An amentoflavone derivative induces apoptosis and interferes with cell proliferation in melanoma by inhibition of the JAK2/STAT3 signaling pathway

by

Jiemin HUANG

Master of Science

2017

Institute of Chinese Medical Sciences

University of Macau

An amentoflavone derivative induces apoptosis and interferes with cell proliferation in melanoma by inhibition of the JAK2/STAT3 signaling pathway

by

Jiemin HUANG

SUPERVISOR: Dr. Chung−Hang LEUNG

Master of Science

in

Chinese Medicinal Science

2017

Institute of Chinese Medical Sciences

University of Macau

一种穗花杉雙黃酮衍生物通過抑制 JAK2/STAT3 細胞通路誘導黑色素瘤細胞凋亡以及細胞周期阻滞

黃傑敏

指導老師: 梁重恒 副教授

理學碩士(中藥學)

2017

中華醫藥研究院

澳門大學

Declaration

I declare that the thesis here submitted is original except for the source materials explicitly acknowledged and that this thesis as a whole or any part of this thesis has not been previously submitted for the same degree or for a different degree.

I also acknowledge that I have read and understood the Rules on Handling Student Academic Dishonesty and the Regulations of the Student Discipline of the University of Macau.

Signature:_____ Date:_____

Acknowledgements

It is my privilege to have the opportunity for pursuing my master degree in ICMS. During the study here, I was inspired by so many people. Here, I would like to give my great appreciations to them.

Firstly, I want to present my deepest thanks to my supervisor, Dr. Chung−Hang Leung, for the patient guidance, genuine enlightenment as well as the tireless support which inspired me to complete this project.

I want to express my thanks to our group members, Ms. Haijing Zhong, Ms. Lijuan Liu, Mr. Chao Yang, Mr. Tianshu Kang, Mr. Guodong Li, Mr. Guanjun Yang, Mr. Kejia Wu and Ms. Jiaxin Liang. Thank you so much for encouraging and helping me in the past two years. They generously assist me in finishing the project.

My sincere thanks also go to Mr. Wei Guo and Ms. Hui Yang, from The University of Hong Kong and Hong Kong Baptist University respectively, for their excellent work in the synthesis of amentoflavone analogues.

In addition, I am beneficial from a well-organized lab. Herein, I want to give my thanks to the lab technicians, Wing, Sandy, Joanna, Thomas, Chris, Jackie, Dorian, Keith, Samuel, Hada and others for fulfilling their duties to offer the best technique support. I am also very appreciated for the service support that the members of administrative staff Ada, Hattie, Jeff, Katrina and Chloe have provided. Additionally, my sincere thanks go to all the teachers and students in ICMS.

Last but not the least, I would like to show the most thanks to my father, mother and my sister for their encouragement and love without end. Hope you all good luck!

Abstract

Melanoma is regarded as the most aggressive type of skin malignancy with a poor prognosis. Melanoma cells show the characteristics of chemoresistance and metastasis. Currently, clinical treatment of malignant melanoma mainly relies on surgery. However, postoperative recurrence and cancer metastasis are commonly seen. Meanwhile, the JAK2/STAT3 pathway contributes to tumorigenesis in various cancers and is a promising target for the discovery and development of antitumor therapeutics. Significantly, JAK2/STAT3 signal pathway's inhibition has been validated that it can induces apoptosis in melanoma cells. Derivatives of amentoflavone have previously been discovered as Type II inhibitors of JAK2. These compounds inhibited JAK2 activity and down-regulated STAT3-directed expression. In this study, we investigated the antitumor potency of amentoflavone derivatives against melanoma. We found that compound **1** showed potent inhibition of JAK2 autophosphorylation and also suppressed the growth of human melanoma cells A375 with an IC_{50} value of 2.31 μM. Importantly, compound **1** also showed inhibition of STAT3 target proteins Bcl-2, Bax, c-myc, and cyclin D1, which are associated with the regulation of apoptosis and the cell proliferation. Taken together, these results showed that compound **1** represents a promising antitumor agent for melanoma therapy.

