

Global gene expression changes in glioblastoma cell line U87 by Zyflamend®, a mixture of standardized extracts from common herbs and spices

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Abstract

Diet is considered as one of the most important modifiable factors in coronary heart disease and cancer development. Recent evidence indicates that modulation of inflammation by compounds found in many herbs and spices may be one of the mechanisms by which diet influences development and progression of these common chronic conditions. A wide spectrum of human malignancies aberrantly overexpress pro-inflammatory cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO) enzymes. Inhibitors of these molecules may be, therefore, useful in cancer chemoprevention and treatment. In this study, we examined the effects of Zyflamend® herbal preparation (New Chapter, Inc., Brattleboro, VT) on apoptosis and global gene expression of an established human glioblastoma cell line U87. Zyflamend consists of ten standardized herbal extracts in olive oil (rosemary, turmeric, ginger, holy basil, green tea, Hu zhang, Chinese goldthread, barberry, oregano, and *Scutellaria baicalensis*) that are among the richest sources of naturally occurring and chemically diverse COX-2 and 5-LO inhibitors. Zyflamend, diluted in DMSO at 0.1 microliters/ml, induced apoptosis of U87 cells compared to olive oil/DMSO control at 48h as determined by the TUNEL analysis. In two independent experiments, total RNA was isolated from U87 cells at 2h and 24h after treatment with Zyflamend, was processed using standard Affymetrix protocols and were hybridized to GeneChip® Human Genome U133A Affymetrix oligonucleotide microarray. In total, at 2h after Zyflamend treatment, we identified seven genes that were up-regulated (five of them belonging to the heat shock protein family) and two that were down-regulated more than two-fold in both experiments compared to the DMSO-treated control cells. At 24h, the expression of 84 genes was increased and of 65 genes was decreased. The expression of eight genes influenced by Zyflamend treatment has been confirmed by the real-time PCR analysis and immunoblotting evaluation is in progress. Unexpectedly, the expression of interleukin-1alpha and IL-1beta genes, potent mediators of inflammation and immunity, was significantly induced (up to 30-fold) at 24 h after Zyflamend treatment of U87 cells. The IL-1 cytokine is known to be produced in response to cell injury, but it remains to be demonstrated that it plays a role in induction of apoptosis by Zyflamend in U87 glioblastoma cells. The obtained results suggest that naturally occurring COX-2 and 5-LO inhibitors (along with other active phytonutrients) from Zyflamend herbal preparation modulate the expression of functionally diverse groups of genes, including those involved in regulation of apoptosis and inflammation, that collectively induce apoptosis in glioblastoma cell line U87.

Introduction

Cyclooxygenase-2 (COX-2) and 5-Lipoxygenase (5-LO) are novel molecular targets for cancer prevention and treatment

- COX-2 and 5-LO oxidize arachidonic acid, a nutritionally-relevant omega-6 polyunsaturated fatty acid to produce eicosanoids.
- Prostaglandins and leukotrienes are the main eicosanoids that regulate cell proliferation, survival, adhesion and motility, as well as angiogenesis, vascular permeability and inflammation, thus playing important roles in development and progression of cancer, coronary artery disease and other chronic conditions (Funk, 2001).
- Both COX-2 and 5-LO are aberrantly overexpressed in a variety of human malignancies.
- Synthetic inhibitors of COX-2 and 5-LO activity have chemopreventive and antitumorigenic effects in preclinical cancer models and COX-2 inhibitors are being examined in clinical trials for a variety of malignancies.

Naturally occurring inhibitors of COX-2 and 5-LO are present in herbs and spices

- Plant foods and especially certain herbs and spices contain a spectrum of anti carcinogenic phytochemicals, including chemically diverse naturally occurring inhibitors of COX-2 and 5-LO. These inhibitors may modulate inflammation and account for protective effects of plant-based diets for many chronic conditions.
- Zyflamend® is an herbal nutritional supplement made by New Chapter, Inc. of Brattleboro, VT. It is a patented COX-2 inhibiting herbal complex consisting of ten solvent-free and potency-assured, standardized herbal extracts (Figure 1).
- These extracts were chosen based on a large volume of epidemiological and experimental data suggesting their potential to inhibit COX-2 and 5-LO and to exert anti-cancer activities (Table 1).

