown

These two results demonstrate that resveratrol modulates the cell cycle *in vitro*. The phase affected by resveratrol treatment depends on the concentration used.

3.5 Modulation of the size of cell nuclei

The effect of resveratrol on the size of HL-60 cell nuclei was studied as described in 'Materials and methods', section 2.6.1. Based on the observations of the cell cycle modulating effects of resveratrol, an increase in the size of the nucleus was expected only for HL-60 cells treated with 20 µM resveratrol, and HepG2 cells treated with 40 µM resveratrol. The reasoning was that these concentrations

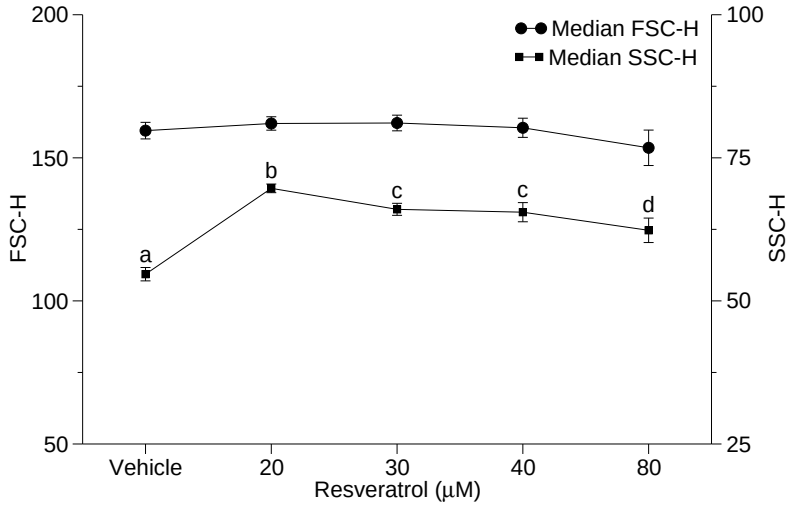


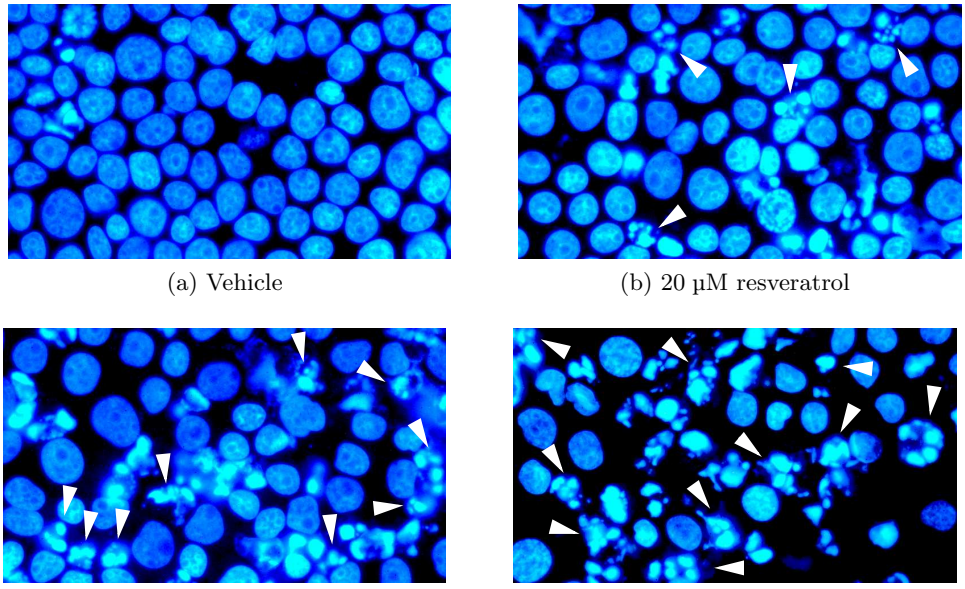
Figure 3.11: Resveratrol stimulates an increase in granularity but does not alter cell size in HL-60 cells. Cells were treated with vehicle or 20, 30, 40 or 80 µM resveratrol for 24 h. The size (FSC-H; left axis) and granularity (SSC-H; right axis) of the cells was examined by flow cytometry. The experiment was performed on 3 occasions and results of a typical experiment are shown, and presented as mean ±SD, $n = 3$. Data points not sharing a letter are significantly different ($p \leq 0.001$)

stimulated S phase accumulation of cells, while increased doses stimulated accumulation in the G_1 phase of the two cell lines.

Resveratrol stimulates an increase of the size of HL-60 nucleus Modulation of cell cycle progression of HL-60 cells caused by resveratrol was correlated with an increase in the size of the nucleus (figure 3.10). This correlation of the nucleus size and the cell cycle phase was possible to evaluate as cellular membranes were removed by the use of the non-ionic detergent. It is predominantly cells in the G_1 phase which display enlarged nuclei, but other stages of the cell cycle are also affected. The observation derives from studying the iso-lines of the contour plots of figure 3.10 as a function of particle size (FSC-H) and bound PI (FL2-H). A putative sub-G_1 population of dead cells is visible in the lower left corner. An increase in size of nucleus was not observed in HepG2 cells (data not shown).

These results indicate that resveratrol stimulates an increase in the nuclear size of HL-60 cells.

Increase in nucleus is not accompanied by an increased cell size To examine whether the increase in the size of the nucleus was paralleled by an increase in the volume of the cell, HL-60 cells were treated with resveratrol for 24 h and analysed by flow cytometry, as described in 'Materials and methods', section 2.6.2. It was demonstrated that resveratrol had no effect on cell size of HL-60 cells as indicated by the median of the forward scatter (figure 3.11).



