Molecular mechanisms of the anti-leukemic properties of Non-steroidal Anti Inflammatory Drugs

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ॐ असतो मा सद्गमय ।

तमसो मा ज्योतिर्गमय ।।

मृत्योर्मामृतं गमय ।

ॐ शान्ति शान्ति शान्ति ।।

- बृहदारण्यक उपनिषद् 1.3.28

Translation

om asato mā sadgamaya

tamaso mā jyotirgamaya

mṛtyor mā amṛtaṁ gamaya

OM śānti śānti śānti

– bṛhadāraṇyak upaniṣad 1.3.28

Translation

Lead me (by giving knowledge) from the unreal to the real;

From darkness (of ignorance) to the light (of knowledge);

From fear of death (sense of limitation) to the Knowledge immortality (limitless liberation)

OM - Let There Be Peace Peace Peace.

– Brihadaranyaka Upanishad 1.3.28

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Abbreviations

AAD	7-Amino-Actinomycin D
%	Percentage
°C	Degree Celsius
Ab	Antibody
ABTS	2,2'-azino-di-(3-athylbenzthiazolin sulfonal)
AML	Acute myeloid leukaemia
APS	Ammonium Per-sulphate
ATP	Adenosine-Tri-Phosphate
BM	Bone Marrow
BrdU	Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
BSA	Bovine Serum Albumin
bp	Base pairs
cAMP	Cyclic adenosine mono phosphate
CDK	Cyclin dependent kinase
CDS	Coding Sequence
CFU	Colony forming unit
CKI	Cyclin dependent kinase inhibitor
CLP	Common Lymphoid Progenitor
CML	Chronic myeloid leukaemia
Cy5	Cyanine 5 Dye
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
E.coli	*Escherichia coli*
ECL	Enhanced chemo luminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbant assay
Epo	Erythropoietin

e.g.	For example (exempli gratia)
EtBr	Ethidiumbromide
EtOH	Ethanol
ERKs	Extracellular signal regulated kinases
FACS	Fluorescence Activated Cell Sorting (Flow Cytometry)
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FITC	Fluoresceine Isothiocyanate
FSC	Forward Scatter
G-CSF	Colony stimulating factor 3 (granulocyte)
GM-CSF	Granulocyte-Macrophage Colony-Stimulating-Factor
HSC	Haematopoietic Stem Cell
IL	Interleukin
IP	Immunprecipitation
JNK	*c-Jun N-terminal kinase*
kB	Kilo base
kDa	Kilo Dalton
L-Glu	L-Glutamine
LB	Luria broth
MAPK	*Mitogen-activated protein kinase*
MEP	Myeloid-Erytheroid Progenitors
mg	Milligram
MNCs	Mononuclear cells
MTS	MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NP-40	Nonidet P-40
NSAIDs	Non-steroidal Anti Inflammatory Drugs
N-terminal	Amino terminal
OD	Optical Density

ORF	Open Reading Frame
PAA	Polyacrylamide
PB	Peripheral blood
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
pmol	Pico-mole
PE	Phycoerythrin
POD	Peroxidase
qRT-PCR	Quantitative Real-time PCR
RNAse	Ribonuclease
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SSC	Side Scatter
SDS	Sodium Dodecyl Sulfate
TNFα	*Tumor necrosis factor α*
Taq	*Thermus aquaticus*
TBE	Tris/boric acid/EDTA
TBS	Tris-buffered saline
TBS/T	Tris-buffered saline/Tween20
TE	Tris/EDTA
TEMED	N,N,N´,N´-Tetramethylethylendiamine
TPO	Thyroid Peroxidase
Tris	Tris(hydrocymethyl)amino methane
UV	Ultraviolet
µg	Micro-gram
µl	Micro-litre
µM	Micro-molar

Zusammenfassung

Die akute myeloide Leukämie (AML) ist eine häufige Erkrankung bei Erwachsenen. Sie zeichnet sich durch ein schnelles Wachstum anormaler myeloider Zellen aus und ist weiterhin durch chromosomale Veränderungen, Translokationen, Duplikationen, Inversionen, Deletionen charakterisiert. In der AML wird die Differenzierung (Blastenkrise) geblockt und Zellen können nicht in ihre verschiedenen Linien ausdifferenzieren. Dadurch reifen diese Zellen nicht aus und entkommen dem Apoptose-Signalweg. Eine Repression dieser Stammzellen ist für eine AML-Therapie notwendig. Der übliche Therapieansatz für die Behandlung der AML ist eine intensive Chemotherapie. Dennoch sterben ca. 50% dieser Patienten. Außerdem sind die Standardtherapien sehr toxisch und werden von älteren Patienten schlecht vertragen.

In verschiedenen Studien wurde die Rolle von nicht-steroidalen anti-inflammatorischen Medikamenten (NSAIDs) überprüft, die eine Proliferations-Inhibierung und eine Apoptose-Induzierung bei verschiedenen Krebszellen *in-vivo* und *in-vitro* bewirken. Deshalb wurde dieser Ansatz verwendet, um mögliche Effekte einer Behandlung von $CD34^+$ AML- Zellen aus dem Knochenmark von Patienten sowie AML Zelllinien mit NSAIDs zu untersuchen. Dabei wurden die Zellen mit einer physiologisch erreichbaren Konzentration der NSAIDs (OSI-461, Sulindac Sulfide und Diclophenac) behandelt. Eine DMSO-Behandlung mit der gleichen Konzentration diente als Kontrolle. Viabilität, Apoptose, Zelldifferenzierung sowie zugrunde liegende molekulare Mechanismen wurden untersucht. Es konnte eine beständige Apoptose-Induzierung sowie bis zu einem gewissen Grad eine myeloide Differenzierungskapazität in NSAIDs behandelten Zellen nachgewiesen werden.

Außerdem waren ein G2/M Zellzyklus-Arrest und die Inhibierung der *cyclin dependent kinase-1 (CDK1*) bei OSI-461 behandelten Zellen zu sehen. AML Zellen, die mit 1 µM OSI-461 behandelt wurden, waren nicht in der Lage, ihr proliferatives Potential wieder zu erlangen, wenn sie nach einer initialen 48 Stunden Behandlung in ein 1 µM OSI-461 freies Medium gegeben wurden, selbst in bis zu acht Tagen nicht. Außerdem zeigten auch normale $CD34^+$ Zellen einen Anstieg der Apoptoserate nach einer initialen 48-stündigen Behandlung mit 1 µM OSI-461. Aber sie waren in der Lage, nach einer 48-stündigen Behandlung mit OSI-461 ihr normales Proliferationspotential wieder zu erlangen. Bei Leukämien ist der Schutz der normalen gesunden Stamm- und Progenitorzellen immer nötig, da gesunde Zellen für die Erholung nach der Therapie essentiell sind.

Es wurden vergleichende Protein- und Genexpressionsanalysen von Diclophenac-behandelten Zellen verwendet, um den Einfluss einer NSAID-Behandlung zu untersuchen und weitere mechanistische Einblicke in anti-leukämische Aktivitäten der NSAIDs als Reaktion auf die Behandlung zu erhalten. Die Resultate zeigen eine transkriptionale Aktivierung von *GADD45α* sowie dem nachgeschalteten *MAPK/JNK* Signalweg sowie erhöhte Proteinlevel des CASPASE 3 - Precursors. Das deutet auf eine Rolle der *c-Jun NH2-terminal kinase (JNK)* in NSAID vermittelter Apoptose hin, die von der JNK-Aktivität abhängt und eine Ergänzung zu einer durch eines spezifischen *JNK*-Inhibitor erzielten Apoptose ist.

Mitglieder der AP-1 Familie sind bei in der AML herunter reguliert. Eine transkriptionale Aktivierung der AP-1 Transkriptionsfaktoren Familienmitglieder *c-Jun, JunB* und *Fra2* nach einer Behandlung der AML-Zellen mit NSAIDs konnte gezeigt werden. Eine Re-Expression dieser Transkriptionsfaktoren führte zu einer Aktivierung von *GADD45α* sowie zu einer Apoptose-Induktion. Zusätzlich konnte gezeigt werden,

dass die durch OSI-461 erzielten antiproliferativen Effekte in AML Zellen mit der Induktion der pro-apoptotischen Zytokine *MDA-7/IL-24* und der Aktivierung des Wachstumsarrests sowie der *DNA-damage inducible genes (GADD) 45α* and *45γ* assoziiert sind. Diese Daten deuten auf ein Potential der NSAIDs hin, den Zelleintritt zu Signalwegen zu regulieren, was zu Apoptose versus Proliferationsarrest führt und dass manche von ihnen möglicherweise therapeutisches Potential für einen selektiven Angriff auf leukämische Zellen haben.

Zusammengefasst wurde *GADD45α* als neuer Differenzierungs und Apoptose-Induzierer in der humanen AML identifiziert, der das Ziel der *AP-1* Familienmitglieder *c-Jun, JunB* und *Fra2* ist. Die Ergebnisse zeigten, dass NSAIDs eine Expression der *AP-1* Genfamilie re-induzieren könnten, wobei sie die AML Proliferation inhibieren und eine Differenzierung induzieren. Damit erhält man eine neue Einsicht in die AML Pathophysiologie und eine neue Strategie, Apoptose und Differenzierung in der AML zu induzieren.

ABSTRACT

AML is described by a rapid growth of abnormal myeloid cells, which are further characterized by chromosomal abbreviations, translocations, duplications, inversions and deletions. In AML, there is a block in differentiation (blast crisis) of stem cells. Stem cells are not able to divide into different lineage. Therefore, these stem cells do not mature and escape the apoptotic pathway. Eradication of these leukemic stem cells is required for the treatment of AML. The common approach for the treatment of AML is the use of intensive chemotherapy. Yet, approximately 50% of these patients die from their leukaemia. Furthermore, standard treatments are very toxic and poorly tolerated especially in elderly patients.

Several studies have proven the role of Non-steroidal anti-inflammatory drugs (NSAIDs) to inhibit proliferation and induce apoptosis in various cancer cells *in-vitro* and *in-vivo*. Therefore, this approach was used to investigate the potential effects of NSAIDs treatment on $CD34^+$ AML cells derived from patients´ bone marrow and AML cell lines. These cells were treated with the physiologically achievable concentration of NSAIDs (OSI-461, Sulindac Sulfide and Diclofenac). Cells treated with equal amounts of DMSO were used as control. Viability, apoptosis and differentiation were analysed. The molecular mechanisms involved were also observed. In present study, a consistent induction of apoptosis and to some extent an increased myeloid differentiation capacity in NSAIDs treated AML cells was observed.

The G2/M cell cycle arrest was also observed along with Inhibition of the *cyclin dependent kinase-1 (CDK1)* in OSI-461 treated AML cells. AML cells treated with OSI-461 1 µM was not able regain their proliferative potential when after initial 48 hour treatment they were placed in a medium free of OSI-461 1 µM, even up-to 8 days. Whereas, although normal $CD34^+$ cells also showed the increase in apoptosis after initial 48 hour OSI-461 1 µM treatment. But they were able to regain their normal proliferative potential, when they were placed in OSI-461 free cell culture medium. In leukaemia protection of normal healthy stem and progenitor cells is always needed as healthy cells are essential for recovery after therapy.

Comprehensive protein and gene expression profiling of Diclofenac treated AML cells was utilized to study the impact of NSAIDs treatment with the aim of gaining further mechanistic insights into the anti-leukemic activities of NSAIDs. The findings demonstrate a transcriptional activation of *DNA-damage inducible genes (GADD) 45α* and *MAPK/JNK* pathway as well as, increased protein levels of the CASPASE-3 precursor. This signifies the role of *c-Jun NH2-terminal kinase (JNK)* in NSAIDs mediated apoptosis. Indeed this NSAIDs mediated apoptosis is dependent on JNK activity. Addition of a specific *JNK*-inhibitor abrogated apoptosis.

The *AP-1* family members are commonly down regulated in AML. It was found that NSAID treatment of AML cells leads to activation of *AP-1* transcription factor family members: *c-Jun, JunB* and *Fra2*. Re-expression of these transcription factors results in activation of *GADD45α* and induction of apoptosis. In addition, the OSI-461 mediated anti-proliferative effects observed in AML are associated with the induction of the pro-apoptotic cytokine *MDA-7/IL-24* and activation of the growth arrest and *GADD45α* and *GADD45γ*. These data indicate that NSAIDs are able to regulate AML cell entry to apoptotic pathways, which leads to apoptosis versus proliferation arrest, and that some of these findings may have therapeutic potential for the selective targeting of leukemic cells.

In summary, *GADD45α* is identified as a novel inducer of differentiation and apoptosis in human AML, targeted by the *AP-1* family member's *c-Jun, JunB* and *Fra2*. The results demonstrated that NSAIDs can re-induce expression of *AP-1* family genes, thereby inhibiting AML proliferation and inducing differentiation. These findings provide novel insights into AML pathophysiology and provide a new strategy to induce apoptosis and differentiation in the AML.

Citations of publications in this dissertation

The publications belonging to this cumulative dissertation are cited in the following way. Please see section 3 for complete references.

Serial Number	Citation	Journal	Status
1.	Singh et al. (2011)	Apoptosis	Published
2.	Singh et al. (2011)	Annals of Hematology	Published

1. INTRODUCTION

1.1 Perfective aspect

Due to developments in the field of stem cell biology in 20th century, many questions like, how a single cell grows in a multi-cellular and highly sophisticated organism can be addressed. In multicellular organism there is a continuous turnover of the cells, due to cell death. Hence there is a need to replenish the depleted cells. To make up the for loss of these cells and to insure maintenance and development throughout the life of an organism small number of organ-specific cells are present, known as **stem cells** (Cairns, 1981).

All cellular blood components are derived from haematopoietic stem cells (HSCs). HSCs are the first and best-studied cells in terms of differentiation and hierarchical order. Haematopoietic cells are short lived so there is a regular need of their regeneration throughout life of an organism (Cumano and Godin, 2007). In mammals the development of various pre, pro-precursor and mature cells is known as "***Haematopoiesis***" (Figure 1).

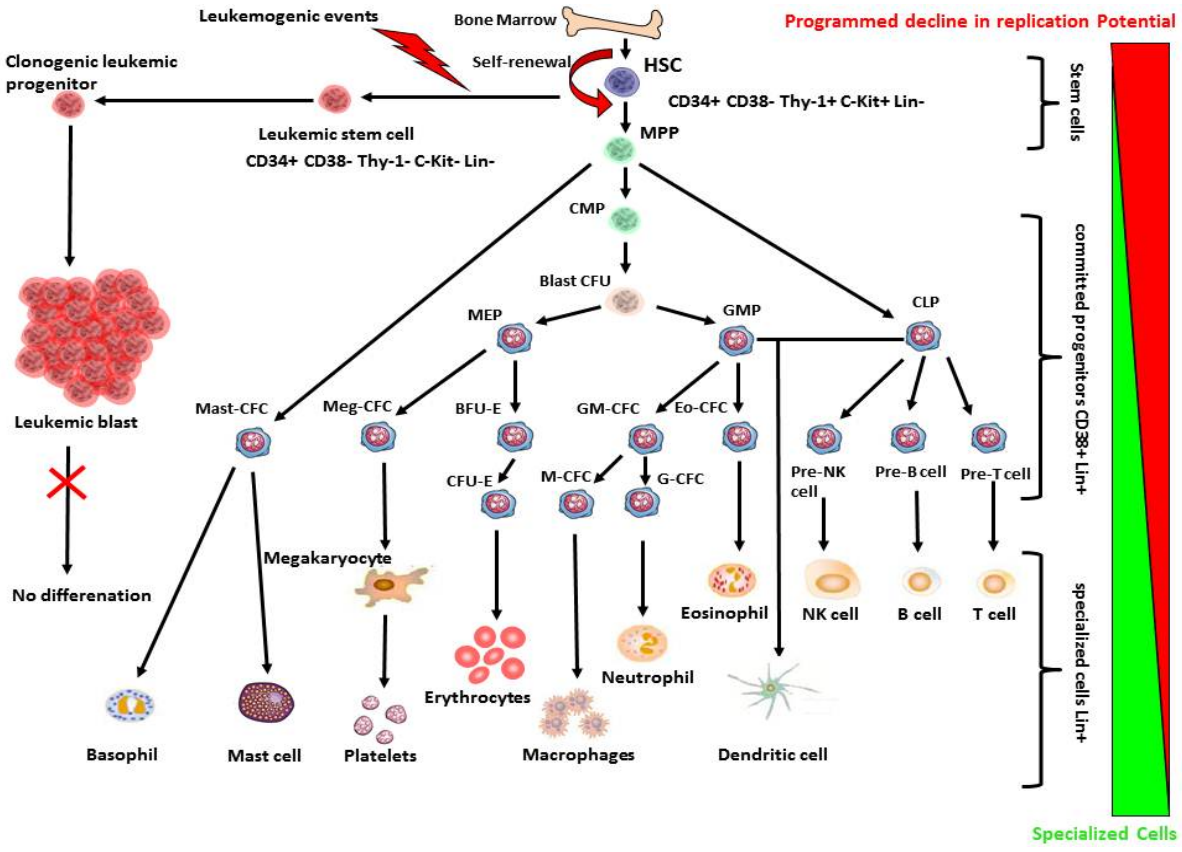

Figure 1: ***Haematopoiesis.*** Lineage tree, showing areas where branching can occur. A single haematopoietic cell can differentiate and can give rise to different lineage progenitor cells, which can further proliferate and mature, finally giving rise to mature blood cells. (HSC = Haematopoietic stem cell, MPP = Multi-Potential Progenitors, CMP= Common Myeloid Progenitor, CLP= Common Lymphoid Progenitor, Blast CFU = Blast colony-forming unit/cells, MEP = Megakaryocyte-Erythroid Progenitor cell, GMP = Granulocyte Macrophage Progenitors, CFC = Colony-forming cells, CFU = Colony-Forming Unit, CFC = Colony-forming cells, Colony-forming cells granulo-monocyte = GM- CFC, Colony-forming cell Eosinophil = Eo-CFC). Adapted from (Dalerba et al., 2007; Lobo et al., 2007)

In general, all blood cells can be found in bone marrow (BM), which is a primary site for synthesis of various Haematopoietic cells. The foundation work done by *Metcalf and Moore* (Metcalf, 1970; Moore and Metcalf, 1970), *Till and Mc Cullock* (Till and Mc, 1961) in their respective studies showed that blood stem cells or multi-potent progenitors (MPP) are located in BM. MPP are responsible for constant make up of blood cells. Different cells of haematopoietic stem cells (HSCs) and steps involved in HSCs differentiation and maturation are shown in Figure 1.

1.2 Leukaemia

Rudolf Virchow coined the term *"Leukaemia"* in *1856*, he has described that, "there is an accumulation of abnormal White Blood Cells in patients". **"Leukaemia"** is a Greek word meaning "White Blood" to describe the condition of the blood in the patient samples.

As per the definition, leukaemia can be defined as the disease of blood in which there is a blocking of differentiation **(*blast crisis*)** and cells are unable to divide into different lineage. These cells do not mature and escape apoptotic pathway, leading to over production of abnormal white blood cells.

Primary cause of origin of leukaemia can be contributed due to constant accumulation of chromosomal abbreviations, translocations, duplications, inversions and deletions (Lowenberg, 2008; Mrozek and Bloomfield, 2006; Tallman et al., 2005). The events which induce leukaemia are suppression of apoptosis, deregulation of cell-cycle to support uncontrolled expansion and invasion. The deregulation of cell-cycle provides a mechanism to support further neoplastic progression.

Leukaemia can divided into two basic types

- **Myeloid Leukaemia**
- **Lymphoid Leukaemia or Lymphocytic Leukaemia**

Myeloid Leukaemia: - If there is a differentiation blockage of the myeloid cells i.e. *monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes*, *megakaryocytes / platelets* and *dendritic* cells, it is known as myeloid leukaemia. In some cases, these cells can still advance to maturation.

Lymphoid Leukaemia or Lymphocytic Leukaemia: - In lymphoid leukaemia or lymphocytic leukaemia as name suggests majority of the effected cells belong to lymphoid lineage i.e. *T-cells*, *B*-cells*, Nκ*-cells.

Leukaemia can also be divided according to progression and lineage type of the disease. Most of the present day leukaemia falls in one of the following major classes.

- **Acute Myeloid Leukaemia (AML)**
- **Acute Lymphocytic Leukaemia (ALL)**
- **Chronic Myeloid Leukaemia (CML)**

- **Chronic Lymphocytic Leukaemia (CLL)**

AML is related to this present study and is discussed further here.

1.3 Acute myeloid leukaemia (AML)

In 1889, Wilhelm Ebstein coined the term "acute leukaemia" to differentiate between fatal and rapidly progressive leukaemia and mild "chronic leukaemia". AML can be defined as a maturation arrest in BM cells which are not able to perform their normal activity. AML is heterogeneous disease of precursor stage myeloid cell multiagency. There is a rapid increase in immature blast cells. AML develops quickly, if not treated can be fatal in few months of its onset. AML is associated with a wide spread of deformities at the DNA level and in signalling pathways. AML is the second most common type of leukaemia in USA. It is estimated that about 12,330 people were diagnosed with AML in 2010.

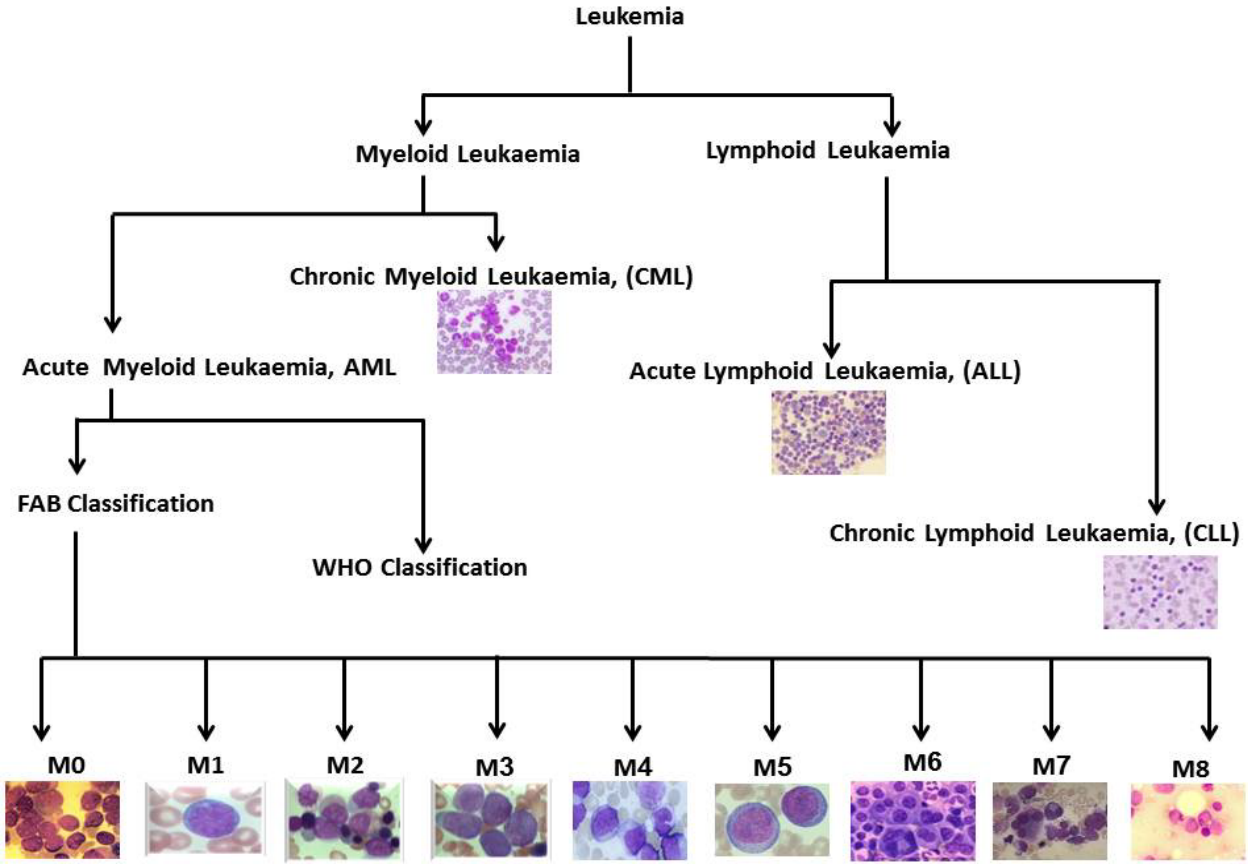

Figure 2: ***Classification of Leukaemia***. Leukaemia can be divided based on clinical and pathologically into variety of large groups i.e. according to which kind of blood cell is affected. Thus, first division can be between its Myeloid and Lymphoid forms. Further, Leukaemia can be subdivided. This division makes difference between acute and chronic forms. (Adapted from www.info-medicine.com/aml-french-american-british-fab-classification-m0.html and www.med-ed.virginia.edu/.%20../wcd/myeloid1.cfm, web-sites last visited on 28th of July, 2011)

AML is a heterogeneous disease due to the combination of the mutations in chromosome. These mutations play a role in the prediction of the disease. Unlike most other cancers, which are classified based on a stage or progression, AML is classified on bases of various aspects of the disease i.e. genomic mutations, age of patient, cytological disorders, etc. (Figure 2).

Abiotic and biotic sources have been reported to cure AML, including Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) (Gullett et al., 2010), Green Tea (Yang and Wang, 2010), Vitamin A (Retinoic Acid) (Shimizu et al., 2004), and curcumin powder (Das et al., 2010). But among these, NSAIDs stand apart due to most extensively studied group of drugs in relation to cancer.

1.4 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) belong to structurally diverse group, but similar in action. Felix Hoffmann (1897) oxidised salicylic acid to Acetylsalicylic Acid (ASA) for his father who was suffering from the pain of arthritis (Cuzick et al., 2009). Later "Bayer" marketed this product under trade name *"ASPRIN",* thus giving rise to first commercially marketed NSAID. NSAIDs are normally used as anti-inflammatory, anti-rheumatic, anti-pyretic, and as analgesic. NSAIDs are weak acids and they dissolve in acidic environment of stomach giving high bioavailability. These drugs have greater affinity to bind to the surface molecule on ribosomes, thus further increasing their action. From the beginning of early 20th century it is known that NSAIDs arrest many tumour progressions.

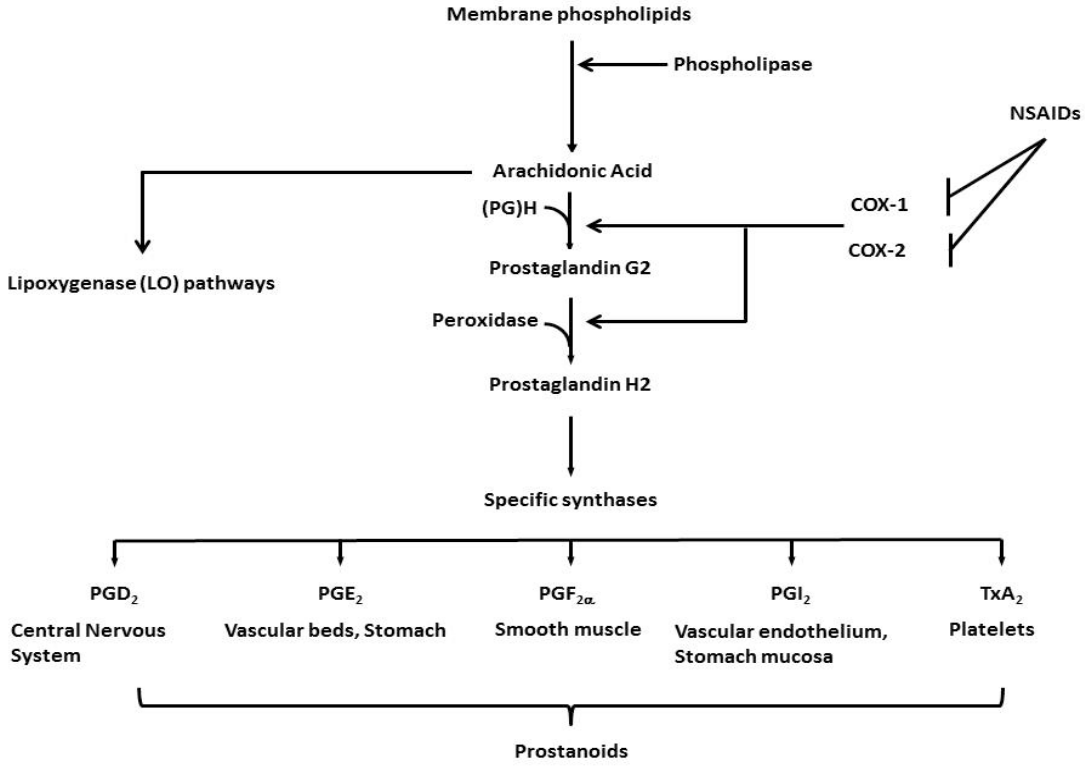

Figure 3: Metabolic pathway of arachadonic acid and cyclooxygenase (COX) pathway. Arachidonic acid, released from membrane phospholipids by phospholipase A2 (*PLA2*), is metabolized by cyclooxygenases to prostaglandin H2 (*PGH2*) in two steps. *PGH2* is converted to a variety of prostanoids by specific isomerases. COX = cyclooxygenase; *PG* = prostaglandin; NSAIDs = Nonsteroidal Anti-Inflammatory Drugs; *PGD2* = prostaglandin D2; *PGE2* = prostaglandin E2; *PGF2* = prostaglandin F2; *PGI2* = prostaglandin I2; *TxA2* = Thromboxane A2. Adapted from (Thun et al., 2002; Ting and Khasawneh, 2010) and Expert Reviews in Molecular Medicine © 2003 Cambridge University Press.

Around 1940, it was first found that aspirin (NSAID) has effect on various vascular events (Xu, 2002). Not much is known about mechanism involved in the pharmacokinetics of the NSAIDs. In 1971, *John Vane* and his colleagues demonstrated that aspirin and other NSAIDs exert their pharmacologic

activities by inhibiting the *Cyclo-Oxygenase (COX)*. *COX* further causes the production of *prostaglandin* (Figure 3). *Prostaglandin*s are involved in inflammation, blood clotting, immune response and many other physiological processes. It was discovered that *COX* exists in other forms *COX2 and COX3* too. But later it was found that *COX3* is splice variant of *COX1* (Ulrich et al., 2006). NSAIDs can also act by inducing physical modification on lipid bi-layer membrane, *NFκB* signalling, Apoptosis signalling, *APL*-β-*Catenin* pathway, *PPARα/γ/δ* target genes and by release of *MPR4* etc. (Ferreira et al., 2005; Sousa et al., 2008).

There are many safety data reports on use of NSAIDs, but there are also reports about adverse effects due to regular, prolonged, or excessive use of NSAIDs. In these reports NSAIDs are shown to cause lymphoid malignancies like non–hodgkin lymphoma, asthma, effects on gastric intestine (GI) even up to bleeding, peptic ulcer and lesions, etc.(Lanas, 2009; Robak et al., 2008)

1.5 Effects of NSAIDs on Cancer

Various experimental, epidemiological and clinical data were churned out in 1980's.These data showed that NSAIDs acted positively in decreasing the development and prevention of the induction of cancers, when NSAIDS were taken occasionally or regularly (i.e. 5 to 7 times per week) or prolonged (>2 years) at clinical approved dose (Harris et al., 2005; Ruegg et al., 2003; Umar et al., 2003). NSAIDs were reported to decrease the size and number of intestinal adenomas in-patient with Familial Adenomatous (FAP) (Giardiello et al., 1993; Spagnesi et al., 1994; Steinbach et al., 2000). There was up to 50 per-cent reduction in incidence of colorectal cancer in people who took NSAIDs regularly (DuBois and Smalley, 1996; Smalley and DuBois, 1997).

Table 1 ***Effects of various NSAIDs on different types of cancers in clinical trials.***

Disease	Number of Patients	Duration	Drug used in study	Phase	Results	Reference
FAP	77	6 months	Celecoxib	II	Celecoxib significantly decreases the No. of colon polyps	(Steinbach et al., 2000)
FAP	10	4 months	Sulindac	III	Polyps regressed completely in 6 patients, partly in 3	(Labayle et al., 1991)
FAP	22	9 months	Sulindac	III	Sulindac decreased No. of polyps by 56% and size by 65%	(Giardiello et al., 1993)
FAP	24	6 months	Sulindac	III	Duodenal polyps <2 mm regressed in 9 of 11 patients treated with sulindac	(Nugent et al., 1993)
Previous adenomatous polyps	44	4 months	Sulindac	III	Sulindac did not statistically significantly decrease number or size of poylps	(Ladenheim et al., 1995)

In studies on various animal models with induced or transplanted cancers, NSAIDs had inhibitory and protective effects on FAP, esophageal, stomach, lung, skin, breast, prostate, urinary bladder, colorectal cancer (Jacoby et al., 2000; Oshima et al., 1996; Wang et al., 2004), colon and pancreatic (Fujimura et al., 2006; Gonzalez-Perez et al., 2003; Thun et al., 2002; Xu, 2002). In various clinical trials NSAID has shown its effects on various cancers also. Few of the randomised clinical trials are listed in Table 1.

It is widely accepted that NSAIDs causes apoptosis and G2/M phase growth arrest (Czibere et al., 2005; Czibere et al., 2006; Zerbini et al., 2006). NSAID (Exisulind) was reported to affect on primary leukemic cells and leukemic cell lines such as KG1, THP-1 & SKM-1, through an induction of apoptosis by *JNK* pathway (Czibere et al., 2005; 2006ab). Recent studies have shown that Aspirin (NSAID) caused apoptosis in various human primary *T & CLL-B leukemic cells* and various cell lines (Iglesias-Serret et al., 2010; Ou et al., 2010).

NSAIDs are believed to act through blockage of *COX* activity or independently through other targets. These targets include β–*Catenin* (Lu et al., 2005), *Ras* (Herrmann et al., 1998), *Nuclear factor κB* (Bren-Mattison et al., 2008; Yamamoto et al., 1999), *Cyclin GMP* (Rice et al., 2006), *ERK1/2* (Rice et al., 2006), *Peroxisome proliferation activated receptor δ (PPARδ)* (He et al., 1999), *NSAIDs activated Gene- 1* (Kim et al., 2005), *GADD45* (Zerbini et al., 2006), *IL-24* (Zerbini et al., 2006), *MKK4* (Czibere et al., 2005; Dai et al., 2010; Zerbini et al., 2006),(Rice et al., 2006), *JNK* (Czibere et al., 2005; Zerbini et al., 2006), *Epidermal growth factor receptor* (Pangburn et al., 2005), *prostate apoptosis response gene -4* (Herrmann et al., 1998) and *CSK/SRC*, etc. (Kunte et al., 2008).

1.6 NSAIDs used in present study

1.6.1 Sulindac Sulfide

Sulindac Sulfide is *(Z)-5-Fluoro-2-methyl-1-[p-(methylthio) benzylidene] indene-3-acetic acid* with **$C_{20}H_{17}FO_2S$** molecular formula (Figure 4) and 340.41 is the molecular weight. Sulindac Sulfide and Sulindac Sulfone are metabolites of Sulindac. Sulindac is known to cause apoptosis in different cancer cell lines (Chan et al., 1998; Herrmann et al., 1998; Lim et al., 1999; Shiff et al., 1995; Zhang et al., 2000). Sulindac Sulfide causes apoptosis in solid tumour cell lines through activation of *JNK* pathway via *GADDs* (Zerbini et al., 2006). Sulindac Sulfide is shown to have anti-cancer effects in mice models (Boolbol et al., 1996; Chiu et al., 1997). Role of Sulindac Sulfide in Familial Adenomatous Polyposis (FAP) regression of has also been reported (Giardiello et al., 1993; Labayle et al., 1991). Further studies are required to understand the role and pathways involved in anticancer activates.

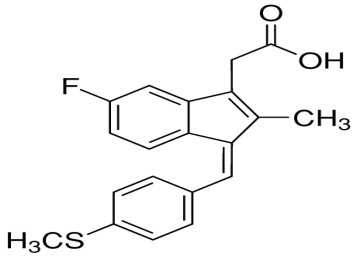

Figure 4: ***Molecular structure of Sulindac Sulfide*** (Source: http://www.sigmaaldrich.com/catalog/ProductDetail.do ?D7=0&N5= SEARCH_CONCAT_PNO|BRAND_KEY&N4=S3131|SIGMA&N25=0&QS=ON&F=SPEC, web-site last visited on 11th of August, 2011)

1.6.2 *Diclofenac*

Diclofenac is *2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid sodium salt*. **$C_{14}H_{10}Cl_2NNaO_2$** is the molecular formula (Figure 5) and 296.148 is the molecular mass. It is mainly used to reduce pain, inflammation and used as analgesic agent. It is a research molecule of "Ciba-Geigy" (now Novartis) and was discovered in 1973. Diclofenac treatment causes 60% decrease in tumour size in mouse model for pancreatic cancer due to the increase in apoptosis in tumours cells (Mayorek et al., 2010). Diclofenac treatment has anti-proliferative effects on various human tumours and causes apoptosis (Zerbini et al., 2006).