Figure 1

Supplement Facts

Serving size 2 Softgels

Two softgels contain

	%DV
Rosemary , (<i>leaf</i>), 100 mg supercritical extract and 50 mg extract (23% total phenolic antioxidants [TPA]-34.5 mg)	150 mg •
Turmeric , (<i>rhizome</i>), 10 mg supercritical extract (45% turmerones-4.5 mg) and 100 mg ethanolic extract (7% curcuminoids-7 mg)	110 mg •
Ginger , (<i>rhizome</i>) 54 mg supercritical extract, organic (30% pungent compounds-16.2 mg, 8% zingiberene-4.3 mg) and 46 mg ethanolic extract (3% pungent compounds-1.4 mg)	100 mg •
Holy Basil , (<i>leaf</i>), extract (2% ursolic acid-2 mg)	100 mg •
Green Tea , (<i>leaf</i>), extract (45% polyphenols-45 mg) (contains 10 mg naturally occurring caffeine)	100 mg •
Hu Zhang , (<i>Polygonum cuspidatum</i>), (<i>root & rhizome</i>) extract, (8% resveratrol-6.4 mg)	80 mg •
Chinese Goldthread , (<i>root</i>), extract (6% berberine-2.4 mg)	40 mg •
Barberry , (<i>root</i>), extract (6% berberine-2.4 mg)	40 mg •
Oregano , (<i>leaf</i>), supercritical extract (0.8% TPA-0.32 mg)	40 mg •
Baikal Skullcap , (<i>Scutellaria baicalensis</i>) (<i>root</i>), hydroethanolic extract (17-26% baicalein complex including baicalein and baicalin - 3.4-5.2 mg, and 0.4-0.9% wogonin - 0.08-0.18 mg)	20 mg •

- Daily Value not established

Other ingredients: Olive oil-extra virgin, maltodextrin, silica and yellow beeswax.

Capsule: Gelatin, vegetable glycerine, purified water, and carob.

Table 1

Herb	Active ingredients	Inhibition of COX-2	Inhibition of 5-LO	References
Rosemary (<i>Rosmarinus officinalis</i>)	Betulinic acid	+		Fulda S et al, 1999
Ginger (<i>Zingiber officinale</i>)	Shogaols	+		Tjendraputra E et al, 2001
	Melatonin		+	Badria FA et al, 2002
	Gingerols	+	+	Kiuchi H et al, 1992
Turmeric (<i>Curcuma longa</i>)	Curcuminoids	+	+	Rao CV et al, 1995
Holy basil (<i>Ocimum sanctum</i>)	Ursolic acid	+		Subbaramaiah K et al, 2000
Oregano (<i>Origanum vulgare</i>)	Apigenin	+		Raso GM et al, 2001
Green tea (<i>Camellia sinensis</i>)	Catechins	+	+	Hong J et al, 2001
Baikal skullcap (<i>Scutellaria baicalensis</i>)	Baicalein	+	+	Nakahata N et al, 2003
	Melatonin	+	+	Reiter Rj et al, 2002
Barberry (<i>Berberis vulgaris</i>)	Berberine	+		Fukuda K et al, 1999
Chinese goldthread (<i>Coptis chinensis</i>)	Berberine	+		Li X-K et al, 2000
Hu zhang (<i>Polygonum cuspidatum</i>)	Resveratrol	+		Gusman J et al, 2001

Anti-cancer activities of Zyflamend herbal preparation

- Zyflamend induces apoptosis of human prostate cancer cell lines in vitro (Bemis et al., 2002). It may be, therefore, effective in other types of cancer cells that overexpress COX-2 and 5-LO, including glioblastoma multiforme (GBM).
- GBM is the most malignant brain tumor that is characterized by highly infiltrative and neurologically destructive growth patterns and a very poor prognosis in spite of the most sophisticated therapies (Nathoo et al., 2004).
- It is well established that some COX-2 and 5-LO inhibitors that are present in Zyflamend pass the blood brain barrier and are, therefore, likely to reach the GBM tumor tissue.