(a) Vehicle (b) 20 µM resveratrol

(c) 40 µM resveratrol (d) 10 µM camptothecin

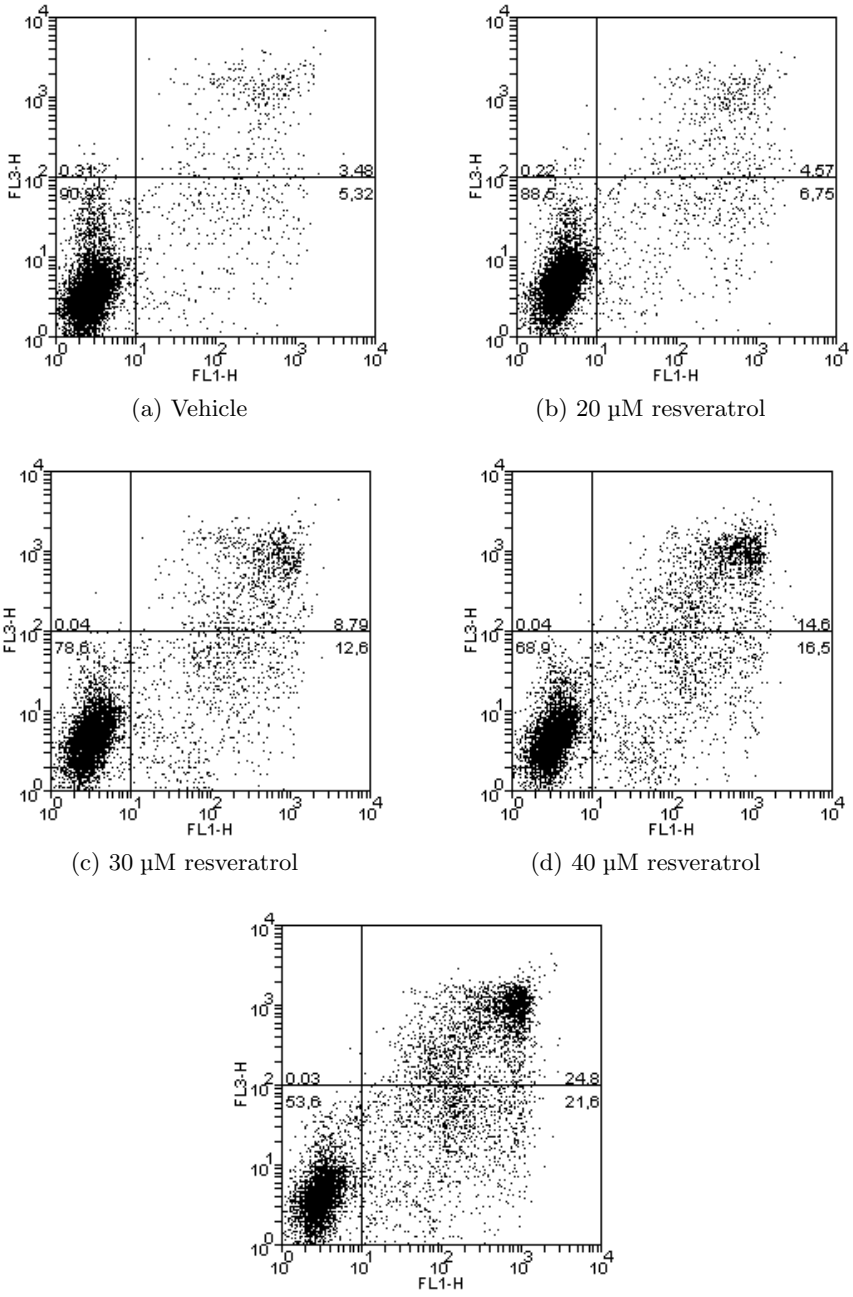
Figure 3.12: Resveratrol stimulates morphological changes of the cell nucleus of HL-60 in a dose dependent manner. Cells were treated with vehicle (a), 20 µM (b), or 40 µM (c) resveratrol for 18 h. After treatment, cells were stained with DAPI and the nuclei visualised under a fluorescence microscope. 10 µM camptothecin (d) (4 h incubation) was used as positive control. Morphological changes are indicated by white arrowheads. The experiment was performed on 4 occasions and results of a typical experiment are shown

Resveratrol induces an increase in cell granularity of HL-60 cells Low doses of resveratrol (20 µM) induced a larger granularity (i.e. altered the amount and/or type of cytoplasmic granules) in HL-60 cells, as measured by the median of the SSC-H, compared to control cells (figure 3.11). Higher doses counteracted the increase in granularity to a minor yet significant degree compared to that of 20 µM resveratrol.

These results demonstrate that the observed increase in nucleus size of HL-60 does not correlate to an increase in cell size. However, resveratrol stimulates an increase in cellular granularity in a dose dependent manner.

3.6 Apoptosis

With the aim of studying the effect of resveratrol on apoptosis in HL-60 cells, several assays were applied. It was found that resveratrol stimulated condensation and fragmentation of the cell nucleus, increased the binding of Annexin-V, and stimulated cleavage of Procaspase-3 and stimulated the activity of Caspase-8.



(a) Vehicle

(b) 20 µM resveratrol

(c) 30 µM resveratrol

(d) 40 µM resveratrol

(e) 80 µM resveratrol

Figure 3.13: Resveratrol stimulates an increase in Annexin-V binding in a dose dependent manner in HL-60 cells. Cells were treated with vehicle (a), 20 (b), 30 (c), 40 (d), or 80 µM (e) resveratrol for 18 h. After resveratrol treatment, cells were double stained with Annexin-V and PI, and examined by flow cytometry. Data is presented as PI (FL3-H) versus Annexin-V-EGFP (FL1-H), with the numbers indicating the percentage of cells in each quadrant. The experiment was performed on 3 occasions and results of a typical experiment are shown. The data are summarized in figure 3.14

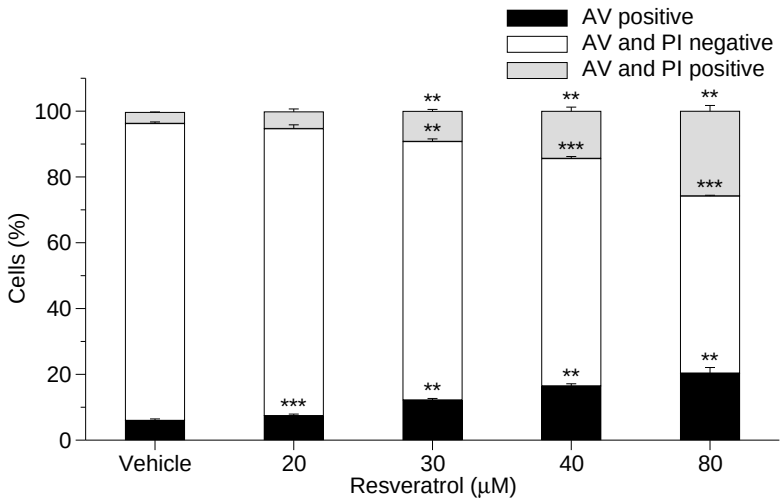
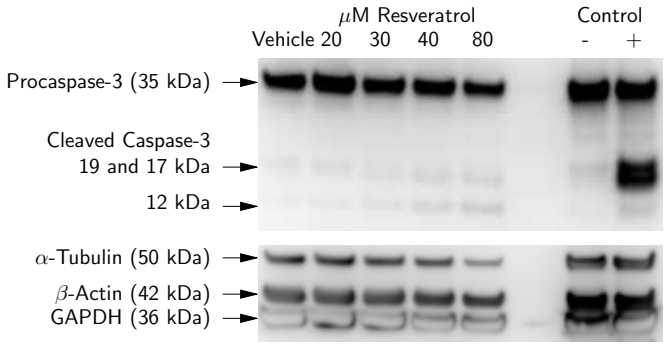


Figure 3.14: Resveratrol stimulates an increase in Annexin-V binding in a dose dependent manner. Graphical representation of the results in figure 3.13. Data is presented relative to untreated cells. The experiment was performed on 3 occasions and results of a typical experiment are shown, and presented as mean ±SD, $n = 3$ (** represents $p \leq 0.01$, *** represents $p \leq 0.001$ compared to vehicle treated cells)

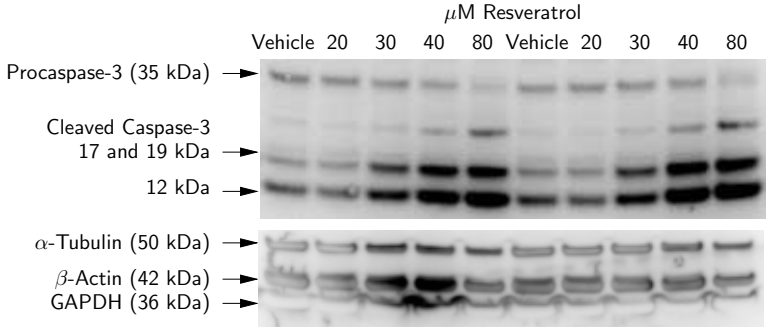
Resveratrol induces changes in nuclear morphology The ability of resveratrol to modulate apoptosis was initially studied by examining nuclear morphological changes associated with apoptosis as described in 'Materials and methods', section 2.7. In a preliminary study, the optimal incubation time of HL-60 cells with resveratrol was found to be 12 to 18 h (data not shown). When treated with resveratrol for 18 h, there was an obvious dose dependent increase in the number of HL-60 nuclei with a condensed and fragmented morphology (figure 3.12). This demonstrates a dose-dependent stimulation of apoptotis by resveratrol.