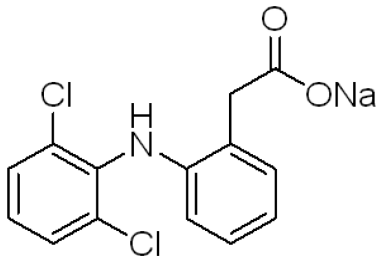

Figure 5: ***Molecular structure of Diclofenac*** (Source: http://www.sigmaaldrich.com/catalog/ProductDetail.do?D7=0&N5=SEARCH_CONCAT_PNO|BRAND_KEY&N4=D6899|SIGMA&N25=0&QS=ON&F=SPEC, web-site last visited on 11th of August, 2011)

1.6.3 OSI-461

OSI-461 was earlier known as **CP-461**, and is *Z-5-fluro-2methyl-(-4 pyridene)-3-(-N-benzyl) -indenyacetmide hydrochloride*. OSI-461 (Figure 6) is a new generation drug and synthetic derivative of Exisulind. OSI-461 lacks both COX-1 and COX-2 inhibition activity i.e. independent of *COX-1* and *COX-2* inhibition. OSI-461 has induced apoptosis and arrest of the cell cycle in G2/M phase in variety of human cancer cells (Shimizu et al., 2004). OSI-461 has shown a slight antitumor activity in a Phase II pilot study with patients having hormone-refractory prostate cancer (Resta et al., 2011).

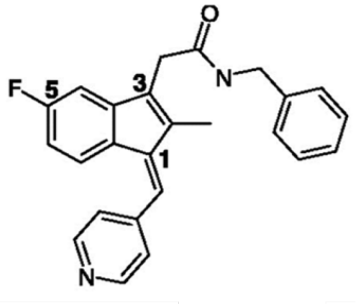

Figure 6: ***Molecular structure of OSI-461*** *Source*: (Xiao et al., 2006)

1.7 Cell-cycle

Different events are required for the cellular progression and development of organisms, which includes cell division, cell-cycle, differentiation and apoptosis. Among these one of the important events is cell-cycle. In cell-cycle genetic material is replicated in a semi-conservative way. Cell has to

pass through many stages, these are different stages of the preparation and cell division and are known as **"*Cell-cycle*"** (Figure 7). Generally, cell divides into daughter cells, according to the requirement of the body and in this process, passes its genetic information to the daughter cells.

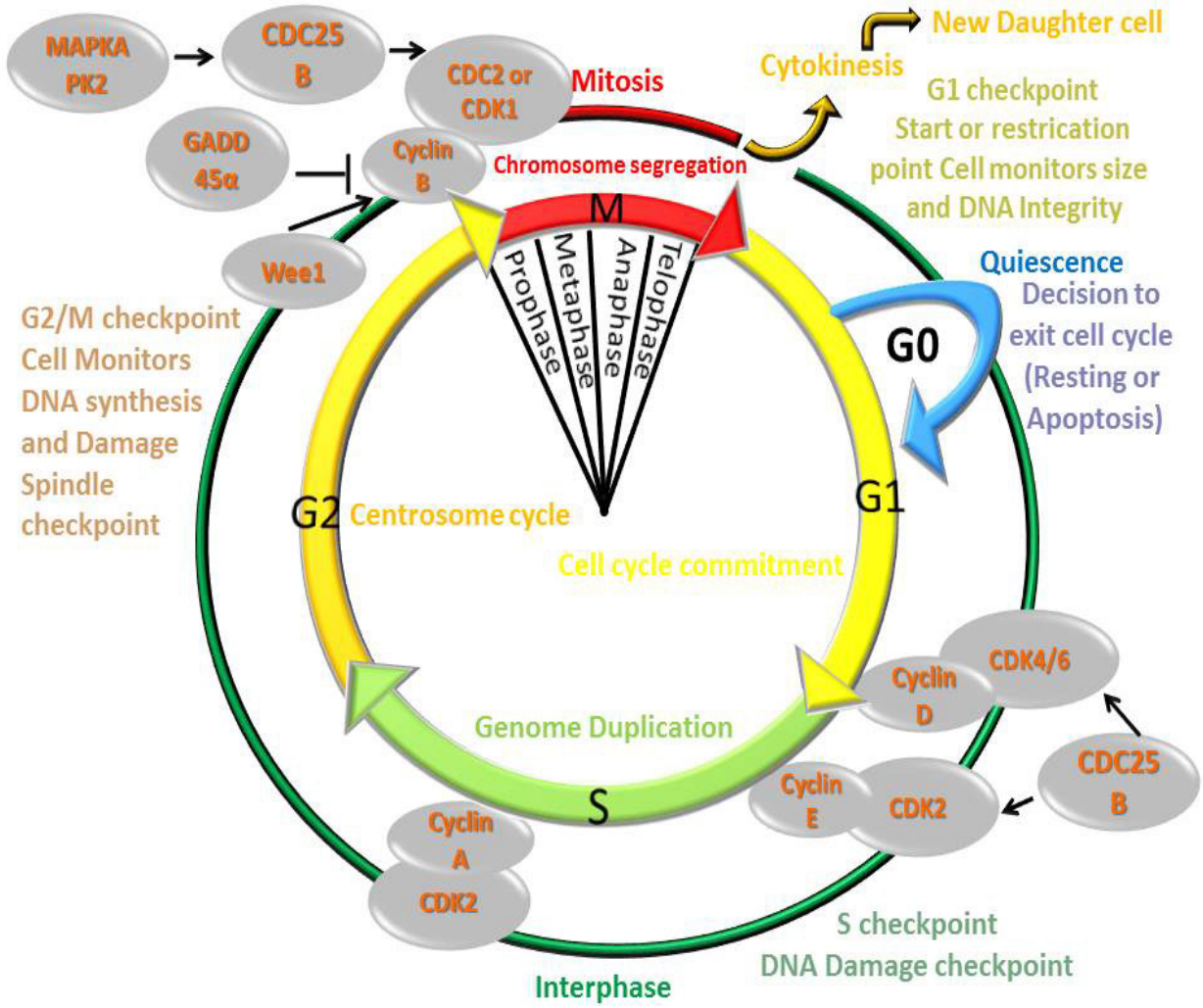

Figure 7: *Cell-cycle.* The different phases of cell cycle are represented with regulators and cell cycle checkpoints. Adapted from (Thornton and Rincon, 2009; Vermeulen et al., 2003)

1.7.1 Cell-cycle regulation

Cell-cycle is a tightly regulated mechanism. Any abnormality in single cell organism leads to its death. It causes malignancies in case of multicellular organism. To maintain cellular integrity and to pass an intact copy of the genetic material, accurate cell division is essential. Maintenance of genetic material is beneficial for cell survival and is required for cancer avoidance. The cells invest huge resources for maintenance of the genetic material. For development of cancer, cells undergo many uncontrolled cell divisions and escape apoptosis, due to these genetic modifications. Cells have regulatory mechanisms to avoid these genetic modifications, deformities and alterations (Figure 7).

1.7.2 Cell-cycle regulators

Few of the cell-cycle regulators are described below

Cyclins: -There are at least 13 *cyclins* known to date. Different *cyclins* are required to regulate cell cycle at different stages of cell-cycle. *CyclinA* expression is required for cells to leave G1 phase arrest (Bonda et al., 2010) while *cyclinA/E* is required in G1 to S phase transit and again in S to G2-phase arrest. *CyclinB* is required in G2 to M phase transit arrest (Bucher and Britten, 2008). *Cyclins* are classified on the basis of their time of expression and activation. All cyclins contain a 100 amino acids homologous region, known as "Cyclin-Box". *Cyclins* bind to *cyclin dependent kinase (**CDK**)* at this homologues region and degeneration of *cyclin* leads to inactivation of *CDKs*.

CDKs: - *Cyclin Dependent Kinases (**CDKs**)* are activated by the formation of the complex between *cyclin*s and *CDKs*. In mammalians 12 loci are known for encoding *CDKs* but only *CDK1, CDK2, CDK3, CDK4* and *CDK6* are directly involved in cell cycle. *CDK1* is expressed in late cell-cycle and is considered as mitotic kinases while other kinases are known to act in the Interphase (Chen et al., 2006a; Malumbres and Barbacid, 2005). Mutations or over-expression of *CDKs* is associated with a number of human cancers.

CKIs: - *CKIs* is short form of *CDK inhibitors* and indirectly control cell-cycle. Seven different *CKIs* are known in mammals, which belong to different classes. *p21, p27* & *p57 CKIs* act in G1/S phase and are also known as first class. *P15, p16, p18* and *p19* are another class of *CKI* and are known as second *CKIs*. *CKIs* act by inhibiting c*yclins complex (CDKs)*.

1.7.3 Cell-cycle check points

Checkpoints can be defined as the point of cell division with further no return, In other words if a cell has crossed this point, it is committed to next step in cell-cycle. Experiments on cells, which were starved of serum before G0 phase did not advance for cell-cycle for division, but in another scenario even starvation of serum after G0 phase did not inhibit them from cell-cycle division and to go into mitosis. These cells appeared to be committed and continued cell-cycle (Vermeulen et al., 2003).

1.7.3.1 Major cell cycle check points

G1 Check Point: - *CyclinB* and *CDK4* complex mediates the progression of cells in early G1-phase. The G1 checkpoint is first line of defence against DNA damage; it delays and stops the cells with DNA damage from entering into S-Phase. The S-phase entry of the cells is mediated by two different ways following the DNA damage. The first and reversible pathway is *CDC25A* mediated de-phosphorylation at *Try15/Thr14* of *CDK2-cyclinE/A*. By this action *CDK2-cyclinE/A* is activated (Figure 7 and 8). The

second pathway is slow and sustained. G1 arrest is caused by *p53* which is induced by *p21*. The *p21* inhibits *CDK2* leading to maintenance arrest of cell-cycle (Houtgraaf et al., 2006; Warmerdam and Kanaar, 2010). *CDC25A* plays a role in this checkpoint by the phosphorylation of the *CDK2*. If there are DNA double-stranded breaks (DSBs) then there is activation of *ATM* mediated phosphorylation of *Chk2*. DSBs cause inhibition of *CDC45*. However, single-strand breaks are repaired by activation of *rad17, RFC.9-1-1* complex and *ATR* through *Chk1*. In this pathway *CDK1* cause G1 arrest by phosphorylation of *CDC25A*. *ATM* and *ATR* phosphorylated *p53*, causes accumulation of *p53* and increased activity of the transcription factor *p53* (Houtgraaf et al., 2006). *p53* leads to expression of *p21*, an inhibitor of *CDK* (Figures 7 and 8). *p21* reduces activity of *cyclinD/CDK4* complex by inhibiting *CDK4* (Kraft et al., 2009). *AP-1* transcription factors also play role as they help in expression of *cyclinD1,D2, E* & *CDK4* (Kraft et al., 2009).

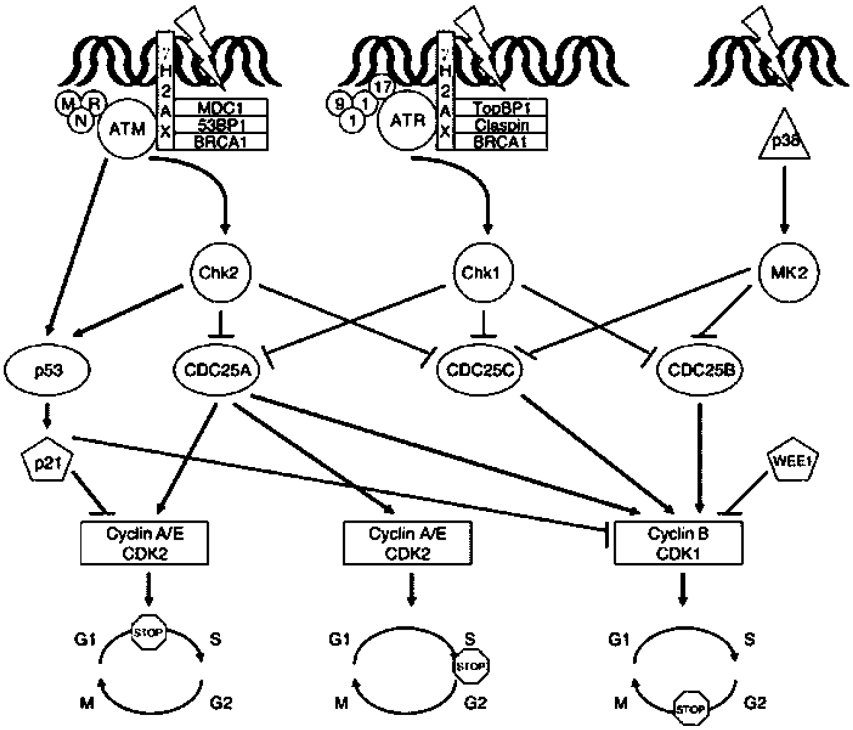

Figure 8: ***Cell cycle checkpoint pathways.*** Once DNA damage is identified with the help of sensors, the checkpoint transducers *ATM* and *ATR* undergo conformational change and/or localisation, resulting in their activation. Together with their mediators, *ATM* and *ATR* activate a series of downstream molecules, including the checkpoint transducer kinases. *Checkpoint kinase-2* and *Chk1* inactivate *CDC25* phosphatases, culminating in cell cycle arrest. Adapted from (Bucher and Britten, 2008)

S-phase checkpoint: - Integrity of DNA is maintained in S-phase checkpoint. This checkpoint monitors the DNA-damage and errors occurred during replication in S-phase (Dai and Grant, 2010). *CDK2* activity is increased in S-phase and thus contributes to the homologous recombination and DNA repair (Warmerdam and Kanaar, 2010). *CDK1*, is also known as *CDC2*, plays an important role in this checkpoint (Kraft et al., 2009). During S phase *CDK1 or CDC2* is activated by ATR and is the key regulator. Two pathways are reported which acted in S-phase checkpoint. First is *ATR.ATM-Chk2.Chk1-CDC25A-CDK2.* Second is *ATM.Nbs1.MRE11.SMC1* (Dai and Grant, 2010; Falck et al., 2002).

In S-phase, *Chk1* (checkpoint kinase 1) down regulation can abrogate S-phase arrest (Xiao et al., 2003). In the first pathway *cyclinE/A-CDK2* are inhibited by the degradation of *CDC25A-ATM/CHK2 & ATR/CHK1* due to DNA damage (Bartek et al., 2004). In the second pathway *ATM* recruit to DNA damage site with the help of *MDC1* via senor *MRN* (Watrin and Peters, 2006). *ATM* is activated on DNA

damaged site and it phosphorylates *SMC1*. *SMC1* is thought to function in DNA repair and S-phase progression. *PCNA* also plays a role in S-phase checkpoint via *CHK1* (Bucher and Britten, 2008; Dai and Grant, 2010).

G2/M checkpoint: - In most cancers, G1 checkpoint is defective, so G2/M checkpoint and S checkpoint are critical in cancer. However, S-phase checkpoint is known only to slow down cell-cycle rather than the arrest. G2/M checkpoint controls the DNA damage which has passed through G1 and S-phase checkpoint. It forces the respective cells with DNA damage to apoptosis (Bucher and Britten, 2008). G2/M checkpoint ensures cell division is blocked, if damaged DNA or deformities are present in DNA (Burgoyne et al., 2009). G2/M checkpoint stops the cells with the damaged DNA from entering into M-phase. *ATM-CHK2-CDC25/CDK1* pathway is activated due to double-stranded breaks and *ATR-CHK1-CDC25* by DNA lesions (Houtgraaf et al., 2006). G2/M checkpoint is mainly controlled by *CDK1/ cyclinB* complex, *p21, Cip* & *p27* regulators (Wang et al., 2009). The entry of cell in M phase is controlled by *cyclinB/CDK1* complexes which are also involved in nuclear envelope breakdown. While in M phase *CDK7*, a *CDK* activating kinase phosphorylates *CDK1* of *cyclinB/CDK1* complex and activate it. In interphase *cyclinB/CDK1* complex is activated by phosphorylation on Tyrosin 15 (Try15) by *Myt1* and Threonin 14 (Thr14) by *Wee1* (Liang et al., 2003). G2/M checkpoint works by inhibition of *cyclinB/CDK1* complex by *CDC25A, CDC25B* and *CDC25C* dual specificity phosphatases that is required to dephosphorylate these sites to activate the *cyclinB/CDK1* complex and progress in mitosis (Ganzinelli et al., 2008; Nigg, 2001; Raleigh and O'Connell, 2000). *CDK1* is dephosphorylated by *CDC25A* in G2/M checkpoint. *CDC25A* kinase is phosphorylated at Ser 76/124 and *CDC20C* at Ser216 by *Chk1*. This further leads to *CDC20A* proteosomal degradation and ubiquitination via *APC/C* and *Skp1/Cullin/F-Box protein/SCF* and *ubiquition* ligases (Figures 7 and 8).

In G2/M, *ATR* activates *Chk1* by phosphorylation of *CDC25A, B, and C.* This phosphorylation prevents the activation of *cyclinB/Cdk1* and results in G2/M phase arrest (Boutros et al., 2006). Further sustained G2/M phase arrest is required for the induction of the transcription factors which endogenously inhibited *CDK1* e.g. *GADD45s, p21, p53 14-3-3j* (Dai and Grant, 2010; Tse et al., 2007). The stress induced activation of *p38 MAPK/MK2* and inactivation of *CDC25B/C,* is thought to be another mechanism involved in G2/M phase arrest (Bucher and Britten, 2008).

1.8 Cell cycle and apoptosis

Many physiological processes are balanced in human body. These processes include proper tissue development and homeostasis. Proper balance is always required between cell cycle and apoptosis. In fact there are many positive and negative regulations identical in cell cycle and apoptosis. Morphological common features are shrinkage of nucleolus and cell, chromatin condensation, membrane blebbing etc. Many cell-cycle genes i.e. *p53, GADD45s, cyclins, CDKs, EF2* etc. participate in apoptosis too. A normal human body needs proper balance in apoptosis and cell proliferation by cell cycle. On average normal human body makes up to $60X10^9$ cells every day. Some of these cells die or used for maintenance and homeostasis. Uncontrolled cell division with lack of apoptosis cause further complications like tumour formation and cancer in human body (Alenzi, 2004).

1.9 Apoptosis

The apoptosis is a programmed cell death and plays a crucial role in regulation of many physiological processes during embryological development as well as in adult organism. Apoptosis is highly conserved throughout evolution (Fulda, 2009). Apoptosis is essential for the removal of unwanted, damaged, or infected cells. Escape from apoptosis is a signal for many cancers.

Cell death by apoptosis was described in 1972 in ground-breaking publication by ***Kerr, Wyllie and Currie*** **(Kerr et al., 1972).** Apoptosis is a Greek word, which is referred to falling of leaves from trees in autumn. As its meaning suggest word was chosen for cell death in mammalian cells to represent desirable death of cells for better survival for the host (Cohen et al., 1992). The process of physiological cell death has been discovered more or less five times independently by various researchers in past 160 years (Cotter, 2009).

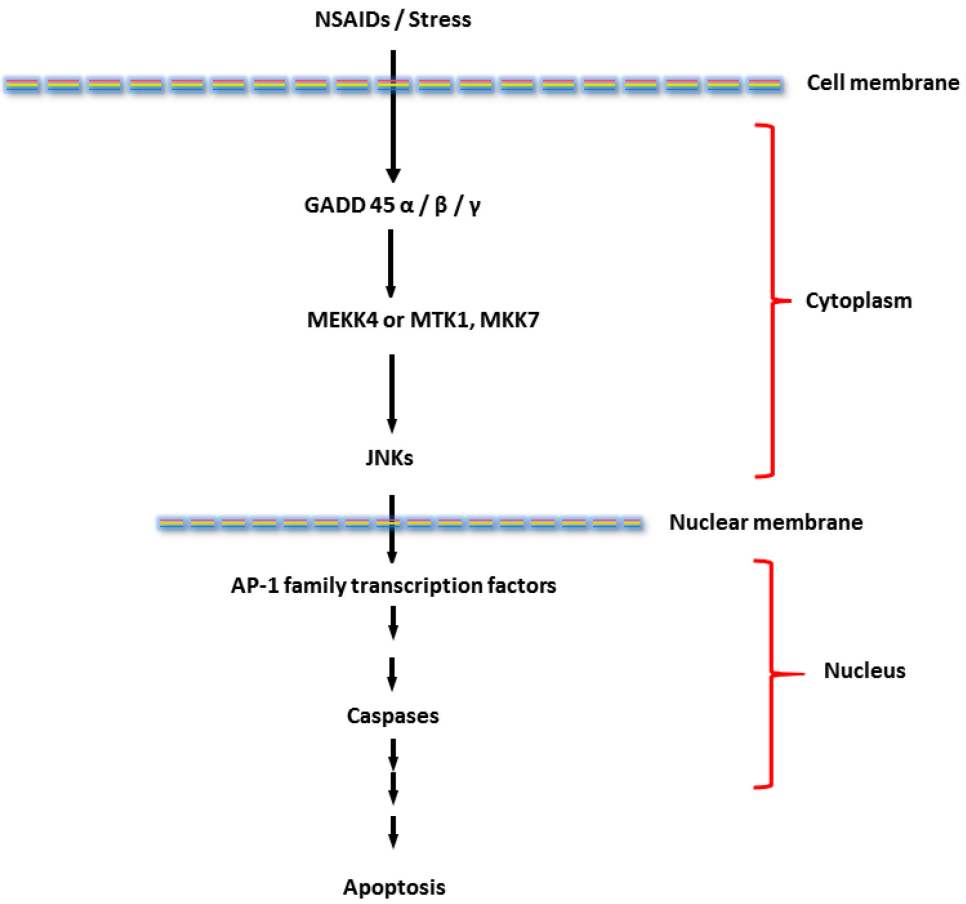

Figure 9: ***Signalling pathway-showing apoptosis.*** *GADDs, JNKs* and *AP-1* members leading to apoptosis. NSAIDs cause activation of *GADDs* and they bind to *MEKK4, MKK7* those results in *JNK*s phosphorylation via *GADDs*. *JNKs* leads to phosphorylation of *c-Jun (AP-1 family transcription factors)* and translocated to nucleus. *c-Jun* through *Caspases* induce apoptosis. Adapted from (Eferl and Wagner, 2003).

Apoptosis is caused by *caspases* (Figure 9). *Caspases* are cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases and are involved in apoptosis, necrosis, and inflammation. About 14 *caspases* are known for their role in Apoptosis in humans. Depending upon their role in human apoptosis, *caspases* can be divided into two groups.

Caspases 2, 8, 9 and 10 are known as initiator *caspases* and mediate the interaction of *caspases* with upstream adaptors and effectors (Noy, 2010).

Caspases 3, 6 and 7 are known as effecter *caspases* or executioner *caspases* and catalyse the downstream steps of apoptosis by mediating & regulating DNA repair, structural proteins & cell-cycle related proteins (Noy, 2010).

1.9.1 Apoptosis can occur through two pathways

The intrinsic apoptotic pathway: - Intrinsic apoptotic pathway start from inside the cell in response to cellular signals from DNA damage, a defective cell-cycle or stress signals. This pathway is mediated by mitochondria and endoplasmic reticulum (ER). *Bcl-2* family members regulate this process. The *Bcl-2* family is also involved in anti-apoptotic process. Other important players of this pathway are *Cytochrome C, Apaf-1* and *pro-caspases9* (Noy, 2010).

The extrinsic pathway:-This pathway is activated from external signals by activation pro-apoptotic receptors, which are on the cell surface (Figure 9). These cell surface death receptors include *TNFα, Fas* and *TRAIL* etc. These receptors further bind to *Caspases8* and initiate downstream signalling of apoptotic pathway. *JNK* and *NFκB* are known target of extrinsic pathway (Figure 9). Extrinsic pathway unlike the intrinsic pathway, triggers apoptosis independent of the p53 protein (Del Principe et al., 2005).

1.10 JNKs

JNKs are members of *MAPK (mitogen-activated protein kinase)* super-family that also includes the *ERK* and the *p38 MAP kinases*. The *c-Jun N-terminal kinase (JNK)* pathway and its components plays central role in mediating the apoptotic effect of NSAIDs. *JNKs* are known to target *AP-1* (*Activator Protein – 1*) transcription factors phosphorylation especially of *c-Jun* and related molecules (Weston and Davis, 2007). Generally, *JNKs* phosphorylates *c-Jun* on transcriptional activation domain at Ser 63 or Ser 73 sites in response to oncogenic expression (Figure 9) (Heasley and Han, 2006) and enhance the initiation of apoptosis. *JNKs* are generally activated in response to various stresses, UV or γ radiation, inflammation, growth factors, and oncogenes (Heasley and Han, 2006). Until now 10 JNK, isoforms are known spliced form of three *JNK* Genes, *JNK1, JNK2* and *JNK3*. *JNK1* and *JNK2* are expressed ubiquitously, and *JNK3* is expressed up to some extent in testis and heart but mainly in brain. Main target of *JNKs* is *c-Jun*, but they can also be phosphorylated by *JunD* but in lesser extent and phosphorylation of *JunB* via *JNKs* is still not clear (Shaulian, 2010).

Phosphorylation of *c-Jun* via *JNKs* was required for development and neuronal apoptosis in mice. Single *JNK* knockout mice in any of three *JNK* did not show any development defects. These knockout mice were more sensitive to skin cancer and induced lung tumorigenesis (Heasley and Han, 2006). *JNKs* exhibited role in cell-cycle via *c-Jun*, which further controls *cyclinD* (Shaulian, 2010). *JNKs* appeared to act in both pro and anti-apoptotic functions depending on the various factors i.e. tumour type, *JNK* isoform etc. (Heasley and Han, 2006).

The apoptotic pathway, mediated by *JNKs* is initiated by diverse signals i.e. stress or oncogenic signals. These signals activated *MAP Kinase Kinase Kinase (MP3Ks)* which in turn phosphorylated and activates

MKK4/MTK1 and MKK7, the isoforms of *MAP2K*. The phosphorylated *MKK4/MTK1* and MKK7 activates *JNK* (Weston and Davis, 2007). Once activated *JNKs* were translocated to the nucleus, where they phosphorylated *c-Jun* at Ser 63 and Ser 73 at N-terminal transactivation domain. By this transactivation of *c-Jun* expression of *c-Jun* is increased and there was also activation of *c-Jun* dependent apoptotic pathway (Li et al., 2004; Zerbini et al., 2004) (Figure 9).

1.11 AP-1 family

The *activator protein-1* (*AP-1*) is a heterodimeric protein. *AP-1* transcription factor was discovered in 1987 in Hela cell line nuclear extracts and identified by DNA element 5'-TGAGCTCA-3' (TRE or TPA response element). *AP-1* is the first transcription factor identified in mammals and is one of the extensively studied transcription factor, functions of which are yet to be fully understood. *AP-1* is a redox sensitive transcription factor and can sense stress. It can transduce changes in cellular redox status by modulating gene expression according to the stress. The AP-1 is a family of proteins, which are recognised by presence of basic leucine zipper (bZip), later is essential for DNA binding (Nair et al., 2010; Shaulian, 2010). The *AP-1* has following sub families (Nair et al., 2010; Shaulian, 2010).

***Jun*: -** This sub-family includes *c-Jun, JunB*, and *JunD*

***Fos*: -** This sub-family includes *c-Fos, FosB, Fra1* and *Fra-2*

Besides these other sub-families are:

Activating transcription factor (ATF) family includes, *ATF1, ATF2, ATF3, ATF4, ATF5, ATF6 & ATF7*.

Jun dimerization protein (Jdp) family, includes *Jdp1* and *Jdp2*

Musculoaponeurotic Fibrosarcoma (Maf) family, includes, *c-Maf, MafA, MafB and MafG/F/K*.

AP-1 family members are known for the Dimer formation, which can occurs between same sub-family members or in the two sub-families to regulate the genes and the expression. *Jun* family can make both homo-dimers (formed by two identical molecules) and hetero-dimers (formed by two different macromolecules), whereas *FOS* members can make only hetero-dimers but. It is proved that *AP-1* transcription factors are active in cell proliferation, information, differentiation, apoptosis, and cellular migration and wound healing (Shaulian, 2010).

Major genes related to present study are described below

1.11.1 c-Jun

The *c-Jun* was earlier known as *Jun*. *c-Jun* is an early response transcription factor of *AP-1* transcription family and *Jun* sub-family. *c-Jun* is activated by various *stimuli* and mediates the transcriptional regulation response (Black et al., 1994). *c-Jun* is phosphorylated at Ser 63 and Ser 73 by the *JNK* pathway (Kallunki et al., 1994). *c-Jun* is essential for development of mice and knock down of *c-Jun* is lethal for mice at E12.5 (Vesely et al., 2009). Knock-in for *c-Jun* with *JunB* or *JunD* rescue embryonic

lethality until birth suggesting the overlapping functions between *Jun* family members (Eferl and Wagner, 2003).

c-Jun is known as an enhancer of proliferation, and tumour promoter and oncogene, but there are many studies, which show that *c-Jun* is involved in prevention of the cancer, e.g. *c-Jun* induces apoptosis in UV exposed cells, killing the cells with damaged DNA. *c-Jun* is also involved in regulation of tumour suppressor via $p14^{ARF}/p19^{ARF}$ (Durchdewald et al., 2009).

1.11.2 JunB

In last decades several studies on animal and clinical observations has shown *JunB* as a tumour suppressor. Expression of cyclinA by any protein was first reported through JunB and later *through* c-Jun, Fra1 etc. *JunB* is required for the re-entry in cell cycle after quiescence (Shaulian, 2010). The data in previous studies suggested that there is a co-operation between *JunB* and *c-Jun*. *JunB* rescues and improves survival of *c-Jun* deficient mice embryos (Nair et al., 2010; Shaulian, 2010). The knockout mice for *JunB* was embryonic lethal and embryos died between E 8.5 and E 10.0 day due to defect in placentation. Embryos of conditional knockout mice for JunB were without obvious abnormalities, but adult mice developed osteopenia and myelo-proliferative diseases (Vesely et al., 2009). Transgenic mice lacking *JunB* in myeloid lineage developed a myeloid disease, which resembled to Human CML (Shaulian, 2010; Zenz and Wagner, 2006). In AML patients, *JunB* expression is generally down regulated in haematopoietic stem cells (HSCs) (Shaulian, 2010).

1.11.3 Fra2

Fra2, also known as *FOSL2* or *FOS-like antigen 2* is the member of *AP-1* transcription factor family and *FOS* sub family. *FOS* sub family consists of three other members namely, *FOSB, FOSL1* or *Fra1, C-FOS or FOS*. The *Fra2/FOSL2* proteins form heterodimers with *Jun* proteins and then bind to DNA. The *Fra2* knockout is lethal at birth in mice (Eferl and Wagner, 2003). In a study on mice adenocarcinoma, CSMLO cell line, it was observed that these cells do not express *Fra2/FOSL2* and very low expression of *c-Fos* (Tkach et al., 2003). In other recent study on *Fra2/FOSL2*, deficient mice had a defect in differentiation in osteoblast cells (Bozec et al., 2010). However, the over expression of *Fra2/FOSL2*, increases tumour cell motility and invasion in breast cancer, colorectal cancer and mesothelioma (Milde-Langosch et al., 2008).

1.12 GADDs

The *Growth Arrest and DNA Damage* or *GADD* members are mostly localized in nucleus. Five *GADD* family members are known until now: *GADD153, GADD34, GADD45α (GADD45alfa), GADD45*β *(GADD45beta)* and *GADD45γ (GADD45gamma)*. *GADD45* family members are identified as regulatory molecules, which protect cell and its survival by the process of cell-cycle arrest, DNA repair, and apoptosis (Reddy et al., 2008; Rosemary Siafakas and Richardson, 2009). *GADD45α* was earlier known as *GADD45* and was discovered in Chinese hamster ovary cells (CHO) (Fornace et al., 1988). The *GADD45*β was known as *MyD118* as it was discovered as myeloid differentiation primary gene (Zhan et al., 1994). The *GADD45γ* was earlier known as *CR6* as it was isolated by using *cDNA* homologues to mice gene that is known as *CR6* (Zhang et al., 1999).

*GADD45α, GADD45*β and *GADD45γ* have shown 55% homology to each other at amino acid level and are conserved throughout evolution. GADD45 are highly acidic with pH around 4. *GADD45s* functions are not well understood (Abdollahi et al., 1991; Vairapandi et al., 2002; Zhang et al., 1999). *GADD45α, GADD45*β and *GADD45γ* are strongly induced by stress through independent or *p53* dependent way. There was a failure of G2/M checkpoint in human cells exposed to UV and is devoid of endogenous *GADD45s* (Liebermann and Hoffman, 1998; Vairapandi et al., 2002; Wang et al., 1999). Interaction and inhibition of *CyclinB1/CDC2* complex with *GADD45s* also causes G1 cell-cycle arrest, which is may be via *p21*. *GADDs* play a role in DNA repair and de-methylation also (Cretu et al., 2009). In their interaction with *MKK4* which is upstream regulator of *JNK*, they induce apoptosis which was validated in Hela cells (Takekawa and Saito, 1998). Further induction of apoptosis via *GADD45* dependent is shown in M1 leukemic, lung cancer etc. *GADD45γ* plays a significant role in apoptosis of neuronal cells and UV irradiated keratinocytes (Cretu et al., 2009). Deficiency of *GADD45*β in haematopoietic cells induces apoptosis. The *GADD45*β had a role in mice embryo fibroblast cell survival through *NfκB*. The *GADD45*β promotes cell survival through inhibition of *MKK4-JNK* stress response apoptotic pathway (Gupta et al., 2006). *GADD45s* repaired DNA and promotes survival through *PCNA* (Cretu et al., 2009).

The *GADD45* members especially *GADD45γ* play an important role in cellular apoptosis (Bulavin et al., 2003; Hollander et al., 1999). Mice which were null for *GADD45*β and *GADD45γ* were more prone to ionizing radiations, chemical carcinogens and hence had higher mutation rates and chances to have cancer (Hollander et al., 1999). The down regulation of *GADD45α* and *GADD45γ* is essential for survival of cancer cells. The loss of *GADD45α* expression contributes to tumour formation, tumour growth and decreases the rate of apoptosis, decreased rate of senescence via *JNK* (Tront et al., 2006). In many primary human tumours and cell lines of breast, prostate, pituitary, adenomas, hodgkin and non-hodgkin lymphoma, nasopharyngeal, cervical, oesophageal, lung carcinoma, and pancreatic cancer; methylation and mutations were observed in *GADD45* promoter and genes. The major step to escape from apoptosis in tumour cells was through activation of *NFκB*, which causes repression of *GADD45α* and *GADD45γ*. In recent studies, it is observed that in AML there is a down regulation of *GADD45s*. *GADD45α* is reported a role in mice for suppression of leukemogenesis (Cretu et al., 2009; Zerbini and Libermann, 2005).

1.13 MDA-7/IL-24

Interleukin-24 (IL-24) is also known as *Melanoma differentiation-associated-7 (MDA-7)* and was discovered by subtraction hybridization of *cDNA* in human melanoma cells (Jiang et al., 1995). *MDA-7/IL-24* belongs to *IL-10* family of cytokines and like *IL-10* family of cytokines can exist as monomer or dimer. *MDA-7/IL-24* mediates its biological effects through two heterodimer receptors, *IL-20R1/IL-20R2* and *IL-22R1/IL-20R2* (Sarkar et al., 2002). The transcription of *MDA-7/IL-24* is regulated by the *AP-1* and *C/EBP* transcription factor families (Madireddi et al., 2000). The *MDA-7/IL-24* has antitumor activity in many cancers via *JNK/MAPK* dependent or other signalling pathways and result in apoptosis of tumour cells (Chada et al., 2004; Inoue et al., 2006). Expression of *MDA-7/IL-24* via adenoviral vector carrying the *MDA-7 gene* (*Ad-MDA-7*) cause growth suppression and apoptosis in cancer cells not to normal cells (Otkjaer et al., 2010). Phase one clinical trial using *Ad-MDA-7* is reported to have tumour suppressor effect (Cunningham et al., 2005; Tong et al., 2005).

2. Aim of this study

Acute myeloid leukaemia (AML) is characterized by disturbed differentiation and rapid expansion of leukemic cells. *AP-1* transcription family members are known as key regulators of myeloid differentiation. *AP-1* family members are generally down regulated in AML and thus play an important role in pathophysiology of the disease. NSAIDs cause initiation of apoptosis effectively and G2/M phase growth arrest in various types of cancer cells. *GADD45α* and *GADD45γ* activation *in-vitro* and *in-vivo* plays a major role in neo-plastic effect of NSAIDs (Czibere et al., 2005; Czibere et al., 2006).

Further studies should be carried-out to understand their role in AML and especially in-connection to *GADD45s* and *JNKs*. Elucidation of pathways involved in AML rescue in NSAIDs dependent way can be translated into clinical prospective. These pathways and their target genes can be used as therapeutic agents for designing new safer, low cost drugs, which are able to overcome drug resistance.

However, there are some questions which need to be addressed about the pathways like, role of different genes and involvement of transcription factors. How the NSAIDs dependent orchestra of pathways works in the rescue of AML and cause arrest in cell cycle and apoptosis? What is role of NSAID in apoptosis and differentiation?

Main purpose of this study was to investigate the effects of NSAIDs in AML cells and to know the pathways activated in AML cells after treatment with NSAIDs. In present study, evidence is provided that OSI-461 induces apoptosis and a G2/M cell cycle arrest in AML cells through *MDA-7/IL-24* pathway and *GADD45α* and *GADD45γ* activation in AML patients. It has been observed that treatment of AML cells with Sulindac Sulfide and Diclofenac leads to a consistent transcriptional activation of the *AP-1* transcription factor gene and *GADD45α* with consecutive induction of apoptosis through a *c-Jun NH2-terminal kinase (JNK)* dependent pathway.