Aim

- The aim of this study was to gain an insight into the inflammation and carcinogenesis-associated pathways that may be modulated by anti carcinogenic phytochemicals, including naturally occurring COX-2 and 5-LO inhibitors present in Zyflamend herbal preparation.
- This was done by determining a global gene expression profile of the established human glioblastoma cell line U87 treated with Zyflamend.

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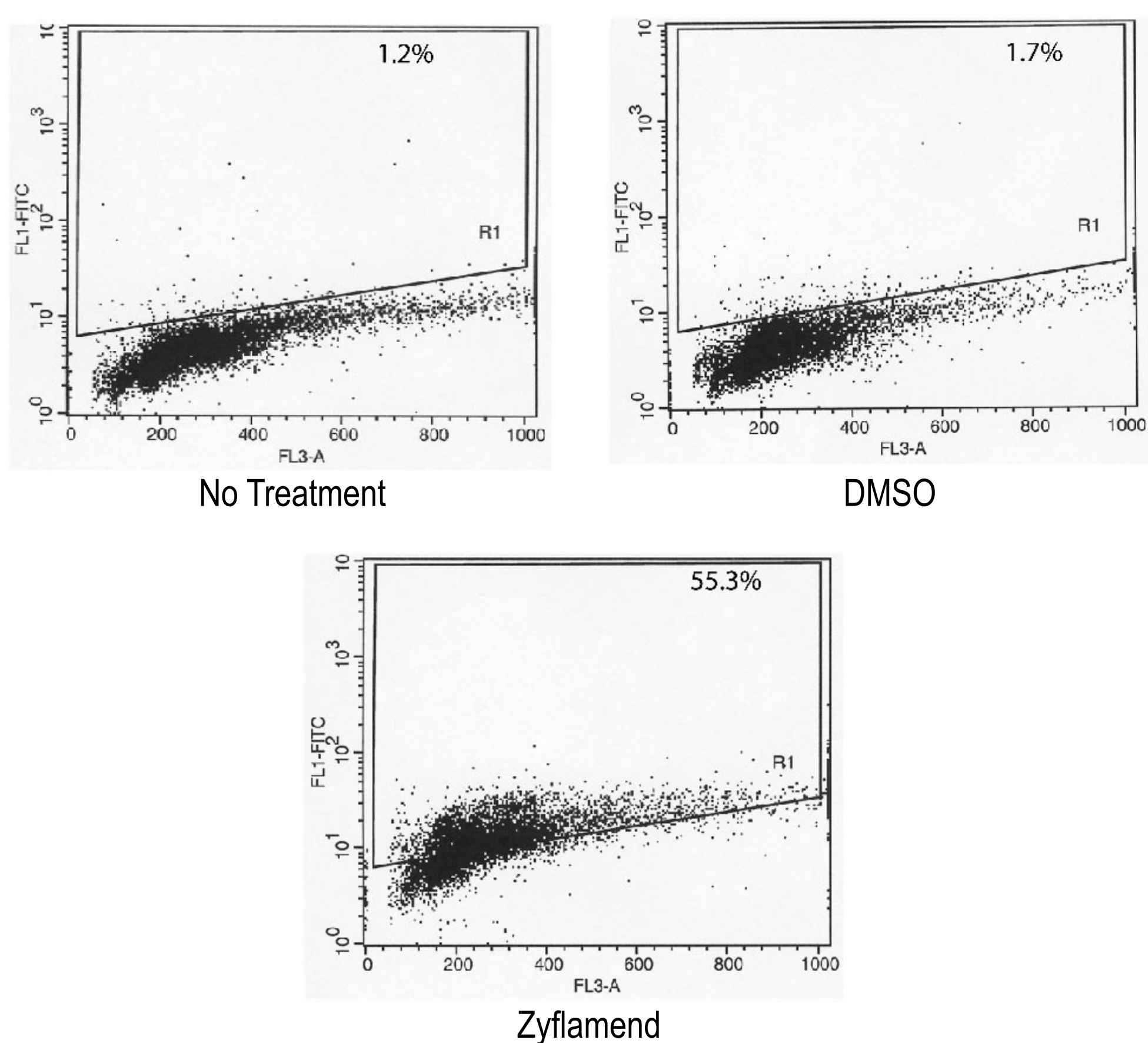
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Methods