Binding of Annexin-V increases with increasing resveratrol concentration To further study the apoptosis modulating effect in HL-60 cells by resveratrol, the Annexin-V binding assay was applied as described in 'Materials and methods', section 2.6.3. Figure 3.13 shows the result of flow cytometric analysis of binding of EGFP conjugated Annexin-V. These results are summarised in figure 3.14. Apoptotic cells in this assay binds Annexin-V but exclude the nuclear binding dye PI, i.e. cells with lost membrane asymmetry but intact membranes.

HL-60 cells treated with resveratrol for 18 h showed a concentration dependent increase in Annexin-V positive/PI negative cells (figure 3.13). Loss of membrane integrity occurred as a function of resveratrol concentration at the same rate as Annexin-V positive only cells.



(a) Anti-Procaspase-3



(b) Anti-cleaved Caspase-3

Figure 3.15: Resveratrol stimulates cleavage of Procaspase-3 in HL-60 cells. Cells were treated with specified resveratrol concentrations for 18 h and prepared for Western blot. The controls were Jurkat cells with (+) or without (−) treatment with Cytochrome *c* and were provided with the antibodies. Immobilised protein was probed with anti-Procaspase-3 (a) or anti-cleaved Caspase-3 (b), where cell lysate was loaded twice to the same SDS-PAGE gel. The amount of antigen was analysed by density measurements of bands and is summarised in figure 3.16

As shown in figure 3.14 only a minor effect of 20 µM resveratrol on HL-60 cells was found, while higher concentrations greatly stimulated Annexin-V binding and membrane permeability.

Resveratrol stimulates Caspase-3 cleavage in a dose dependent fashion The observed changes in nuclear morphology and increased binding of Annexin-V suggest involvement of Caspase-3 in resveratrol stimulated apoptosis. Western blot detecting cleaved Caspase-3 was applied to study the effect of resveratrol on cleavage of Caspase-3 as described in 'Materials and methods', section 2.9. Resveratrol had no significant effect on the levels of Procaspase-3 (figure 3.15a), but exerted a stimulating effect on cleavage of Procaspase-3 into the larger and smaller catalytic subunits of 17 and 12 kDa (figure 3.15b).

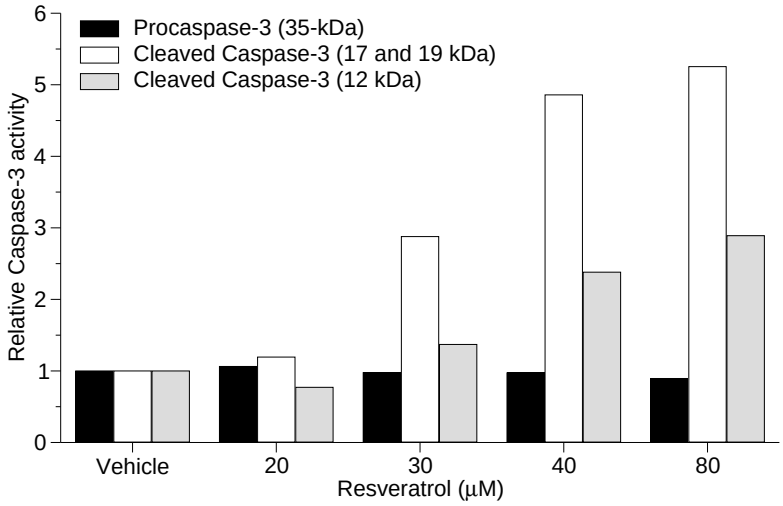
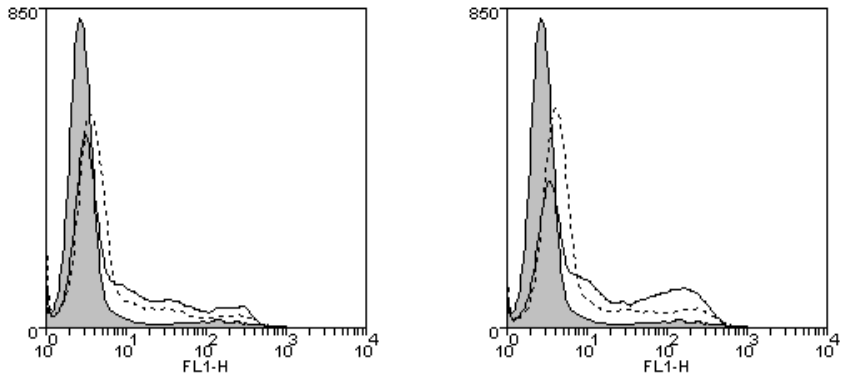


Figure 3.16: Summary of resveratrol stimulated cleavage of Caspase-3 in HL-60 cells. The amount of antigen shown in figure 3.15 was analysed by density measurements of the bands. Caspase-3 cleavage is expressed in relative values with vehicle controls arbitrarily set as 1. The experiment was performed on 3 occasions and results of a typical experiment are shown



(a) 18 h (b) 24 h

Figure 3.17: Resveratrol stimulates Caspase-8 activity in HL-60 in a dose and time dependent manner. Cells were treated with resveratrol for 18 (a) and 24 h (b), incubated with FAM labelled Caspase-8 inhibitor, and analysed by flow cytometry. Data is presented as counts as a function of fluorochrome bound to Caspase-8. The result of a single experiment is shown.
Legend: Gray area is cells treated with vehicle, dotted line is 20 µM, and full line is 80 µM resveratrol

Negligible effect of 20 µM resveratrol on the levels of catalytic Caspase-3 subunits was observed, while higher concentrations of resveratrol clearly stimulated cleavage of Procaspase-3 (figure 3.16).

Resveratrol stimulates Caspase-8 activity To study the involvement of the extrinsic pathway in the apoptosis modulating effects of resveratrol, Caspase-8 activity was assayed by flow cytometry as described in 'Materials and methods', section 2.6.4. In a preliminary study incubation with resveratrol for 18 h (figure 3.17a) and 24 h (figure 3.17b) showed a stimulated binding of a specific Caspase-8 inhibitor labelled with the FAM fluorophor. This binding was increased with increased resveratrol concentration and longer incubation time. Thus an increased Caspase-8 activity as a function of resveratrol concentration and treatment time can be observed.