摘要

黑色素瘤是一類惡性程度極高，疾病進程較快的皮膚癌。由於黑色素瘤容易發生轉移同時對放化療治療不敏感，因此臨床治療黑色素瘤一般以手術為主，然而病人术後也極容易出現癌症復發或者癌細胞轉移。目前癌症靶向治療越來越受到人們的青睞，對此的研究也越發深入。有研究顯示 JAK2/STAT3 細胞通路能夠影響多種癌症的惡化進程，對下一代抗癌藥物的研發有積極的影響。研究顯示抑制 JAK2/STAT3 通路能夠誘導黑色素瘤細胞凋亡，從而影響癌細胞的增殖。穗花杉雙黃酮的衍生物作為經過優化的 JAK2 二型抑制劑，具有明顯的拮抗 JAK2 激酶磷酸化的作用並能顯著影響其下游蛋白 STAT3 的表達。本文探討了上述化合物在黑色素瘤細胞增殖過程中的抗癌活性。結果顯示，在衆多的衍生物中，化合物 **1** 能夠抑制黑色素瘤細胞 A375 生長（IC_{50} = 2.31 μM），並且對 JAK2 蛋白的活化也有較為明顯的抑制。與此同時，對 JAK2 的下游蛋白 STAT3 靶向基因的表達存在相應的影響。例如能夠調控促凋亡蛋白 Bcl-2 和抗凋亡 Bax 的基因表達，以及對能夠影響調控細胞週期的蛋白 cyclin D1 和與細胞生長緊密聯繫的蛋白 c-myc 的基因表達。給葯處理後的黑色素瘤細胞 A375 最終表現出促細胞凋亡和抗癌細胞增殖的特點，因此化合物 **1** 有希望給黑色素瘤的治療帶來助力。

關鍵字：JAK2/STAT3，黑色素瘤，二型 JAK2 抑制劑

Table of Contents

Declaration ii
Acknowledgements iii
Abstract iv
Table of Contents vi
List of Tables and Figures viii
Glossary ix
List of Abbreviations x
Chapter 1 Introduction 1
1.1 Malignant melanoma 1
1.1.1 Therapeutic targets for malignant melanoma 1
1.1.2 Clinical pharmacotherapy 2
1.1.3 Drug resistance 2
1.2 The JAK2/STAT3 pathway 3
1.2.1 Mechanism for JAK2 protein 4
1.2.2 Mechanism of JAK2/STAT3 signaling 4
1.2.3 JAK2/STAT3 signal pathway in melanoma 6
1.3 Significant for Type II inhibitors 9
1.4 Structure-based discovery and optimization of Type II JAK2 inhibitors 11
1.4.1 Identification of natural product scaffolds as Type II JAK2 inhibitor 11
1.4.2 Optimization of natural product-like Type II JAK2 inhibitors 13
1.5 Hypothesis 14
Chapter 2 Methods and Materials 15
2.1 Research methodology 15
2.1.1 Determination of JAK2 inhibitor cytotoxicity 15
2.1.2 Inhibition of downstream protein expression 15
2.1.3 Detection of apoptosis 16
2.1.4 Detection of cell proliferation 16
2.1.5 Statistical analysis 17
2.2 Materials 17
2.2.1 Reagents 17
2.2.2 Cell culture 18
2.3 Methods 18

2.3.1 Cell viability assay 18
2.3.2 Western blot analysis 19
2.3.3 Flow cytometric analysis of apoptosis 19
2.4 Result 20
2.4.1 Amentoflavone analogues inhibit melanoma cell viabilities 20
2.4.2 Effect of compound **1** in human melanoma cells A375 21
2.4.3 Induction of apoptosis in melanoma cells 24
2.4.4 Interrupting the cell proliferation of melanoma cells 28
Chapter 3 Conclusions 31
References 32
Appendices 39
Publications 41

List of Tables and Figures

Table 1.1 Recent drugs in clinical or pre-clinical trials for targeted melanoma therapy 2

Table 1.2 General comparisons between Type I/II inhibitors 11

Table 1.3 Conventional binding feature between Type I and Type II JAK2 inhibitors 11

Table 1.4 Structures of amentoflavone analogous. 14

Fig. 1.1 Schematic drawing for STAT3-induced carcinogenesis 6

Fig. 1.2 The JAK2-STAT3 signaling pathway in melanoma 7

Fig. 1.3 The chemical structure of amentoflavone. 12

Fig. 2.1 Cell viability assay of amentoflavone analogues in melanoma cells and normal kidney cell .. 21