This study provides the evidences that non-steroidal anti-inflammatory drugs (NSAIDs) can be effective in chemoprevention or treatment of AML.

3. Personal bibliography

3.1 Publications of dissertation

The non-steroidal anti-inflammatory drugs Sulindac Sulfide and Diclofenac induce apoptosis and differentiation in human acute myeloid leukemia cells through an AP-1 dependent pathway.

Published "**Apoptosis**" 2011 July 8

***Singh R**, Cadeddu RP, Fröbel J, Wilk CM, Bruns I, Zerbini LF, Prenzel T, Hartwig S, Bruennert D, Schroeder T, Lehr S, Haas R and Czibere A*

The novel compound OSI-461 induces apoptosis and growth arrest in human acute myeloid leukemia cells.

Published "**Annals of Hematology**" 2011 Jun 30

Singh R, *Fröbel J*, *Cadeddu RP*, *Bruns I*, *Schroeder T*, *Bruennert D*, *Wilk CM*, *Zerbini LF*, *Haas R* and *Czibere A*

3.2 Other publications

***Low RPS14 expression is common in myelodysplastic syndromes without 5q- aberration and defines a subgroup of patients with prolonged survival**.*

"**Haematologica**" 2009 Oct;94(10):1453-5

*Czibere A, Bruns I, Junge B, **Singh R**, Kobbe G, Haas R, Germing U*

The hematopoietic stem cell in chronic phase CML is characterized by a transcriptional profile resembling normal myeloid progenitor cells and reflecting loss of quiescence.

"**Leukemia**" 2009 May; 23(5):892-9

*Bruns I, Czibere A, Fischer JC, Roels F, Cadeddu RP, Buest S, Bruennert D, Huenerlituerkoglu AN, Stoecklein NH, **Singh R**, Zerbini LF, Jäger M, Kobbe G, Gattermann N, Kronenwett R, Brors B, Haas R*

Pegylated granulocyte colony-stimulating factor mobilizes CD34+ cells with different stem and progenitor subsets and distinct functional properties in comparison with unconjugated granulocyte colony-stimulating factor.

"**Haematologica**" 2008 Mar; 93(3):347-55

*Bruns I, Steidl U, Fischer JC, Czibere A, Kobbe G, Raschke S, **Singh R**, Fenk R, Rosskopf M, Pechtel S, von Haeseler A, Wernet P, Tenen DG, Haas R, Kronenwett R*

4. Publications of the dissertation

4.1 The non-steroidal anti-inflammatory drugs Sulindac Sulfide and Diclofenac induce apoptosis and differentiation in human acute myeloid leukemia cells through an AP-1 dependent pathway.

Apoptosis
DOI 10.1007/s10495-011-0624-y

ORIGINAL PAPER

The non-steroidal anti-inflammatory drugs Sulindac sulfide and Diclofenac induce apoptosis and differentiation in human acute myeloid leukemia cells through an AP-1 dependent pathway

Raminder Singh · Ron-Patrick Cadeddu · Julia Fröbel · Christian Matthias Wilk · Ingmar Bruns · Luiz Fernando Zerbini · Tanja Prenzel · Sonja Hartwig · Daniela Brünnert · Thomas Schroeder · Stefan Lehr · Rainer Haas · Akos Czibere

Abstract Acute myeloid leukemia is a heterogeneous disease with varying genetic and molecular pathologies. Non-steroidal anti-inflammatory drugs (NSAIDs) have been proven to possess significant anti-proliferative potential in various cancer cells in vitro and in vivo. Hence, treatment with these agents can be utilized to study disease specific anti-proliferative pathways. In this study, a total number of 42 bone marrow derived $CD34^+$ selected de novo AML patient samples and the AML cell lines THP-1 and HL-60 were treated with the NSAIDs Sulindac sulfide and Diclofenac. We analyzed viability, apoptosis, differentiation and addressed the molecular mechanisms involved. We found a consistent induction of apoptosis and to some extent an increased myeloid differentiation capacity in NSAID treated AML cells. Comprehensive protein and gene expression profiling of Diclofenac treated AML cells revealed transcriptional activation of GADD45α and its downstream MAPK/JNK pathway as well as increased protein levels of the caspase-3 precursor. This pointed towards a role of the c-Jun NH_2-terminal kinase (JNK) in NSAID mediated apoptosis that we found indeed to be dependent on JNK activity as addition of a specific JNK-inhibitor abrogated apoptosis. Furthermore, the AP-1 transcription factor family members' c-Jun, JunB and Fra-2 were transcriptionally activated in NSAID treated AML cells and re-expression of these transcription factors led to activation of GADD45α with induction of apoptosis. Mechanistically, we demonstrate that NSAIDs induce apoptosis in AML through a novel pathway involving increased expression of AP-1 heterodimers, which by itself is sufficient to induce GADD45α expression with consecutive activation of JNK and induction of apoptosis.

Keywords AML · NSAID · GADD45α · Growth arrest · Apoptosis · AP-1

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Introduction

Acute myeloid leukemia (AML) represents a heterogeneous group of myeloid neoplasias which are driven by varying genetic and/or molecular alterations in clonally evolving hematopoietic stem and progenitor cells (HSPCs) [1–3]. As a consequence of this diversity, treatment regimens are nowadays refined and tailored towards the specific genetic/molecular alterations that occur in individual patients. In the current understanding of AML pathology the concept is prevailing that at some point before disease onset, the leukemic clone gains a proliferative advantage over normal HSPCs while the ability of terminal myeloid

differentiation is lost [4]. A common event in all these distinct types of leukemia is that despite eminent genetic or molecular alterations the cell intrinsic safety mechanisms to prevent survival of genetically "damaged" cells fail. Genomic instability, recurrent translocations or complex karyotype aberrations are fairly common in all types of cancer cells, including those originating from the hematopoietic system [5]. Even if there was an attempt to repair any given DNA-damage, which obviously failed, the consequence in the leukemia initiating cells is not activation of programmed cell death [6]. One may hypothesize that some of these cell intrinsic programs still exist in malignant cells and it is intriguing to speculate that they could be selectively targeted and activated through pathways that are commonly effective in all different types of AML or cancer cells in general. One example for such a re-activation of a cell intrinsic apoptosis program in cancer cells is the activation of the p38/MAPK pathway as a consequence of high-level expression of the pro-apoptotic cytokine mda-7/IL-24 [7–10]. In these studies, independently from the origin of the cancer cells, the exact same anti-proliferative pathways were activated once mda-7/IL-24 reached a certain transcriptional threshold in these cells. Surprisingly, this induction of high-level mda-7/IL-24 expression could be achieved by treatment of cancer cells with several, structurally not related non-steroidal anti-inflammatory drugs (NSAIDs) in vitro and in vivo [11–13]. The purpose of this study was to evaluate, if these pathways can also be activated in AML cells and thereby render a potentially novel therapeutic strategy for the treatment of all subtypes of AML. We found that treatment of AML cells with NSAID leads to a consistent transcriptional activation of the AP-1 transcription factor family members' c-Jun, JunB and Fra-2. As a consequence the growth-arrest and DNA damage inducible gene (GADD) 45α is activated with consecutive induction of apoptosis through a c-Jun NH_2-terminal kinase (JNK) dependent pathway.

Materials and methods

Patient samples

Bone marrow (BM) cells from AML patients and healthy donors were obtained after written informed consent according to an approved protocol. $CD34^+$ cells were selected from BM mononuclear cells (MNCs) using the MACS immunomagnetic separation system (Miltenyi Biotec). Patient and disease characteristics are shown in Online Resource 1. $CD34^+$ cells were cultured in serum free HPGM (Hematopoietic Progenitor Growth Medium; Lonza) supplemented with recombinant human IL-3, IL-6 (both 10 ng/ml) and SCF (25 ng/ml) (all from Peprotech). Cells were maintained at 37°C for 48 h under humidified conditions with 5% CO_2.

Cell culture

THP-1, PC-3 and HL-60 cells were obtained from the American Type Culture Collection and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, respectively. Leukemic cell lines were cultured in RPMI1640 (Sigma-Aldrich) and PC-3 cells in HAMS F-12 medium (BioWhitaker) containing 10% fetal bovine serum (Biochrom), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all Sigma-Aldrich), respectively. Moreover, medium for THP-1 cells additionally contained 0.05 mM β-mercaptoethanol (Invitrogen). Cells were cultured at 37°C, 5% CO_2 under humidified conditions.

Reagents

Sulindac sulfide and Diclofenac were purchased from Sigma-Aldrich and dissolved in DMSO. For all assays, cells were treated for 48 h with 100 µM Sulindac sulfide, 100 µM Diclofenac or equal amounts of DMSO (less than 0.1% final concentration). The JNK inhibitor JNKII SP6001 was purchased from Calbiochem and used at final concentrations of 40 nM.

Kinase assays and western blot analysis

The SAPK/JNK kinase assay was performed according to the manufacturer's protocol (Cell Signaling Technology). Briefly, treated cells were washed twice with ice-cold PBS and transferred into cell lysis buffer (Cell Signaling Technology) containing complete protease inhibitor cocktail (Roche) at 4°C. Protein concentrations were determined using the BCA protein kit according to the manufacturer's instructions (Pierce Biotechnology). Western blot analysis following SDS-PAGE gel separation was performed as described [11] using 30 µg of protein per sample. Antibodies used were anti-phospho-c-Jun and anti-phospho histone H1 (all Cell Signaling Technology). Bands were visualized by chemiluminescence detection (Amersham ECL™ Advance Western Blotting Detection Kit, GE Healthcare).

Apoptosis and viability assay

Viability of treated cells was assessed by hemocytometry following trypan blue staining. Rate of apoptosis was measured by utilizing the cell death detection ELISA-PLUS kit according to the manufacturer's guidelines (Roche). Absorbance was measured at 405 nm on a Wallac multilabel counter 1420 (Perkin Elmer) as described before [14].

Flow cytometry analysis

Expression of the cell surface markers CD11b, CD14, CD15 and CD114 was determined using a FACS Calibur (Becton–Dickinson). Cells were collected and fixed in phosphate buffered saline (PBS; Sigma-Aldrich) containing 0.1% formaldehyde. Subsequently, cells were stained for 30 min at 4°C in the dark with anti-CD11b (APC-Cy7), anti-CD14 (FITC), anti-CD15 (APC) and anti-CD114 (PE) antibodies (all BD Biosciences). Samples were washed two times with PBS containing 0.1% formaldehyde to remove residual antibodies. Data was analyzed using the Cell Quest Software package (Becton–Dickinson).

RNA isolation

Total RNA was extracted from cells using QIAshredder and RNeasy Mini Kit in combination with RNase-Free DNase Set (all Qiagen) according to the manufacturer's instructions. RNA was quantified using the NanoDrop spectrophotometer (NanoDrop Technologies).

Quantitative real-time polymerase chain reaction

cDNA was generated from 100 ng RNA using the M-MLV reverse transcriptase (Invitrogen). RT-PCR was performed on a LightCycler 1.2 utilizing the LightCycler® FastStart DNA Master SYBR Green I kit (both Roche). After an initial denaturation step of 5 min at 94°C, conditions for cycling were 40 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C. Relative gene expression levels were calculated as the difference of CT values of the gene of interest and the housekeeping gene hGAPDH as control (ΔCT). The delta–delta CT method was used for calculation of expression differences of the respective genes. All experiments were performed in duplicates. The following primers were used in this study: GAPDH sense-TCCATGACAACTTTG GTATCG, GAPDH antisense-GTCGCTGTTGAAGTCAG AGGA; GADD45α sense-TGCTGACGCGCAGGATGTT, GADD45α antisense-GCTGCTCAACGTCGACCC; Fra-2 sense-TTGCCAAACGCCTAATTACC, Fra-2 antisense-CAGGAGACGCCCTACTCAAG; c-Jun sense-TTGCCA AACGCCTAATTACC, c-Jun antisense-CACCTGTTCCC TGAGCATGTTG; JunB sense-ATGGAACAGCCCTTCT ACCACG, JunB antisense-AGGCTCGGTTTCAGGAGT TTG.

Two-dimensional difference gel electrophoresis (2D-DIGE™)

Cells were solubilized, sonicated and purified as described previously [15]. Protein labeling with cyanine dyes was performed according to the manufacturer's (GE-Healthcare) instructions. Labeled samples were combined (50 µg each labeled with Cy3, Cy5 and Cy2 per physical gel) and applied adjacent to the acidic end of Immobiline™ DryStrips (24 cm, pH 4-7 and pH 6-9 linear) by cup loading. IEF was performed on a MultiPhor II electrophoresis unit (GE Healthcare) as previously described [15]. Prior to second-dimension separation using 12.5% polyacrylamide gels in a Laemmli buffer system, IPG strips were equilibrated and protein separation by size was performed on an EttanDalt 12 system (GE Healthcare) as depicted before [15]. Subsequently, gels were scanned using a Typhoon 9400 laser scanner (GE Healthcare) and protein spot abundances and statistics were determined using Proteomweaver 4.0 image analysis software (Bio-Rad) as previously described [16]. Selection criteria for detection of significant changed protein spots were set as followed: (1) protein spots have to be present in all analyzed gels; (2) the standardized average spot volume ratio exceeds 1.8-fold; (3) *P*-value lower than 0.05 (Student's *t*-test).

Protein identification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)

For spot picking the cyanine dye-labeled gels were re-stained with a ruthenium fluorescent stain as previously described [17] and protein spots of interest were excised from the 2-D gels using a Gelpix spot picker (Genetix). Prior to MS analysis gel pieces were washed, the proteins trypsin digested and the resulting peptides eluted as depicted before [15]. Subsequently, 4 µl of extracted peptides were applied to a MALDI Prespotted AnchorChip target (Bruker Daltonics), incubated for 30 s and removed quantitatively. Samples were analyzed in an Ultraflex-Tof/Tof mass spectrometer (Bruker Daltonics) and acquired mass spectra were automatically calibrated and annotated using Compass 1.1 software (Bruker Daltonics). Protein identification via peptide mass fingerprinting (PMF) was performed on-the-fly using Biotools 3.0 (Bruker Daltonics) by searching the human subset of SwissProt (SwissProt_49.1) and NCBInr (NCBInr_20070326.fasta) non-redundant databases with Mascot (Version 1.9; Matrix Science). Proteins were considered as identified, when they were assigned with a Mascot score higher than 56 ($\hat{=}\ P < 0.05$) on two different gels.

Gene expression profiling

Total RNA from treated THP-1 and HL-60 cells was harvested as described above. Respective cRNA was generated and hybridized to HG-U133A gene arrays (Affymetrix), washed and scanned according to the manufacturer's instructions. Array images were analyzed by dChip following smoothing-spline normalization [18]. A gene was considered significantly differentially

expressed between the DMSO and Diclofenac treated groups, if the 90% lower confidence bound of the fold change between these groups was >1.2 [19]. Experiments were done in duplicates and THP-1 and HL-60 cells were analyzed together. Pathway and network analysis was done using Ingenuity web tools (Ingenuity Systems) Array data have been stored in the gene expression omnibus database (www.ncbi.nlm.nih.gov/geo/; accession no: GSE28185) according to MIAME standards.

AP-1 and GADD45α expression and luciferase vectors

The GADD45α expression plasmid and the pGL3-GADD45α-luciferase vector have been described before [20]. c-Jun, JunB and Fra-2 plasmids were generated by inserting PCR products of the respective coding sequence into pcDNA3 vectors using appropriate restriction sites. Mammalian cells were transfected using Nucleofector I according to the manufacturer's (Amaxa) instructions. Transfection efficiency was measured using a GFP expressing plasmid. Luciferase assays were performed utilizing the BrightGlo Luciferase Assay System (Promega) according to the manufacturer's instructions. Experiments were done in duplicates and empty vector controls were used as reference.

RNA interference experiments

The ShRNA duplexes were designed with BLOCK-iT™ RNAi Designer (Invitrogen). The ShRNA sequences used are Fra-2 top strand-CACCGAACCTCGTCTTCACC TATCCCGAAGGATAGGTGAAGACGAGGTTC; Fra-2 bottom strand-AAAAGAACCTCGTCTTCACCTATCCT TCGGGATAGGTGAAGACGAGGTTC; c-Jun top strand-CACCGCCTTCGTTAACTGTGTATGTCGAAACATAC ACAGTTAAAGGC; c-Jun bottom strand-AAAAGCCT TCGTTAACTGTGTATGTTTCGACATACACAGTTAAC GAAGGC; JunB top strand-CACCGCTCAAACAGAA GGTCACCGAAGTCATGACCTTCTGTTTGAGC; JunB bottom strand-AAAAGCTCAAACAGAAGGTCATGACT TCGGTCATGACCTTCTGTTTGAGC. LacZ ShRNA was used as the positive, non-silencing control (top strand-CACCGCTACACAAATCAGCGATTTCGAAAAATCGC TGATTTGTGTAG; bottom strand-AAAACTACACAAAT CAGCGATTTTTCGAAATCGCTGATTTGTGTAGC). Further steps were performed according to the manufacturer's protocol. In brief, equal amounts (50 μM) of top and bottom strand oligonucleotides were annealed and cloned into the pENTR™/U6 vector (Invitrogen). To confirm the correct orientation and sequence of the double-strand oligonucleotides vectors were sequenced. Plasmids were transfected in primary AML cells using Nucleofector I (Amaxa) and were tested for specificity and efficiency by RT-PCR.

Statistical analysis

Statistical significance was tested using paired, two-tailed Student's *t*-test to assess the significance levels of treated samples compared to their respective DMSO controls and *P*-values below 0.05 were considered significant.

Results

NSAIDs inhibit survival of acute myeloid leukemia cells through induction of apoptosis and myeloid differentiation

To investigate a potentially anti-proliferative effect of the NSAIDs Sulindac sulfide and Diclofenac in AML, we treated HL-60 and THP-1 cells as well as 25 bone marrow derived $CD34^+$ enriched de novo AML samples (see Online Resource 1 for patient demographics). We found a significant induction of apoptosis in THP-1 and HL-60 cells by 3.90 and 4.01-fold for Sulindac sulfide and by 6.30 and 5.44-fold for Diclofenac, respectively (Fig. 1a). The mean fold of apoptosis induction for the patient samples tested was 2.44 and 3.34 for Diclofenac and Sulindac sulfide, respectively (Fig. 1b). These pro-apoptotic effects were accompanied by a significant decrease of viable cells in all samples tested (Fig. 1c, d). The mean number of viable THP-1 cells decreased by 30 and 35% and viable HL-60 cells decreased by 30 and 40% when cells were treated with Sulindac sulfide or Diclofenac compared to DMSO treated controls, respectively. The mean proportion of viable cells from the 9 de novo AML patient samples analyzed decreased by 23 and 31% when treated with Sulindac sulfide or Diclofenac, respectively. Further, we asked if treatment with NSAIDs also possesses differentiation inducing capacities. Therefore, we tested cell surface expression of CD11b, CD14, CD15 and CD114 on samples from five individual AML patients via flow cytometry. Here, we found that Sulindac sulfide induced myeloid differentiation was more pronounced than in Diclofenac treated cells. Following treatment with Sulindac sulfide 3 of 5 patient samples showed an increase in the expression of CD11b and 4 of 5 patients showed increased expression of CD14, CD15 and CD114 by at least 1.5-fold, respectively (Fig. 1e). Thus, AML cells respond to in vitro treatment with NSAIDs with a significant induction of apoptosis and increased expression of myeloid differentiation markers.

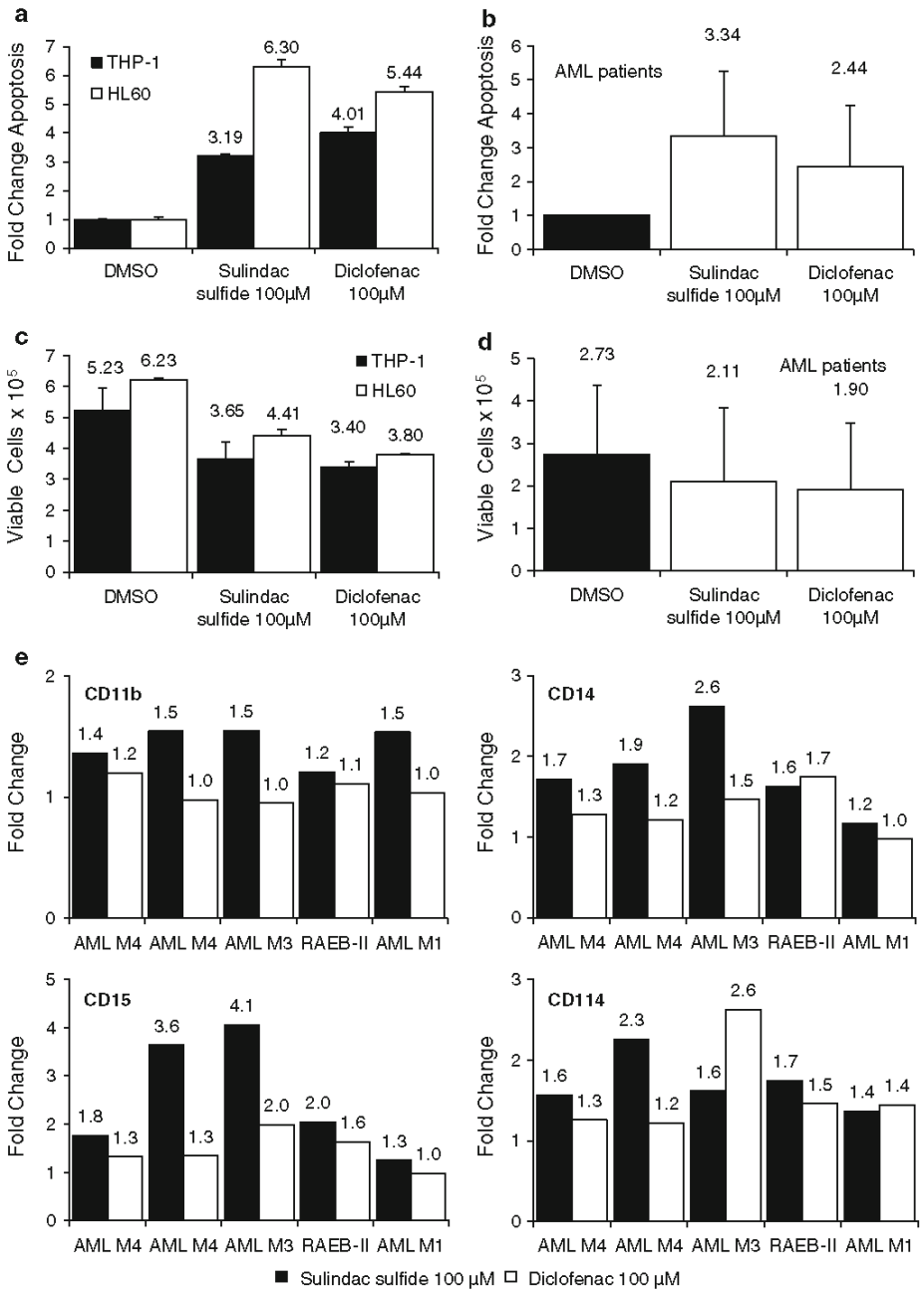

Fig. 1 Sulindac sulfide and Diclofenac induce apoptosis and differentiation in AML. All samples were treated with 100 µM Sulindac sulfide, 100 µM Diclofenac or DMSO for 48 h. **a** NSAIDs significantly induce apoptosis in HL-60 and THP-1 AML cells. Data is shown as mean fold change compared to the respective DMSO control from triplicate experiments + SD ($P < 0.05$). **b** Data from 25 different bone marrow derived AML samples is shown as mean fold increase + SD ($P< 0.05$). **c**, **d** Viability analysis performed by hemocytometric analysis following trypan blue staining. HL-60, THP-1 cells (**c**) and nine patient samples (**d**) were analysed. Shown are the mean viable cell numbers following 48 h of the indicated treatment + SD ($P < 0.05$). **e** Flow cytometric analysis of the cell surface expression of the myeloid differentiation markers CD11b, CD14, CD15 and CD114 after 48 h of the indicated treatment. Individual data from each analysed patient with respective fold increase as compared to the respective DMSO control is shown

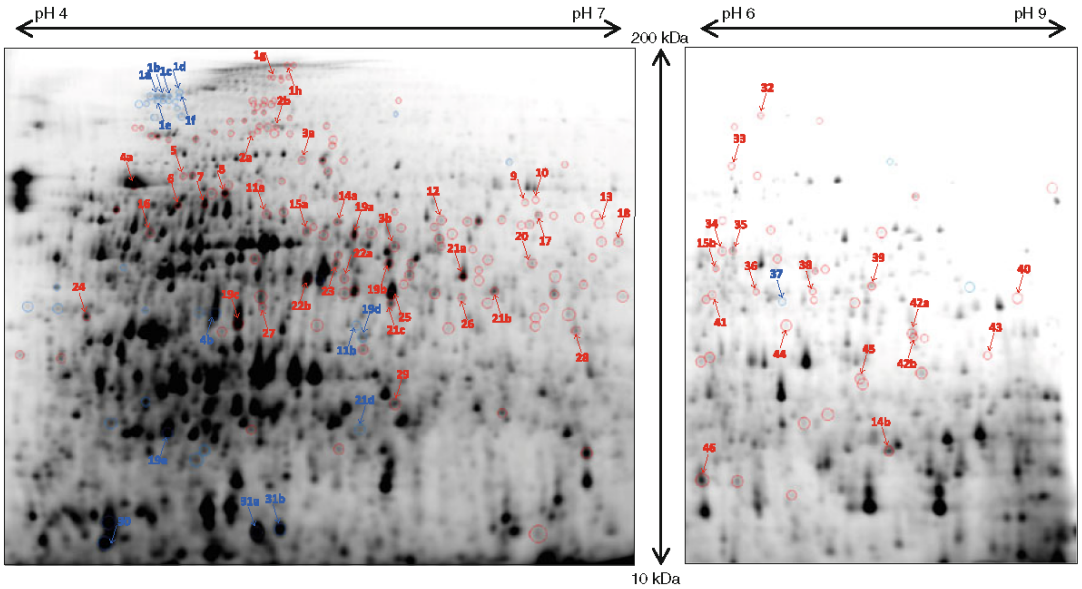

Fig. 2 Differential proteome analysis of HL-60 cells treated with 100 μM Diclofenac for 48 h. Representative 2-D gel image of HL-60 cell lysates (pH ranges 4–7, 6–9) showing the 211 protein spots with altered concentrations after Diclofenac treatment. *Red circles* represent the spots with higher and *blue circles* with lower expression levels after treatment. The *serial numbers* showing the position of the identified proteins are identical to the numbers given in Online Resource 2. *Letters* following numbers are used to distinguish between different spots representing the same protein (Color figure online)

Differential proteomic analysis of total cell lysates from NSAID treated AML cells reveals activation of pro-apoptotic signaling pathways

To elucidate changes on the protein level of AML cells as a consequence of NSAID treatment, we performed differential 2-D gel analysis of Diclofenac or DMSO treated HL-60 cells followed by MALDI-TOF mass spectrometry of trypsin digested peptides for protein identification. Overall, we found that the expression of 211 potential protein spots changed in response to Diclofenac treatment. Searching the Swiss-Prot non-redundant database, peptide mass information revealed the identity of 69 protein spots (Fig. 2, numbers correspond to respective proteins in Online Resource 2). These 69 identified protein spots comprised 46 distinct proteins. Of these 46 proteins, 42 proteins had a higher concentration and four proteins showed a lower concentration in Diclofenac treated HL-60 cells. Among the differentially expressed proteins were components of the cytoskeleton such as the various actin and tubulin isoforms as well as numerous proteins involved in cell metabolism. These findings correlate well with the observed increase of apoptosis and differentiation in AML cells treated with NSAIDs as both processes require the reorganization of the cellular structure. In line with this assumption, we also found that the caspase-3 precursor was induced on the protein level following treatment with Diclofenac. These results point towards an activation of the MAPK/JNK pathway as a potential mechanism for induction of apoptosis in AML in response to NSAID treatment.

Gene expression profiling reveals activation of the MAPK/JNK pathway with induction of GADD45α and AP-1 family transcription factors

To gain a more comprehensive insight into the molecular mechanisms involved in NSAID induced apoptosis and differentiation in AML, we performed gene expression analysis of THP-1 and HL-60 cells treated with Diclofenac or DMSO. We found a total number of 253 genes that significantly changed their level of expression in response to Diclofenac in both cell lines with a lower bound of fold change of >1.2 (Fig. 3a). Of these 253 genes, 114 had a higher and 139 a lower expression as a consequence of NSAID treatment (Online Resource 3). Among the activated genes were GADD45α and the AP-1 transcription factor family members c-Jun, JunB and Fos. Consecutive Ingenuity network and pathway analysis revealed transcriptional changes in the network involved in transcription and cancer progression with the AP-1 family members JUN and FOS as center molecules (Fig. 3b). Furthermore, activation of the MAPK/JNK pathway was found to be the most significantly changed pathway ($P < 0.001$, not shown). Quantitative RT-PCR analysis confirmed the increased expression of GADD45α and the AP-1 family transcription factors c-Jun, JunB and Fra-2 in response to Sulindac sulfide or Diclofenac

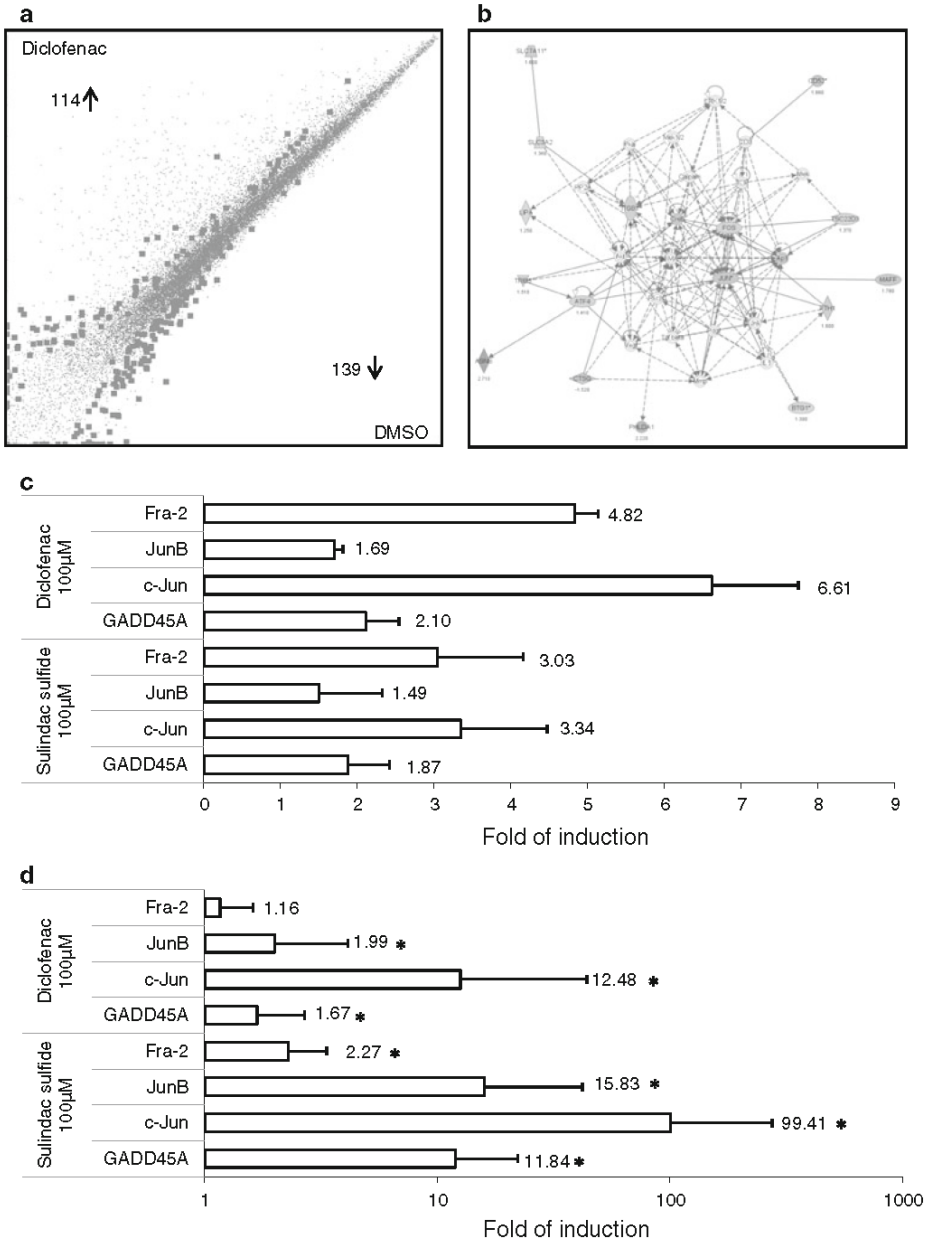

Fig. 3 Gene expression profiling of Diclofenac treated AML cells. Prior gene expression profiling, HL-60 and THP-1 AML cells were treated with 100 µM Diclofenac for 48 h. **a** M/A plot illustrating the 253 differentially expressed genes in Diclofenac and DMSO treated THP-1 and HL-60 cells. **b** Ingenuity pathway analysis reveals a network of genes involved in transcription and cancer progression to be the top altered network following Diclofenac treatment. **c** Quantitative RT-PCR analysis of GADD45α and AP-1 family transcription factor expression in NSAID treated HL-60 cells. All samples were treated with 100 µM Sulindac sulfide, 100 µM Diclofenac or equal amounts of DMSO for 48 h prior harvest of total RNA. Data is shown as mean fold of induction from triplicate experiments + SD. **d** Quantitative RT-PCR analysis of GADD45α and AP-1 family transcription factor expression in NSAID treated AML patient samples. Data is shown as mean fold of induction + SD ($n = 14$, $^*P < 0.05$)

treatment in HL-60 cells and 14 bone marrow derived de novo AML samples. Diclofenac and Sulindac sulfide treatment induced the expression of GADD45α by 2.10 and 1.87-fold, of c-Jun by 6.61 and 3.34-fold, of JunB by 1.69 and 1.49-fold, and of Fra-2 by 4.82 and 3.03-fold in HL-60 cells, respectively (Fig. 3c). Mean fold of induction in the 14 AML patient samples following treatment with Sulindac sulfide or Diclofenac was 11.84 and 1.67-fold for GADD45α, 99.41 and 12.48-fold for c-Jun, 15.83 and 1.99-fold for JunB, and 2.27 and 1.66-fold for Fra-2, respectively

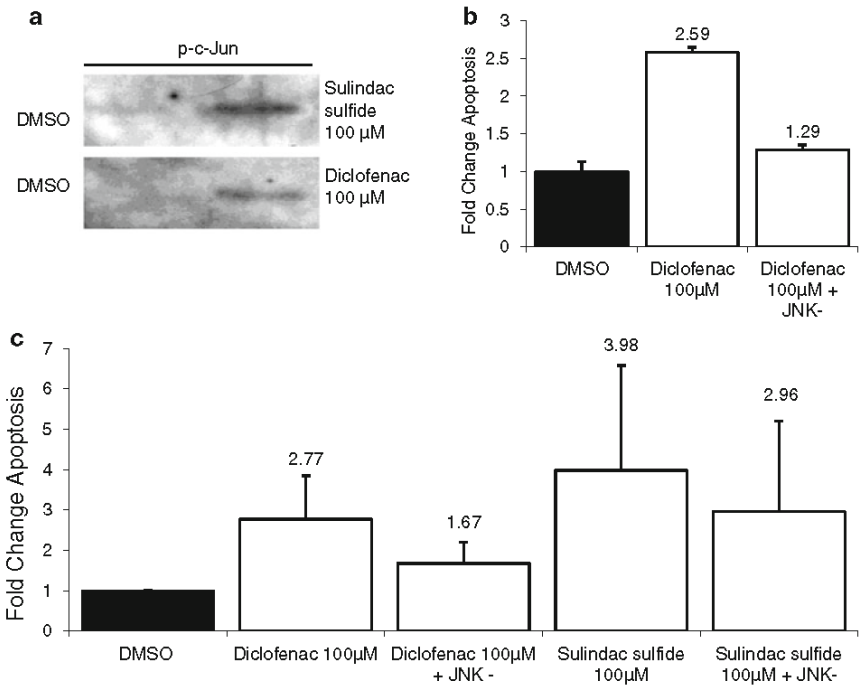

Fig. 4 NSAID mediated apoptosis in AML depends on activation of JNK. **a** Kinase assay showing induction of JNK kinase activity in cell lysates from Diclofenac and DMSO treated THP-1 cells. **b**, **c** Addition of 40 nM of a specific JNK-inhibitor (JNK-) decreased Diclofenac induced apoptosis in THP-1 cells ($P< 0.05$, panel b) and in Sulindac sulfide and Diclofenac treated de novo AML samples ($n = 6$, $P< 0.05$, panel c). All samples were treated with 100 µM of the indicated drugs for 48 h. Data is shown as mean + SD

(Fig. 3d). We also tested other AP-1 transcription family members such as Fra-1, Fos and FosB, but did not find a significant increase in expression in response to treatment with NSAIDs (data not shown). Overall, treatment of AML samples with NSAIDs led to a pronounced transcriptional activation of GADD45α, c-Jun, JunB and Fra-2 suggesting a role for these genes in NSAID induced apoptosis and differentiation in AML.