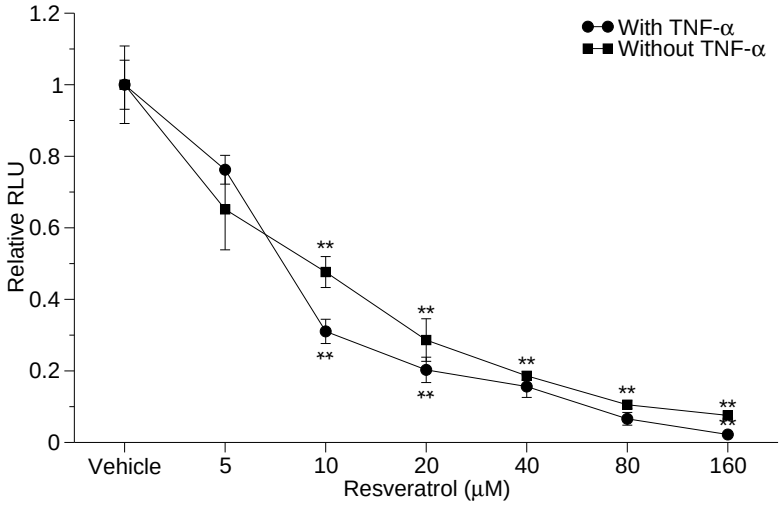
3.7 Phosphorylation of NF-κB

The possible role of NF-κB in the stimulation of apoptosis by resveratrol was studied. It was found that resveratrol modulated the product of a NF-κB controlled reporter gene in HeLa-pNF-kB-Luc cells. It was also found that resveratrol does not modulate the phosphorylation of NF-κB in HL-60 cells as studied by the use of Western blot for phosphorylated RelA/p65.

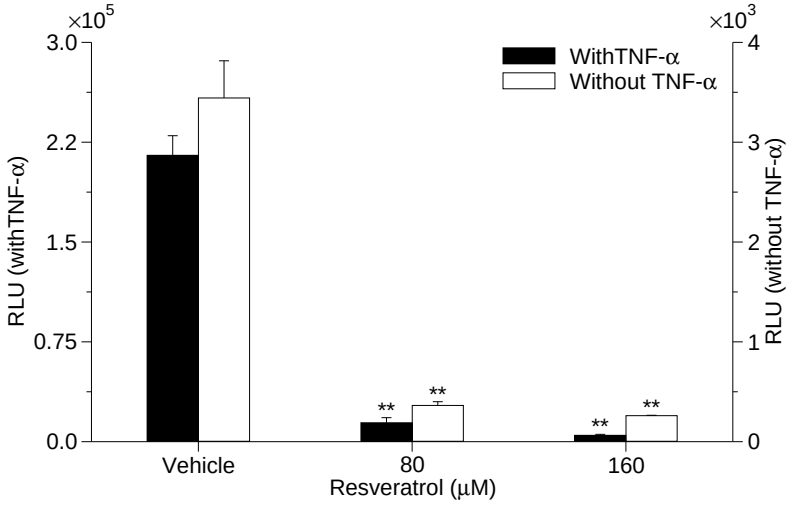
Resveratrol reduces levels of NF-κB controlled *luciferase* gene product HeLa-pNF-kB-Luc cells are HeLa cells stably transfected with a luciferase reporter gene under the transcriptional control of the NF-κB promoter. The *luciferase* gene expression assay was performed as described in 'Materials and methods', section 2.8.

It was found that 5 ng/ml tumor necrosis factor (TNF-α) induced more than 60 fold increase in *luciferase* products (data not shown). The effect of resveratrol on TNF-α induced *luciferase* product was subsequently examined (figure 3.18). Resveratrol was capable of reducing the levels of *luciferase* product (figure 3.18a), with an IC_{50} value of 10 µM in both TNF-α stimulated and unstimulated cells, as measured by RLU. However, when co-treated with TNF-α, the inhibitory effect of resveratrol was 40 fold, compared to a 10 fold inhibition of unstimulated cells (figure 3.18b). Preincubation with resveratrol for 4 h did not enhance the effect (data not shown).

RelA/p65 is rapidly phosphorylated and dephosphorylated in HL-60 and HeLa-pNF-kB-Luc cells To establish the time curve of TNF-α stimulated RelA/p65 phosphorylation in HL-60, cells were treated with 5 ng/ml TNF-α for various durations between 0 and 4 h, and submitted to Western blot for anti-RelA/p65 and anti-phosphorylated RelA/p65, with HeLa-pNF-kB-Luc as a positive control (figure 3.19).

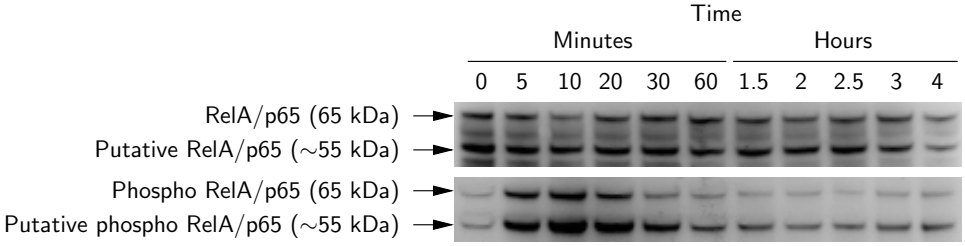


(a) Relative to untreated

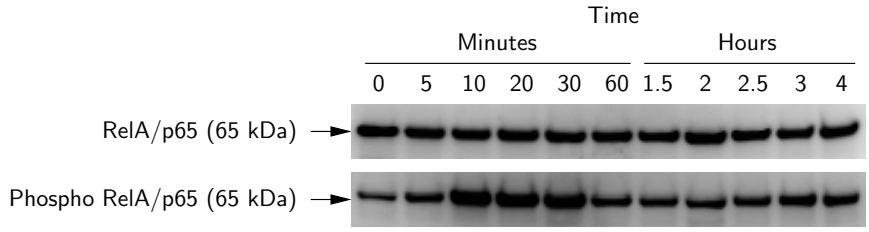


(b) Reduction in RLU

Figure 3.18: Resveratrol inhibits TNF-α stimulated transcription of the *luciferase* gene in HeLa-pNF-kB-Luc cells. Cells were treated with specified concentrations of resveratrol, or vehicle alone in the presence or absence of 0.5 ng/ml TNF-α. Levels of expressed *luciferase* gene was examined by luminescence. Data is presented as RLU relative to untreated cells (a) and RLU (b). The experiment was performed on 3 occasions and results of a typical experiment are shown, and presented as mean $\pm$SD, $n = 3$. Overlapping data points are annotated with the lowest common p-value (** represents $p \leq 0.01$ compared to vehicle treated cells)