- U87 cells were grown in duplicate 10 cm dish in DMEM medium with 1% fetal bovine serum (FBS). A soft gel capsule of Zyflamend® herbal mixture in olive oil was cut by scalpel and diluted 1:10 in DMSO, sterile filtered and added to cell culture at final concentration of 0.1 microl/ml (1:10,000 dilution). As a control, olive oil was diluted 1:10 in DMSO, sterile filtered and added at final concentration of 0.1 microl/ml to cells or the cells were left un-treated.
- In two independent experiments, total RNA was isolated from U87 GBM cells at 2 and 24 hours after treatment with Zyflamend or DMSO and in vitro processed using standard Affymetrix protocol and hybridized to a GeneChip® Human Genome U133A Affymetrix oligonucleotide microarray that contains over 22,000 well characterized human genes. Affymetrix Microarray suite 5.0, Microsoft Access 2000 and GeneSpring 5.1 programs were used for data analysis.
- The expression of candidate genes was confirmed by the real-time PCR quantitation analysis on the Perkin Elmer/ABI PRISM™ 7700 Sequence Detection System.

Results - 1

Figure 2. Zyflamend induces apoptosis of U87 GBM cells.



Exponentially grown U87 cells in DMEM with 1% FBS were treated with Zyflamend or olive oil at final concentration of 0.1 microl/ml, or left untreated. After 24 hours, floating and attached cells were collected, fixed in 1% paraformaldehyde in phosphate buffered saline (PBS), washed in PBS, resuspended in 70% ethanol and analyzed by flow cytometry. The APO-BRDU kit, a two color staining method for labeling DNA breaks and total cellular DNA was used to detect apoptotic cells. Percentage of apoptotic cells is indicated in the upper right corner.

Results - 2

Table 2

At 2 hours after Zyflamend treatment, seven genes were up-regulated and two were down-regulated more than 2-fold by the Zyflamend treatment in both experiments.