When combining the datasets of our gene expression profiling and differential proteomic studies for a comprehensive network and signaling pathway analysis using Ingenuity IPA 9.0, we found that the genomic and proteomic approach complemented each other fairly well. Two of the top three networks evolved around caspase-3 and JNK signaling (Online Resource 4). With regards to the top altered biological functions in our combined analysis approach, we found that the most significantly changed pathways all involve cellular growth, proliferation, apoptosis and cancer development. A full list of these pathways and involved genes can be found in Online Resources 5–9.

NSAID induced apoptosis is mediated by activation of JNK

It is well established that activation of GADD45α and the MAPK/JNK pathway leads to activation of JNK with consecutive induction of cellular apoptosis through phosphorylation of c-Jun followed by activation of the caspase-3 dependent apoptosis pathway. We had already identified an increased protein concentration of the caspase-3 precursor in our proteomics analysis and an increase in GADD45α expression, so we examined if JNK is involved in NSAID mediated apoptosis observed in AML. First, we tested if JNK is activated as a consequence of Sulindac sulfide or Diclofenac treatment in THP-1 AML cells and found a consistent increase in JNK activity as demonstrated by an increased c-Jun phosphorylation in our JNK kinase assay (Fig. 4a). Next, we treated THP-1 cells and 6 individual AML patient samples with the aforementioned NSAIDs and added a specific JNK inhibitor that blocks JNK activity and thereby inhibits JNK-dependent apoptosis. Here, we found that addition of the inhibitor significantly decreased the pro-apoptotic potency of Sulindac sulfide and Diclofenac in THP-1 cells and the 6 AML patient samples tested. In THP-1 cells, addition of a JNK inhibitor decreased apoptosis by 51% when cells were treated with Diclofenac (Fig. 4b). Within the patient samples, apoptosis was decreased by an average of 40 and 26% when a JNK inhibitor was added to the Sulindac sulfide or Diclofenac treated cells (Fig. 4c). These data indicate that activation of JNK plays an integral role in NSAID mediated apoptosis in AML.

Transient expression of GADD45α and AP-1 family transcription factors is sufficient to induce apoptosis in AML

To evaluate if the NSAID mediated increased expression of GADD45α and the AP-1 transcription factor family members itself is sufficient to induce apoptosis in AML, we transiently overexpressed these proteins in 8 de novo bone

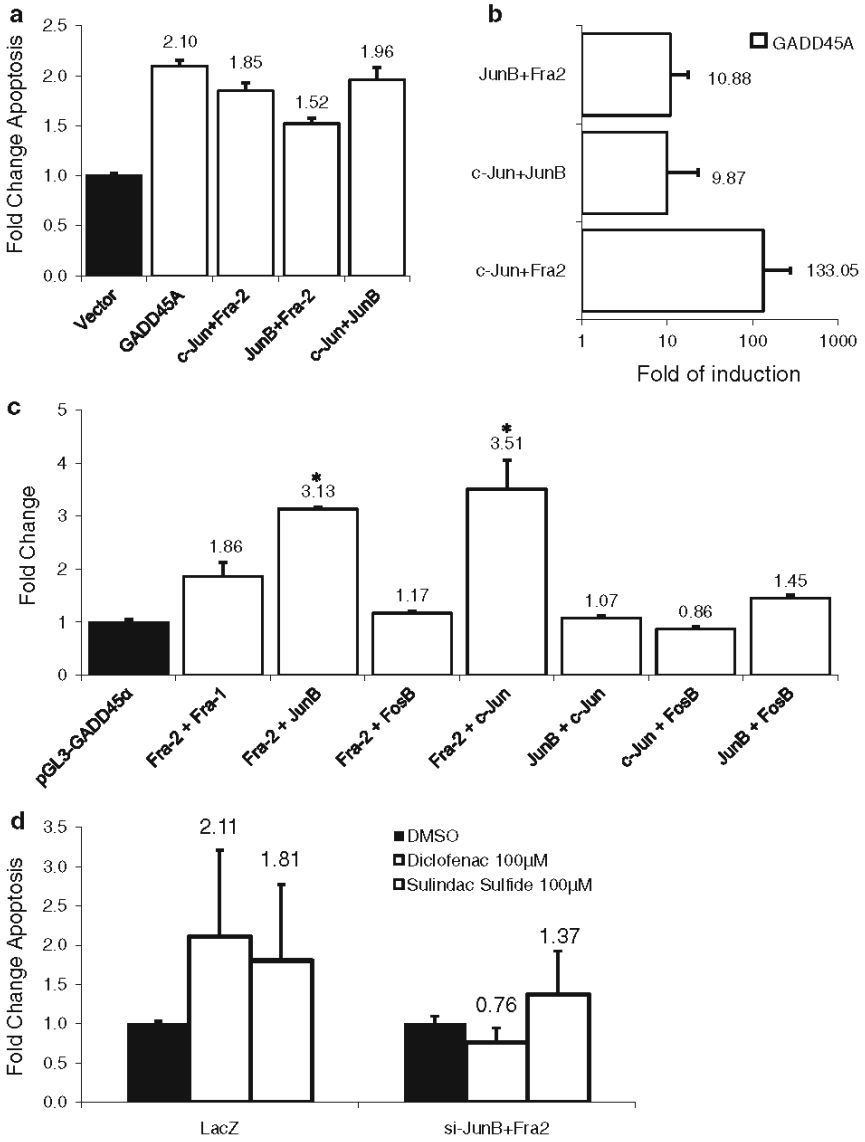

Fig. 5 AP-1 factors and GADD45A are sufficient to induce apoptosis in AML. **a** Overexpression of GADD45α or the shown AP-1 heterodimer combinations is sufficient to induce apoptosis in AML. Data is shown as mean fold increase of photometric extinction at 405 nm + SD as compared to the respective DMSO treated controls ($n = 8$ patients; $P < 0.05$). **b** RT-PCR showing induction of GADD45α following transfection with AP-1 heterodimers in AML patient samples. Data is shown as mean fold increase in expression + SD as compared to empty vector transcfections as controls ($n = 5$, $P < 0.05$). **c** Luciferase enhancer assay showing transactivation capacity of AP-1 heterodimers co-transfected with the pGL3-GADD45α promoter construct. Experiment done in triplicates and data is presented as fold of induction in luciferase activity + SD (*$P < 0.05$). **d** RNAi of JunB + Fra-2 prior Sulindac sulfide or Diclofenac treatment decreased the apoptotic potential of the aforementioned drugs. Data is shown as mean fold increase of photometric extinction at 405 nm + SD as compared to the respective DMSO treated controls ($n = 2$ patients)

marrow derived AML samples and performed consecutive apoptosis analyses 48 h after transduction. We did not detect an increased rate of apoptosis when c-Jun, JunB or Fra-2 were transfected alone (data not shown), but found a 1.85-fold increase when c-Jun + Fra-2, a 1.52-fold when JunB + Fra-2 and a 1.96-fold when c-Jun + JunB were co-transfected, respectively. We also found that expression of GADD45α alone led to an average 2.1-fold increase in apoptosis in the AML samples tested (Fig. 5a). Co-transfection of AP-1 factors in five patients with de novo AML also led to an activation of transcription of GADD45α by a 133.05-fold increase when c-Jun + Fra-2, by a 10.88-fold when JunB + Fra-2 and still by 9.87-fold when c-Jun + JunB were added, respectively (Fig. 5b). This indicates that GADD45α may be a downstream target of these AP-1 transcription factors in NSAID treated AML cells. We also looked for an increased expression of the differentiation markers CD11b, CD14, CD15 and CD114 in AML patient samples following co-transfection with c-Jun + Fra-2, c-Jun + JunB and JunB + Fra-2. Here, only 2 out of 8 patient samples showed an increased expression of these markers in response to co-transfection of the aforementioned AP-1 transcription factors (data not shown), indicating that other NSAID-related effects may contribute to the overall increased expression of myeloid differentiation markers observed in response to Sulindac sulfide. To further establish GADD45α as a downstream target of AP-1, we performed luciferase transactivation assays in PC-3 cells. Overexpression of AP-1 monomers demonstrated only a mild activation of the GADD45α promoter (data not

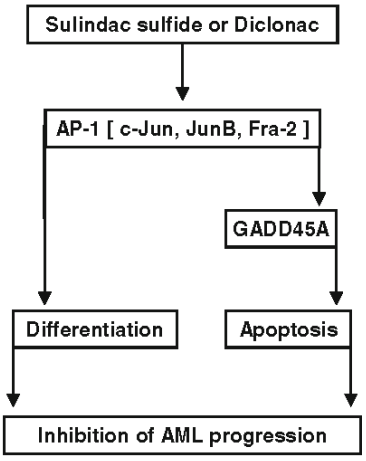

Fig. 6 Proposed mode of action of NSAID in AML. Based on our data we propose that the NSAIDs Sulindac sulfide and Diclofenac lead to an induction of expression of the AP-1 transcription factors c-Jun, JunB and Fra-2. This results in an increased expression of GADD45α with activation of JNK and consecutive induction of apoptosis

shown). This is in line with the minor effects observed following transfection with AP-1 single factors in AML. However, in accordance with the anti-proliferative effects observed when AP-1 heterodimers were transduced, we found a strong induction of the GADD45α promoter by 3.13 and 3.51-fold when Fra-2 + JunB or Fra-2 + c-Jun were co-transfected, respectively (Fig. 5c). Further, we asked if loss of Fra-2 + JunB prior to treatment can abrogate apoptosis in human AML cells. Therefore, we used RNAi to inhibit expression of JunB and Fra-2 following treatment, and found that knockdown of JunB + Fra-2 decreased the induction of apoptosis for Sulindac sulfide from 2.11 to 0.76-fold and for Diclofenac from 1.81 to 1.37 fold, respectivley. Based on the data presented in this manuscript, we hypothesize that NSAIDs exert their anti-proliferative effects in AML through activation of AP-1 family transcription factors c-Jun, JunB and Fra-2, which leads to an increased transcription of GADD45α with activation of JNK (Fig. 6).

Discussion

In this report, we demonstrate that the NSAIDs Sulindac sulfide and Diclofenac are functionally effective in AML cell lines as well as primary cells from AML patients' bone marrow. They exert their anti-proliferative activity through transcriptional activation of the AP-1 transcription factor family members' c-Jun, JunB and Fra-2 as well as through activation of GADD45α. The transcriptional activation of GADD45α leads to an increase in JNK activity with induction of the caspase-3 dependent apoptosis program. To some extent, an increased expression of the myeloid differentiation markers CD11b, CD14, CD15 and CD114 was observed in AML patient samples treated with Sulindac sulfide. This is noteworthy as these AML samples were $CD34^+$ selected leukemic cells cultured for 48 h in liquid suspension cultures without any G-CSF or GM-CSF. Under normal conditions, up to 10 days of culture in the presence of myeloid differentiation promoting cytokines are necessary to induce expression of the aforementioned differentiation markers. Still, the most profound effect observed was the significant and consistent induction of apoptosis throughout all different types of AML tested. This included BM samples from patients with complex aberrant karyotypes, FLT3-ITD mutations, MLL-mutations and recurrent translocations such as t(15,17). There is also increasing evidence that long-term low dose intake of NSAID may prevent certain types of cancers which is in line with numerous studies showing a profound anti-cancer effect of NSAID in vitro and in vivo [14, 21–24]. We do not suggest that NSAIDs should be used as single agents for the treatment of patients who suffer from AML. We consider them rather as additional compounds to increase the susceptibility to conventional cytotoxic drugs. Moreover, the cell intrinsic pathways activated through NSAID in AML that inhibit proliferation through induction of apoptosis may be exploited through novel agents or become feasible targets for future drug discovery studies. To shed some light on the potential mechanisms involved in the NSAID mediated anti-proliferative effects in AML we performed comprehensive protein and gene expression profiling studies. Results from these examinations were validated using primary cells from patients with de novo AML. At a first glance the correlation between our two types of studies was relatively weak. Still, both datasets complemented each other well. A likely reason for the low overlap between genomics and proteomics data may rely on technical reasons such as different sensitivities of the two different methods [25, 26]. Still, we identified 33% of the differentially expressed potential protein spots, implying that the actual overlap may be higher, but remains masked due to the limitations of this approach. Nonetheless, the increased expression of the caspase-3 precursor and proteins related to cellular structure and metabolism that we identified in the Diclofenac treated AML cells pointed towards activation of apoptosis and/or differentiation. Caspase-3 is a key molecule in the MAPK/JNK pathway and has been demonstrated numerous times as a critical mediator of drug induced apoptosis [27–30]. Reorganization of the cellular skeleton and a high metabolic activity are associated with both apoptosis and differentiation [31–34]. In line with these observations, gene expression profiling of Diclofenac treated THP-1 and HL-60 AML cells

revealed that the MAPK/JNK pathway was the most significantly altered pathway in response to this treatment. The activation of this pathway can lead to activation of JNK with increased phosphorylation of c-Jun [35]. This leads to its translocation to the nucleus and activation of the caspase-3 dependent apoptosis pathway [36, 37]. In line with this, we found that induction of apoptosis in NSAID treated AML patient samples and cell lines depend, at least partially, on JNK activity. Not only did we observe an activation of JNK in NSAID treated AML cells, we also found that addition of a specific JNK-inhibitor significantly decreased the apoptotic potential of NSAIDs in AML.

Our gene expression analysis also showed that GADD45α was transcriptionally induced upon NSAID treatment. It is well established that GADD45α is upstream of the MAPK/JNK pathway [38, 39]. GADD45α is mostly inactive in cancer cells, while it can be induced through either inhibition of NF-κB or activation of mda-7/IL-24 [11, 20]. We did not find evidence for both of these mechanisms in our data sets. Still, we found an increased expression of the AP-1 family transcription factors c-Jun and JunB. Both transcription factors have been studied extensively with respect to their role in malignant hematopoiesis and could be linked to PU.1 as critical downstream targets in a murine AML model [40]. In this model, re-expression of c-Jun and JunB restored myeloid differentiation and also inhibited proliferation of the PU.1 deficient leukemic cells. In line with these observations, we also observed that transient re-expression of c-Jun and JunB blocked proliferation through induction of apoptosis, although the effects on terminal differentiation of the patient derived AML cells were limited. The AP-1 family of transcription factors act as heterodimers and their function on target genes depends on the composition of the dimer [41, 42]. In line with this, we found an increased expression of Fra-2, another AP-1 family member. It is a Fos-related transcription factor that forms dimers with AP-1 family members from the Jun family such as c-Jun and JunB [42]. It is likely to assume that Fra-2 pairs with c-Jun or JunB in NSAID treated AML samples as we observed the most profound increase in GADD45α promoter activation when Fra-2 was co-transfected with c-Jun or JunB. Also, transfections of single AP-1 factors did not induce apoptosis or GADD45α expression, indicating that activation of several AP-1 factors through NSAID is required to induce apoptosis in AML. Looking for a potential link between AP-1 and GADD45α we found a potential AP-1 transcription factor binding site in the GADD45α promoter. Subsequent transactivation luciferase reporter assays revealed that the GADD45α promoter is strongly induced by AP-1 heterodimers such as JunB + Fra-2 or c-Jun + Fra-2, but not when the two Jun factors are co-transfected. Again, single AP-1 factor transfections had no effect on the GADD45α promoter, which fits well with our previous observation of lack of apoptosis induction. This observation implies for the first time a direct link between AP-1 factors and GADD45α that interact in a novel pathway to induce apoptosis in AML. Overall, we propose a model where activation of several AP-1 family transcription factors such as c-Jun, JunB and Fra-2 through NSAID treatment induces GADD45α transcription in AML and to some degree myeloid differentiation. As a further consequence, GADD45α activates JNK, which than leads to induction of apoptosis. These effects were observed in a broad variety of different AML subtypes indicating that targeting this pathway may be suitable to improve anti-proliferative treatment modalities for patients with AML.

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Supplementary Data

Apoptosis

The non-steroidal anti-inflammatory drugs Sulindac sulfide and Diclofenac induce apoptosis and differentiation in human acute myeloid leukemia cells through an AP-1 dependent pathway

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Online Resource 1. Patient and disease characterists

No.	Diagnosis	Age	Gender	Cytogenetics	% Blasts BM	Molecular Diagnosis
1	AML M1	27	male	46, XY	41	N/A
2	sAML/MDS	67	female	45,XX,t(3,3)(q21;q26),-7[17]/46,XX[3]	13	N/A
3	sAML/MDS	52	male	46,XY	17	MLL-PTD
4	sAML/MDS	33	male	46, XY, t(9;22)(q34;q11) [14]	25	N/A
5	AML M2	67	male	Complex	27	N/A
6	AML M5	43	male	N/A	99	N/A
7	AML M2	30	female	Complex	80	N/A
8	MDS RAEBI	74	male	45, XY, -7 [18], 46, XY, dup (1)(q34q25)	7	N/A
9	AML M5	55	male	45, XY, -7 [24]	60	N/A
10	AML M2	53	female	Complex	44	N/A
11	sAML/MDS	70	female	46, XX	30	N/A
12	AML M1	55	female	46, XX	84	FLT3-ITD, MLL-PTD
13	AML M2	27	male	46, XY	94	N/A
14	AML M4	23	male	46, XY inv16	46	N/A
15	AML M1	78	female	46, XY	40	N/A
16	AML M4	63	female	46, XX	37	FLT3-ITD
17	AML M3	55	male	46, XY, t(15;17) [35]	95	PML-RARalpha
18	MDS RAEB II	67	male	46, XY	12	N/A
19	AML M1	57	female	46, XX, t(6;9)(p23;q34)	80	FLT3-ITD
20	sAML/MDS	72	male	Complex	40	N/A
21	sAML/MDS	46	male	45, X, -Y[22]. 46, XY [1]	68	N/A
22	MDS RAEB II	87	male	N/A	N/A	N/A
23	AML M4	78	male	N/A	N/A	N/A
24	sAML/MDS	72	male	46, XY	90	N/A
26	AML M3	28	female	46, XX ,t(15;17) [23]	88	PML-RARalpha
27	AML M2	52	male	46, XY	20	N/A
28	sAML M4 (MDS)	63	female	46, XX 5q-, +8	28	N/A
30	AML M1	54	female	N/A	65	N/A
31	CMML	36	male	N/A	N/A	N/A
32	AML M4	59	female	46, XX	84	FLT3-ITD
34	CMML	72	female	N/A	6	N/A
35	AML M4	40	male	46 XY, t(3;21)	62	N/A
36	sAML/PV	80	female	N/A	30	N/A
39	AML M1	77	female	N/A	N/A	N/A
41	CMML	53	male	47 XY, +8 [7/23]; 46 XY [16/23]	15	N/A
42	AML M1	49	female	N/A	66	N/A

N/A: Not available; sAML/MDS: secondary AML after MDS; FAB classification is used

Apoptosis

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Ramincer Singh[1], Ron-Patrick Cadodcu[1], Julia Proedl[1,2], Christian Matthias Wilk[1], Itamar Bruns[1], Luiz Fernando Zerbini[4], Ian Sonja Hartwig[2], Daniela Dröllnert[1], Manoj Bhasin[3], Thomas Schroeder[1], Stefan Lehr[2], Daniel Geoffrey Tenen[3], Rainer Haas[1],

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Online Resource 2. Full list of identified proteins

ID	Name	Annotation	MW	Fold Change
1a	Filamin-A	FLNA_HUMAN	283192	-3,77
1b	Filamin-A	FLNA_HUMAN	283192	-3,73
1c	Filamin-A	FLNA_HUMAN	283192	-3,47
1d	Filamin-A	FLNA_HUMAN	283192	-2,84
1e	Filamin-A	FLNA_HUMAN	283192	-2,84
1f	Filamin-A	FLNA_HUMAN	283192	-2,11
1g	Filamin-A	FLNA_HUMAN	283192	2,14
1h	Filamin-A	FLNA_HUMAN	283192	3,77
2a	Alpha-actinin-4	ACTN4_HUMAN	105245	2,18
2b	Alpha-actinin-4	ACTN4_HUMAN	105245	1,86
3a	Stress-70 protein	GRP75_HUMAN	73920	2,22
3b	Stress-70 protein	GRP75_HUMAN	73920	2,57
4a	Protein disulfide-isomerase precursor	PDIA1_HUMAN	57480	2,95
4b	Protein disulfide-isomerase precursor	PDIA1_HUMAN	57480	-2,24
5	Transitional endoplasmic reticulum ATPase	TERA_HUMAN	89819	2,15
6	Vimentin	VIME_HUMAN	53545	2,14
7	ATP synthase beta chain	ATPB_HUMAN	56525	2,47
8	Alpha-tubulin 6	TBA6_HUMAN	50540	5,05
9	Filamin/filamin-like protein 1A	FRWIA_HUMAN	70248	4,30
10	60S ribosomal protein L34	RL34_HUMAN	13382	2,29
11a	Protein disulfide-isomerase A3 precursor	PDIA3_HUMAN	57146	2,36
11b	Protein disulfide-isomerase A3 precursor	PDIA3_HUMAN	57146	-1,92
12	Fumarylacetoacetase	FAAA_HUMAN	46743	2,85
13	NADH-ubiquinone oxidoreductase	NISM_HUMAN	21737	2,45
14a	Sulfotransferase 1C2	ST1C2_HUMAN	35882	2,00
14b	Sulfotransferase 1C2	ST1C2_HUMAN	35882	2,12
15a	Coronin-1A	CORA_HUMAN	51678	2,01
15b	Coronin-1A	CORA_HUMAN	51678	2,61
16	Plastin-2	PLSL_HUMAN	70015	1,95
17	Hemolysin protein GSH-1	GSH1_HUMAN	26378	4,08
18	Glucose-6-phosphate 1-dehydrogenase	G6PD_HUMAN	59553	2,56
19a	Alpha-enolase	ENOA_HUMAN	47350	2,18
19b	Alpha-enolase	ENOA_HUMAN	47350	4,01
19c	Alpha-enolase	ENOA_HUMAN	47350	1,81
19d	Alpha-enolase	ENOA_HUMAN	47350	-2,40
19e	Alpha-enolase	ENOA_HUMAN	47350	-2,21
20	CYCL3	MIP2B_HUMAN	11563	1,80
21a	Phosphoglycerate kinase 1	PGK1_HUMAN	44854	1,95
21b	Phosphoglycerate kinase 1	PGK1_HUMAN	44854	2,23
21c	Phosphoglycerate kinase 1	PGK1_HUMAN	44854	2,78
21d	Phosphoglycerate kinase 1	PGK1_HUMAN	44854	-1,83
22a	Tubulin beta-2 chain	TBB2_HUMAN	50095	1,93
22b	Tubulin beta-2 chain	TBB2_HUMAN	50095	2,02
23	Protein disulfide-isomerase A6 precursor	PDIA6_HUMAN	48490	2,29
24	Proliferating cell nuclear antigen (PCNA)	PCNA_HUMAN	29092	2,00
25	Pyruvate kinase isozymes M1/M2	KPYM_HUMAN	58339	2,43
26	Cytokeratin 1	K2C1_HUMAN	65018	1,98
27	Spermidine synthase	SPEE_HUMAN	34373	2,01
28	Hepcidin precursor	HEPC_HUMAN	9815	2,67
29	Ras-related protein Rab-11A	RB11A_HUMAN	24361	1,99
30	Gamma-actin	ACTG_HUMAN	42108	-2,33
31a	Beta-actin	ACTB_HUMAN	42052	-1,92
31b	Beta-actin	ACTB_HUMAN	42052	-1,83
32	Hornerin	HORN_HUMAN	282140	1,97
33	Keratin, type I cytoskeletal 9A	K2C9A_HUMAN	60162	4,36
34	Glutamine synthetase	GLNA_HUMAN	42534	2,86
35	Tudor domain-containing protein 3	TDRD3_HUMAN	73425	2,33
36	Transketolase	TKT_HUMAN	68519	1,97
37	DNA topoisomerase 2-alpha	TOP2A_HUMAN	175317	-1,05
38	Filamin/FRR-repeat protein 3	FRXI3_HUMAN	49474	2,65
39	Poly(rC)-binding protein 1	PCBP1_HUMAN	37987	1,90
40	Brain derived neurotrophic factor precursor	BDNF_HUMAN	28199	1,86
41	Ubiquinol-cytochrome c reductase core protein II	gi|50592900	40504	1,06
42a	Fructose-bisphosphate aldolase A	ALDOA_HUMAN	39720	2,33
42b	Fructose-bisphosphate aldolase A	ALDOA_HUMAN	39720	2,73
43	Citrate synthase	CISY_HUMAN	51908	2,06
44	Caspase-3 precursor	CASP3_HUMAN	32044	2,38
45	Adenylate kinase isoenzyme 2	KAD2_HUMAN	26558	1,81
46	Keratin, type I cytoskeletal 1	K2C1_HUMAN	65018	1,80

MW: Molecular Weight in Dalton

Apoptosis

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Online Resource 3. Full list of differentially expressed genes

Probe Set ID	EntrezGene ID	Gene Name	LBFC	FC
212488_at	1289	COL5A1: collagen, type V, alpha 1	4.09	8,83
220616_at	N/A	g5453588	3.36	3,36
208122_s_at	5663	PSD: pleckstrin and Sec7 domain containing	3.35	3,35
204687_at	1043	CD52: CD52 molecule	3.21	4.4
205822_at	5062	PAK2: p21 (CDKN1A)-activated kinase 2	2.57	4,61
214456_at	2702	GJA5: gap junction protein, alpha 5, 40kDa	2.46	5,93
206553_at	4939	OAS2: 2'-5'-oligoadenylate synthetase 2, 69/71kDa	2.32	3,86
207473_at	3726	JUNB: jun B proto-oncogene	2.29	2,43
208671_s_at	6711	SPTBN1: spectrin, beta, non-erythrocytic 1	2.27	5,62
209122_at	123	ADFP: adipose differentiation-related protein	2.14	2,41
211223_at	5625	PROP1: prophet of Pit1, paired-like homeodomain transcription factor	2.12	5,13
227273_at	860	RUNX2: runt-related transcription factor 2	2.12	5,25
207052_at	2040	STOM: stomatin	2	3,33
37547_at	27241	BBS5: Bardet-Biedl syndrome 5	1.95	1,95
216254_s_at	3913	LAMB2: laminin, beta 2 (laminin S)	1.74	5,23
213324_at	6714	SRC: v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	1.73	4,87
203153_at	3434	IFIT1: interferon induced protein with tetratricopeptide repeats 1	1,7	3,48
205047_s_at	440	ASNS: asparagine synthetase	1.68	3,91
213958_at	923	CD6: CD6 molecule	1.67	1,67
202887_s_at	54541	DDIT4: DNA damage inducible transcript 4	1.65	2,38
219658_at	79870	PTCD3: pentatricopeptide repeat domain 3	1.65	2,98
207201_s_at	6580	SLC22A1: solute carrier family 22 (organic cation transporter), member 1	1.64	3,76
34640_at	816	CAMK2B: Calcium/calmodulin-dependent protein kinase II beta	1.64	5,23
209422_at	333	APLP1: amyloid beta (A4) precursor-like protein 1	1,6	2,56
207476_at	3174	HNF4G: hepatocyte nuclear factor 4, gamma	1.59	3,01
207670_at	3881	KRT35: keratin 35	1.57	2,59
220177_s_at	64699	TMPRSS3: transmembrane protease, serine 3	1.56	2,66
203507_at	968	CD68: CD68 molecule	1.55	2,75
203540_at	2670	GFAP: glia fibrillary acidic protein	1.54	2,61
217036_at	27445	PCLO: piccolo (presynaptic cytomatrix protein)	1.54	1,54
219894_at	10520	ARID3B: AT rich interactive domain 3B (BRIGHT-like)	1.52	3,87
206857_s_at	2995	GYPA: glycophorin A (MNS blood group)	1.51	3,48
204880_x_at	5675	PSG5: pregnancy specific beta-1-glycoprotein 5	1,5	1,5
209547_s_at	57794	SF4: splicing factor 4	1.49	1,49
202409_at	3481	IGF2: insulin like growth factor 2 (somatomedin A)	1.47	1,47
206548_at	79938	FLJ23556: hypothetical protein FLJ23556	1.47	2,82
31837_at	91289	TMEM112B: transmembrane protein 112B	1.45	3,13
207281_at	1261	CNGA3: cyclic nucleotide gated channel alpha 3	1.44	1,44
204439_at	10964	IFI44L: interferon-induced protein 44-like	1.43	2,13
205179_s_at	101	ADAM8: ADAM metallopeptidase domain 8	1.43	2,69
201893_x_at	1634	DCN: decorin	1.43	2,93
219519_s_at	6614	SIGLEC1: sialic acid binding Ig-like lectin 1, sialoadhesin	1.43	2,6
218990_s_at	6707	SPRR3: small proline-rich protein 3	1.42	2,56
214211_at	2495	FTH1: ferritin, heavy polypeptide 1	1.41	1,64
201466_s_at	3725	JUN: jun oncogene	1,4	1,92
205247_at	4855	NOTCH4: Notch homolog 4 (Drosophila)	1,4	2,55
209524_at	50810	HDGFRP3: hepatoma-derived growth factor, related protein 3	1.39	2,89
214319_at	10129	FRY: furry homolog (Drosophila)	1.39	1,93
215282_at	25847	ANAPC13: anaphase promoting complex subunit 13	1.39	1,89
34210_at	1043	CD52: CD52 molecule	1.38	1,7
206315_at	9244	CRLF1: cytokine receptor like factor 1	1.38	5,76
220301_at	79839	CCDC102B: coiled coil domain containing 102B	1.38	2,39
203483_at	57715	SEMA4C: sema domain, immunoglobulin domain, transmembrane domain and short cytoplasmic domain 4C	1.37	3,41
217678_at	23657	SLC7A11: solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	1.37	1,59

230068_at	728	C5AR1: complement component 5a receptor 1	1,37	2,16
2C4364_s_at	65055	REEP1: receptor accessory protein 1	1,36	3,13
21[illegible]520_at	5600	MAPK11: mitogen-activated protein kinase 11	1,36	2,93
206624_at	8287	USP9Y: ubiquitin specific peptidase 9, Y-linked (fat facets-like, Drosophila)	1,36	3,37
219532_at	6785	ELOVL4: elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	1,36	3,19
206786_at	3347	HTN3: histatin 3	1,35	2,42
213443_at	8717	TRADD: TNFRSF1A-associated via death domain	1,34	2,08
205997_at	10863	ADAM28: ADAM metallopeptidase domain 28	1,33	2,02
217359_s_at	4684	NCAM1: neural cell adhesion molecule 1	1,33	2,04
2C7563_s_at	8473	OGT: O-linked N-acetylglucosamine (GlcNAc) transferase	1,33	2,13
221114_at	258	AMDN: amelobastin (enamel matrix protein)	1,33	3,34
221147_x_at	51741	WWOX: WW domain containing oxidoreductase	1,33	1,33
213281_at	3725	JUN: jun oncogene	1,32	1,86
2C1925_s_at	1604	CD55: CD55 molecule, decay accelerating factor for complement (Cromer blood group)	1,31	1,6
2C2847_at	5106	PCK2: phosphoenolpyruvate carboxykinase 2 (mitochondrial)	1,31	1,54
210726_at	1576	CYP3A4: cytochrome P450, family 3, subfamily A, polypeptide 4	1,31	1,70
36125_at	9905	RUTBC1: RUN and TBC1 domain containing 1	1,31	3,16
205785_at	3684	ITGAM: integrin, alpha M (complement component 3 receptor 3 subunit); CD11b	1,3	1,88
210228_at	1437	CSF2: colony stimulating factor 2 (granulocyte-macrophage)	1,3	2,12
2C2869_at	4938	OAS1: 2',5'-oligoadenylate synthetase 1, 40/46kDa	1,29	1,88
214284_s_at	8817	FGF18: fibroblast growth factor 18	1,29	5,89
203725_at	1647	GADD45A: growth arrest and DNA-damage-inducible, alpha	1,29	1,61
219224_x_at	79797	ZNF408: zinc finger protein 408	1,29	1,29
208773_s_at	1831	TSC22D3: TSC22 domain family, member 3	1,28	1,43
203547_at	920	CD4: CD4 molecule	1,28	4,06
216994_s_at	860	RUNX2: runt-related transcription factor 2	1,28	1,85
200886_s_at	5660	PSAP: prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	1,27	1,64
208510_s_at	5468	PPARG: peroxisome proliferator-activated receptor gamma	1,27	1,6
214644_at	8330	HIST1H2AK: histone cluster 1, H2ak	1,27	1,72
217502_at	8433	IFIT2: interferon-induced protein with tetratricopeptide repeats 2	1,27	1,65
218484_at	56901	NDUFA4L2: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	1,27	1,58
206884_s_at	8796	SCEL: sciellin	1,26	3,54
213293_s_at	10346	TRIM22: tripartite motif containing 22	1,26	1,48
2C1821_s_at	10440	TIMM17A: translocase of inner mitochondrial membrane 17 homolog A (yeast)	1,26	2,4
217213_at	6530	SLC6A2: solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	1,26	1,7
217738_at	10135	PBEF1: pre-B cell colony enhancing factor 1	1,26	1,48
213683_at	10762	NUP50: nucleoporin 50kDa	1,26	2,09
2C1926_s_at	1604	CD55: CD55 molecule, decay accelerating factor for complement (Cromer blood group)	1,25	1,48
2C4271_s_at	1910	EDNRB: endothelin receptor type B	1,25	3,12
2C4415_at	2537	IFI6: interferon, alpha-inducible protein 6	1,25	2,02
217767_at	653879	LOC653879: similar to Complement C3 precursor	1,25	1,6
220892_s_at	29968	PSAT1: phosphoserine aminotransferase 1	1,25	1,51
2C1749_at	1889	ECE1: endothelin converting enzyme 1	1,24	1,63
203821_at	1839	HBEGF: heparin-binding EGF-like growth factor	1,24	1,65
211915_s_at	720613	TUBB8: tubulin, beta polypeptide 4, member Q	1,24	1,5
209924_s_at	6526	SLC3A2: solute carrier family 3, member 2	1,23	1,45
2C5369_x_at	1629	DBT: dihydrolipoamide branched chain transacylase E2	1,23	1,67
216434_at	55020	FLJ20609: hypothetical protein FLJ20609	1,23	1,23
2C5193_at and	23764	MAFF: v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	1,23	1,96
2C3913_s_at	3218	HPGD: hydroxyprostaglandin dehydrogenase 15 (NAD)	1,22	2,26
218145_at	57761	TRIB3: tribbles homolog 3 (Drosophila)	1,22	1,47
2C3525_s_at	324	APC: adenomatosis polyposis coli	1,21	3,27
2C6270_at	5588	PRKCG: protein kinase C, gamma	1,21	3,49
2C9749_s_at	1636	ACE: angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	1,21	3,7
210682_at	4025	LPO: lactoperoxidase	1,21	1,21
214696_at	84961	MGC14376: hypothetical protein MGC14376	1,21	1,72
219256_s_at	54436	SH3TC1: SH3 domain and tetratricopeptide repeats 1	1,21	1,62
221346_at	26476	OR10J1: olfactory receptor, family 10, subfamily J, member 1	1,21	1,21
221377_s_at	11317	RDPJL: recombination signal binding protein for immunoglobulin kappa J region-like	1,21	2,06
217739_x_at	7329	UBE2I: ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	1,21	1,41
207736_s_at	7142	TNP2: transition protein 2 (during histone to protamine replacement)	1,2	1,46
2C5685_at	942	CD86: CD86 molecule	1,2	1,79
2C6995_x_at	8578	SCARF1: scavenger receptor class F, member 1	-1,2	-1,4
216078_at	6648	SOD2: superoxide dismutase 2, mitochondrial	-1,2	-2,12
216239_at	400084	LOC100084: hypothetical gene supported by AK057532; AL137270; BC057846	1,2	2,42
220650_at	54859	TMEM106B: transmembrane protein 106B	-1,2	-1,58
221331_x_at	1493	CTLA4: cytotoxic T-lymphocyte-associated protein 4	1,2	1,43
207351_at	26959	HBP1: HMG-box transcription factor 1	1,21	1,64
2C7477_at	N/A	Hs.283046.0	-1,21	-2,5
206187_s_at	395	ARHGAP6: Rho GTPase activating protein 6	-1,21	-2,8