(a) HL-60

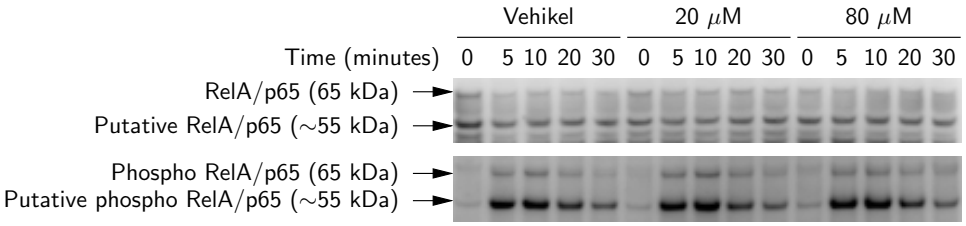


(b) HeLa-pNF-kB-Luc

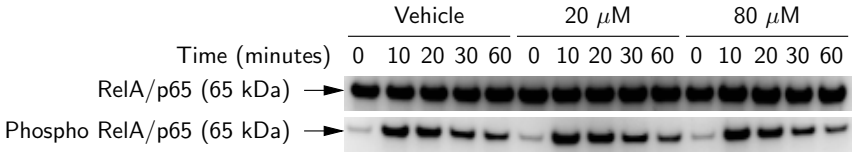
Figure 3.19: TNF-α stimulates rapid and transient phosphorylation of RelA/p65 in HeLa-pNF-kB-Luc and HL-60 cells. HL-60 (a) and HeLa-pNF-kB-Luc (b) cells were treated with 5 ng/ml TNF-α for the specified times and prepared for Western blot. Immobilised protein was probed with anti-RelA/p65 or anti phosphorylated RelA/p65. The result of a single experiment is shown

Phosphorylation of RelA/p65 in HL-60 cells RelA/p65 appears to exist mainly in a truncated form of 55 kDa in HL-60 cells (figure 3.19a). Total levels of both the 65 kDa and 55 kDa fragments declined between 3 and 4 h after addition of 5 ng/ml TNF-α. Both fragments had low background levels of RelA/p65 phosphorylation. The phosphorylation of both fragments peaked after 10 min with TNF-α and then declined over the course of 4 h after 30 min.

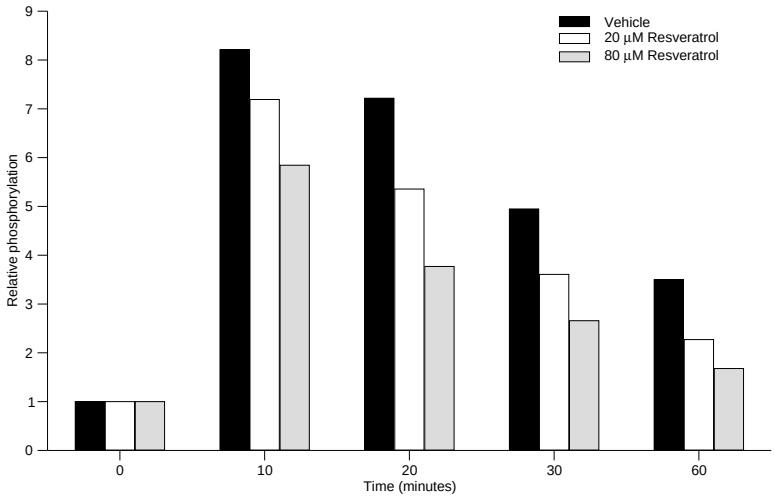
Phosphorylation of RelA/p65 in HeLa-pNF-kB-Luc cells The TNF-α stimulated RelA/p65 phosphorylation reaches maximum levels in HeLa-pNF-kB-Luc cells 10 min after addition of the ligand (figure 3.19b). The background levels of RelA/p65 phosphorylation are easily detectable, and the level is slightly increased after stimulation with 5 ng/ml TNF-α for 4 h.



(a) HL-60



(b) HeLa-pNF-kB-Luc



(c) Summary of (b)

Figure 3.20: Resveratrol has no effect on TNF-α stimulated RelA/p65-phosphorylation in HL-60 cells. Cells were treated with vehicle or 20 or 80 µM resveratrol for the specified times in the presence of 5 ng/ml TNF-α and prepared for Western blot. Immobilised protein from HL-60 cells was probed with anti-RelA/p65 or anti-phosphorylated RelA/p65 (a). The experiment was performed on 4 occasions and results of a typical experiment are shown Immobilised protein from HeLa-pNF-kB-Luc cells was probed with anti-RelA/p65 or anti-phosphorylated RelA/p65 (b). The experiment was performed on 2 occasions and results of a typical experiment are shown, and summarised in (c), where data is presented as fold induction of phosphorylation relative to cells treated for 0 h. Relative phosphorylation is determined as the relative phosphorylation compared to untreated over relative levels of total RelA/p65 compared to untreated

Resveratrol does not inhibit phosphorylation of RelA/p65 in HL-60 cells The focus for this part of the present study was to evaluate the immediate effect of resveratrol on phosphorylation of RelA/p65. Neither for the 65 kDa nor the 55 kDa fragment, was an effect of resveratrol on TNF-α stimulated RelA/p65 phosphorylation observed (figure 3.20a).

As a positive control, TNF-α stimulated RelA/p65 phosphorylation in HeLa-pNF-kB-Luc was studied (figure 3.20b and summarised in figure 3.20c). Resveratrol seemed to limited the TNF-α induced phosphorylation of RelA/p65 in a dose dependent manner and at all measured time points.

3.8 Summary

In summary the following results regarding the effects of resveratrol were obtained:

DNA synthesis Even low concentrations and short treatment times inhibited DNA synthesis

Cell cycle progression The phase affected depended on the resveratrol concentration used

Apoptosis Resveratrol stimulated apoptosis in a dose dependent manner, with Caspase-3 activation. The extrinsic pathway was also activated

Activation of NF-κB No inhibitory effect on phosphorylation of the 65 or 55 kDa RelA/p65 fragment in HL-60

Furthermore, it was found that levels of resveratrol decreased over time in the presence of FCS. Resveratrol stimulated an increase in the nuclear size, which was not paralleled by an increase in cell size.

Chapter 4

Discussion and conclusion

4.1 Purpose

The purpose of this study was to identify the effect of resveratrol on DNA synthesis and cell cycle progression. The apoptosis stimulating properties of resveratrol were also studied, as well as the possible role of NF-κB in cell cycle modulation and apoptosis.

4.2 Inhibition of thymidine incorporation

The dose dependent effect on cell growth measured by thymidine incorporation after 2 h was a very surprising and most interesting observation. To the best of my knowledge, this is the first report to show that short term treatment with low concentrations of resveratrol affects the growth of HL-60 and HepG2 cells in culture. The observation has only been paralleled by observations in the human prostate cancer cell line LNCap (Kuwajerwala et al., 2002). Because of lack of detailed description of experimental procedures, it is impossible to compare the IC_{50} value reported by Kuwajerwala et al. (2002) with those observed in the present study. The IC_{50} value at 24 and 48 h is in concordance with what has been reported earlier (Horvath et al., 2005; Roy et al., 2002).

In HL-60 cells, an IC_{50} value of 20 µM has been reported after treatment for 8 h (Surh et al., 1999).This value is in agreement with the results obtained in this study of 15 µM. The observed IC_{50} value in HepG2 is in disagreement with observations made by Delmas et al. (2000), who observed no decrease in incorporation of thymidine. The difference may be due to different experimental conditions.