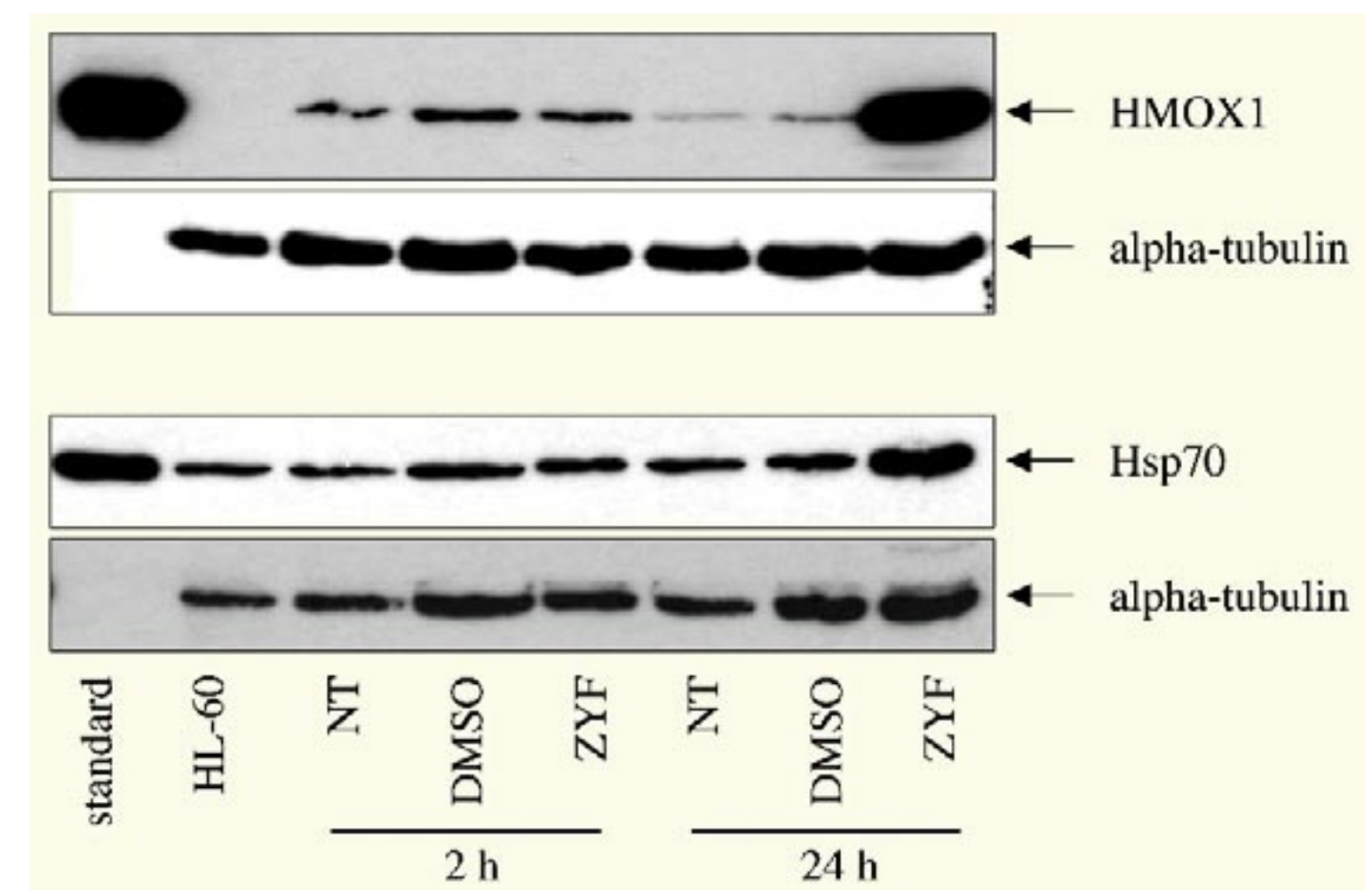
Gene Name	Gene Symbol	Gene Bank Number	Microarray		Biological Process	Molecular Function
			Fold Change	EXP1		
heat shock 70kDa protein 1A	HSPA1A	NM_005345	4.9	7.0	mRNA catabolism, protein folding	heat shock protein activity
heat shock 70kDa protein 6 (HSP70B)	HSPA6	NM_002155	3.5	5.7	protein folding	heat shock protein activity, ATP binding
aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	AKR1C2	U05598	4.9	5.3	lipid metabolism, transport, digestion, canalicular bile acid transport	bile acid transporter activity, binding, electron transporter activity, oxidoreductase activity, trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity
heat shock 70kDa protein 1B	HSPA1B	NM_005346	3.5	4.9	mRNA catabolism, protein folding, response to unfolded protein, heme oxidation	ATP binding, unfolded protein binding, heme oxygenase (decyclizing) activity, signal transducer activity, oxidoreductase activity
heme oxygenase (decyclizing) 1	HMOX1	NM_002133	4.3	4.6	positive regulation of I-kappaB kinase/NF-kappaB cascade	activity, signal transducer activity, oxidoreductase activity
solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	AB040875	2.3	2.3	protein complex assembly, transport, amino acid transport	cystine glutamate antiporter activity, amino acid permease activity
DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1	NM_006145	2.0	2.1	protein folding, response to unfolded protein	unfolded protein binding
dual specificity phosphatase 6	DUSP6	BC003143	-2.5	-2.6	regulation of cell cycle, inactivation of MAPK protein amino acid, dephosphorylation, apoptosis	protein serine/threonine phosphatase activity, protein tyrosine phosphatase activity, hydrolase activity, MAP kinase phosphatase activity, DSPc:protein tyrosine/serine/threonine phosphatase activity; 2.3e-23
basic helix-loop-helix domain containing, class B, 2	BHLHB2	NM_003670	-2.8	-3.7	regulation of transcription, DNA-dependent	transcription factor activity