208529_at	630	BTF3L1: basic transcription factor 3, like 1	1,21	2,61
207978_s_at	8013	NR4A3: nuclear receptor subfamily 4, group A, member 3	1,21	1,5
211945_s_at	3688	ITGB1: integrin, beta 1	-1,21	-1,8
216310_at	57551	TAOK1: TAO kinase 1	-1,21	-2,18
205415_s_at	4287	ATXN3: ataxin 3	-1,21	-1,54
203623_at	55558	PLXNA3: plexin A3	-1,22	-1,63
206553_x_at	5371	PML: promyelocytic leukemia	-1,22	-4,8
219127_at	63827	BCAN: brevican	-1,22	-1,89
220116_at	3781	KCNN2: potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	-1,22	-1,5
204790_s_at	9572	THRA: thyroid hormone receptor, alpha	-1,22	-1,63
206569_at	11009	IL24: interleukin 24	-1,23	-1,58
206814_at	4803	NGFB: nerve growth factor, beta polypeptide	-1,23	-2,05
207695_s_at	3547	ICSF1: immunoglobulin superfamily, member 1	-1,23	-4,52
203459_s_at	64601	VPS16: vacuolar protein sorting 16 homolog (S. cerevisiae)	-1,23	-2,29
211702_at	27307	MAG: myelin associated glycoprotein	-1,23	-2,55
200614_at	1213	CLTC: clathrin, heavy chain (Hc)	-1,24	-2,78
202340_x_at	3164	NR4A1: nuclear receptor subfamily 4, group A, member 1	1,24	1,88
211218_at	29021	PRO1848: PRO1848 protein	1,24	2,84
216549_s_at	55333	TBC1D22B: TBC1 domain family, member 22B	1,24	2,02
206281_at	116	ADCYAP1: adenylate cyclase activating polypeptide 1 (pituitary)	-1,25	-1,5
204323_x_at	4763	NF1: neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease)	-1,25	-2,24
204989_s_at	3691	ITGB4: integrin, beta 4	-1,25	-1,91
217215_s_at	N/A	Hs.225954.0	-1,25	-1,93
205527_at	22999	ARHGEF15: Rho guanine nucleotide exchange factor (GEF) 15	-1,25	-1,9
220085_at	64102	TNMD: tenomodulin	-1,25	-2,36
216086_at	22987	SV2C: synaptic vesicle glycoprotein 2C	-1,26	-1,9
219862_s_at	26502	NARF: nuclear prelamin A recognition factor	-1,26	-1,43
220598_at	79092	CARD14: caspase recruitment domain family, member 14	-1,26	-3,46
220893_at	57399	LOC57399: uncharacterized gastric protein ZA52D	-1,26	-2,06
205152_at	2567	SLC6A1: solute carrier family 6 (neurotransmitter transporter, GABA), member 1	-1,27	-1,76
205544_s_at	1380	CR2: complement component (3d/Epstein Barr virus) receptor 2	-1,27	-1,73
207145_at	2666	GDF8: growth differentiation factor 8	-1,27	-3,1
207442_at	1440	CSF3: colony stimulating factor 3 (granulocyte)	1,27	2,18
208545_x_at	6874	TAF1: TAF1 RNA polymerase II, TATA box binding protein (TBP) associated factor, 135kDa	1,27	4,23
205872_x_at	9659	PDE4DIP: phosphodiesterase 4D interacting protein (myomegalin)	-1,27	-3,5
216225_at	N/A	Clone: MAGE 25997	1,27	2,54
219524_s_at	N/A	gi13129143	-1,27	-1,73
220821_at	2587	GALR1: galanin receptor 1	-1,27	-2,06
204782_at	**4291**	**MLF1: Myeloid leukemia factor 1**	-1,28	-2,11
204083_s_at	7169	TPM2: tropomyosin 2 (beta)	-1,28	-1,64
220936_s_at	81626	C1orf14: chromosome 1 open reading frame 14	-1,28	-3,09
219710_at	79628	SH3TC2: SH3 domain and tetratricopeptide repeats 2	-1,29	-1,99
221908_at	84900	TMEM113: transmembrane protein 113	-1,29	-1,85
209469_at	2822	GPM6A: glycoprotein M6A	-1,3	-2,45
206651_s_at	1361	CPD: carboxypeptidase D (plasma)	-1,31	-13,92
207658_s_at	2291	FOXG1 /// FOXG1B: forkhead box G1B /// forkhead box G1	-1,31	-2,36
209827_s_at	4784	NFIX: nuclear factor I/X (CCAAT-binding transcription factor)	-1,31	-2,21
216447_at	N/A	CLNA: FLJ20872 fis, clone ADKA02604	-1,31	-1,93
218829_s_at	55636	CHD7: CHromodomain helicase DNA binding protein 7	-1,31	-1,68
221457_s_at	56244	BTNL3: butyrophilin like 2 (MHC class II associated)	1,31	1,67
211636_at	3507	IGHA1: immunoglobulin heavy constant alpha 1	1,32	3,2
214547_at	55811	SAC: testicular soluble adenylyl cyclase	-1,32	-1,91
215018_at	85459	KIAA1731: KIAA1731	-1,32	-2,2
205043_at	1080	CFTR: cystic fibrosis transmembrane conductance regulator	-1,33	-2,83
210329_at	9400	RECQL5: RecQ protein-like 5	-1,33	-2,17
203157_at	4130	MAP1A: microtubule-associated protein 1A	-1,33	-2,3
205959_at	4322	MMP13: matrix metallopeptidase 13 (collagenase 3)	-1,34	-2,7
221182_at	80133	C1orf129: chromosome 1 open reading frame 129	-1,34	-2,69
216239_at	27241	BBS9: Bardet-Biedl syndrome 9	-1,34	-1,64
207410_s_at	1552	DDO: D-aspartate oxidase	-1,35	-5,65
202163_at	3985	LIMK2: LIM domain kinase 2	-1,35	-1,75
205654_at	722	C4BPA: complement component 4 binding protein, alpha	-1,36	-1,79
205186_at	7802	DNALI1: cytoplasmic dynein 1 intermediate chain 1	-1,37	-2,38
205820_at	6519	SLC3A1: solute carrier family 3	-1,37	-2,29
210144_at	25771	TBC1D22A: TBC1 domain family, member 22A	1,37	2,26
207864_at	6332	SCN7A: sodium channel, voltage-gated, type VII, alpha	-1,38	-1,77
220771_at	51152	LOC51152: melanoma antigen	1,38	1,99
203734_at	8476	CDC42BPA: CDC42 binding protein kinase alpha (DMPK-like)	1,39	3,37
219820_s_at	79896	THNSL1: threonine synthase-like 1 (bacterial)	-1,39	-2,31
205396_at	4088	SMAD3: SMAD family member 3	-1,4	-1,4

207669_at	3883	KRT83: keratin 83	1,44	4
203020_at	11075	STMN2: stathmin-like 2	1,41	2,98
214517_at	3846	KRTAP5-9: keratin associated protein 5-9	-1,42	-3,24
215520_at	92017	LOC92017: similar to RIKEN cDNA 4933437K13	-1,42	-1,42
220634_at	9496	TBX4: T-box 4	-1,42	-2,04
204329_at	1583	CYP1A1: cytochrome P450, family 1, subfamily A, polypeptide 1	-1,43	-1,79
203724_s_at	22902	RUFY3: RUN and FYVE domain containing 3	-1,44	-3,06
219896_at	50632	DRD1IP: dopamine receptor D1 interacting protein	-1,44	-2,7
220314_at	728302	HSFX1: heat shock transcription factor family, X-linked 1	-1,44	-2,79
221172_at	80099	FLJ21075: hypothetical protein FLJ21075	-1,44	-2,93
207802_at	10321	CRISP3: cysteine-rich secretory protein 3	-1,45	-2,85
212977_at	57007	CXCR7: chemokine (C-X-C motif) receptor 7	-1,45	-2,16
215276_at	90199	WFDC8: WAP four-disulfide core domain 8	-1,45	-2,21
212817_at	25822	DNAJB5: DnaJ (Hsp40) homolog, subfamily B, member 5	-1,46	-1,46
216824_at	N/A	Hs.307150.0	-1,46	-1,46
217562_at	339479	FAM5C: family with sequence similarity 5, member C	-1,46	-2,73
208235_x_at	1444	CSHL1: chorionic somatomammotropin hormone-like 1	1,47	3,2
219780_at	51333	ZNF771: zinc finger protein 771	1,48	2,56
214079_at	10202	DHRS2: dehydrogenase/reductase (SDR family) member 2	1,51	5,62
206517_at	1014	CDH16: cadherin 16, KSP-cadherin	-1,51	-1,51
207729_at	5563	PRKAA2: protein kinase, AMP-activated, alpha 2 catalytic subunit	-1,52	-2,28
220069_at	51807	TUBA8: tubulin, alpha 8	-1,52	-3,1
205177_at	7135	TNNI1: troponin I type 1 (skeletal, slow)	-1,54	-1,54
205388_at	7125	TNNC2: troponin C type 2 (fast)	-1,54	-3,06
216511_s_at	6934	TCF7L2: transcription factor 7-like 2 (T-cell specific, HMG-box)	-1,54	-3,99
215032_at	6239	RFX1: regulatory factor X, 1 (influences HLA class II expression)	-1,54	-3,26
220087_at	N/A	gi|22538	-1,54	-2,6
215647_at	84062	RBM28: RNA binding motif protein 28	-1,55	-3,17
213910_at	3490	IGFBP7: insulin-like growth factor binding protein 7	-1,59	-2,84
211156_at	1029	CDKN2A: cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	-1,6	-4,58
220025_at	53829	P2RY13: purinergic receptor P2Y, G-protein coupled, 13	-1,6	-3,59
207295_at	6340	SCNN1G: sodium channel, nonvoltage-gated 1, gamma	-1,62	-3,06
207938_at	6480	ST6GAL1: ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	1,62	3,7
221138_s_at	N/A	gi|7662605	1,62	3,11
206221_at	22821	RASA3: RAS p21 protein activator 3	-1,64	-1,64
217661_x_at	147912	SIX5: sine oculis homeobox homolog 5 (Drosophila)	1,66	3,51
221302_at	28999	KLF15: Kruppel-like factor 15	-1,69	-2,25
213880_at	8549	LGR5: leucine-rich repeat-containing G protein-coupled receptor 5	-1,7	-4,32
206970_at	6900	CNTN2: contactin 2 (axonal)	-1,72	-1,72
220185_at	57731	SPTBN4: spectrin, beta, non-erythrocytic 4	-1,76	-5,8
209756_s_at	4613	MYCN: v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-1,78	-1,78
220913_at	N/A	gi|3443021	-1,79	-1,79
218370_at	22861	NLRP1: NLR family, pyrin domain containing 1	-1,8	-1,8
220718_at	80072	C15orf24: chromosome 15 open reading frame 24	-1,82	-4,47
205838_at	2997	GYPA: glycophorin A (MNS blood group)	-1,93	-2,85
217452_s_at	8707	B3GALT2: UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	-1,96	-3,72
212670_at	2006	ELN: elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)	-2,06	-2,06
208257_x_at	5669	PSG1: pregnancy specific beta-1-glycoprotein 1	-2,07	-3,06
211062_s_at	8532	CPZ: carboxypeptidase Z	-2,51	-2,51
222384_at	284649	DKFZP564C196: DKFZP564C196 protein	2,57	5,31
213138_at	23114	NFASC: neurofascin homolog (chicken)	2,63	5,99
204200_s_at	5155	PDGFB: platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	-2,65	-4,09
217687_x_at	3811	KIR3DL1: killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	-2,81	-7,41
206994_at	1472	CST4: cystatin S	-2,88	-5,36
206326_at	2922	GRP: gastrin-releasing peptide	-2,94	-5,91
207622_s_at	10061	ABCB2: ATP-binding cassette, sub-family B (MDR/TAP), member 2	-3,08	-3,08
204922_at	79703	C11orf80: chromosome 11 open reading frame 80	-3,59	-9,64
211645_x_at	N/A	Immunoglobulin kappa light chain (IGKV) mRNA variable region, joining region, and constant region	-3,82	-6,52
206230_at	3975	LHX1: LIM homeobox 1	-4,03	-10,9

Full list of genes that had at least a 90% lower confidence bound (LB FC) of 1.2 for the respective fold change (FC)

FC: Fold Change (Diclofenac treated HL-60 and THP-1 cells / DMSO treated HL-60 and THP-1 cells)

LBFC: Lower Bound of Fold Change (Diclofenac treated HL-60 and THP-1 cells / DMSO treated HL-60 and THP-1 cells)

Apoptosis

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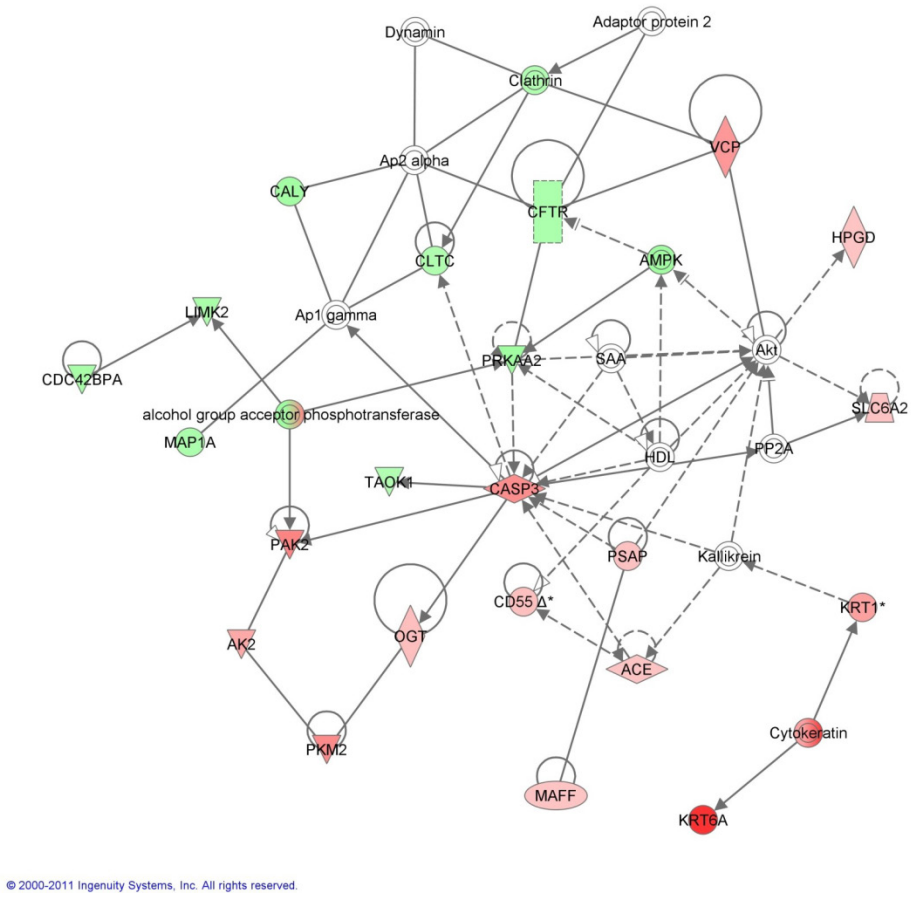
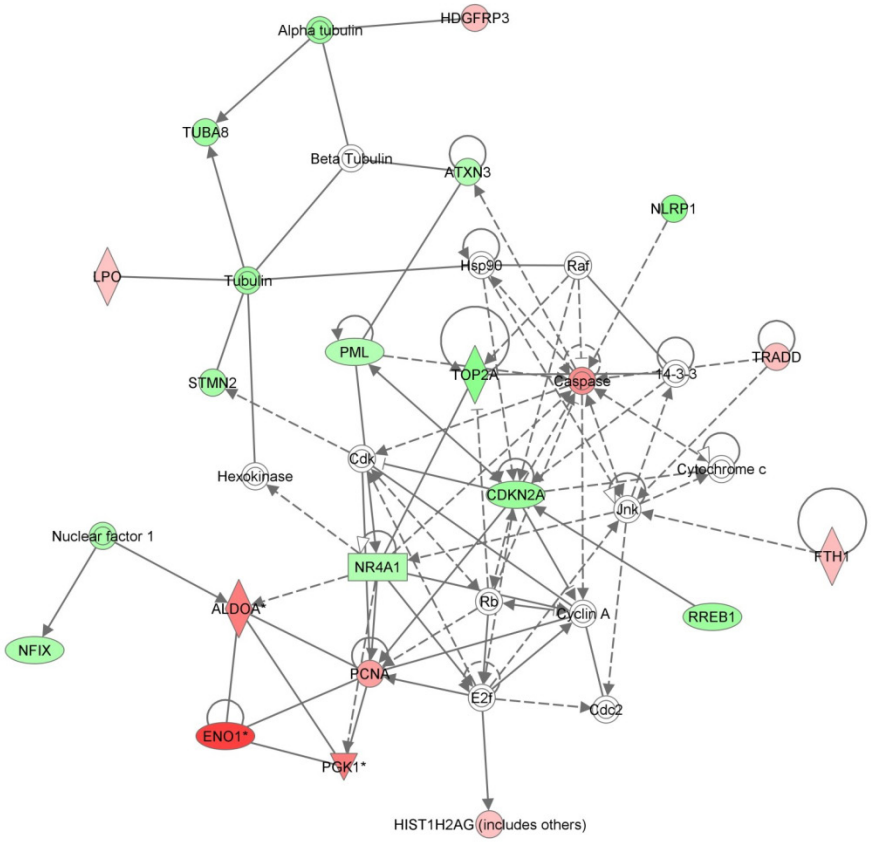
Online Resource 4. Ingenuity network analysis of the genomic and proteomic approach datasets
Ingenuity pathway analysis of the gene expression profiling and differential proteomic analysis datasets together reveals that 2 of the top 3 altered networks following Diclofenac treatment evolved around JNK signaling with Caspase-3 as centre molecule.

Network 2



Network 3



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Online Resource 5. Ingenuity pathway analysis regarding Apoptosis

Category	Functions Annotation	p-Value	Molecules	# Molecules
Cancer	tumor	7.41E-12	ACTG1, AK2, ALDOA, APC, BCAN, BDNF, BTRC, C4BPA, CAMK2B, CASP3, CD4, CD52, CD55, CD86, CDC42BPA, CDKN2A, CSF2, CTLA4, CXCR7, CYP3A4, DCN, DNALI1, EDNRB, ENO1, FAM5C, FGF18, FLNA, FOXG1, FTH1, G6PD, GALR1, GFAP, GPM6A, HAMP, HBEGF, HPGD, IFI44L, IFIT1, IGF2, IGFBP7, IL24, ITGB1, ITGB4, JUN, LOC51152, MMP13, MYCN, NCAM1, NF1, NGF, NOTCH4, NUP50, P4HB, PCNA, PDGFB, PFN1, PGK1, PKM2, PML, PPARG, PSAT1, RREB1, RUNX2, SCN7A, SIGLEC1, SLC6A2, SLC7A11, SMAD3, SOD2, SPTBN1, SRC, SRM, STMN2, STOM, TAF4, TAOK1, TBX4, TNNC2, TOP2A, TRADD, TUBA8, VCP, VIM, WWOX	84
Cellular Growth and Proliferation	proliferation of normal cells	2.31E-10	ADCYAP1, AMBN, APC, BDNF, BTNL2, BTRC, CASP3, CD4, CD6, CD86, CDKN2A, CORO1A, CPB2, CR2, CSF2, CSF3, CTLA4, DCN, EDNRB, ELN, FGF18, FOXG1, G6PD, GALR1, HBEGF, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, MSTN, MYCN, NAMPT, NCAM1, NF1, NGF, NR1D1, NR4A3, PDGFB, PGK1, PML, PPARG, PRKCG, RUNX2, SIGLEC1, SLC7A11, SMAD3, SOD2, SPTBN1, SRC, ST6GAL1, TNMD, TSC22D3	56
Cellular Growth and Proliferation	growth of cells	4.60E-10	ACTB, ACTG1, ACTN4, AK2, APC, ATP5B, BDNF, C5AR1, CASP3, CD4, CD55, CD86, CDKN2A, CORO1A, CPB2, CR2, CSF2, CSF3, CTLA4, CXCR7, DCN, ENO1, FGF18, FOXG1, FTH1, GJA5, GRP, HBEGF, HBP1, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, LAMB2, LCP1, MAFF, MSTN, MYCN, NF1, NFIX, NGF, NOTCH4, NR4A3, PDGFB, PDIA3, PFN1, PGK1, PKM2, PLIN2, PML, PPARG, PSAP, RUNX2, SLC22A1, SLC3A2, SLC7A11, SMAD3, SOD2, SRC, TAF4, TPM2	65
Cellular Growth and Proliferation	growth of eukaryotic cells	4.29E-09	ACTN4, APC, BDNF, C5AR1, CASP3, CD4, CD55, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, CXCR7, DCN, FGF18, FOXG1, FTH1, GRP, HBEGF, HBP1, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, LCP1, MSTN, MYCN, NF1, NFIX, NGF, NOTCH4, NR4A3, PDGFB, PDIA3, PFN1, PML, PPARG, RUNX2, SLC3A2, SLC7A11, SMAD3, SOD2, SRC, TAF4, TPM2	50
Cell Death	apoptosis of eukaryotic cells	3.37E-08	ADCY10, ADCYAP1, ALDOA, APC, ASNS, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, DDIT4, EDNRB, ENO1, FTH1, G6PD, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, P4HB, PAK2, PCNA, PDIA3, PML, PPARG, PRKAA2, PRKCG, PSAP, RUNX2, SMAD3, SOD2, SRC, ST6GAL1, TOP2A, TRADD, TRIB3, VCP, WWOX	62

Cell Death	cell death of eukaryotic cells	4.85E-08	ACTB, ADCY10, ADCYAP1, ALDOA, APC, ASNS, ATXN3, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CR2, CSF2, CSF3, CTLA4, CYP3A4, DCN, DDIT4, EDNRB, ENO1, FLNA, FTH1, G6PD, GFAP, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, P4HB, PAK2, PCNA, PDGFB, PDIA3, PML, PPARG, PRKAA2, PRKCG, PSAP, RUNX2, SLC7A11, SMAD3, SOD2, SRC, ST6GAL1, TAF4, TOP2A, TRADD, TRIB3, VCP, WWOX	70
Cellular Development	differentiation of cells	5.69E-08	ADCYAP1, APC, BDNF, C5AR1, CASP3, CD4, CD52, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, DCN, EDNRB, ENO1, FGF18, FOXG1, GLUL, HBEGF, HBP1, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, LIMK2, MAFF, MMP13, MSTN, MYCN, NAMPT, NCAM1, NF1, NGF, NOTCH4, NR1D1, NR4A1, NR4A3, PML, PPARG, RASA3, RUNX2, SCEL, SMAD3, SOD2, SRC, STMN2, TCF7L2, TRIB3, TSC22D3, WWOX	56
Cellular Movement	invasion of eukaryotic cells	6.48E-08	BCAN, BDNF, CASP3, CDKN2A, CSF2, CSF3, EDNRB, FAM5C, FLNA, GRP, HBEGF, HBP1, IGF2, ITGB1, ITGB4, JUN, LCP1, MMP13, MYCN, NF1, NGF, PPARG, RUNX2, SOD2, SRC, VIM, WWOX	27
Cell Morphology	shape change of eukaryotic cells	2.21E-07	APC, BDNF, CASP3, CDKN2A, CFTR, CORO1A, CSF2, DCN, EDNRB, FLNA, FOXG1, ITGAM, ITGB1, JUN, NGF, PFN1, RUNX2, SOD2, SRC, TAOK1, VIM	21
Cellular Development	shape change of eukaryotic cells	2.21E-07	APC, BDNF, CASP3, CDKN2A, CFTR, CORO1A, CSF2, DCN, EDNRB, FLNA, FOXG1, ITGAM, ITGB1, JUN, NGF, PFN1, RUNX2, SOD2, SRC, TAOK1, VIM	21
Cell Death	apoptosis	2.74E-07	ACE, ACTN4, ADCY10, ADCYAP1, ALDOA, APC, ASNS, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, DDIT4, DHRS2, ECE1, EDNRB, ENO1, FTH1, G6PD, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, LAMB2, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, OAS1, P4HB, PAK2, PCNA, PDIA3, PKM2, PML, PPARG, PRKAA2, PRKCG, PROP1, PSAP, RUNX2, SMAD3, SOD2, SRC, ST6GAL1, TOP2A, TRADD, TRIB3, VCP, WWOX	70
Gene Expression	activation of synthetic promoter	3.92E-07	ACTB, APC, BDNF, BTRC, CASP3, CD4, CDKN2A, CSF2, CSF3, DCN, HBEGF, HBP1, IL24, JUN, KLF15, MAPK11, MYCN, NGF, NOTCH4, PLIN2, PML, PPARG, RUNX2, SIX5, SMAD3, TCF7L2, TRADD, WWOX	28
Tissue Morphology	quantity of cells	7.85E-07	ACTN4, ADCYAP1, APLP1, BDNF, C5AR1, CASP3, CD4, CD86, CDKN2A, CFTR, CNTN2, CR2, CRLF1, CSF2, CSF3, CTLA4, DCN, EDNRB, FGF18, HBEGF, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, JUN, LHX1, MMP13, NF1, NGF, NOTCH4, NR4A1, NR4A3, PDGFB, PML, PPARG, PSAP, RUNX2, SIGLEC1, SMAD3, SOD2	42
Cellular Growth and Proliferation	growth of cell lines	8.20E-07	ACTN4, APC, CDKN2A, CSF2, CSF3, CXCR7, DCN, FGF18, FOXG1, FTH1, GRP, HBEGF, HBP1, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, LCP1, MSTN, NF1, NFIX, NGF, NOTCH4, PDGFB, PDIA3, PFN1, PML, PPARG, RUNX2, SLC3A2, SLC7A11, SOD2, SRC, TPM2	38
Cellular Movement	migration of cells	9.48E-07	ADCYAP1, ALDOA, APC, BDNF, C5AR1, CD4, CD86, CDKN2A, CNTN2, CORO1A, CSF2, CSF3, CTLA4, CXCR7, DCN, EDNRB, ELN, FAM5C, G6PD, GRP, HBEGF, IGF2, ITGAM, ITGB1, ITGB4, JUN, KRT6A, MAPK11, MMP13, NCAM1, NF1, NFIX, NGF, NR1D1, PAK2, PDGFB, PPARG, PRKCG, RUNX2, SLC3A2, SMAD3, SOD2, SRC, ST6GAL1, TCF7L2, WWOX	46
Organismal Survival	death of animal	1.08E-06	ACE, ADCYAP1, APC, APLP1, BDNF, C5AR1, CASP3, CD4, CDH16, CDKN2A, COL5A1, CRLF1, CSF2, CSF3, CTLA4, CYP11A1, DCN, HAMP, HBEGF, IGF2, ITGAM, ITGB1, ITGB4, LAMB2, MAFF, MYCN, NF1, NFIX, NR4A3, PDGFB, PFN1, PPARG, RUNX2, SMAD3, SOD2, SRC, TKT, WWOX	38
Cell Cycle	G1 phase	1.78E-06	ADCYAP1, APC, BTRC, CAMK2B, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, ITGB1, JUN, NGF, PPARG, RUNX2, SOD2, SRC	19
Cell Cycle	entry into cell division process of normal cells	2.64E-06	CDKN2A, CSF3, CTLA4, ITGB1, MYCN, PPARG, RUNX2, SMAD3, SOD2	9
Cell Cycle	cell division process	3.07E-06	ADCYAP1, APC, ASNS, BTRC, CAMK2B, CASP3, CD4, CDKN2A, CLTC, CSF2, CSF3, CTLA4, DCN, FAM5C, FOXG1, GRP, GYPA, HBEGF, HBP1, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, MYCN, NAMPT, NF1, NGF, NR4A1, NR4A3, PAK2, PCNA, PDGFB, PML, PPARG, PSAP, RAB11A, RUNX2, SMAD3, SOD2, SRC, TCF7L2, TOP2A, WWOX	47

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Skeletal and Muscular Disorders	skeletal and muscular disorder of mice	3.29E-06	BDNF, C5AR1, CASP3, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, GFAP, ITGB1, MMP13, NGF, PPARG, RUNX2, ST6GAL1	16
Organismal Survival	death of mice	3.62E-06	ACE, ADCYAP1, APC, APLP1, BDNF, CASP3, CD4, CDH16, CDKN2A, COL5A1, CRLF1, CSF2, CSF3, CTLA4, CYP11A1, DCN, HAMP, IGF2, ITGAM, ITGB1, ITGB4, LAMB2, MAFF, MYCN, NF1, NFIX, NR4A3, PDGFB, PFN1, PPARG, RUNX2, SMAD3, SOD2, SRC, TKT, WWOX	36
Skeletal and Muscular System Development and Function	skeletal and muscular process of eukaryotic cells	4.44E-06	BTF3L1, CDKN2A, CSF2, IGF2, ITGB1, JUN, PDGFB, PPARG, RUNX2, SMAD3, TSC22D3	11
Hematopoiesis	hematopoiesis	5.36E-06	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, HAMP, IGF2, IGHM, ITGB1, JUN, MYCN, NGF, NOTCH4, NR4A1, NR4A3, PDIA3, PML, RUNX2, SMAD3, SOD2, TOP2A	32
Hematological System Development and Function	hematopoiesis	5.36E-06	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, HAMP, IGF2, IGHM, ITGB1, JUN, MYCN, NGF, NOTCH4, NR4A1, NR4A3, PDIA3, PML, RUNX2, SMAD3, SOD2, TOP2A	32
Cell Cycle	interphase of normal cells	6.32E-06	CDKN2A, CSF2, CTLA4, DCN, IGFBP7, IGHM, ITGB1, JUN, MYCN, PPARG, RUNX2, SOD2	12
Cell Cycle	G1 phase of eukaryotic cells	7.63E-06	APC, BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, ITGB1, JUN, NGF, PPARG, RUNX2, SOD2	16
Protein Synthesis	expression of protein	8.06E-06	ACE, CDKN2A, CSF3, CTLA4, GRP, IGHM, ITGB1, JUN, MSTN, PPARG, RUNX2, SOD2, SRC	13
Cell Death	apoptosis of normal cells	8.33E-06	ADCY10, ADCYAP1, ALDOA, APC, BDNF, CASP3, CD4, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, IGF2, IGHM, IL24, ITGAM, ITGB1, JUN, MAPK11, MYCN, NAMPT, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, P4HB, PDIA3, PML, PPARG, RUNX2, SMAD3, SOD2, SRC, TOP2A, TRADD	38
Cell Death	apoptosis of endothelial cells	9.36E-06	ADCY10, BDNF, CASP3, CSF3, ITGB1, MAPK11, NR4A3, PPARG, RUNX2, SRC	10
Cellular Development	developmental process of epithelial cells	1.02E-05	APC, CASP3, CDKN2A, ECE1, EDNRB, HBEGF, HBP1, IGF2, IL24, ITGB1, JUN, KRT6A, MAFF, PPARG, RUNX2, SCEL, SMAD3	17
Tissue Development	ossification of connective tissue	1.05E-05	CASP3, FGF18, MMP13, NFIX, RUNX2	5
Skeletal and Muscular System Development and Function	ossification of connective tissue	1.05E-05	CASP3, FGF18, MMP13, NFIX, RUNX2	5
Cancer	hematologic cancer	1.40E-05	CD52, CD86, CDKN2A, CSF2, CTLA4, CYP3A4, ITGB1, JUN, MAPK11, MYCN, NCAM1, NF1, PDIA6, PML, RUNX2, SLC6A2, SOD2, SRC, TOP2A, TUBA8	20
Hematological Disease	hematologic cancer	1.40E-05	CD52, CD86, CDKN2A, CSF2, CTLA4, CYP3A4, ITGB1, JUN, MAPK11, MYCN, NCAM1, NF1, PDIA6, PML, RUNX2, SLC6A2, SOD2, SRC, TOP2A, TUBA8	20
Hematopoiesis	development of blood cells	1.62E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, IGF2, IGHM, ITGB1, JUN, NOTCH4, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, SOD2, TOP2A	28
Hematological System Development and Function	development of blood cells	1.62E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, IGF2, IGHM, ITGB1, JUN, NOTCH4, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, SOD2, TOP2A	28
Cellular Development	development of blood cells	1.62E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, IGF2, IGHM, ITGB1, JUN, NOTCH4, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, SOD2, TOP2A	28
Tissue Development	developmental process of tissue	1.70E-05	APC, BDNF, BTF3L1, CASP3, CDKN2A, CHD7, ECE1, ELN, FGF18, FOXG1, GJA5, HBEGF, IGF2, ITGB1, JUN, KRT6A, LHX1, MMP13, MSTN, NF1, NFIX, NGF, NR4A3, PDGFB, PFN1, PKM2, PPARG, PTCD2, RUNX2, SMAD3, SRC, TBX4, TCF7L2, TNNI1, WWOX	35
Cellular Development	differentiation of tumor cell lines	1.77E-05	ADCYAP1, BDNF, CSF2, CSF3, ENO1, IGF2, ITGAM, JUN, LIMK2, MSTN, NCAM1, NGF, PML, PPARG, RUNX2, SRC, STMN2	17
Cellular Development	developmental process of T lymphocytes	2.05E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD55, CD6, CD86, CDKN2A, CHD7, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, PPARG, RUNX2, SMAD3, TOP2A	25

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Tissue Development	development of tissue	2.07E-05	APC, BDNF, CASP3, CDKN2A, CHD7, ECE1, ELN, FGF18, FOXG1, GJA5, HBEGF, IGF2, ITGB1, JUN, KRT6A, LHX1, MMP13, MSTN, NF1, NFIX, NGF, NR4A3, PDGFB, PFN1, PPARG, PTCD2, RUNX2, SMAD3, TBX4, TCF7L2, TNNI1	31
Cellular Growth and Proliferation	growth of tumor cell lines	2.21E-05	ACTN4, APC, CDKN2A, CSF2, CXCR7, DCN, FTH1, GRP, HBEGF, HBP1, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, LCP1, NF1, NGF, PDIA3, PFN1, PPARG, RUNX2, SLC3A2, SLC7A11, SOD2, SRC	29
Cellular Development	growth of tumor cell lines	2.21E-05	ACTN4, APC, CDKN2A, CSF2, CXCR7, DCN, FTH1, GRP, HBEGF, HBP1, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, LCP1, NF1, NGF, PDIA3, PFN1, PPARG, RUNX2, SLC3A2, SLC7A11, SOD2, SRC	29
Cellular Development	developmental process of blood cells	2.40E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD55, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, IGF2, IGHM, ITGAM, ITGB1, JUN, MYCN, NGF, NOTCH4, NR4A1, NR4A3, PDIA3, PML, PPARG, RUNX2, SMAD3, SOD2, TOP2A	34
Cellular Development	developmental process of leukocytes	2.46E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD55, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, IGF2, IGHM, ITGAM, ITGB1, JUN, MYCN, NGF, NR4A1, NR4A3, PDIA3, PML, PPARG, RUNX2, SMAD3, TOP2A	31
Cell Cycle	interphase of eukaryotic cells	2.64E-05	APC, BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PML, PPARG, PSAP, RUNX2, SOD2, SRC	21
Cancer	adenocarcinoma	2.75E-05	ALDOA, BTRC, C4BPA, CDKN2A, CTLA4, EDNRB, ENO1, FGF18, FTH1, NOTCH4, PCNA, PGK1, PKM2, PPARG, RUNX2, SMAD3, SRM, TRADD, TUBA8, VIM	20
Cancer	head and neck tumor	2.80E-05	APC, CDKN2A, FOXG1, GFAP, HBEGF, IGF2, JUN, MYCN, NF1, PCNA, PDGFB, PPARG, RUNX2, SLC6A2, SRC, TAOK1, TOP2A, TUBA8, VIM	19
Hematological System Development and Function	quantity of leukocytes	3.40E-05	C5AR1, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, HBEGF, IGF2, IGHM, ITGAM, MMP13, NF1, NGF, NOTCH4, NR4A1, PML, PPARG, PSAP, RUNX2, SIGLEC1, SMAD3	23
Tissue Morphology	quantity of leukocytes	3.40E-05	C5AR1, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, HBEGF, IGF2, IGHM, ITGAM, MMP13, NF1, NGF, NOTCH4, NR4A1, PML, PPARG, PSAP, RUNX2, SIGLEC1, SMAD3	23
Cell Cycle	cell stage	4.09E-05	ADCYAP1, APC, BTRC, CAMK2B, CD4, CDKN2A, CLTC, CSF2, CSF3, CTLA4, DCN, FOXG1, GRP, GYPA, HBEGF, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PAK2, PML, PPARG, PSAP, RAB11A, RUNX2, SMAD3, SOD2, SRC, TOP2A	33
Cell Cycle	interphase	4.53E-05	ADCYAP1, APC, BTRC, CAMK2B, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PML, PPARG, PSAP, RUNX2, SOD2, SRC	23
Cell Cycle	cell division process of normal cells	5.10E-05	CDKN2A, CSF2, CSF3, CTLA4, DCN, HBEGF, IGF2, IGFBP7, IGHM, ITGB1, ITGB4, JUN, MYCN, NGF, NR4A3, PPARG, RUNX2, SMAD3, SOD2	19
Tissue Morphology	quantity of mononuclear leukocytes	5.42E-05	C5AR1, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, IGF2, IGHM, ITGAM, NGF, NOTCH4, NR4A1, PML, PSAP, RUNX2, SIGLEC1, SMAD3	19
Hematological System Development and Function	quantity of mononuclear leukocytes	5.42E-05	C5AR1, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, IGF2, IGHM, ITGAM, NGF, NOTCH4, NR4A1, PML, PSAP, RUNX2, SIGLEC1, SMAD3	19
Developmental Disorder	developmental disorder	5.62E-05	ACE, APLP1, BBS9, BDNF, BTRC, CSF2, CSF3, CYP11A1, EDNRB, ELN, FLNA, GFAP, GRP, GYPA, HBEGF, IGF2, ITGB1, JUN, KLF15, MMP13, MSTN, MYCN, NF1, NFIX, NGF, PML, PPARG, PROP1, RUNX2, SCN7A, SLC3A1, SMAD3, SOD2, SRC	34
Cell Morphology	shape change of normal cells	5.66E-05	BDNF, CDKN2A, CORO1A, CSF2, DCN, FOXG1, ITGAM, ITGB1, JUN, NGF, RUNX2, SRC	12
Cellular Development	shape change of normal cells	5.66E-05	BDNF, CDKN2A, CORO1A, CSF2, DCN, FOXG1, ITGAM, ITGB1, JUN, NGF, RUNX2, SRC	12
Hematological System Development and Function	development of leukocytes	6.19E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	25
Hematopoiesis	development of leukocytes	6.19E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	25
Cellular Development	development of leukocytes	6.19E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	25