The reduced proliferation rate of HL-60 and HepG2 cells after short time incubation, could indicate interference with the replication machinery. This interference may be a direct or a indirect inhibition of formation or movement of the DNA polymerase. Resveratrol has been shown to interfere with the DNA polymerase (Stivala et al., 2001; Sun et al., 1998) and inhibit the

ribonucleotide reductase (Fontecave et al., 1998; Pozo-Guisado et al., 2002). In full concordance, resveratrol has been shown to reduce intracellular levels of dNTPs in HL-60 cells (Horvath et al., 2005). Although treatment with resveratrol for 24 h has no effect on assembly of replication complexes (Stivala et al., 2001), it cannot be ruled out as an immediate effect. The mechanism by which resveratrol inhibits synthesis of DNA is not yet fully understood, but may depend on the DNA polymerase involved (Stivala et al., 2001).

The effect of resveratrol on thymidine incorporation in HepG2 cells presented here, is in disagreement with observations made by Goldberg et al. (1995a). The authors observed no inhibition of DNA synthesis or viability, when HepG2 cells were treated with 1, 5, or 50 µM resveratrol for 1, 3, and 7 days. This discrepancy is yet unexplained.

In conclusion, short term treatment with resveratrol results in a straight forward dose dependent interference with the cell proliferation. This effect of resveratrol is previously unreported.

4.3 Stability of resveratrol

When HL-60 cells were treated with resveratrol and incorporation of thymidine was measured, the IC_{50} value for growth inhibition increased as a function of incubation time (figure 3.1). This increase could be explained by the cells causing detoxification of resveratrol. Such a detoxification of resveratrol would be expected to require gene activation. The fact that IC_{50} increased between 4 to 24 h, but not between 2 and 4 h suggests the need for activation of a set of genes and thus supports the idea of detoxification. However, treatment for 8 h may not be enough time for gene activation alone to account for the observed effect. This indicates some additional effect e.g. degradation or inactivation of *trans*-resveratrol as the cause of the observed increase in IC_{50} values.

Resveratrol is labile as a result of an extracellular effect To evaluate whether the cells were actively involved in the observed increase in IC_{50} values the thymidine incorporation assay was modified from concurrent to asynchronous resveratrol addition, as described in 'Materials and methods', section 2.3.1. The reasoning being that if cellular activity e.g. metabolism was needed for the increase in IC_{50} values the increase would not be affected by the modified assay. If, on the other hand, the increase was due to degradation or inactivation of resveratrol in the medium a reduced difference in IC_{50} values would be expected.

As shown in figure 3.2 the actual outcome of the experiment was a collapse of all IC_{50} values into a single value. This result, clearly demonstrates that the observed increase in IC_{50} values is related to an extracellular effect.

FCS is associated with reduction in levels of *trans*-resveratrol Light absorption was used as a method to determine levels of resveratrol in solutions. This method is possible, because *trans*-resveratrol has a distinct absorption spectrum with an absorption maximum of approximately 308 nm (Trela and Waterhouse, 1996).

To analyse if the decreased levels of active *trans*-resveratrol was caused by FCS in the media, resveratrol was diluted in PBS with (figure 3.4b) or without (figure 3.4a) FCS. When diluted in PBS alone and left in centrifugation tubes in the dark in a standard incubator, resveratrol was stable and concentrations retained for at least 7 days. This clearly rules out any adsorption to the plastic of the tube.

It can, however, be observed from the result in figure 3.4 that the presence of FCS causes the absorption at 310 nm to be reduced with time. This demonstrates for the first time that *trans*-resveratrol in a FCS containing solution disappears as a function of time.

The result presented in figure 3.4c shows that an increase in FCS from 10 to 20 % has little effect on levels of reduction in absorbance, indicating that some maximum has been reached at 10 % FCS. In support of this, 5 % FCS has a lesser effect on the levels of resveratrol than 10 % FCS. However, the light-absorption scan is a semiqualitative method, and only tendencies can be extracted. To further study the degradation of resveratrol in solution, analysis by HPLC would be appropiate. This would give valuable information on specific resveratrol quantities in solution as well as possible metabolites formed.

Enzymatic activity of compounds of FCS can be ruled out as a cause of the reduction, since the FCS used was heat inactivated. Resveratrol has previously been shown to bind to BSA (Jannin et al., 2004; Lançon et al., 2004), thus direct interaction with other proteins of the FCS is a likely event. Binding to proteins in the FCS could affect resveratrol in several possible ways. 1) The compound of FCS which binds resveratrol in turn might adsorb to the plastic of the tube. However, this does not explain the observed tail in the visible light range. 2) Binding of resveratrol to some component of FCS could change the light-absorption of resveratrol. The absorption of resveratrol bound to a compound must then shift towards the visual range to accommodate the observed tail.

The role of cellular activity When HepG2 cells were treated with resveratrol an increase in IC_{50} value of incorporated thymidine was observed from 2 to 24 h (figure 3.3). This strongly suggests the role of cellular activity e.g. metabolism in addition to the effect of FCS. Given the fast increase in IC_{50} values between 2 and 4 h, the cellular activity may not require gene activation.

Metabolism of resveratrol in HepG2 cells is not well studied. It has been shown that resveratrol stimulated expression of the *Cyp1A1* gene (Allen et al.,

2001), while no effect on the expression of the *Cyp3A4* gene has been observed (Raucy, 2003).

Glucuronidation of resveratrol has been observed in human liver samples (Santi et al., 2000a), and HepG2 cells has furthermore been shown to metabolise quercetin, another polyphenol, to quercetin glucuronide (O'Leary et al., 2003). It is therefore possible that some cells actively inactivate resveratrol by metabolism.

In conclusion, FCS stimulates a reduction in the levels of *trans*-resveratrol, an effect previously unreported. In addition cellular activity in some cell lines may also contribute to the reduction. Further studies with HPLC or other more discriminative methods are needed to asses the rate of reduction and production of possible metabolites.

4.4 The effect on the cell cycle

A striking result of treating HL-60 and HepG2 cells with different concentrations of resveratrol, is the dose dependent phase specific accumulation of cells (figures 3.8 and 3.9).

20 µM resveratrol for 24 h resulted in nearly a doubling of the number of cells in the S phase compared to untreated HL-60 cells. The same was observed for 40 µM in HepG2 cells. The result presented here demonstrates that low levels of resveratrol prolong the S phase.

Is has been suggested that the accumulation of cells in the S phase shows a stimulatory effect of low levels of resveratrol on DNA synthesis (Dorai and Aggarwal, 2004). However, the only valid explanation of the accumulation of cells in the S phase is that the phase is prolonged.

Treatment with 80 µM doubles the number of cells in the G_1 phase compared to HL-60 and HepG2 cells treated with 20 and 40 µM, respectively. This result clearly suggests that high levels of resveratrol stimulate accumulation of HL-60 cells in the G_1 phase and thus prevent cells from entering the S phase. This view is in concordance with observations where resveratrol inhibited production of polyamines by interfering with the Ornithine Decarboxylase (ODC) (Schneider et al., 2000). Polyamines are found in all studied eucaryotic cells and are heavily involved in cell progression to the S phase. The rate limiting step in polyamine production is catalysed by ODC (Wolter et al., 2004). Resveratrol is cabable of preventing entry into the S phase.