Table 3

Gene Name	Gene Symbol	Gene Bank Number	Microarray		Q-PCR		Biological Process	Molecular Function
			Fold Change	EXP1	EXP2	Fold Change		
interleukin 1, beta	IL1B	NM_000576	7.7	27.9	N.D.	49.8	Apoptosis; negative regulation of cell proliferation; inflammatory response; regulation of cell cycle; signal transduction; cell-cell signaling; cell proliferation	signal transducer activity; interleukin-1 receptor binding
heme oxygenase (decyclizing) 1	HMOX1	NM_002133	8.6	11.3	19.6	14.4	heme oxidation; positive regulation of I-kappaB kinase/NF-kappaB cascade	heme oxygenase (decyclizing) activity; signal transducer activity; oxidoreductase activity
interleukin 1, alpha	IL1A	M15329	4.3	8.0	15.6	11.2	negative regulation of cell proliferation; apoptosis; anti-apoptosis; chemotaxis; inflammatory response; immune response; cell-cell signaling	signal transducer activity; interleukin-1 receptor binding
colony stimulating factor 3 (granulocyte)	CSF3	NM_000759	5.7	8.0	N.D.	N.D.	positive regulation of cell proliferation; immune response; cellular defense response cell surface receptor-linked signal transduction; cell-cell signaling; development	cytokine activity; granulocyte colony-stimulating factor receptor binding; interleukin-6 receptor binding
leukemia inhibitory factor (cholegric differentiation factor)	LIF	NM_002309	7.0	4.0	N.D.	N.D.	immune response; cell surface receptor-linked signal transduction; cell-cell signaling; development; positive regulation of cell proliferation	cytokine activity; leukemia inhibitory factor; receptor binding; oncostatin-M receptor binding; growth factor activity
serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	SERPIN2	NM_002575	3.7	7.0	N.D.	N.D.	anti-apoptosis	serine-type endopeptidase inhibitor activity; plasminogen activator activity
hypothetical protein FLJ22761	FLJ22761	NM_025130	3.5	5.7	N.D.	N.D.	glycolysis	hexokinase activity; ATP binding
neuregulin 1	NRG1	NM_013959	3.5	4.0	N.D.	N.D.	transmembrane receptor protein tyrosine kinase ligand binding; neurogenesis; embryonic development; cell differentiation	receptor binding growth factor activity; transmembrane receptor protein tyrosine kinase activator activity
aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	AKR1C2	U05598	4.6	3.0	19.2	15.2	lipid metabolism; transport; digestion; canalicular bile acid transport	bile acid transporter activity; binding; electron transporter activity; oxidoreductase activity; trans-1,2-dihydrogenase activity
fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	FABP3	NM_004102	-3.7	-17.1	N.D.	N.D.	negative regulation of cell proliferation; transport	fatty acid binding; lipocalin; transporter activity
collagen, type 1, alpha 2	COL1A2	NM_000089	-3.5	-4.9	N.D.	N.D.	perception of sound; phosphate transport; skeletal development	extracellular matrix structural constituent; structural constituent of bone
single-stranded DNA binding protein 2	SSBP2	NM_012446	-3.5	-4.6	-10.6	-3.2	regulation of transcription	single-stranded DNA binding; transcription regulator activity
lysyl oxidase-like 1	LOXL1	NM_005576	-4.3	-4.3	N.D.	N.D.	protein modification	copper ion binding; electron transporter activity; oxidoreductase activity; protein-lysine 6-oxidase activity
leucine rich repeat containing 17	LRR17	NM_005824	-3.5	-4.0	N.D.	N.D.		
thioredoxin interacting protein	TXNIP	NM_006472	-4.3	-3.5	N.D.	N.D.	arrestin; signal transduction	
chromosome 5 open reading frame 13	C5orf13	NM_004772	-3.7	-3.5	N.D.	N.D.		

Results - 3

Figure 3. Zyflamend up-regulates expression of HMOX1 and Hsp70 protein in U87 GBM cell lines after 24 hours of stimulation.



Protein extracts were prepared from human U87 GBM cells treated with Zyflamend (ZYF), DMSO control or non-treated (NT) cells at 2 and 24 hours after stimulation. Immunoblotting with anti-Heme Oxygenase-1 (HMOX1) and anti-Heat shock protein 70 (Hsp70) monoclonal antibodies (Stressgen Biotechnology, Canada) was done according to recommended protocol.

Summary

- Naturally occurring phytochemicals, including COX-2 and 5-LO inhibitors that are present in Zyflamend herbal preparation modulate the expression of functionally diverse groups of genes, including those involved in regulation of apoptosis and inflammation, that collectively induce apoptosis in glioblastoma cell line U87.
- It remains to be determined whether the expression of candidate genes is mediated by COX-2 and/or 5-LO inhibition or whether Zyflamend modulates gene expression independently of COX-2 and/or 5-LO inhibitory activity.

References

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