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Gene Expression	activation of Smad binding sequence	8.51E-05	RUNX2, SMAD3, TCF7L2, WWOX	4
Cancer	development of tumor	8.58E-05	CD4, CD86, CDKN2A, CTLA4, JUN, MYCN, NOTCH4, PML, RUNX2, SMAD3	10
Hematological System Development and Function	T cell development	8.89E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	23
Cell-mediated Immune Response	T cell development	8.89E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	23
Cellular Function and Maintenance	T cell development	8.89E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	23
Hematopoiesis	T cell development	8.89E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	23
Cellular Development	T cell development	8.89E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	23
Cellular Growth and Proliferation	proliferation of osteocytes	1.24E-04	CSF2, FGF18, HBEGF, IGF2, RUNX2, SRC	6
Cell Cycle	delay in cell division process of eukaryotic cells	1.63E-04	CDKN2A, IGFBP7, PCNA, PPARG, RUNX2, SOD2, TOP2A	7
Cellular Development	differentiation of osteocytes	1.98E-04	APC, C5AR1, CSF2, FGF18, JUN, NAMPT, NF1, PPARG, RUNX2, SMAD3, SRC, WWOX	12
Connective Tissue Development and Function	differentiation of osteocytes	1.98E-04	APC, C5AR1, CSF2, FGF18, JUN, NAMPT, NF1, PPARG, RUNX2, SMAD3, SRC, WWOX	12
Cell Cycle	delay in G1 phase of cell lines	2.16E-04	CDKN2A, IGFBP7, RUNX2, SOD2	4
Cell Morphology	sprouting	2.43E-04	BDNF, DCN, IGF2, JUN, NCAM1, NGF, RUNX2	7
Developmental Disorder	hypertrophy	2.83E-04	ACE, BDNF, CSF2, CSF3, ELN, GFAP, GRP, HBEGF, JUN, KLF15, MSTN, NF1, NGF, PPARG, RUNX2, SRC	16
Cellular Development	sprouting of normal cells	3.07E-04	BDNF, DCN, JUN, NGF, RUNX2	5
Cell Morphology	sprouting of normal cells	3.07E-04	BDNF, DCN, JUN, NGF, RUNX2	5
Organismal Functions	healing	3.95E-04	COL5A1, HBEGF, IL24, NF1, RUNX2, SCNN1G, SMAD3, SPRR3	8
Tissue Development	aggregation of cells	4.01E-04	BDNF, C4BPA, CD4, CD6, IGHM, ITGAM, ITGB1, NCAM1, NF1, NFASC, RUNX2, SCARF1, SLC3A2	13
Tissue Development	formation of tissue	5.78E-04	BDNF, DCN, IGF2, ITGB1, JUN, LHX1, NCAM1, NR4A3, NUP50, PPARG, RUNX2, SMAD3, SRC, WWOX	14
Cell Cycle	cell stage of eukaryotic cells	6.66E-04	APC, BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PML, PPARG, PSAP, RUNX2, SOD2, SRC, TOP2A	22

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Apoptosis

The non-steroidal anti-inflammatory drugs Sulindac sulfide and Diclofenac induce apoptosis and differentiation in human acute myeloid leukemia cells through an AP-1 dependent pathway

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Online Resource 6. Ingenuity pathway analysis regarding Cancer

Category	Functions Annotation	p-Value	Molecules	# Molecules
Cancer	tumorigenesis	5.77E-15	ACE, ACTB, ACTG1, ADAM8, AK2, ALDOA, ANAPC13, APC, BCAN, BDNF, BTRC, C4BPA, CAMK2B, CASP3, CD4, CD52, CD55, CD68, CD86, CDC42BPA, CDKN2A, CFTR, CSF2, CSF3, CTLA4, CXCR7, CYP11A1, CYP3A4, DCN, DDIT4, DNALI1, EDNRB, ENO1, FAM5C, FGF18, FLNA, FOXG1, FRY, FTH1, G6PD, GALR1, GFAP, GLUL, GPM6A, GRP, HAMP, HBEGF, HBP1, HPGD, IFI44L, IFIT1, IFIT2, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, KIR3DL1, KRT1, KRT6A, LOC51152, MAPK11, MMP13, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NOTCH4, NR4A1, NR4A3, NUP50, P4HB, PAK2, PCNA, PDGFB, PDIA3, PDIA6, PFN1, PGK1, PKM2, PLIN2, PML, PPARG, PSAT1, RREB1, RUNX2, SCN7A, SIGLEC1, SLC22A1, SLC6A2, SLC7A11, SMAD3, SOD2, SPTBN1, SRC, SRM, STMN2, STOM, SULT1C2, TAF4, TAOK1, TBX4, TCF7L2, TNNC2, TOP2A, TPM2, TRADD, TTC38, TUBA8, VCP, VIM, WWOX	116
Cancer	cancer	4.60E-14	ACE, ACTB, ACTG1, ADAM8, AK2, ALDOA, ANAPC13, APC, BCAN, BTRC, C4BPA, CASP3, CD52, CD55, CD68, CD86, CDC42BPA, CDKN2A, CFTR, CSF2, CSF3, CTLA4, CXCR7, CYP3A4, DCN, DDIT4, DNALI1, EDNRB, ENO1, FGF18, FLNA, FOXG1, FRY, FTH1, GFAP, GLUL, GPM6A, GRP, HAMP, HBEGF, HBP1, HPGD, IFI44L, IFIT1, IFIT2, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, KIR3DL1, KRT1, KRT6A, LOC51152, MAPK11, MMP13, MYCN, NAMPT, NCAM1, NF1, NLRP1, NOTCH4, NR4A1, NR4A3, NUP50, P4HB, PAK2, PCNA, PDGFB, PDIA3, PDIA6, PFN1, PGK1, PKM2, PLIN2, PML, PPARG, PSAT1, RREB1, RUNX2, SCN7A, SIGLEC1, SLC22A1, SLC6A2, SLC7A11, SMAD3, SOD2, SPTBN1, SRC, SRM, STMN2, STOM, SULT1C2, TAF4, TAOK1, TBX4, TNNC2, TOP2A, TPM2, TRADD, TTC38, TUBA8, VCP, VIM, WWOX	107
Cancer	tumor	7.41E-12	ACTG1, AK2, ALDOA, APC, BCAN, BDNF, BTRC, C4BPA, CAMK2B, CASP3, CD4, CD52, CD55, CD86, CDC42BPA, CDKN2A, CSF2, CTLA4, CXCR7, CYP3A4, DCN, DNALI1, EDNRB, ENO1, FAM5C, FGF18, FLNA, FOXG1, FTH1, G6PD, GALR1, GFAP, GPM6A, HAMP, HBEGF, HPGD, IFI44L, IFIT1, IGF2, IGFBP7, IL24, ITGB1, ITGB4, JUN, LOC51152, MMP13, MYCN, NCAM1, NF1, NGF, NOTCH4, NUP50, P4HB, PCNA, PDGFB, PFN1, PGK1, PKM2, PML, PPARG, PSAT1, RREB1, RUNX2, SCN7A, SIGLEC1, SLC6A2, SLC7A11, SMAD3, SOD2, SPTBN1, SRC, SRM, STMN2, STOM, TAF4, TAOK1, TBX4, TNNC2, TOP2A, TRADD, TUBA8, VCP, VIM, WWOX	84
Cancer	benign tumor	9.19E-11	AK2, ALDOA, APC, BDNF, BTRC, CAMK2B, CDKN2A, CSF2, CXCR7, DCN, EDNRB, FAM5C, FTH1, GALR1, GPM6A, IFI44L, IFIT1, IGF2, IGFBP7, JUN, NF1, NOTCH4, NUP50, PCNA, PML, PPARG, RREB1, SCN7A, SMAD3, SOD2, SRC, STMN2, TAF4, TNNC2, TOP2A, TUBA8, VCP	37

Cancer	carcinoma	2.91E-09	ACTG1, ALDOA, APC, BCAN, BTRC, C4BPA, CASP3, CD55, CDC42BPA, CDKN2A, CSF2, CTLA4, CYP3A4, DNALI1, EDNRB, ENO1, FGF18, FLNA, FTH1, HAMP, HBEGF, IFIT1, IGF2, ITGB4, JUN, LOC51152, MMP13, MYCN, NCAM1, NF1, NOTCH4, P4HB, PCNA, PFN1, PGK1, PKM2, PML, PPARG, PSAT1, RUNX2, SIGLEC1, SLC7A11, SMAD3, SOD2, SPTBN1, SRC, SRM, STMN2, STOM, TBX4, TNNC2, TOP2A, TRADD, TUBA8, VIM, WWOX	56
Cancer	malignant tumor	4.53E-09	ACTG1, ALDOA, APC, BCAN, BTRC, C4BPA, CASP3, CD52, CD55, CDC42BPA, CDKN2A, CSF2, CTLA4, CYP3A4, DCN, DNALI1, EDNRB, ENO1, FGF18, FLNA, FOXG1, FTH1, HAMP, HBEGF, IFIT1, IGF2, IL24, ITGB4, JUN, LOC51152, MMP13, MYCN, NCAM1, NF1, NOTCH4, P4HB, PCNA, PFN1, PGK1, PKM2, PML, PPARG, PSAT1, RUNX2, SCN7A, SIGLEC1, SLC7A11, SMAD3, SOD2, SPTBN1, SRC, SRM, STMN2, STOM, TAF4, TAOK1, TBX4, TNNC2, TOP2A, TRADD, TUBA8, VIM, WWOX	63
Cancer	uterine cancer	1.81E-07	AK2, ALDOA, BTRC, CDKN2A, CSF2, EDNRB, GPM6A, IFI44L, IFIT1, IGF2, JUN, MMP13, NUP50, PCNA, PML, PPARG, RREB1, SCN7A, SRC, TOP2A, TUBA8, VCP	22
Cancer	breast cancer	1.96E-07	ACE, ALDOA, ANAPC13, APC, BTRC, CASP3, CD68, CDKN2A, CTLA4, CYP3A4, DDIT4, DNALI1, ENO1, HBEGF, HBP1, IFIT1, JUN, KRT6A, MMP13, NAMPT, NOTCH4, NR4A1, NR4A3, P4HB, PCNA, PDGFB, PFN1, PGK1, PLIN2, PML, PPARG, SLC6A2, SMAD3, SRC, TAOK1, TOP2A, TTC38, TUBA8, WWOX	39
Cancer	uterine tumor	8.83E-07	AK2, ALDOA, BTRC, CDKN2A, CSF2, EDNRB, GPM6A, IFI44L, IFIT1, IGF2, JUN, MMP13, NUP50, PCNA, PPARG, RREB1, SCN7A, TUBA8, VCP	19
Cancer	central nervous system tumor	9.55E-07	APC, CDKN2A, EDNRB, FOXG1, GFAP, HBEGF, IGF2, MYCN, NF1, PDGFB, PML, PPARG, SLC6A2, SRC, TAOK1, TOP2A, TUBA8	17
Cancer	hyperproliferation	2.11E-06	APC, CASP3, CD86, CDKN2A, CSF2, CSF3, CYP11A1, GRP, HBEGF, ITGB1, MMP13, MYCN, NF1, NGF, PPARG, SMAD3, SRC, TAF4, TCF7L2	19
Cancer	brain tumor	2.59E-06	APC, CDKN2A, FOXG1, GFAP, HBEGF, IGF2, MYCN, NF1, PDGFB, PPARG, SLC6A2, SRC, TAOK1, TOP2A, TUBA8	15
Cancer	disease of tumor	2.92E-06	APC, BTRC, CD86, CDKN2A, CSF2, DCN, IGF2, MYCN, NCAM1, NF1, PML, PPARG, SRC, VIM	14
Cancer	lung cancer	3.74E-06	ACE, ALDOA, APC, BTRC, CDKN2A, CSF3, CTLA4, ENO1, GRP, HPGD, ITGB1, ITGB4, JUN, NCAM1, PCNA, PML, PPARG, SOD2, SRC, SRM, TAOK1, TOP2A, TUBA8, VIM, WWOX	25
Cancer	developmental process of tumor	5.83E-06	APC, CASP3, CD4, CD86, CDKN2A, CSF2, CTLA4, IGF2, IL24, ITGB1, JUN, MYCN, NOTCH4, PML, PPARG, RUNX2, SMAD3, SOD2	18
Cancer	tumorigenesis of colon	6.41E-06	APC, CDKN2A, PPARG, SMAD3	4
Cancer	digestive organ tumor	7.79E-06	ACTG1, APC, BTRC, C4BPA, CASP3, CDKN2A, CTLA4, FTH1, HAMP, HBEGF, IGF2, ITGB4, JUN, NF1, P4HB, PFN1, PGK1, PKM2, PML, PPARG, SRC, TBX4, TOP2A, TUBA8, VIM, WWOX	26
Cancer	head and neck cancer	8.19E-06	ADAM8, APC, CDKN2A, FOXG1, GFAP, HBEGF, IGF2, JUN, KRT1, MYCN, NF1, PAK2, PCNA, PDGFB, PPARG, RUNX2, SIGLEC1, SLC6A2, SRC, TAOK1, TOP2A, TUBA8, VIM	23
Cancer	leiomyoma	8.34E-06	AK2, ALDOA, EDNRB, FTH1, GPM6A, IFI44L, IFIT1, IGF2, JUN, NUP50, PCNA, PPARG, RREB1, SCN7A, SOD2, VCP	16
Cancer	sarcoma	9.52E-06	APC, CDKN2A, CTLA4, ITGB4, JUN, MMP13, MYCN, NCAM1, NF1, PML, PPARG, SRC, TAOK1, TOP2A, TUBA8, WWOX	16
Cancer	neurofibrosarcoma	1.05E-05	CDKN2A, ITGB4, MMP13, NF1, SRC	5
Cancer	quantity of tumor	1.39E-05	APC, CDKN2A, CSF2, EDNRB, HPGD, IGF2, JUN, NGF, TAF4	9
Cancer	hematologic cancer	1.40E-05	CD52, CD86, CDKN2A, CSF2, CTLA4, CYP3A4, ITGB1, JUN, MAPK11, MYCN, NCAM1, NF1, PDIA6, PML, RUNX2, SLC6A2, SOD2, SRC, TOP2A, TUBA8	20
Cancer	metastasis	1.47E-05	ACTB, CASP3, CD86, CDKN2A, CSF2, CTLA4, CXCR7, EDNRB, FRY, GLUL, IGFBP7, ITGB4, NCAM1, NLRP1, PPARG, SLC7A11, SMAD3, SRC, STMN2, TOP2A, TUBA8, VIM	22
Cancer	uterine leiomyoma	1.58E-05	AK2, ALDOA, EDNRB, GPM6A, IFI44L, IFIT1, IGF2, JUN, NUP50, PCNA, PPARG, RREB1, SCN7A, VCP	14
Cancer	transformation	1.61E-05	APC, CD4, CDKN2A, CLTC, FOXG1, FTH1, G6PD, HBP1, ITGB1, ITGB4, JUN, MYCN, NGF, PAK2, PDGFB, PML, PPARG, PRKCG, SLC3A2, SRC, TAF4, TSC22D3	22
Cancer	myeloid leukemia	2.61E-05	CD86, CDKN2A, CSF2, CTLA4, CYP3A4, JUN, MYCN, NCAM1, NF1, PML, SRC, TOP2A	12

Cancer	adenocarcinoma	2.75E-05	ALDOA, BTRC, C4BPA, CDKN2A, CTLA4, EDNRB, ENO1, FGF18, FTH1, NOTCH4, PCNA, PGK1, PKM2, PPARG, RUNX2, SMAD3, SRM, TRADD, TUBA8, VIM	20
Cancer	head and neck tumor	2.80E-05	APC, CDKN2A, FOXG1, GFAP, HBEGF, IGF2, JUN, MYCN, NF1, PCNA, PDGFB, PPARG, RUNX2, SLC6A2, SRC, TAOK1, TOP2A, TUBA8, VIM	19
Cancer	lymphomagenesis	3.14E-05	CASP3, CD52, CDKN2A, CSF2, CTLA4, DCN, JUN, NCAM1, NF1, PPARG, SRC, TAOK1, TOP2A, TUBA8, WWOX	15
Cancer	transformation of cells	3.15E-05	APC, CD4, CDKN2A, CLTC, FOXG1, FTH1, G6PD, HBP1, ITGB1, ITGB4, JUN, MYCN, NGF, PAK2, PDGFB, PML, PPARG, PRKCG, SLC3A2, SRC, TSC22D3	21
Cancer	prostate cancer	3.43E-05	BTRC, CD52, CDKN2A, CTLA4, CYP3A4, EDNRB, FLNA, IFI44L, IGHM, ITGB4, PCNA, PGK1, PML, PPARG, PSAT1, RUNX2, SIGLEC1, SLC6A2, SOD2, SRC, TOP2A, TRADD, TUBA8, WWOX	24
Cancer	tumorigenesis of neurons	3.49E-05	CASP3, CDKN2A, MYCN	3
Cancer	fibrosarcoma	3.52E-05	CDKN2A, CTLA4, ITGB4, MMP13, NF1, SRC	6
Cancer	soft tissue sarcoma	3.68E-05	CDKN2A, CTLA4, ITGB4, MMP13, MYCN, NCAM1, NF1, PPARG, SRC, TOP2A, TUBA8	11
Cancer	tumorigenesis of benign tumor	5.00E-05	APC, BTRC, CDKN2A, NF1, PML	5
Cancer	hyperproliferation of eukaryotic cells	5.45E-05	CASP3, CD86, CDKN2A, CSF2, CSF3, MYCN, NF1, NGF, PPARG, SMAD3, SRC	11
Cancer	squamous-cell carcinoma	6.86E-05	CD55, CDC42BPA, CDKN2A, IFIT1, MYCN, PCNA, SLC7A11, SMAD3, SPTBN1, SRC, STMN2, TNNC2, TOP2A, TUBA8, WWOX	15
Cancer	neuroepithelial tumor	7.12E-05	CDKN2A, GFAP, HBEGF, IGF2, NF1, PDGFB, PML, PPARG, SRC, TAOK1, TOP2A	11
Cancer	pancreatic cancer	7.77E-05	CASP3, CDKN2A, CFTR, CTLA4, ENO1, HBEGF, ITGB4, PCNA, PML, SRC, TPM2, TUBA8, VIM	13
Cancer	transformation of eukaryotic cells	8.34E-05	APC, CD4, CDKN2A, CLTC, FOXG1, FTH1, G6PD, HBP1, ITGB4, JUN, MYCN, NGF, PAK2, PDGFB, PPARG, PRKCG, SRC, TSC22D3	18
Cancer	development of tumor	8.58E-05	CD4, CD86, CDKN2A, CTLA4, JUN, MYCN, NOTCH4, PML, RUNX2, SMAD3	10
Cancer	myelodysplastic syndrome	9.68E-05	CD52, CD86, CTLA4, MAPK11, NF1, PDIA3, PML, SRC, TOP2A	9
Cancer	transformation of fibroblast cell lines	1.28E-04	CD4, CDKN2A, CLTC, FTH1, G6PD, HBP1, ITGB4, JUN, MYCN, PDGFB, PRKCG, SRC, TSC22D3	13
Cancer	transformation of cell lines	1.31E-04	APC, CD4, CDKN2A, CLTC, FTH1, G6PD, HBP1, ITGB4, JUN, MYCN, NGF, PDGFB, PRKCG, SRC, TSC22D3	15
Cancer	glioma	1.57E-04	CDKN2A, GFAP, HBEGF, IGF2, NF1, PDGFB, PPARG, SRC, TAOK1, TOP2A	10
Cancer	lymphoma	1.89E-04	CASP3, CD52, CDKN2A, CTLA4, DCN, JUN, NCAM1, PPARG, SRC, TAOK1, TOP2A, TUBA8, WWOX	13
Cancer	hyperplasia	2.21E-04	APC, CASP3, CD86, CDKN2A, CYP11A1, GRP, HBEGF, ITGB1, MMP13, NF1, NGF, PPARG, TAF4, TCF7L2	14
Cancer	hyperproliferation of normal cells	2.24E-04	CASP3, CD86, CDKN2A, CSF2, CSF3, MYCN, NF1, NGF, PPARG, SMAD3	10
Cancer	hyperproliferation of tunica intima	2.34E-04	CDKN2A, NF1	2
Cancer	tumorigenesis of endocrine gland tumor	2.80E-04	CDKN2A, MYCN, VIM	3
Cancer	hyperproliferation of blood cells	2.80E-04	CD86, CDKN2A, CSF2, CSF3, MYCN, NF1	6
Cancer	tumorigenesis of organ	3.11E-04	APC, CDKN2A, EDNRB, GRP, HBEGF, JUN, PPARG, SMAD3, TCF7L2	9
Cancer	leukemia	4.33E-04	CD52, CD86, CDKN2A, CSF2, CTLA4, CYP3A4, ITGB1, JUN, MYCN, NCAM1, NF1, PML, SLC6A2, SRC, TOP2A, TUBA8	16
Cancer	chronic myeloid leukemia	4.37E-04	CDKN2A, CSF2, CTLA4, JUN, NF1, SRC	6
Cancer	liver tumor	4.96E-04	ACTG1, BTRC, CASP3, CDKN2A, CTLA4, HAMP, IGF2, JUN, PFN1, PML, TOP2A, TUBA8	12
Cancer	lymphoid cancer	6.13E-04	CASP3, CD52, CDKN2A, CTLA4, DCN, JUN, NCAM1, PCNA, PML, PPARG, SRC, TAOK1, TOP2A, TUBA8, WWOX	15
Cancer	prostatic tumor	6.13E-04	BTRC, CDKN2A, CTLA4, CYP3A4, EDNRB, FLNA, ITGB4, PPARG, PSAT1, RUNX2, SIGLEC1, SOD2, TOP2A, TRADD, TUBA8	15
Cancer	renal cancer	6.27E-04	APC, CDKN2A, CTLA4, EDNRB, IGF2, ITGB1, PML, PPARG, SIGLEC1, TOP2A, TUBA8	11

Apoptosis

The non-steroidal anti-inflammatory drugs Sulindac sulfide and Diclofenac induce apoptosis and differentiation in human acute myeloid leukemia cells through an AP-1 dependent pathway

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Online Resource 7. Ingenuity pathway analysis regarding Cell Cycle

Category	Functions Annotation	p-Value	Molecules	# Molecules
Cell Cycle	cell division process of cell lines	4.49E-08	APC, BTRC, CDKN2A, CSF2, CSF3, DCN, FOXG1, GRP, GYPA, HBEGF, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NF1, NGF, NR4A1, NR4A3, PAK2, PCNA, PML, PPARG, PSAP, RUNX2, SMAD3, SOD2, SRC, TOP2A, WWOX	33
Cell Cycle	cell cycle progression	8.81E-08	ADCYAP1, APC, ASNS, BTRC, CASP3, CD4, CDKN2A, CLTC, CSF2, CSF3, CTLA4, FAM5C, FOXG1, GRP, GYPA, HBEGF, HBP1, HPGD, IGF2, IGFBP7, IGHM, ITGB1, ITGB4, JUN, NAMPT, NF1, NGF, NR4A1, NR4A3, PAK2, PCNA, PML, PPARG, SMAD3, SRC, TCF7L2, TOP2A, WWOX	38
Cell Cycle	cell cycle progression of eukaryotic cells	2.15E-07	BTRC, CDKN2A, CSF2, CSF3, CTLA4, FOXG1, GRP, HBEGF, HPGD, IGF2, IGHM, ITGB1, ITGB4, NF1, NGF, NR4A1, NR4A3, PCNA, PML, PPARG, SMAD3, WWOX	22
Cell Cycle	entry into cell division process of eukaryotic cells	9.79E-07	APC, CDKN2A, CSF3, CTLA4, HPGD, ITGB1, MYCN, PPARG, PSAP, RUNX2, SMAD3, SOD2, SRC	13
Cell Cycle	cell cycle progression of cell lines	1.50E-06	BTRC, CDKN2A, CSF2, FOXG1, GRP, HBEGF, HPGD, IGHM, NF1, NGF, NR4A1, NR4A3, PCNA, PML, PPARG, SMAD3, WWOX	17
Cell Cycle	G1 phase	1.78E-06	ADCYAP1, APC, BTRC, CAMK2B, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, ITGB1, JUN, NGF, PPARG, RUNX2, SOD2, SRC	19
Cell Cycle	cell division process of eukaryotic cells	2.51E-06	APC, BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, FOXG1, GRP, GYPA, HBEGF, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, MYCN, NF1, NGF, NR4A1, NR4A3, PAK2, PCNA, PML, PPARG, PSAP, RUNX2, SMAD3, SOD2, SRC, TOP2A, WWOX	35
Cell Cycle	entry into cell division process of normal cells	2.64E-06	CDKN2A, CSF3, CTLA4, ITGB1, MYCN, PPARG, RUNX2, SMAD3, SOD2	9
Cell Cycle	cell division process of tumor cell lines	2.83E-06	APC, BTRC, CDKN2A, CSF2, CSF3, DCN, HBEGF, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, NF1, NGF, NR4A1, PCNA, PML, PPARG, PSAP, SOD2, SRC, TOP2A, WWOX	24
Cell Cycle	cell division process	3.07E-06	ADCYAP1, APC, ASNS, BTRC, CAMK2B, CASP3, CD4, CDKN2A, CLTC, CSF2, CSF3, CTLA4, DCN, FAM5C, FOXG1, GRP, GYPA, HBEGF, HBP1, HPGD, IGF2, IGFBP7, IGHM, IL24, ITGB1, ITGB4, JUN, MYCN, NAMPT, NF1, NGF, NR4A1, NR4A3, PAK2, PCNA, PDGFB, PML, PPARG, PSAP, RAB11A, RUNX2, SMAD3, SOD2, SRC, TCF7L2, TOP2A, WWOX	47
Cell Cycle	interphase of tumor cell lines	3.67E-06	APC, BTRC, CDKN2A, CSF2, CSF3, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, NGF, PML, PPARG, PSAP, SOD2, SRC	17
Cell Cycle	interphase of normal cells	6.32E-06	CDKN2A, CSF2, CTLA4, DCN, IGFBP7, IGHM, ITGB1, JUN, MYCN, PPARG, RUNX2, SOD2	12
Cell Cycle	interphase of cell lines	6.35E-06	APC, BTRC, CDKN2A, CSF2, CSF3, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PML, PPARG, PSAP, RUNX2, SOD2, SRC	20
Cell Cycle	G1 phase of eukaryotic cells	7.63E-06	APC, BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, ITGB1, JUN, NGF, PPARG, RUNX2, SOD2	16

Cell Cycle	entry into interphase of eukaryotic cells	1.27E-05	APC, CDKN2A, CTLA4, ITGB1, MYCN, PPARG, PSAP, RUNX2, SOD2, SRC	10
Cell Cycle	G1 phase of normal cells	1.41E-05	CDKN2A, CSF2, CTLA4, DCN, IGFBP7, IGHM, JUN, PPARG	8
Cell Cycle	delay in G1 phase of eukaryotic cells	1.74E-05	CDKN2A, IGFBP7, PPARG, RUNX2, SOD2	5
Cell Cycle	re-entry into cell division process of cell lines	2.24E-05	CDKN2A, CSF2, FOXG1, MYCN, NGF, PPARG	6
Cell Cycle	entry into interphase of normal cells	2.24E-05	CDKN2A, CTLA4, ITGB1, MYCN, PPARG, RUNX2, SOD2	7
Cell Cycle	interphase of eukaryotic cells	2.64E-05	APC, BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PML, PPARG, PSAP, RUNX2, SOD2, SRC	21
Cell Cycle	cell cycle progression of tumor cell lines	2.76E-05	CDKN2A, CSF2, HBEGF, HPGD, IGHM, NF1, NGF, NR4A1, PCNA, PML, PPARG, WWOX	12
Cell Cycle	cell stage of tumor cell lines	3.53E-05	APC, BTRC, CDKN2A, CSF2, CSF3, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, NGF, PML, PPARG, PSAP, SOD2, SRC, TOP2A	18
Cell Cycle	arrest in cell cycle progression of eukaryotic cells	3.99E-05	BTRC, CDKN2A, CSF2, CTLA4, IGHM, ITGB1, NGF, NR4A1, NR4A3, PML, PPARG, SMAD3	12
Cell Cycle	cell stage	4.09E-05	ADCYAP1, APC, BTRC, CAMK2B, CD4, CDKN2A, CLTC, CSF2, CSF3, CTLA4, DCN, FOXG1, GRP, GYPA, HBEGF, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PAK2, PML, PPARG, PSAP, RAB11A, RUNX2, SMAD3, SOD2, SRC, TOP2A	33
Cell Cycle	interphase	4.53E-05	ADCYAP1, APC, BTRC, CAMK2B, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PML, PPARG, PSAP, RUNX2, SOD2, SRC	23
Cell Cycle	entry into S phase of eukaryotic cells	4.91E-05	APC, CDKN2A, CTLA4, ITGB1, MYCN, PPARG, PSAP, SOD2, SRC	9
Cell Cycle	cell division process of normal cells	5.10E-05	CDKN2A, CSF2, CSF3, CTLA4, DCN, HBEGF, IGF2, IGFBP7, IGHM, ITGB1, ITGB4, JUN, MYCN, NGF, NR4A3, PPARG, RUNX2, SMAD3, SOD2	19
Cell Cycle	cell stage of cell lines	5.71E-05	APC, BTRC, CDKN2A, CSF2, CSF3, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PML, PPARG, PSAP, RUNX2, SOD2, SRC, TOP2A	21
Cell Cycle	G1 phase of tumor cell lines	6.07E-05	APC, BTRC, CDKN2A, CSF2, DCN, IGF2, IGFBP7, IGHM, ITGB1, NGF, SOD2	11
Cell Cycle	cell cycle progression of normal cells	6.75E-05	CDKN2A, CSF2, CSF3, CTLA4, IGF2, IGHM, ITGB1, ITGB4, NR4A3, PPARG, SMAD3	11
Cell Cycle	arrest in cell cycle progression of cell lines	6.80E-05	BTRC, CDKN2A, CSF2, IGHM, NGF, NR4A1, NR4A3, PML, PPARG, SMAD3	10
Cell Cycle	arrest in cell division process of eukaryotic cells	8.93E-05	BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, NGF, NR4A1, NR4A3, PML, PPARG, SMAD3, SRC	19
Cell Cycle	arrest in cell division process of cell lines	9.13E-05	BTRC, CDKN2A, CSF2, CSF3, DCN, IGF2, IGHM, IL24, ITGB1, JUN, NGF, NR4A1, NR4A3, PML, PPARG, SMAD3, SRC	17
Cell Cycle	G1 phase of cell lines	1.00E-04	APC, BTRC, CDKN2A, CSF2, CSF3, DCN, IGF2, IGFBP7, IGHM, ITGB1, NGF, RUNX2, SOD2	13
Cell Cycle	cell division process of lymphoma cell lines	1.17E-04	CSF2, IGFBP7, IGHM, SOD2, WWOX	5
Cell Cycle	cell cycle progression of blood cells	1.26E-04	CDKN2A, CSF2, CSF3, CTLA4, IGHM, ITGB1, SMAD3	7
Cell Cycle	arrest in cell division process	1.35E-04	APC, BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, NGF, NR4A1, NR4A3, PML, PPARG, SMAD3, SRC, TCF7L2	21
Cell Cycle	entry into S phase of normal cells	1.54E-04	CDKN2A, CTLA4, ITGB1, MYCN, PPARG, SOD2	6
Cell Cycle	delay in cell division process of eukaryotic cells	1.63E-04	CDKN2A, IGFBP7, PCNA, PPARG, RUNX2, SOD2, TOP2A	7
Cell Cycle	entry into S phase of tumor cell lines	1.82E-04	APC, CDKN2A, PPARG, PSAP, SRC	5
Cell Cycle	entry into cell division process of tumor cell lines	1.90E-04	APC, CDKN2A, HPGD, PPARG, PSAP, SRC	6
Cell Cycle	delay in G1 phase of cell lines	2.16E-04	CDKN2A, IGFBP7, RUNX2, SOD2	4
Cell Cycle	delay in G1 phase of lymphoma cell lines	2.34E-04	IGFBP7, SOD2	2
Cell Cycle	arrest in cell cycle progression	2.71E-04	APC, BTRC, CDKN2A, CSF2, CTLA4, IGHM, ITGB1, NGF, NR4A1, NR4A3, PML, PPARG, SMAD3, TCF7L2	14
Cell Cycle	delay in cell stage of eukaryotic cells	3.36E-04	CDKN2A, IGFBP7, PPARG, RUNX2, SOD2, TOP2A	6
Cell Cycle	delay in cell division process of cell lines	4.01E-04	CDKN2A, IGFBP7, PCNA, RUNX2, SOD2, TOP2A	6
Cell Cycle	S phase of tumor cell lines	4.05E-04	APC, CDKN2A, ITGB1, PML, PPARG, PSAP, SRC	7
Cell Cycle	arrest in G1 phase of normal cells	5.44E-04	CDKN2A, CSF2, DCN, IGFBP7, IGHM	5
Cell Cycle	arrest in cell division process of blood cells	5.44E-04	CDKN2A, CTLA4, DCN, IGHM, ITGB1	5

Cell Cycle	cell stage of eukaryotic cells	6.66E-04	APC, BTRC, CDKN2A, CSF2, CSF3, CTLA4, DCN, IGF2, IGFBP7, IGHM, IL24, ITGB1, JUN, MYCN, NGF, PML, PPARG, PSAP, RUNX2, SOD2, SRC, TOP2A	22
Cell Cycle	arrest in cell cycle progression of neuronal hybrid cells	6.96E-04	NR4A1, NR4A3	2
Cell Cycle	entry into S phase of colon cancer cell lines	6.96E-04	APC, CDKN2A	2

Apoptosis

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Online Resource 8. Ingenuity pathway analysis regarding Cell Death

Category	Functions Annotation	p-Value	Molecules	# Molecules
Cell Death	cell death of cell lines	1.15E-09	ACTB, ADCYAP1, APC, ASNS, ATXN3, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CSF2, CSF3, CTLA4, CYP3A4, DDIT4, EDNRB, ENO1, FTH1, G6PD, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR4A1, P4HB, PAK2, PCNA, PDGFB, PDIA3, PML, PPARG, PRKAA2, PRKCG, PSAP, SLC7A11, SOD2, SRC, ST6GAL1, TOP2A, TRADD, TRIB3, VCP, WWOX	59
Cell Death	cell death of tumor cell lines	2.34E-09	APC, ASNS, ATXN3, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CSF2, CYP3A4, DDIT4, FTH1, G6PD, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR4A1, P4HB, PAK2, PCNA, PML, PPARG, PRKAA2, PSAP, SLC7A11, SOD2, SRC, ST6GAL1, TOP2A, TRADD, VCP, WWOX	49
Cell Death	apoptosis of tumor cell lines	8.54E-09	APC, ASNS, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CSF2, DDIT4, G6PD, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, MAPK11, MYCN, NCAM1, NGF, NLRP1, NR4A1, P4HB, PAK2, PCNA, PML, PPARG, PRKAA2, PSAP, SOD2, SRC, ST6GAL1, TOP2A, TRADD, VCP, WWOX	43
Cell Death	apoptosis of cell lines	1.23E-08	ADCYAP1, APC, ASNS, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CSF2, CSF3, CTLA4, DDIT4, EDNRB, FTH1, G6PD, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, MAPK11, MYCN, NCAM1, NGF, NLRP1, NR4A1, P4HB, PAK2, PCNA, PML, PPARG, PRKAA2, PRKCG, PSAP, SOD2, SRC, ST6GAL1, TOP2A, TRADD, TRIB3, VCP, WWOX	50
Cell Death	apoptosis of eukaryotic cells	3.37E-08	ADCY10, ADCYAP1, ALDOA, APC, ASNS, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, DDIT4, EDNRB, ENO1, FTH1, G6PD, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, P4HB, PAK2, PCNA, PDIA3, PML, PPARG, PRKAA2, PRKCG, PSAP, RUNX2, SMAD3, SOD2, SRC, ST6GAL1, TOP2A, TRADD, TRIB3, VCP, WWOX	62
Cell Death	survival of eukaryotic cells	4.03E-08	ACE, ADCYAP1, BDNF, CAMK2B, CASP3, CD4, CD55, CD86, CDC42BPA, CDKN2A, CRLF1, CSF2, CSF3, CXCR7, CYP3A4, HBEGF, HTN3, IGF2, IL24, ITGB1, JUN, MMP13, MYCN, NF1, NGF, NR4A1, NR4A3, PCNA, PDIA3, PML, PPARG, PRKAA2, PRKCG, SLC22A1, SMAD3, SOD2, SRC	37
Cell Death	cell death of eukaryotic cells	4.85E-08	ACTB, ADCY10, ADCYAP1, ALDOA, APC, ASNS, ATXN3, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CR2, CSF2, CSF3, CTLA4, CYP3A4, DCN, DDIT4, EDNRB, ENO1, FLNA, FTH1, G6PD, GFAP, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, P4HB, PAK2, PCNA, PDGFB, PDIA3, PML, PPARG, PRKAA2, PRKCG, PSAP, RUNX2, SLC7A11, SMAD3, SOD2, SRC, ST6GAL1, TAF4, TOP2A, TRADD, TRIB3, VCP, WWOX	70