The result obtained for HepG2, where the dose dependent phase specific accumulation of cells is less pronounced, may in part be explained by some cellular detoxification activity of the HepG2 cells, as discussed in section 4.3.

The hypothesis of a dose dependent cell cycle arrest in HL-60 and HepG2 cells is in agreement with what has been observed in a range of other cell lines (Bernhard et al., 2000; Delmas et al., 2002; Kuwajerwala et al., 2002;

Matsuoka et al., 2001; Opipari et al., 2004; Pozo-Guisado et al., 2002; Stivala et al., 2001; Wolter et al., 2001), see table 1.4.

The G_1 phase accumulation of HL-60 cells observed in the present study is in agreement with findings reported by Kang et al. (2003), who found that 100 µM resveratrol for 24 h stimulated an accumulation of HL-60 cells in the G_1 phase. This result is in disagreement with an earlier report by Joe et al. (2002). The authors found that 300 µM resveratrol for 24 h stimulated an accumulation of cells in the S phase. However, the resveratrol concentration used by Joe et al. was so high that a direct comparison is difficult.

The cell cycle analysis explain the reduced cell growth at 24 h, but does not reveal if there is a dose dependent slowdown of cell cycle progression or a dose dependent cell cycle arrest point. Cells caught in an extended S phase may initiate the process of apoptosis. However, to the best of my knowledge, no report yet has shown cell cycle phase specific stimulation of apoptosis by resveratrol.

More systematic studies are required to elucidate the effects of resveratrol on the cell cycle. This applies for short and long term stimulation as well as studies of cell cycle phase specific apoptosis.

In conclusion, treatment of HL-60 and HepG2 cells with resveratrol for 24 h causes a complex pattern of effects; low doses cause a slow down in progression through the cell cycle, while higher doses induces a phase specific cell cycle arrest.

4.5 Nuclear size and cell granulation

An increase in the size of the cell nucleus was observed in HL-60 but not HepG2 cells. The increase in nuclear size was primarily observed in the G_1 and early S phases of the cell cycle (figure 3.10). An increase in nuclear size has previously been reported with the HIV protease inhibitors indinavir and nelfinavir in adipose cells (Caron et al., 2003). To the best of my knowlegde, no compound has been reported to stimulate nuclear enlargement in leukemia cells.

One could speculate that the increase in the nuclear size could be due to changes in the chromatin structure and nuclear laminin content. Modifications of the chromatin structure by histone acetylation is necessary for gene transcription (reviewed in Eberharter and Becker, 2002). Though no effect has been observed of resveratrol on acetylation or deacetylation of histones in A549 cells (Donnelly et al., 2004), this effect may be cell specific. The observed effect could also be explained by artefacts risen from incomplete cell lysis by the detegernt, due the to apoptotic effects of resveratrol. Further studies are needed to elucidate the effect of resveratrol on the size of cell nuclei in HL-60 cells.

Resveratrol stimulates an increase in cellular granularity The increase in size of the nucleus is not accompanied by an increase in cell size, but by an increase in granularity or density of the cells after treatment with resveratrol for 24 h (figure 3.11). The increase in cell granularity (i.e. alteration of the amount and/or type of cytoplasmic granules) has previously been reported for ovarian cancer cell lines (Opipari et al., 2004). In concordance, an increase in mitochondrial mass has been reported in the colon carcinoma cell line HCT-116 treated with resveratrol (Mahyar-Roemer et al., 2001). These observations, together with observations presented in this study demonstrate that resveratrol has a stimulating effect on the amount of cellular organelles.

100 µM resveratrol has previously been shown to stimulate an increase in cell size in HCT-116 cells after treatment for 24 h (Mahyar-Roemer et al., 2001). The discrepancy between observations presented in the present study and the observations made by Mahyar-Roemer et al. (2001) regarding cell size may be explained by the different cell lines used.

Low concentrations of resveratrol has a significantly greater effect on the density of treated HL-60 cells than has higher resveratrol concentrations. This surprising effect is previously unreported, and demonstrates that the effects of high resveratrol concentration could counteract the effects of lower resveratrol concentrations.

In conclusion, two previously unreported and surprising observations were made regarding resveratrol stimulated increase in nucleus size and cell complexity in the present study.

1) The increase in nuclear size is correlated to the phases of the cell cycle. 2) The effects of low concentrations of resveratrol is counteracted by the effect of higher concentrations

4.6 Apoptosis

Change in nuclear morphology and loss of membrane asymmetry are two hallmarks of apoptosis, and these were used to study apoptosis stimulating effects of resveratrol.

Nuclear fragmentation and exposure of PS The study of nuclear condensation and fragmentation, and exposure of PS identified a dose dependent effect of resveratrol (figures 3.12 and 3.14). DAPI staining identified a possible pro-apoptotic effect of 20 µM resveratrol as seen by nuclear condensation. However, the same observation could not be made with the Annexin-V binding assay, though higher doses resulted in a correlation between the results of the DAPI staining and the Annexin-V binding assay. Both events are controlled by Caspase-3 activation (Hirata et al., 1998). Thus the difference may be due

to a sequential process with the loss of membrane asymmetry following the nuclear condensation and fragmentation.

When treated with resveratrol, HL-60 cells lost membrane integrity as a function of resveratrol concentration, indicating necrotic death. Depleting cells of ATP results in necrotic rather than apoptotic cells death (Leist et al., 1997). Resveratrol has been found to inhibit extracellular ATP synthesis in human umbilical vein endothelial cells (Arakaki et al., 2003), and to inhibit rat liver and rat brain mitochondrial F0F1-ATPase activity (Zheng and Ramirez, 1999, 2000). It is therefore possible, that resveratrol stimulates necrotic cell death though inhibition of ATP synthesis. Interestingly, low levels of resveratrol (4 to 8 µM) inhibited apoptosis stimulated by H_2O_2 and the anticancer agent vincristine (Ahmad et al., 2004, 2003).

Activation of Caspase-3 and Caspase-8 Data from Western blot analysis for cleaved Procaspase-3 were concordant with the dose dependent stimulation of apoptosis. Furthermore, these results were in full agreement with the Annexin-V assay, where 20 µM resveratrol had no effect on apoptosis in HL-60 cells. Flow cytometric evaluation of HL-60 cells treated with resveratrol revealed that the Caspase-8 activity was stimulated in a dose and time dependent manner.

Activation of Caspase-8 indicates activation of the extrinsic apoptotic pathway, and is in concordance with earlier observations (Clement et al., 1998; Su et al., 2005). However, Pervaiz (2001) observed that 8 to 32 µM resveratrol stimulates Caspase-9 and Caspase-3 activity, but not Caspase-8 activity in HL-60 cells. The authors further found that resveratrol stimulated a drop in the voltage gradient across the mitochondrial membrane as well as stimulated Cytochrome *c* release. The observations by Pervaiz (2001) are supported by similar findings in HL-60 cells by Dörrie et al. (2001). The reason for the discrepancy between the activation of the extrinsic pathway by resveratrol found in the present study and others on one side, and the lack of activation found by others is unknown.

Cleavage and activation of Caspase-3 in the present study clearly shows that resveratrol stimulates caspase dependent apoptosis. Caspase-8 activation occurred later than the other apoptotic markers used in this study. This suggests less activation of the extrinsic pathway compared to the intrinsic pathway, and thus indicates that Caspase-8 may not be needed for stimulation of apoptosis. This would be in concordance with observations made by Mahyar-Roemer et al. (2001). The authors found that Caspase-3 inhibitor, but not Caspase-8 inhibitor had inhibitory effect on resveratrol stimulated apoptosis in HCT-116 cells, after treatment for 24 h with 100 µM. Only a single paper reports a minor effect of resveratrol on apoptosis (Ragione et al., 1998).

In conclusion, resveratrol stimulates apoptosis in a dose dependent manner. Stimulation of apoptosis involves activation of Caspase-3 and Caspase-8. Activation of Caspase-8 is a late occurring response, indicating the intrinsic pathway as the primary pathway for resveratrol stimulated apoptosis. Furthermore, resveratrol may stimulate the necrotic as well as apoptotic cell death.

4.7 Phosphorylation of NF-κB

NF-κB activation in HeLa-pNF-kB-Luc cells Resveratrol is a potent inhibitor of NF-κB transactivation in unstimulated HeLa-pNF-kB-Luc cells as well as in cells stimulated with TNF-α (figure 3.18). The inhibitory effect of resveratrol was 40 fold in stimulated cells compared to 10 fold in unstimulated cells. For this reason, only stimulated cells were used in evaluation of RelA/p65 phosphorylation.

A dose dependent inhibition of phosphorylated RelA/p65 in TNF-α treated HeLa-pNF-kB-Luc cells was observed (figure 3.20c). The time span of TNF-α stimulated RelA/p65 phosphorylation presented in this report is in concordance with earlier reports (Sakurai et al., 2003). Phosphorylation of RelA/p65 may be essential to nuclear translocation (Chen and Greene, 2004; Hu et al., 2004). The observations presented in this work demonstrate that resveratrol reduces transcriptional activity of NF-κB, and that the inhibition in stimulated cells correlates to a reduction in RelA/p65 phosphorylation.

RelA/p65 exists predominantly as a 55 kDa fragment in HL-60 cells Only low levels of unmodified and phosphorylated RelA/p65 were found in HL-60 cells stimulated with TNF-α (figure 3.19a). Surprisingly, a fragment of 55 kDa was found to bind more antibody compared to the 65 kDa RelA/p65. The fragment could be a result of unspecific binding of the anti-RelA/p65 antibodies, and the smear observed for unmodified RelA/p65 indeed argues that this is the case. Less smear was observed with anti-phosphorylated RelA/p65 than with anti-RelA/p65. Treatment of HeLa-pNF-kB-Luc cell lysate with anti-RelA/p65 resulted in a clear, single protein band and no smear. These two observations indicate that the observed smear is cell specific and furthermore suggested, that the 55 kDa fragment of the HL-60 cells is a truncated form of RelA/p65.

Resveratrol does not inhibit RelA/p65 phosphorylation in HL-60 cells HL-60 cells were stimulated with TNF-α and treated with 20 or 80 µM resveratrol (figure 3.20a). No changes in phosphorylation of RelA/p65 or the 55 kDa fragment of HL-60 cells were observed.

The existence of the truncated RelA/p65 suggests that HL-60 is not a very good model for studying NF-κB activation. Reports on NF-κB activation in HL-60 cells by the use of Western blot are sparse, and thus support the

notion of HL-60 cells being a less useful model for this purpose. One study, however, verifies the TNF-α stimulated activation of NF-κB in HL-60 (Takada and Aggarwal, 2004). The authors studied the effect of TNF-α on NF-κB activity by nuclear extracts and electric mobility shift assay (EMSA). To my knowledge, the effect of resveratrol on phosphorylation of RelA/p65 has not previously been studied in HL-60 cells.

In conclusion, resveratrol inhibits immediate TNF-α stimulated RelA/p65 phosphorylation in HeLa-pNF-kB-Luc cells. Furthermore, HL-60 cells may be a less useful model for studying NF-κB phosphorylation.

4.8 Interference with common cytotoxicity assays

As shown in figures 3.5a and 3.6 the MTT and NR assays caused difficulties when cells were treated with resveratrol. Both assays were demonstrated to be reliable in the absence of resveratrol (figure 3.5b and data not shown).

MTT The MTT assay and other variants of tetrazolium salt based assays have previously been used to study the cytotoxicity of resveratrol on several cell lines, among these HL-60 cells (Roy et al., 2002) and HepG2 cells (Delmas et al., 2000).

The results of Hill et al. (1995) and Yoshikawa et al. (1999) were reproduced (figure 3.5b), why it is concluded the MTT assay works and produces valid results. Although the MTT assay has been used to asses cytotoxicity of resveratrol in HL-60 and HepG2 in previous studies, resveratrol in some way interfered with the assay in the present work. As a result, the outcome of the assay is quite unreliable (figure 3.5a). The present observation is in line with Holian and Walter (2001), who found the MTT cytotoxicity data of cells treated with resveratrol to be flawed.

Solving the problem regarding the assay was considered beyond the scope of the present work and was not further explored.

NR Assessing the cytotoxicity of resveratrol was also attempted using the NR assay. The assay is based on active uptake of the NR dye, and was found to work very well with cells in adhesion (HepG2) and suspension (HL-60) cultures (data not shown). The absorption of the dye was furthermore found to be persistent for at least 2 h (data not shown). These initial results served to verify the assay in the present study.

When NR was added to a cell suspension of resveratrol treated HL-60 cells, a rapid (within 30 min) and resveratrol concentration dependent decolouration occurred. The decolouration correlated to a formation of crystals, indicating precipitation of the NR dye. This rendered the assay useless.

To my knowledge, the present study is the first to report this effect of resveratrol on the NR assay. The effect may be due to changes in pH of the cultivation medium induced by resveratrol and the dye in conjunction, resulting in reduced solubility of the dye. However, this was not assessed in detail.

Because of the resveratrol induced decolouration and crystal formation, the NR assay was judged unfit to asses the cytotoxicity of resveratrol.

In conclusion, the MTT and NR assays failed to provide support for the observation made with thymidine incorporation because of interference of resveratrol with the assay. This is the first report on the interference by resveratrol on the NR assay.

4.9 Conclusion

The purpose of this study was to evaluate the effects of resveratrol on DNA synthesis, cell cycle progression, and apoptosis. The possible role of NF-κB was also evaluated. It was found that:

- Short incubation times with resveratrol inhibits DNA synthesis in a dose dependent manner
- The cell cycle is modulated in a complex manner, resulting in accumulation of cells in the S or G_1 phases. The phase affected depends on the concentration of resveratrol
- The apoptosis stimulating effects of resveratrol includes activation of Caspase-3 and Caspase-8. Activation of the extrinsic pathway is a late occurring response compared to other apoptosis markers, indicating the intrinsic pathway as the primary pathway for resveratrol stimulated apoptosis
- The role of NF-κB in cell cycle modulation and stimulation of apoptosis remain inconclusive

Additionally, it was found that:

- Resveratrol stimulates an increase in the size of cell nuclei and cell granularity. The cell size is not affected
- FCS stimulates a reduction in the levels of resveratrol in solution

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