Cell Death	survival of cells	6.89E-08	ACE, ADCYAP1, BDNF, CAMK2B, CASP3, CD4, CD55, CD86, CDC42BPA, CDKN2A, CRLF1, CSF2, CSF3, CXCR7, CYP3A4, HBEGF, HTN3, IGF2, IL24, ITGB1, JUN, MMP13, MYCN, NF1, NGF, NR4A1, NR4A3, OGT, PCNA, PDIA3, PML, PPARG, PRKAA2, PRKCG, SLC22A1, SMAD3, SOD2, SRC, TOP2A	39
Cell Death	cell death of blood cells	1.69E-07	ADCYAP1, CASP3, CD4, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, EDNRB, FTH1, IGHM, ITGAM, ITGB1, JUN, NAMPT, NGF, NR4A1, NR4A3, PDIA3, PML, PPARG, SMAD3, SOD2, TOP2A	25
Cell Death	cell death	2.43E-07	ACE, ACTB, ACTN4, ADCY10, ADCYAP1, ALDOA, APC, ASNS, ATXN3, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CPB2, CR2, CSF2, CSF3, CTLA4, CYP3A4, DCN, DDIT4, DHRS2, ECE1, EDNRB, ENO1, FLNA, FTH1, G6PD, GFAP, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, LAMB2, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, OAS1, P4HB, PAK2, PCNA, PDGFB, PDIA3, PKM2, PML, PPARG, PRKAA2, PRKCG, PROP1, PSAP, RUNX2, SLC7A11, SMAD3, SOD2, SRC, ST6GAL1, TAF4, TOP2A, TRADD, TRIB3, VCP, WWOX	79
Cell Death	apoptosis of blood cells	2.71E-07	ADCYAP1, CASP3, CD4, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, IGHM, ITGAM, ITGB1, JUN, NAMPT, NGF, NR4A1, NR4A3, PDIA3, PML, PPARG, SMAD3, SOD2, TOP2A	23
Cell Death	apoptosis	2.74E-07	ACE, ACTN4, ADCY10, ADCYAP1, ALDOA, APC, ASNS, BDNF, BTRC, C5AR1, CASP3, CD4, CD55, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, DDIT4, DHRS2, ECE1, EDNRB, ENO1, FTH1, G6PD, HBEGF, IFI6, IGF2, IGFBP7, IGHM, IL24, ITGAM, ITGB1, ITGB4, JUN, KIR3DL1, LAMB2, MAPK11, MYCN, NAMPT, NCAM1, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, OAS1, P4HB, PAK2, PCNA, PDIA3, PKM2, PML, PPARG, PRKAA2, PRKCG, PROP1, PSAP, RUNX2, SMAD3, SOD2, SRC, ST6GAL1, TOP2A, TRADD, TRIB3, VCP, WWOX	70
Cell Death	apoptosis of leukocytes	3.03E-07	ADCYAP1, CASP3, CD4, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, IGHM, ITGAM, ITGB1, JUN, NAMPT, NGF, NR4A1, NR4A3, PDIA3, PPARG, SMAD3, SOD2, TOP2A	22
Cell Death	cell death of leukemia cell lines	5.96E-07	C5AR1, CASP3, CD4, CD55, CDKN2A, CSF2, FTH1, ITGAM, ITGB1, JUN, KIR3DL1, NGF, PAK2, PML, SOD2, TOP2A, TRADD	17
Cell Death	cell death of leukocytes	6.66E-07	ADCYAP1, CASP3, CD4, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, FTH1, IGHM, ITGAM, ITGB1, JUN, NAMPT, NGF, NR4A1, NR4A3, PDIA3, PPARG, SMAD3, SOD2, TOP2A	23
Cell Death	survival of cell lines	8.02E-07	ACE, BDNF, CAMK2B, CASP3, CD55, CDC42BPA, CDKN2A, CSF2, CSF3, CXCR7, CYP3A4, HBEGF, IGF2, ITGB1, JUN, MYCN, NGF, NR4A1, NR4A3, PCNA, PML, PRKAA2, PRKCG, SLC22A1, SOD2, SRC	26
Cell Death	apoptosis of leukemia cell lines	2.59E-06	C5AR1, CASP3, CD4, CD55, CDKN2A, CSF2, ITGAM, ITGB1, JUN, KIR3DL1, NGF, PAK2, PML, SOD2, TRADD	15
Cell Death	cell death of lymphocytes	5.38E-06	ADCYAP1, CASP3, CD4, CDKN2A, CR2, CSF2, CSF3, CTLA4, FTH1, IGHM, ITGB1, JUN, NGF, NR4A1, NR4A3, PDIA3, SMAD3, TOP2A	18
Cell Death	cell death of normal cells	5.76E-06	ADCY10, ADCYAP1, ALDOA, APC, ATXN3, BDNF, CASP3, CD4, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, EDNRB, FLNA, FTH1, GFAP, IGF2, IGHM, IL24, ITGAM, ITGB1, JUN, MAPK11, MYCN, NAMPT, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, P4HB, PDIA3, PML, PPARG, RUNX2, SMAD3, SOD2, SRC, TAF4, TOP2A, TRADD	44
Cell Death	survival of normal cells	7.68E-06	ADCYAP1, BDNF, CASP3, CD4, CD86, CRLF1, CSF2, CSF3, HTN3, IGF2, ITGB1, JUN, MMP13, MYCN, NF1, NGF, PCNA, PDIA3, PPARG, PRKCG, SMAD3, SRC	22
Cell Death	apoptosis of normal cells	8.33E-06	ADCY10, ADCYAP1, ALDOA, APC, BDNF, CASP3, CD4, CDKN2A, CR2, CSF2, CSF3, CTLA4, DCN, IGF2, IGHM, IL24, ITGAM, ITGB1, JUN, MAPK11, MYCN, NAMPT, NF1, NGF, NLRP1, NR1D1, NR4A1, NR4A3, P4HB, PDIA3, PML, PPARG, RUNX2, SMAD3, SOD2, SRC, TOP2A, TRADD	38
Cell Death	apoptosis of endothelial cells	9.36E-06	ADCY10, BDNF, CASP3, CSF3, ITGB1, MAPK11, NR4A3, PPARG, RUNX2, SRC	10
Cell Death	apoptosis of pheochromocytoma cell lines	1.56E-05	CASP3, DDIT4, G6PD, JUN, MAPK11, NGF, PSAP, SOD2	8
Cell Death	apoptosis of mononuclear leukocytes	1.79E-05	ADCYAP1, CASP3, CD4, CDKN2A, CR2, CSF2, CTLA4, IGHM, ITGB1, JUN, NGF, NR4A1, NR4A3, PDIA3, SMAD3, TOP2A	16
Cell Death	cell death of cancer cells	2.10E-05	BDNF, CASP3, CSF2, CTLA4, ENO1, IGHM, IL24, ITGB4, JUN, MYCN, NGF, NR4A1	12
Cell Death	survival of neuroglia	2.24E-05	BDNF, IGF2, ITGB1, NF1, NGF, PPARG	6
Cell Death	cell death of pheochromocytoma cell lines	2.30E-05	ATXN3, CASP3, DDIT4, G6PD, JUN, MAPK11, NGF, PSAP, SOD2	9
Cell Death	cell death of tumor cells	4.09E-05	BDNF, CASP3, CDKN2A, CSF2, CTLA4, ENO1, HBEGF, IGHM, IL24, ITGB4, JUN, MYCN, NGF, NR4A1	14

Cell Death	apoptosis of lymphocytes	4.87E-05	ADCYAP1, CASP3, CD4, CDKN2A, CR2, CTLA4, IGHM, ITGB1, JUN, NGF, NR4A1, NR4A3, PDIA3, SMAD3, TOP2A	15
Cell Death	survival of tumor cell lines	5.93E-05	ACE, BDNF, CAMK2B, CASP3, CD55, CDC42BPA, CDKN2A, CSF2, CXCR7, HBEGF, IGF2, ITGB1, MYCN, NGF, PCNA, PML, PRKAA2, SOD2, SRC	19
Cell Death	cell death of macrophages	6.11E-05	CASP3, CDKN2A, CSF2, DCN, NGF, NR4A1, PPARG, SOD2	8
Cell Death	cell death of lymphatic system cells	6.80E-05	CASP3, CD4, CDKN2A, CSF2, CSF3, DCN, ITGAM, NAMPT, NGF, PML	10
Cell Death	cell death of phagocytes	7.12E-05	CASP3, CDKN2A, CSF2, CSF3, DCN, NAMPT, NGF, NR4A1, NR4A3, PPARG, SOD2	11
Cell Death	survival of neurons	7.50E-05	ADCYAP1, BDNF, CRLF1, CSF3, IGF2, JUN, MYCN, NF1, NGF, PDIA3, PRKCG	11
Cell Death	cell death of lymphoma cell lines	7.90E-05	CASP3, CD4, CD55, CSF2, FTH1, IGFBP7, IGHM, PML, PPARG, SOD2, WWOX	11
Cell Death	cell death of antigen presenting cells	8.50E-05	CASP3, CDKN2A, CSF2, DCN, NGF, NR4A1, NR4A3, PPARG, SOD2	9
Cell Death	apoptosis of macrophages	1.16E-04	CASP3, CDKN2A, CSF2, DCN, NGF, PPARG, SOD2	7
Cell Death	survival of central nervous system cells	1.26E-04	ADCYAP1, BDNF, IGF2, JUN, NF1, NGF, PRKCG	7
Cell Death	cell death of connective tissue cells	1.31E-04	CASP3, CDKN2A, DCN, FLNA, IGF2, IL24, ITGB1, JUN, MAPK11, NGF, NR4A1, PML, PPARG, RUNX2, TRADD	15
Cell Death	delay in cell death of normal cells	1.36E-04	BDNF, CASP3, CSF2, CSF3, NGF	5
Cell Death	survival of neutrophils	1.40E-04	CSF2, CSF3, NGF, PCNA	4
Cell Death	apoptosis of phagocytes	1.41E-04	CASP3, CDKN2A, CSF2, CSF3, DCN, NAMPT, NGF, NR4A3, PPARG, SOD2	10
Cell Death	apoptosis of cancer cells	1.49E-04	CASP3, CSF2, CTLA4, ENO1, IGHM, IL24, ITGB4, JUN, NGF, NR4A1	10
Cell Death	cell death of B lymphocytes	1.51E-04	CDKN2A, CR2, CSF2, CSF3, IGHM, NR4A1, NR4A3, SMAD3	8
Cell Death	apoptosis of antigen presenting cells	1.74E-04	CASP3, CDKN2A, CSF2, DCN, NGF, NR4A3, PPARG, SOD2	8
Cell Death	survival of connective tissue cells	1.77E-04	CASP3, HTN3, IGF2, JUN, MMP13, NGF, PPARG	7
Cell Death	apoptosis of lymphatic system cells	1.79E-04	CASP3, CDKN2A, CSF2, CSF3, DCN, ITGAM, NAMPT, NGF, PML	9
Cell Death	apoptosis of tumor cells	1.82E-04	CASP3, CDKN2A, CSF2, CTLA4, ENO1, HBEGF, IGHM, IL24, ITGB4, JUN, NGF, NR4A1	12
Cell Death	apoptosis of bone marrow cells	1.99E-04	CASP3, CDKN2A, CSF2, CSF3, DCN, ITGAM, NAMPT, PML	8
Cell Death	cytotoxicity of neutrophils	2.34E-04	CSF2, ITGAM	2
Cell Death	cell death of neurons	2.36E-04	ADCYAP1, ATXN3, BDNF, CASP3, CD4, CDKN2A, CSF3, GFAP, IGF2, JUN, MAPK11, MYCN, NF1, NGF, NLRP1, NR1D1, P4HB, SOD2, TAF4	19
Cell Death	survival of hippocampal neurons	2.63E-04	ADCYAP1, BDNF, IGF2, JUN	4
Cell Death	survival of cerebral cortex cells	3.07E-04	ADCYAP1, BDNF, IGF2, JUN, PRKCG	5
Cell Death	delay in apoptosis of normal cells	3.18E-04	CASP3, CSF2, CSF3, NGF	4
Cell Death	activation of caspase	3.36E-04	CDKN2A, NLRP1, PML, PPARG, SMAD3, VCP	6
Cell Death	apoptosis of pancreatic cancer cell lines	3.36E-04	ASNS, BTRC, C5AR1, CDKN2A, G6PD, IL24	6
Cell Death	cell death of breast cancer cell lines	3.74E-04	CASP3, DDIT4, IL24, ITGB1, ITGB4, JUN, NGF, NLRP1, PML, PPARG, SOD2, TRADD, VCP	13
Cell Death	activation-induced cell death of cell lines	4.51E-04	CTLA4, ITGB1, KIR3DL1, NR4A1	4
Cell Death	activation-induced cell death	4.75E-04	ADCYAP1, CASP3, CTLA4, ITGB1, KIR3DL1, NR4A1	6
Cell Death	survival of embryonic cell lines	5.31E-04	HBEGF, NR4A1, NR4A3, SLC22A1	4
Cell Death	apoptosis of neuroblastoma cell lines	5.67E-04	BDNF, CASP3, ITGB1, MYCN, NCAM1, NGF, PPARG	7
Cell Death	apoptosis of lymphoma cell lines	5.89E-04	CASP3, CD4, CD55, CSF2, IGFBP7, IGHM, PML, PPARG, WWOX	9
Cell Death	cell death of leukocyte cell lines	6.04E-04	ADCYAP1, CASP3, CSF2, CSF3, CTLA4, IGHM, ITGB1, JUN, NGF, NR4A1, ST6GAL1	11
Cell Death	cell death of splenocytes	6.20E-04	CASP3, CD4, NGF, PML	4

Apoptosis

The non-steroidal anti-inflammatory drugs Sulindac sulfide and Diclofenac induce apoptosis and differentiation in human acute myeloid leukemia cells through an AP-1 dependent pathway

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Online Resource 9. Ingenuity pathway analysis regarding Hematopoiesis

Category	Functions Annotation	p-Value	Molecules	# Molecules
Hematological System Development and Function	hematological process	4.91E-06	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CPB2, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, HAMP, IGF2, IGHM, ITGB1, JUN, MYCN, NGF, NOTCH4, NR4A1, NR4A3, PDGFB, PDIA3, PML, PPARG, RUNX2, SMAD3, SOD2, TOP2A	35
Hematological System Development and Function	hematopoiesis	5.36E-06	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, HAMP, IGF2, IGHM, ITGB1, JUN, MYCN, NGF, NOTCH4, NR4A1, NR4A3, PDIA3, PML, RUNX2, SMAD3, SOD2, TOP2A	32
Hematological System Development and Function	expansion of lymphocytes	1.27E-05	C5AR1, CD4, CD55, CD86, CDKN2A, CR2, CSF2, CTLA4, IGHM, PPARG	10
Hematological System Development and Function	differentiation of leukocytes	1.44E-05	ADCYAP1, APC, C5AR1, CD4, CD52, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, IGF2, IGHM, ITGAM, ITGB1, JUN, MYCN, NGF, PML, PPARG, RUNX2, SMAD3	23
Hematological System Development and Function	development of blood cells	1.62E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, G6PD, IGF2, IGHM, ITGB1, JUN, NOTCH4, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, SOD2, TOP2A	28
Hematological System Development and Function	differentiation of mononuclear leukocytes	2.84E-05	ADCYAP1, APC, C5AR1, CD4, CD52, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, IGF2, IGHM, ITGB1, JUN, MYCN, NGF, PPARG, SMAD3	20
Hematological System Development and Function	T cell homeostasis	3.34E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CORO1A, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	24
Hematological System Development and Function	quantity of leukocytes	3.40E-05	C5AR1, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, HBEGF, IGF2, IGHM, ITGAM, MMP13, NF1, NGF, NOTCH4, NR4A1, PML, PPARG, PSAP, RUNX2, SIGLEC1, SMAD3	23
Hematological System Development and Function	quantity of blood cells	5.12E-05	C5AR1, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, HBEGF, IGF2, IGHM, ITGAM, MMP13, NF1, NGF, NOTCH4, NR4A1, PDGFB, PML, PPARG, PSAP, RUNX2, SIGLEC1, SMAD3	24
Hematological System Development and Function	quantity of mononuclear leukocytes	5.42E-05	C5AR1, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, IGF2, IGHM, ITGAM, NGF, NOTCH4, NR4A1, PML, PSAP, RUNX2, SIGLEC1, SMAD3	19
Hematological System Development and Function	development of leukocytes	6.19E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CSF3, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	25
Hematological System Development and Function	proliferation of leukocytes	6.68E-05	ADCYAP1, BTNL2, CASP3, CD4, CD6, CD86, CDKN2A, CORO1A, CR2, CSF2, CSF3, CTLA4, DCN, IGHM, IL24, ITGAM, ITGB1, MYCN, NF1, NGF, PPARG, SIGLEC1, ST6GAL1, TSC22D3	24
Hematological System Development and Function	T cell development	8.89E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	23
Hematological System Development and Function	development of lymphocytes	9.94E-05	ADCYAP1, APC, C5AR1, CASP3, CD4, CD52, CD6, CD86, CDKN2A, CHD7, CR2, CSF2, CTLA4, FTH1, IGF2, IGHM, ITGB1, JUN, NR4A1, NR4A3, PDIA3, RUNX2, SMAD3, TOP2A	24

Hematological System Development and Function	survival of neutrophils	1.40E-04	CSF2, CSF3, NGF, PCNA	4
Hematological System Development and Function	expansion of T lymphocytes	1.41E-04	C5AR1, CD4, CD55, CD86, CSF2, CTLA4, IGHM, PPARG	8
Hematological System Development and Function	adhesion of leukocyte cell lines	1.82E-04	CSF2, CSF3, ITGAM, ITGB1, SRC	5
Hematological System Development and Function	maturation of neutrophils	1.89E-04	CSF2, CSF3, PML	3
Hematological System Development and Function	differentiation of lymphocytes	2.51E-04	ADCYAP1, APC, C5AR1, CD4, CD52, CD86, CDKN2A, CHD7, CR2, CSF2, CTLA4, IGF2, IGHM, ITGB1, MYCN, NGF, SMAD3	17
Hematological System Development and Function	quantity of lymphocytes	2.97E-04	C5AR1, CD4, CD86, CDKN2A, CR2, CSF2, CSF3, CTLA4, IGF2, IGHM, ITGAM, NGF, NOTCH4, NR4A1, PSAP, RUNX2, SIGLEC1	17
Hematological System Development and Function	stimulation of granulocytes	3.80E-04	C5AR1, CSF2, CSF3, IGF2	4
Hematological System Development and Function	stimulation of neutrophils	3.95E-04	C5AR1, CSF2, CSF3	3
Hematological System Development and Function	T cell response	4.05E-04	C5AR1, CASP3, CD4, CD86, CSF2, CTLA4, PPARG	7
Hematological System Development and Function	cell movement of phagocytes	4.57E-04	C5AR1, CD4, CD55, CD86, CFTR, CORO1A, CSF2, CSF3, EDNRB, ELN, ITGAM, ITGB1, NGF, PDGFB, PPARG, SMAD3	16
Hematological System Development and Function	proliferation of lymphocytes	5.24E-04	ADCYAP1, BTNL2, CASP3, CD4, CD6, CD86, CDKN2A, CORO1A, CR2, CSF2, CTLA4, IGHM, IL24, ITGAM, ITGB1, MYCN, NGF, PPARG, SIGLEC1, ST6GAL1, TSC22D3	21
Hematological System Development and Function	cell movement of leukocytes	6.02E-04	ACTN4, C5AR1, CD4, CD55, CD86, CFTR, CORO1A, CSF2, CSF3, CTLA4, CXCR7, EDNRB, ELN, GFAP, ITGAM, ITGB1, NGF, PDGFB, PPARG, SMAD3	20
Hematological System Development and Function	quantity of T lymphocytes	6.55E-04	C5AR1, CD4, CD86, CDKN2A, CSF2, CSF3, CTLA4, IGF2, ITGAM, NOTCH4, NR4A1, RUNX2, SIGLEC1	13
Hematological System Development and Function	binding of peripheral blood monocytes	6.96E-04	CD4, ITGAM	2
Hematological System Development and Function	elimination of leukocyte cell lines	6.96E-04	CD4, CTLA4	2

4.2 The novel compound OSI-461 induces apoptosis and growth arrest in human acute myeloid leukemia cells.

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ORIGINAL ARTICLE

The novel compound OSI-461 induces apoptosis and growth arrest in human acute myeloid leukemia cells

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Abstract Acute myeloid leukemia (AML) is a heterogeneous hematological malignancy. Treatment of patients suffering from high-risk AML as defined by clinical parameters, cytogenetics, and/or molecular analyses is often unsuccessful. OSI-461 is a pro-apoptotic compound that has been proposed as a novel therapeutic option for patients suffering from solid tumors like prostate or colorectal carcinoma. But little is known about its anti-proliferative potential in AML. Hence, we treated bone marrow derived $CD34^+$ selected blast cells from 20 AML patients and the five AML cell lines KG-1a, THP-1, HL-60, U-937, and MV4-11 with the physiologically achievable concentration of 1 μM OSI-461 or equal amounts of DMSO as a control. Following incubation with OSI-461, we found a consistent induction of apoptosis and an accumulation of cells in the G2/M phase of the cell cycle. In addition, we demonstrate that the OSI-461 mediated anti-proliferative effects observed in AML are associated with the induction of the pro-apoptotic cytokine mda-7/IL-24 and activation of the growth arrest and DNA-damage inducible genes (GADD) 45α and 45γ. Furthermore, OSI-461 treated leukemia cells did not regain their proliferative potential for up to 8 days after cessation of treatment following the initial 48 h treatment period with 1 μM OSI-461. This indicates sufficient targeting of the leukemia-initiating cells in our in vitro experiments through OSI-461. The AML samples tested in this study included samples from patients who were resistant to conventional chemotherapy and/or had FLT3-ITD mutations demonstrating the high potential of OSI-461 in human AML.

Keywords AML · OSI-461 · GADD45 · Growth arrest · Apoptosis

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Introduction

Acute myeloid leukemia is a heterogeneous malignancy originating from the hematopoietic stem and progenitor cell compartment that is characterized by the expansion of an immature blast cell population [1]. Treatment strategies are nowadays based on patients' clinical parameters and cytogenetic as well as molecular analyses. Through extensive research over the past decade, several novel risk factors and treatment strategies could be identified and developed [2]. Unsolved problems include the induction of long-term remissions in patients suffering from high-risk AML as defined by the clinical course of their disease, complex aberrant karyotypes, and/or mutations like FLT3-ITD. Still, approximately 50% of these patients die from their leukemia [2]. A feasible way to improve treatment outcomes in those patients would include sufficient targeting of the leukemia initiating cells. The novel compound OSI-461 has been widely studied in solid tumors, but little is known about its potential in hematological malignancies

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such as AML. We could previously demonstrate that the OSI-461 related compounds sulindac sulfide and sulindac sulfone induce apoptosis and growth arrest in a broad variety of cancer cells through a strictly mda-7/IL-24 dependent pathway involving GADD45α and GADD45γ activation in vitro and in vivo [3, 4]. The anti-cancer potential of the pro-apoptotic cytokine mda-7/IL-24 is currently under investigation for various solid cancer entities, and recent work also demonstrated an anti-proliferative effect of mda-7/IL-24 in AML [5, 6]. Here, we provide evidence that OSI-461 induces apoptosis and a G2/M cell cycle arrest in AML cells, including samples obtained from high-risk AML patients.

Materials and methods

Patient samples Patient and normal bone marrow samples were obtained after written informed consent following an approved protocol. Bone marrow $CD34^+$ cells were selected by immunomagnetic cell separation (Miltenyi Biotec) according to the manufacturer's instructions. Primary $CD34^+$ cells were cultured in serum-free HPGM (hematopoietic progenitor growth medium; Lonza) supplemented with IL-3, IL-6 (both 10 ng/ml), and SCF (25 ng/ml; all from Peprotech). $CD34^+$ cells were cultured at 37°C, 5% CO_2 under humidified conditions. Patients' characteristics are detailed in Table 1.

Cell culture The AML cell lines KG-1a and HL-60 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, whereas THP-1, U-937, and MV4-11 cells were obtained from the American Type Culture Collection. All cell lines were purchased for the purpose of this study and grown in RPMI 1640 (Sigma-Aldrich) supplemented with 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all Sigma-Aldrich). For HL-60 and U-937 cells media was completed with 20%, for KG-1a and THP-1 cells with 10% fetal bovine serum. Cells were cultured at 37°C, 5% CO_2 under humidified conditions.

Reagents OSI-461 was provided by OSI Pharmaceuticals Inc. and was dissolved in DMSO (Sigma-Aldrich). Cells were generally treated with 1 μM OSI-461. Respective controls were treated with equal amounts of DMSO (less than 0.1% final concentration).

Semisolid clonogenic assays Following 48 h of initial treatment with 1 μM OSI-461 or DMSO, cells were seeded in semisolid ready-to-use methylcellulose growth medium (MethoCult H4436, StemCell Technologies) at concentrations of 1×10^3 cells/mL. After 2 weeks of culture at 37°C, 5% CO_2 under humidified conditions, colonies were graded (CFU-GEMM, CFU-G/M/GM, CFU-E, BFU-E) and counted. Each experiment was performed in triplicates.

Table 1 Patients' characteristics

No.	Diagnosis	Sample status	Karyotype	Molecular diagnostics
AML 1	AML M1	Primary diagnosis	N/A	N/A
AML 2	AML M4/M5	Primary diagnosis	47, XY +8	N/A
AML 3	MDS RAEBII	Primary diagnosis	46, XY	MLL-PTD
AML 4	sAML/MDS	Refractory	45, XX, t(3;3), −7/46, XX	None
AML 5	AML M1	Refractory	46, XY	None
AML 6	sAML/MDS	Refractory	46, XY	None
AML 7	CML-BC	Disease progression	46, XY, t(9;22)	BCR-ABL
AML 8	AML M1	Primary diagnosis	46, XX, t(6;9)	FLT3-ITD
AML 9	sAML MDS	Relapse	46, XY	N/A
AML 10	AML M5	Relapse	Complex	None
AML 11	AML M5	Primary diagnosis	46, XX	NPM1
AML 12	sAML/MDS	Primary diagnosis	46, XY	None
AML 13	MDS RAEBII	Primary diagnosis	46, XX	None
AML 14	AML M5b	Primary diagnosis	46, XX inv(16)	None
AML 15	AML M1	Primary diagnosis	N/A	N/A
AML 16	AML M2	Primary diagnosis	N/A	N/A
AML 17	sAML/MDS	Refractory	Complex	None
AML 18	AML M4	Primary diagnosis	46, XX	None
AML 19	AML M4	Relapse	46, XX	FLT3-ITD
AML 20	AML M2	Relapse	46, XX	FLT3-ITD

Apoptosis and cell viability assays Measurement of apoptosis following OSI-461 treatment was performed utilizing the Cell Death Detection ELISAplus kit (Roche). Absorbance was measured at 405 nm on a Victor Wallac multilabel counter 1420 (Perkin Elmer). Viability was assessed by counting in a hemocytometer following trypan-blue staining.

Cell cycle analysis Distribution of cells in the cell cycle was assayed with the FITC BrdU Flow Kit (BD Biosciences) by BrdU and 7-amino-actinomycin D (7-AAD) staining according to the manufacturer's instructions. Briefly, prior analysis 3×10^6 cells were incubated in 6 mL RPMI medium supplemented with 1 μM OSI-461 or 1 μM DMSO for 48 h. Following treatment, cells were pulsed with BrdU every 6 h for 24 h prior staining and flow-cytometric analysis on a FACS Calibur (Becton Dickinson).

Real-time PCR Total RNA was isolated using QIAshredder and RNeasy Mini Kit (both Qiagen) according to the manufacturer's instructions. The amount of extracted RNA was quantified using the NanoDrop spectrophotometer (NanoDrop Technologies). Real-time PCR was performed using the LightCycler-FastStart DNA Master SYBR Green I kit in a LightCycler 1.2 (both Roche Diagnostics). Relative gene expression levels were calculated as the difference of CT values of the gene of interest and the housekeeping gene GAPDH as control (ΔCT). The delta–delta CT method was used for calculation of expression differences of the respective genes. Primer sequences were as follows: mda-7/IL-24 sense—CAAAGCCTGTGGACTTTAGCC; IL-24 antisense—GAATAGCAGAAACCGCCTGTG; human GAPDH sense—TCCATGACAACTTTGGTATCG; human GAPDH antisense—GTCGCTGTTGAAGTCAGAGGA; GADD45α sense—TGCTGACGCGCAGGATGTT; GADD45α antisense—GCTGCTCAACGTCGACCC; GADD45γ sense—CTGCATGAGTTGCTGCTGTC; and GADD45γ antisense—TTCGAAATGAGGATGCAGTG.

Statistical analysis Statistical significance was tested using paired, two-tailed Student *t* test to assess the significance levels of OSI-461 treated samples compared to their respective DMSO controls. *P* values lower than 0.05 were considered as significant.

Results

OSI-461 induces apoptosis and growth arrest in AML

Clinical trials have demonstrated that OSI-461 can reach steady-state plasma concentrations of 2.5 μM in humans [7]. A dose–response analysis of the FLT3-ITD positive human AML cell line MV4-11 revealed that OSI-461 consistently induces apoptosis at concentrations as low as 0.75 μM following 48 h of treatment (Fig. 1a). In a time–response analysis with 1 μM OSI-461 in the human AML cell line KG-1a, we found that induction of apoptosis starts as early as 36 h post-treatment (Fig. 1b). Based on these data, we chose a treatment period of 48 h with 1 μM of OSI-461 for our further experiments. First, the AML cell lines HL-60, THP-1, MV4-11, U-937, and KG-1a were treated with 1 μM OSI-461 for 48 h. Following incubation, changes in apoptosis and cell cycle as a result of OSI-461 treatment were analyzed. We found that treatment with 1 μM OSI-461 led to a consistent induction of apoptosis in all cell lines tested (Fig. 2a). This was

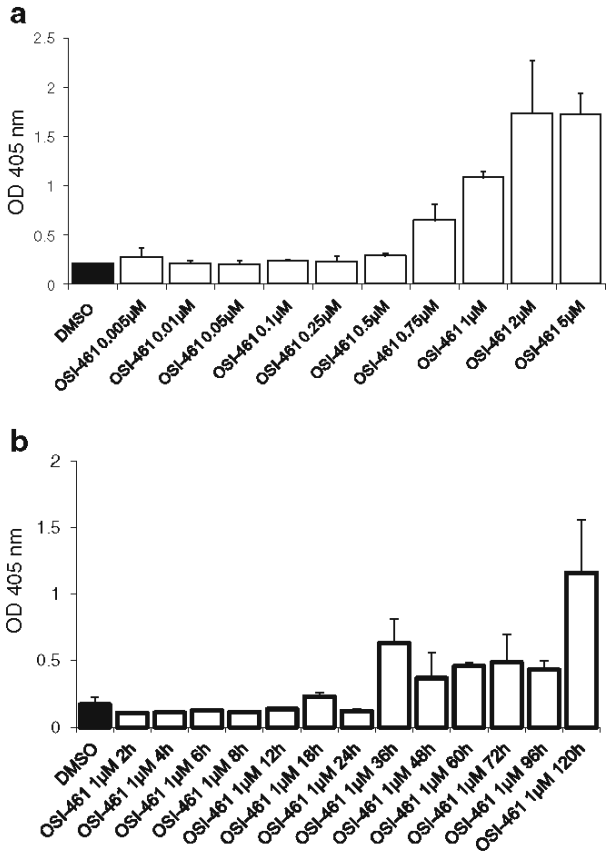
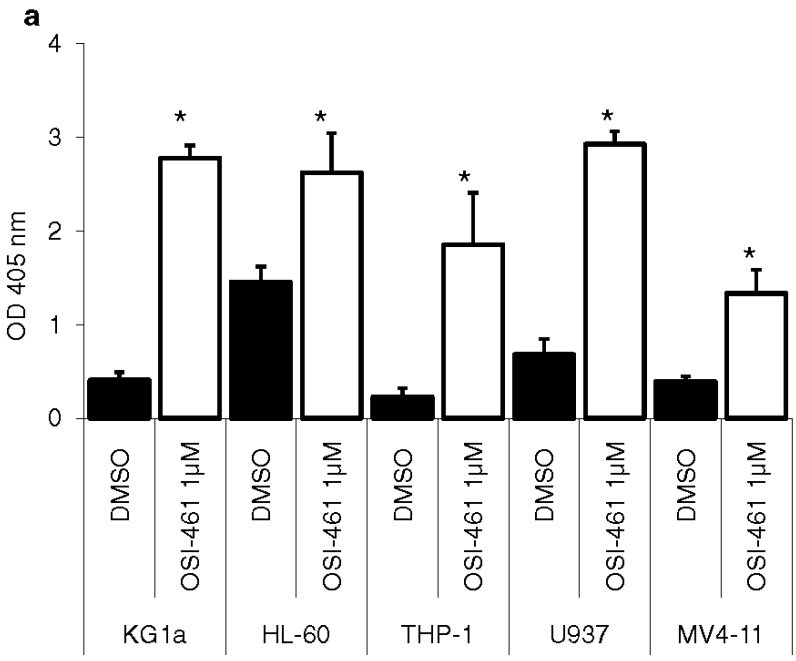
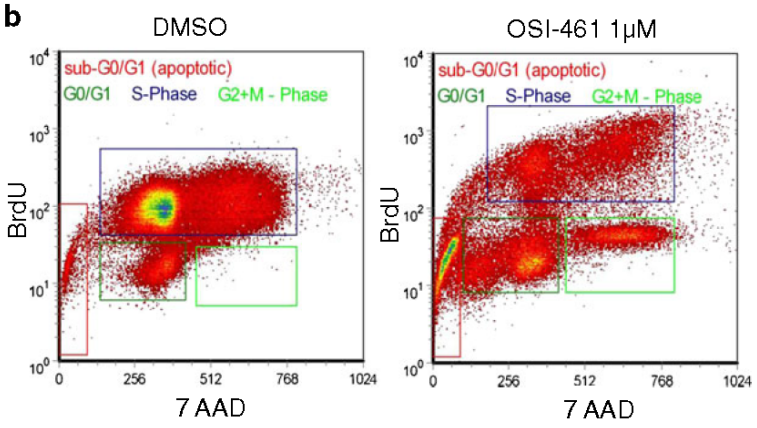


Fig. 1 Dose- and time-dependent response curves to OSI-461 in AML. **a** FLT3-ITD positive MV4-11 cells were treated with increasing OSI-461 concentrations ranging from 0.005 μM to 5 μM for 48 h. We detected a steady increase in apoptotic activity starting at 0.75 μM as measured by an increased extinction at 405 nm. **b** Time–response analysis in KG-1a AML cells treated with 1 μM OSI-461 for a total of 120 h. Apoptosis was measured at the indicated time points and could be detected as early as 36 h post-treatment start





MV4-11	DMSO	OSI-461		THP-1	DMSO	OSI-461
sub-G0	1.91	18.05		sub-G0	0.51	9.97
G0/G1	9.54	27.83	%	G0/G1	7.29	13.75
S	85.38	36.07		S	84.12	56.46
G2/M	0.33	12.09		G2/M	0.50	12.96

Fig. 2 OSI-461 induces apoptosis and growth arrest in AML cell lines. **a** Various AML cell lines were treated with 1 µM OSI-461 for 48 h prior to measurement of apoptotic activity. We detected a consistent increase in apoptotic cells, as indicated by the increased extinction at 405 nm. Data show means±SD of independent triplicate experiments for each treatment (*$P<0.01$). **b** Cell cycle analysis was performed by BrdU and 7-AAD staining following 48 h of treatment with 1 µM OSI-461. The *upper panel* shows one representative experiment in MV4-11 cells. The *lower table* shows the percentage of cells in the respective phases of the cell cycle, demonstrating for both THP-1 and MV4-11 cells a decrease of cells in the proliferative S-phase, and an increase of cells in the G2/M-phase

accompanied by accumulation of the treated cells in the G0/G1 and G2/M-phase of the cell cycle with a decreased proportion of cells in the S-phase (Fig. 2b). Furthermore, we tested if treatment with OSI-461 also led to induction of myeloid differentiation. We evaluated cell surface expression of CD11b, CD14, CD15, and CD114, and performed clonogenic assays, but found no change in the expression of the aforementioned myeloid differentiation markers as well as no mature colonies in our clonogenic assays (data not shown).

OSI-461 induced apoptosis in primary AML blast cells from patients with high-risk AML is durable

Next, we examined the pro-apoptotic capabilities of OSI-461 in 20 bone marrow derived $CD34^+$ enriched AML

patient samples. Following treatment with 1 μM OSI-461 for 48 h, apoptosis increased by an average of 2.2-fold as compared to DMSO treated controls (Fig. 3a, $P<0.01$). Overall, 17 of the 20 patients tested (85%) responded to this in vitro treatment. These data point towards a potential efficacy of OSI-461 as a pro-apoptotic agent in AML. So we next asked if single agent treatment with OSI-461 is sufficient to eradicate the leukemia initiating cells in vitro. For this purpose, we treated bone marrow derived $CD34^+$ enriched leukemia cells from patients AML 17 to AML 20 with 1 μM of OSI-461 for 48 h. Then, cells were transferred into fully supplemented medium (IL-3, IL-6, SCF) without DMSO or OSI-461, and cell numbers and viability were continuously assessed every 48 h for up to 8 days (192 h). We found that none of the OSI-461 treated samples was able to regain its proliferative potential, while the formerly DMSO treated cells continued to expand rapidly. Figure 3b shows data from two patients. These data indicate that 48 h of treatment with 1 μM of OSI-461 is sufficient to inhibit proliferation of the leukemic cells even after cessation of therapy for up to 8 days, indicating that OSI-461 targets the leukemia initiating cells in vitro.

OSI-461 induces apoptosis in normal hematopoietic CD34+ cells, which are able to recover and give rise to differentiated progenitors after cessation of treatment

Although no hematopoietic toxicities were reported from trials investigating OSI-461 for the treatment of patients with solid cancers, we were interested in any potentially toxic effect of OSI-461 on normal $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) in vitro. Therefore, we treated four normal bone marrow derived $CD34^+$ cells with 1 μM OSI-461 for 48 h analog to the primary AML cells and also found an increased rate of apoptosis as a consequence of the treatment with OSI-461 (Fig. 4a). In contrast to primary AML cells, normal $CD34^+$ cells did recover from OSI-461 treatment when maintained in OSI-461-free medium for another 96 h as shown by a steady increase of viable cells (Fig. 4b). We further evaluated if OSI-461 treatment did affect the differentiation capacity of normal $CD34^+$ HSPC in clonogenic assays. Therefore, following 48 h of treatment with 1 μM OSI-461 or DMSO, we transferred 1×10^3 HSPCs/mL into methylcellulose-based medium supplemented with G-CSF, EPO, TPO, IL-

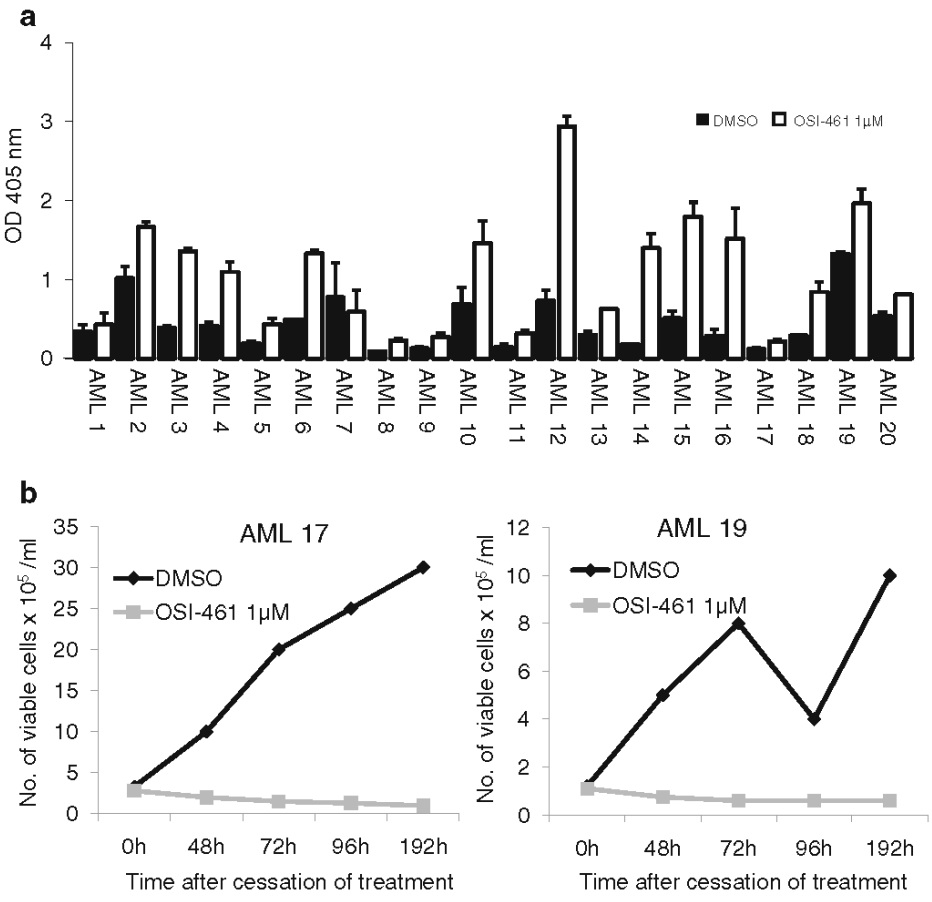


Fig. 3 Anti-proliferative effect of OSI-461 is consistent in human AML samples and maintained after cessation of therapy. **a** Bone marrow derived $CD34^+$ enriched primary AML samples from 20 patients were treated with 1 μM OSI-461 for 48 h. Experiments were carried out in duplicates. Apoptosis was induced by an average fold of 2.2 ($P<0.05$) with 85% of the patients tested responding. **b** Following treatment with 1 μM OSI-461 for 48 h cells were transferred in fresh, OSI-461 or DMSO free, media and cultured for an additional 8 days. OSI-461 treated AML cells did not regain full proliferative potential as compared to the previously DMSO treated cells. The *panel* shows data for two representative patients

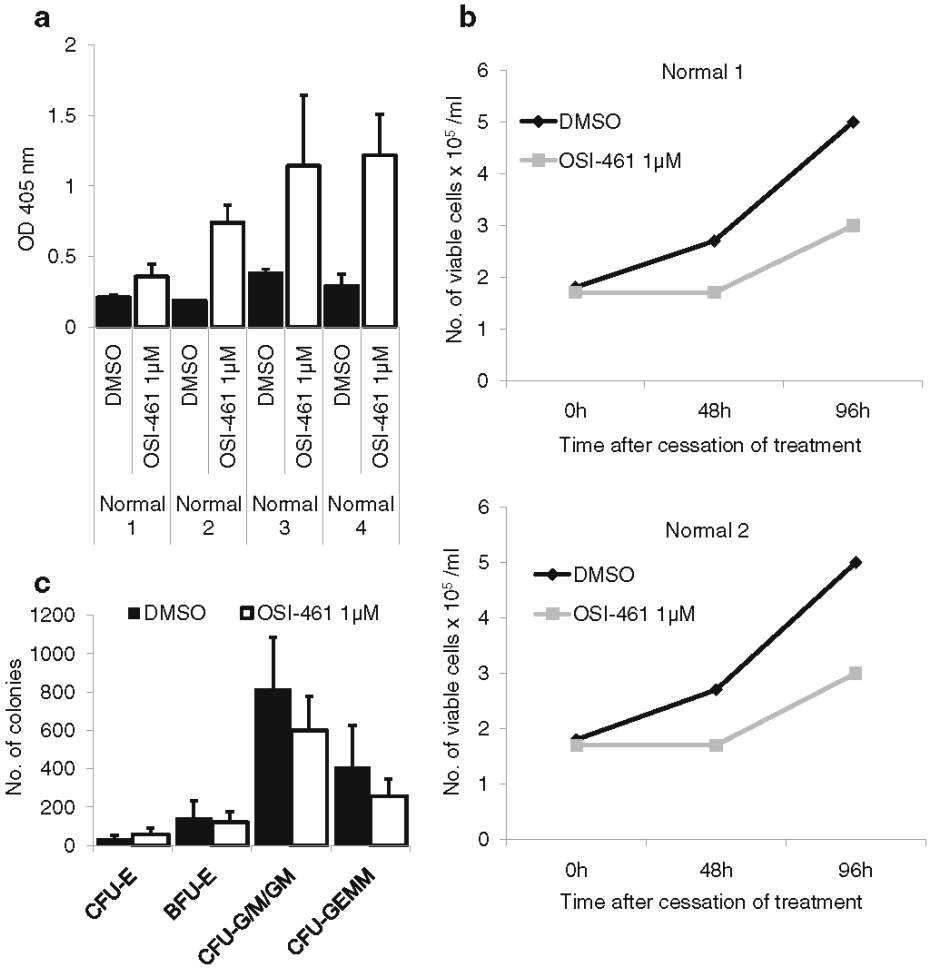


Fig. 4 OSI-461 treatment does not affect the hematopoietic potential of normal CD34$^+$ HSPC. **a** CD34$^+$ hematopoietic stem and progenitor cells (HSPC) from normal donors were treated with 1 μM OSI-461 for 48 h. Apoptosis analysis shows, similar to the AML patient samples, an increase in apoptotic activity in the OSI-461 treated samples. **b** Normal CD34$^+$ HSPC regain their proliferative potential after cessation of 48 h of OSI-461 treatment, as shown by a continuous increase in the number of viable cells in both the DMSO and OSI-461 treated cells 48 h and 96 h post-treatment cessation. **c** Myeloid differentiation potential is not disturbed by OSI-461 treatment as demonstrated by clonogenic assays. No significant difference between DMSO and OSI-461 treated samples could be detected. All experiments were done in triplicates and the data are presented as mean±SD

3, IL-6, and FLT3L. After 14 days in culture, colonies were assessed and counted (Fig. 4c). We found no differences between cells previously treated with DMSO or OSI-461 with regard to their myeloid potential, thus demonstrating that OSI-461 treated normal CD34$^+$ HSPCs retain their full hematological potential.

OSI-461 anti-proliferative activities are accompanied by induction of mda-7/IL-24 transcription with activation of GADD45α and GADD45γ

We have previously reported that related non-steroidal anti-inflammatory drugs (NSAID) that cause apoptosis and a G2/M cell cycle arrest in malignant cells utilize a strictly mda-7/IL-24 dependent signaling pathway through GADD45α and GADD45γ activation. Therefore, we asked if this may be a molecular pathway involved in OSI-461 treated hematological malignancies and found a consistent induction of mda-7/IL-24, GADD45α, and GADD45γ expression in primary AML samples and AML cell lines treated with 1 μM OSI-461 or equal amounts of DMSO for 48 h. Median fold of induction in the nine AML patients analyzed was 8.9 (range 2.7- to 60.3-fold) for mda-7/IL-24, and 2.6 (range 1.4- to 77.2-fold) and 1.7 (range 1.4- to 39.8-fold) for GADD45α and GADD45γ, respectively (Fig. 5b, d). This included AML samples from patients with FLT3-ITD and MLL mutations. Transcriptional activation of mda-7/IL-24, GADD45α, and GADD45γ following OSI-461 treatment was also consistently observed in the AML cell lines tested here (Fig. 5a, c).

Discussion

Here, we report that the novel pro-apoptotic compound OSI-461 induces apoptosis and a G2/M growth arrest in primary cells of patients with acute myeloid leukemia (AML). These anti-proliferative effects of OSI-461 are accompanied by an induction of mda-7/IL-24, GADD45α, and GADD45γ transcription. OSI-461 is an investigational pro-apoptotic agent related to the metabolites of the non-steroidal anti-inflammatory drug sulindac sulfoxide

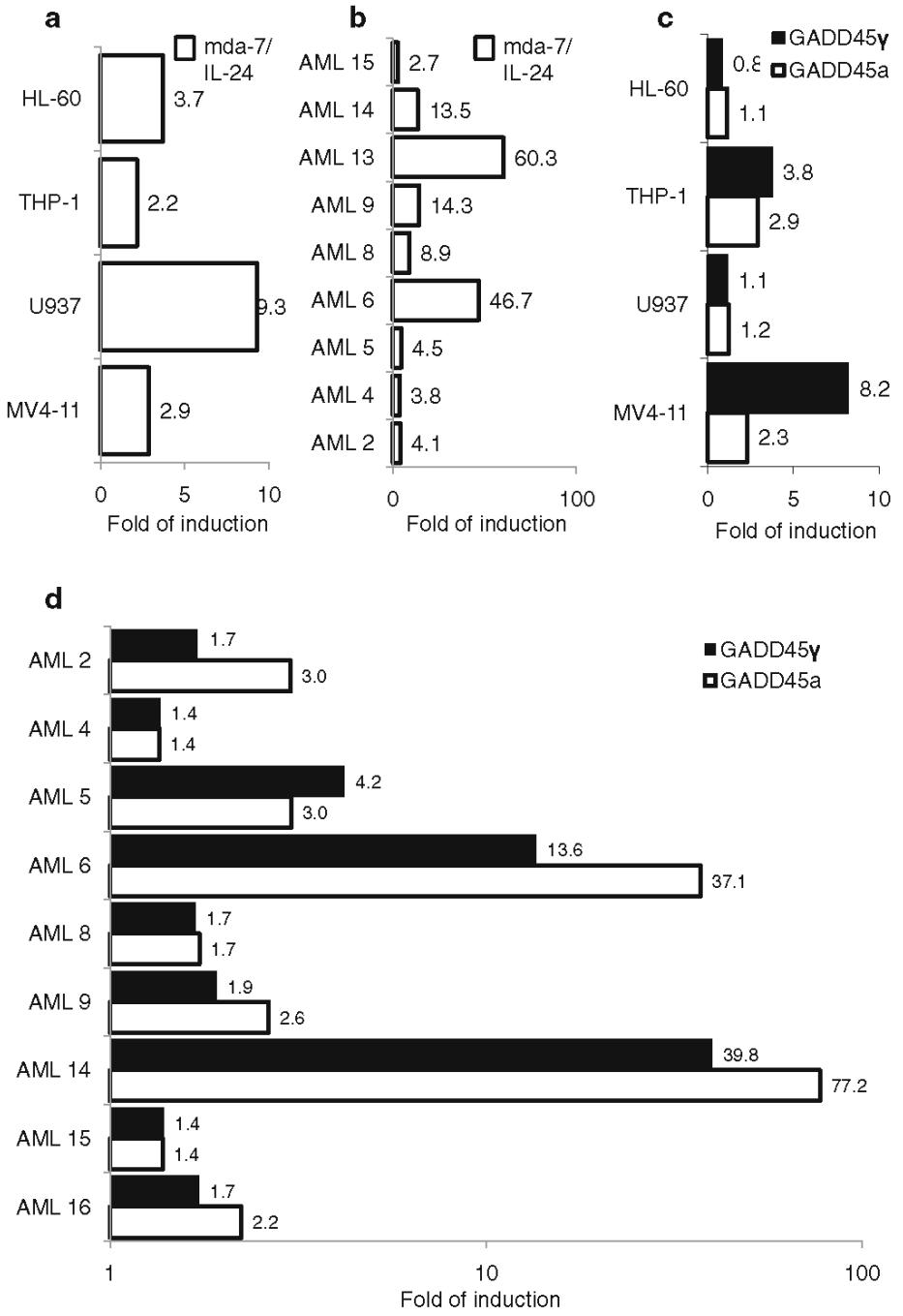


Fig. 5 Changes in gene expression following OSI-461 treatment. Increase in transcript levels of mds-7/IL-24 (**a** and **b**), GADD45α and GADD45γ (**c** and **d**) in AML cell lines (**a** and **c**), and primary AML samples (**b** and **d**), respectively, following treatment with 1 μM OSI-461 or DMSO as a control for 48 h

(Clinoril, Merck) [8, 9]. It does not inhibit cyclooxygenases and is supposed to act through inhibition of cancer-specific phosphodiesterases (PDE) 2 and 5 [9]. There are several promising in vitro studies showing high potency of OSI-461 against solid tumors like colorectal carcinoma, and lung or prostate cancer [10–12]. But following phase I/II trials were largely unsuccessful in patients with these entities [13–16]. We believe this discrepancy is owed to the fact that the drug concentrations used in some of the in vitro studies exceeded the achievable plasma concentration of 2.5 μM in humans [7]. Furthermore, we postulate that the bioavailability of the drug may be insufficient in solid tumors as compared to the highly accessible bone marrow environment. Data obtained from phase I and phase II trials showed that side effects are minor with fatigue being the most severe. There were no toxicities reported for the hematopoietic system when doses between 200 and 800 mg OSI-461 twice daily were given [7]. Hence, we treated all

primary AML cells with 1 μM of OSI-461, which is an easily achievable steady-state concentration in humans, and found a consistent and significant induction of apoptosis. To analyze the selectivity of OSI-461 induced apoptosis and growth arrest on malignant cells, we also treated normal $CD34^+$ cells obtained from mobilized healthy donors. Although we also found an increased rate of apoptosis in OSI-461 treated normal cells, the effect was only transient and the cells were able to overcome the proliferative block after cessation of OSI-461 exposure while maintaining their full myeloid potential. This is of relevance as normal and malignant hematopoiesis can be observed side by side in some patients with myelodysplastic syndromes (MDS), and sparing the healthy stem and progenitor cells is essential for hematological recovery after therapy.

Asking for the mode of OSI-461 action, we found that treatment of AML cells with OSI-461 led to induction of apoptosis and an accumulation of cells in the G2/M phase of the cell cycle accompanied by an increased expression of mda-7/IL-24, GADD45α, and GADD45γ. This is in line with previous studies where we could show that treatment with the OSI-461 related compounds sulindac sulfide or sulindac sulfone caused strong induction of mda-7/IL-24 transcription followed by activation of GADD45α, GADD45γ, and JNK with inhibition of CDK1 [17]. We also found JNK-dependent induction of apoptosis in primary cells from patients with advanced MDS in response to treatment with sulindac sulfone [4]. Although sulindac sulfide and sulindac sulfone were also capable of inducing apoptosis in primary AML samples in our study, induction of mda-7/IL-24 transcription was not observed (data not shown). To the best of our knowledge, OSI-461 appears to be the only available compound that is capable of inducing mda-7/IL-24 transcription in primary AML cells. This is of interest as mda-7/IL-24 is currently under investigation as a potent pro-apoptotic drug for the treatment of different malignant solid tumors, and recent studies highlighted its efficacy as an anti-proliferative cytokine in primary AML cells [5, 6]. The anti-proliferative effects of mda-7/IL-24 do not depend on membrane expression of its physiological receptors IL-20R/IL-22R as it exerts its pro-apoptotic effects through strictly cell intrinsic pathways [18]. Current strategies of mda-7/IL-24 delivery into malignant cells involve the use of viral gene transfer [19]. Many unsolved problems like frequent adverse immune reactions and limitations related to local delivery are still not satisfactorily resolved [19]. Our data provide a new strategy for specific and systemic high-level induction of mda-7/IL-24 that could exceed the efficacy of adenoviral gene transfer approaches.

We believe that OSI-461 may be more efficient for the treatment of hematological malignancies like AML than solid cancers, as concentrations as low as 0.75 μM are sufficient to induce apoptosis even in AML patient cells carrying FLT3-ITD mutations, who have a poor prognosis with early relapses after primary induction chemotherapy [20]. The same is true for patients who are resistant to conventional chemotherapy, as reflected by multiple relapses during the course of the disease [21, 22].

The pro-apoptotic pathways activated by OSI-461 seem to be generally intact in AML irrespective of the various genomic aberrations. Despite this heterogeneity, AML cells still share two major commonalities: they are highly proliferative and have lost their myeloid differentiation capacities. This may be very well where OSI-461 and mda-7/IL-24 interfere. For the GADD45 gene family, implications in proliferation and apoptosis in many cancers have been established, and their downstream signaling pathways leading to JNK activation and CDK1 inhibition can be activated by different upstream regulators like p38, NF-κB, or mda-7/IL-24 [5, 17, 23, 24].

Another reason for a potentially higher efficacy of OSI-461 in AML in comparison to solid tumors could be that AML, as a systemic disease originating mainly from the bone marrow, is more accessible to relevant drug concentrations than cancer cells from solid tumors. Therefore, we strongly emphasize phase II trials to assess the efficacy and safety of OSI-461 for the treatment of high-risk AML patients who carry FLT3-ITD mutations or are resistant to chemotherapy. The drug could also be an alternative or addition for patients suffering from advanced MDS that do not qualify for high dose chemotherapy and are resistant to epigenetic treatment approaches.

Our data also underline the importance of the pro-apoptotic potential of mda-7/IL-24 in proliferative diseases. In summary, OSI-461 might be the right drug for anti-cancer treatment, but to this date, the right disease to be treated has not been found.

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5. SUMMARY AND DISCUSSION

Acute myeloid leukaemia (AML) is a disease in which there is a constant accumulation of various neoplastic transformations, especially genetic alterations in stem and related cells. Studies conducted in the last decade have shed deep insights into the stem cells. It is believed now, that there are specialized cells which cause initiation of leukaemia and known as leukaemia stem cells. It is speculated that these leukaemia stem cells escape the programmed cell death in cell cycle. Leukemic stem cells have high regenerative potential and pluripotency properties. These cells are able to initiate tumours on distant and different places too; whereas normal stem cells are able to divide only at low pace that too in respective niche (Levesque and Winkler, 2009).

Non-steroidal anti-inflammatory drugs (NSAIDs) are diverse group of heterogeneous, chemically different substances which provide anti-inflammatory, anti-pyretic and analgesic effects. NSAIDs are also known for their ability to inhibit proliferation of cancer cells under *in-vitro* and *in-vivo* conditions (Fujimura et al., 2006; Gonzalez-Perez et al., 2003; Thun et al., 2002; Xu, 2002). In a previous study it was shown that NSAIDs cause up-to 70% reduction in colonic adenomas patients (Piazza et al., 2009). Anti-neoplastic effects of NSAIDs have already shown promising results in pre-clinical, clinical and epidemiological studies.

NSAIDs can play an important role in the treatment of leukaemia (Levesque and Winkler, 2009). Different modes of action of NSAIDs have been put forward. One of these is though *COX* (*cyclooxygenase*) inhibitors. *COX1* plays an important role in pathogenesis of leukaemia. *MLL-AF4* is rare genetic mutation of genes and is found in AML. *MLL-AF4* knock-in mice has a lymphoid and myeloid deregulation accompanied by increased number of cells in lymphoid and myeloid compartments (Chen et al., 2006b). It has been shown though micro-array data that *COX1* is the most up regulated gene in GMP-MLL-AF4 leukemic cells when compared to the normal HSCs.

Besides their above said commonalities NSAIDs differ in binding affinity towards iso-enzymes of *COX*. Moreover, NSAIDs are able to inhibit proliferation of cancer cells independently of *COX* activity, through other targets. These targets include *ERK1/2* (Rice et al., 2006), *GADD45s*, *MKK4* and *JNK* (Czibere et al., 2005; Zerbini et al., 2006) etc. In the present study three NSAIDs drugs; OSI-461 (*COX1* and *COX2* independent), Sulindac Sulfide (*COX1* and *COX2* inhibitor) and Diclofenac (selective *COX2* inhibitor) were examined.

The aim of the current study was to explore the role of NSAIDs anti-neoplastic effects in AML. Emphasis was paid to have an inside look into mechanism of induction of pro-apoptosis responses and those pathways which are associated with pro-apoptosis responses (Figure 10). Detailed investigation was carried-out to study the effects of NSAIDs in various human AML cells lines and primary human AML cells with different FAB types and chromosomal abnormalities. All three NSAIDs have shown similar effects on induction of apoptosis (2.2 to 6.30 fold change as compared to control), reduction of proliferation (from 23% to 40% as compared to control) and cell cycle arrest in G2/M phase (from 12.09% to 12.96% accumulation of the cells as compared to control). Also, it is worth mentioning that OSI-461 is both *COX1* and *COX2* independent.

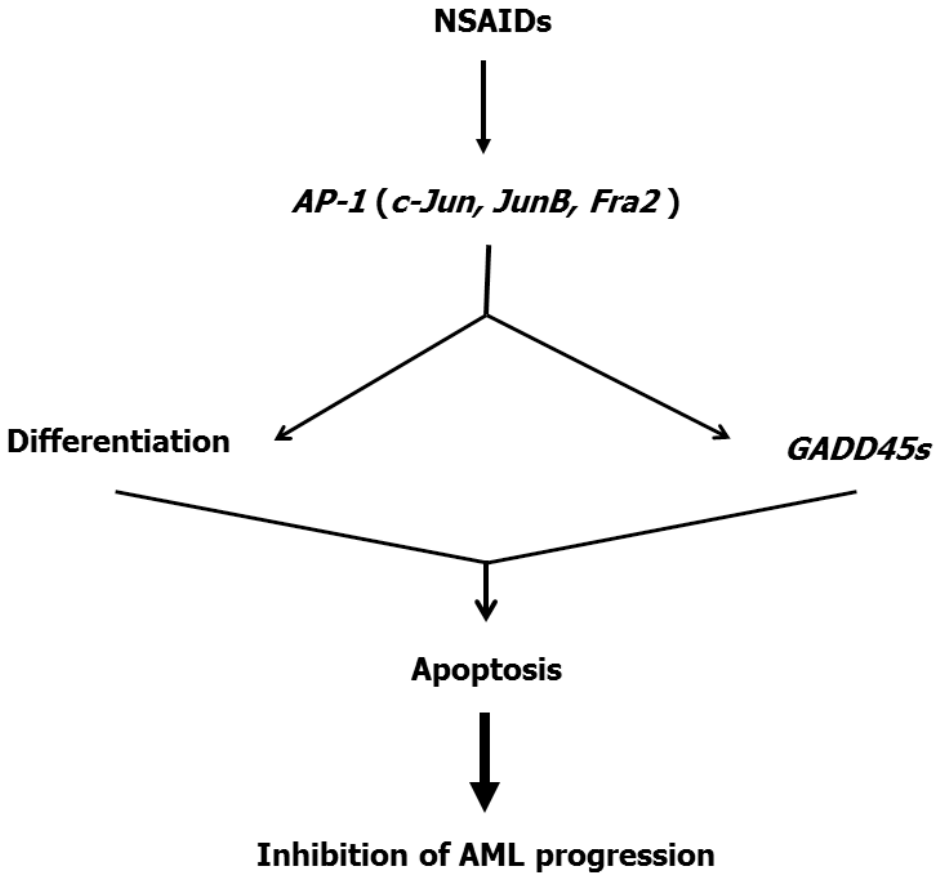

Figure 10: ***Schematic representation of our model of NSAIDs mediated induction of apoptosis in human AML.*** NSAIDs lead to an induction of expression of the *AP-1* transcription factors *c-Jun, JunB* and *Fra2*. This results in activation of *GADDs* and they that results in *JNK*s activation and consecutive induction of apoptosis.

To find-out mode of action of NSAIDs in AML, AML cells were treated with above mentioned NSAIDs. For controls, cells were also cultured in DMSO alone as control by using same amounts of DMSO as of amount of NSAIDs, which was <0.1% of the final concentration. Our results confirmed the earlier studies that NSAIDs induce apoptosis and reduction of proliferation (Zerbini et al., 2006). The expression of *MDA-7/IL-24* was increased when AML cells was treated with OSI-461 (2.2 to 60.3 fold change), but not with Sulindac Sulfide and Diclofenac.

MDA-7/IL-24 is a cytokine and belongs to the *IL-10* family of cytokines. In recent investigations on treatment of maligned solid tumours it was proved that *MDA-7/IL-24* causes the growth suppression and apoptosis in cancer cells but not in normal cells. *MDA-7/IL-24* is considered as a potent anti-neoplastic with high efficacy (Rahmani et al., 2010; Yang et al., 2011). Various studies have been carried out to examine the expression of *MDA-7*/*IL-24* via adenoviral vector carrying the *MDA-7* gene (Ad-*MDA-7*) (Otkjaer et al., 2010). The transfer of *MDA-7/IL-24* through adenovirus is related to the immune reaction and it cannot deliver satisfactory levels of *MDA-7/IL-24* (Sauane et al., 2008). Our results indicate that the level induction of *MDA-7/IL-24* through OSI-461 is higher and could have fewer side effects than the adenoviral vector therapy as induction of *MDA-7/ IL-24* is through the drug rather than adenoviral vector carrying the *MDA-7* gene.

G2/M checkpoint is important as it prevents the cell with damaged DNA from entering mitosis (M-phase) and further cell division and proliferation. In AML there is a rapid increase in number of undifferentiated myeloid cells, which have a defective genomic DNA. In present study, G2/M arrest was observed in OSI-461 treated AML cells. OSI-461 treated AML cells were accumulated in the G2/M phase of the cell cycle. Initiation of apoptosis in NSAIDs treated cells was also observed. Above described data indicates the return of the cells towards normal cell cycle and apoptosis rather than escaping it.

Induction of *MDA-7/IL-24* is not affected in case of Sulindac Sulfide and Diclofenac treated AML cells. To investigate the mechanism involved in Sulindac Sulfide and Diclofenac mediate effects on AML, comprehensive protein and gene expression profiling of Diclofenac treated AML cells was done. There was an increased expression of CASPASE-3 precursor and other related proteins which function in cellular structure and metabolism. Re-organization of the cellular structure and high rate of metabolism is associated with the differentiation and apoptosis (Cohen and Chen, 2008; Grzanka et al., 2003; Launay et al., 2003; Rebillard et al., 2010). CASPASE-3 is a key molecule of *MAPK/JNK* pathway and it plays an important role in induction of apoptosis after NSAIDs treatment (Senthivinayagam et al., 2009). These observations were further verified by flow cytometric analysis.

Gene expression data revealed that the expression of the *MAPK/JNK* pathway is the target of NSAIDs treatment. *GADD45α*, which is an upstream target of *MAPK/JNK* pathway, was significantly up-regulated in NSAIDs treated AML cells. *GADD45* family plays an important role in proliferation and apoptosis in the cancer (Takekawa and Saito, 1998). The *GADD45* is critical in activation of *JNK* and inhibition of *CDK1*. In most of the cancers *GADD45α* is down-regulated (Zerbini et al., 2006).

MAPK/JNK pathway is known to cause activation of *c-Jun NH2-terminal Kinase (JNK)* which in turn increases expression of *c-Jun*. The activation of this pathway can lead to activation of *JNK* with increased phosphorylation of *c-Jun*. The phosphorylation of *c-Jun* by the *MAPK / JNK* pathway, initiates *Caspases-3* dependent apoptosis pathway (Johnson and Lapadat, 2002; Takada et al., 2008) (Figure 9). *MAPK / JNK* pathway is related to mechanisms causing apoptosis. In line with these observations, *MAPK / JNK* pathway was the most significantly altered pathway in response to the NSAIDs treatment. The NSAIDs treatment up-regulated *MAPK / JNK* pathway and there was an activation of apoptosis in AML cells. This proved that activation of this pathway is important for NSAIDs related effects.

JNK is also known for its role in repression of basal transcription level of *p53* oncogene which down-regulate the expression of $p21^{cip1}$ (Cripe et al., 2002). $p21^{cip1}$ has been linked to cell cycle arrest (Tuder et al., 2008). Effect of inhibition of *JNK* on rate of apoptosis in NSAIDs treated AML cells was studied by the addition of *JNK* inhibitor in cell culture medium. Addition of *JNK* inhibitor resulted in significant decrease of the apoptosis rate in AML cells (26% to 50% decrease).

Treatment of primary AML cells with Sulindac Sulfide lead to an increased expression of cell surface differentiation markers like CD11b, CD14, CD15 and CD114 (at-least by 1.5 fold change respectively) after 48 hours. CD11b is a maturation marker i.e. mature granulocytes and macrophages express the high levels of CD11b. CD14 is a marker molecule for monocytes and macrophages. CD15 is a marker for the identification of granulocyte cells. CD114 is granulocyte colony stimulating factor (G-CSF) receptor. G-CSF is known to play a significant role in the hematopoietic cell differentiation into granulocytes. Under normal conditions, 10 days are required post G-CSF or GM-CSF treatment for induction of myeloid differentiation markers like CD11b, CD14, CD15 and CD114. But in present study expression of these cell surface differentiation markers was observed under normal conditions without addition of G-

CSF or GM-CSF and after 48 hours only with the addition of NSAIDs. Differential expression of the myeloid differentiation markers in our study is a significant observation and it was complementary to the results that NSAIDs treatment caused apoptosis in AML cells.

Further the genes which were differently expressed in the human micro-array were verified by the quantitative real time PCR. Expression level of differential expressed genes, *GADD45α* was 1.1 to 77.2 and *GADD45γ* 0.8 to 39.8*, c-Jun* 3.34 to 99.41, *JunB* 1.49 to 15.83 and *Fra-2/FosL2* 1.66 to 4.82 fold respectively as compared to control in quantitative real time PCR.

Another observation of this study was the activation of *AP-1* family transcription factors. In NSAIDs treated AML cells, *c-Jun* and *JunB* were up-regulated. Both of the genes are known for their important role in Leukaemia. *c-Jun* and *JunB* in mice animal model studies are known to be associated with *PU.1* (Steidl et al., 2006). When transcription factor *PU.1* is knockdown in mice, it leads to AML. Restoration of *c-Jun* and *JunB* expression in the *PU.1* knockdown mice cells initiates differentiation and prevents the proliferation of the cells. In agreement with the animal model study, it was observed that when *c-Jun* and *JunB* were re-expressed in the AML cells without treatment, there was induction of apoptosis.

Expression of *Fra2/FOSL2,* another *AP-1* family member was also observed in our study. *Fra2/FOSL2* forms heterodimer with *Jun* family members (Shaulian, 2010). When AML cells were transfected with *GADD45α* or *AP-1* family transcription factor, they have induced apoptosis in heterodimer state (1.52 to 2.1 fold increase in apoptosis rate). Transfection of AML cells with *AP-1* family heterodimer was able to increase the expression level of *GADD45α* (9.87 to 133.5 fold respectively). With knockdown of the *JunB+Fra2/FosL2* with shRNAi, rate of apoptosis was decreased (0.76 to 1.37 fold decrease in apoptosis rate). This observation indicated that more than one transcription factor is required for the initiation of the apoptosis and it can be achieved by NSAIDs treatment. It was also observed that *AP-1* transcription factors and *GADD45* interact and induced apoptosis after NSAIDs treatment.

NSAIDs treatment of AML cells was responsible for apoptosis in leukemic cells even with complex aberrant karyotypes like, *FLT3-ITD* mutations, *MLL*-mutations and recurrent translocations such as *t (15; 17)*. No expression of *p38* or *NF-κB* was observed. *NF-κB* is known for its role in cancer by helping the leukemic cells to escape from programmed cell death or apoptosis. Expression of *NF-κB* leads to repression of *GADDs* and inhibition of *NF-κB* leads to consecutive expression of *JNK* thereof, apoptosis in AML cells (Zerbini et al., 2004). Similar observations were also made in this study confirming the important role played by NSAIDs treatment of the AML cells.

Under the present set of experimental conditions it was observed that after NSAIDs treatment of AML cells there was an activation of *AP-1* transcription factors i.e. *c-Jun, JunB* and *Fra-2/FosL2*. This activation of *AP-1* family members induced *GADD45* transcription factors. *GADD45* further activates *JNK*. The activation of *JNK* initiated apoptosis and myeloid differentiation (Figure 10).

In OSI-461 treated cells initiation of apoptosis was in agreement with earlier studies on *MDA-7/IL-24*. Clinical trials of phase I and phase II, confirmed that OSI-461 was almost free of any major side effects. Minor side effects like fatigue were reported. No haematopoietic toxicities were reported even when doses between 200 and 800 mg OSI-461 twice daily were given (O'Bryant et al., 2009).

In-vitro observations of OSI-461 treated cells do not show any significant clinical benefits to the patients suffering from solid tumours like colorectal carcinoma, lung or prostate cancer (O'Bryant et al., 2009; Resta et al., 2011). Apparent dissimilarities might be due to fact that, the amount of OSI-461

used in those studies was up-to 10 µM which is far more than the achievable plasma concentration in patients. In humans achievable plasma concentration of OSI-461 is 2.5 µM (O'Bryant et al., 2009).

The dose response curve (killing curve) showed that initiation of apoptosis in AML cells started at 0.75 µM concentration of OSI-461 and after 36 hours of treatment. Therefore, AML cells were treated with 1 µM of OSI-461 for 48 hours. 1 µM concentration of OSI-461 is easily achievable steady-state concentration in humans. A consistent and significant induction of apoptosis was observed after 1 µM of OSI-461 treatment.

Higher availability of OSI-461 in AML than in solid tumours can be explained as - AML is a disease of blood and originates from bone marrow. Environment of bone marrow is more accessible to relevant drug concentrations than cancer cells from solid tumours. In a previous study it was shown that intensive chemotherapy has no benefit for patients aged over 60 years (Knipp et al., 2007). OSI-461 can be a safe drug for elderly patients who are resistant to chemotherapy or with high-risk AML like *FLT3-ITD*. OSI-461 can also be an additional or alternative drug for patients suffering from MDS, for whom high dose chemotherapy is not recommended and they are resistant to normal treatment.

Treatment of normal $CD34^+$ cells from healthy donors with OSI-461 has induced apoptosis, but these effects were only transient and when after initial 48 hour treatment the cells were transferred to medium without OSI-461 they overcame effects of drug and started their normal proliferation. Whereas AML $CD34^+$ cells treated with OSI-461 1 µM was not able regain their proliferative potential when after initial 48 hour treatment they were placed in a medium free of OSI-461, even up-to 8 days. This phenomenon is relevant and very important in leukaemia as there is always a need to protect normal healthy stem and progenitor cells. Protection of the healthy cells is essential for recovery after therapy.

In the present study an increase in the rate of apoptosis after NSAIDs treatment in various primary $CD34^+$ AML cells and in AML cell lines was observed. Freshly obtained samples from patients, indicated that above mentioned *MAPK / JNK* pathway in association with *GADDs* can cause apoptosis after NSAIDs treatment in various types of AML. The expression of *GADDs* along with *AP-1* family could be a common reason for the increase in apoptosis due the NSAIDs treatment.

For patients suffering from AML, NSAIDs can bring new hope but nerveless NSAIDs can-not be suggested as the single agent for the treatment. Rather they can be thought of as compliment to the conventional drugs. NSAIDs may increase action of the conventional drugs and thus, saving susceptibility to cytotoxic effects. This study can be useful in future for the designing of the novel chemo-preventive agents or novel agents which can target pathways specifically activated through NSAIDs in AML.

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8 Declaration

I, Raminder Singh confirm that:

1. I have written the present dissertation and has not been submitted in the same or similar form to other institutions myself and independently.
2. I have not previously failed a doctoral examination procedure.
3. This work was done wholly or mainly while in candidature for a Ph.D. degree at this University.
4. Where I have consulted the published work of others, this is always clearly attributed.
5. Where I have quoted from the work of others, the source is always given.
6. I have acknowledged all main sources of help.

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13th October 2011