Fig. 2.2 Inhibitory effect of compound **1** against JAK2 phosphorylation. 22

Fig. 2.3 Compound **1**'s inhibition against p-JAK2 expression in A375 cells. 22

Fig. 2.4 Compound **1** positively regulated the JAK2 downstream protein STAT3 in A375 cells 23

Fig. 2.5 Compound **1** induced apoptosis by down-regulating expression of Bcl-2 and up-regulating the expression of Bax through JAK2/STAT3 cascade.. 24

Fig. 2.6 Compound **1**'s inhibiton against Bcl-2 expression in A375 cells 25

Fig. 2.7 Compound **1** induced apoptosis. 26

Fig. 2.8 Compound **1** influenced caspase 3 cleavage in A375 cells 27

Fig. 2.9 Compound **1** induced apoptosis in A375 melanoma cells 27

Fig. 2.10 Compound **1** decreased cyclin D1 expression 28

Fig. 2.11 Compound **1** decreased expression of c-myc in A375 cells. 29

Fig. 2.12 Compound **1**'s inhibition effect against c-*myc* 30

Fig. S1 The synthetic procedure for amentoflavone analogous 40

Fig. S2 Different IC_{50} values of Amentoflavone analogues 40

Glossary

Virtual screening	A computational technique which used in drug discovery research to identify possible lead structures against a specific pharmaceutical target.
D-type cyclin	General name of cyclins D1, D2, and D3

List of Abbreviations

°C	Degrees Celsius
AKT	Akt murine thymoma viral oncogene
ATP	Adenosine 5′-triphosphate
Bak	Bcl-2-antagonist/killer
Bax	B-cell lymphoma-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-x_L	B-cell lymphoma-extra large
bFGF	Basic fibroblast growth factor
bHLH/LZ	a basic-helix-loop-helix/leucine zipper
BRAF	B-Raf proto-oncogene, serine/threonine kinase
Caspase 3/9	Cysteinyl aspartate specific proteinases 3/9
CCND1	Cyclin D1
Cdc25A	Cell division cycle 25A
Cdks	Cyclin-dependent kinases
c-myc	The Myc proto-oncogene encodes a ubiquitous transcription factor
Cocktail	Protease inhibitor Cocktail
CSC	Cancer stem cells
CXCL10	IFN-γ-inducible protein10
Cyclin D1	Putative G_1 cyclin
Cyt c	Cytochrome c

DFG	Conserved Asp-Phe-Gly motif
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOLHPIN	Deletion-of-loop Asp-Phe-Gly-in
DTIC	Alkylating agent dacarbazine
EC_{50}	Concentration for 50% of maximal effect
Epo	Erythropoietin
ERK	Extracellular-signal-regulated kinase
GNA11	G11α
GNAQ	G protein alpha subunit q
GPCR	G-protein coupled receptor
HCV	Hepatitis C virus
HEK293T	Human embryonic kidney cells 293
HIF-1α	Hypoxia-inducible Factor 1α
HTS	High-throughput screening
IC_{50}	Half maximal inhibitory concentration
ICM	Internal coordinate mechanics
IDO1	Indoleamine 2,3-dioxygenase-1
IFN-γ	Interferon-γ
IL6	Interleukin 6

JAK1	Janus kinase 1
JAK2	Janus kinase 2
JAKs	Janus kinases
MAPK	Ras–mitogen-activated protein kinase
Mcl-1	ML1 myeloid cell leukemia 1
MEK 1/2	MAPK/ERK kinases 1 and 2
MET	The receptor for hepatocyte growth factor
MITF	Microphthalmia-associated transcription factor
MM	Malignant melanoma
MMP	Metalloproteinase
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappa B
Oct 3/4	Octamer-binding transcription factor 3 and 4
P/S	Penicillin and streptomycin
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
Pim	Polymorphic immunodominant molecule

Plk1	Polo-like kinase 1
PMSF	Serine protease inhibitor
PS	Phosphatidylserine
Ras/Raf/MEK	Mitogen-activated protein kinase/ERK kinase
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RT	Room temperature
RTK	Receptor tyrosine kinase
SH2	SRC homology-2
STAT3	Signal transducer and activator of transcription-3
STATs	Signal transducers and activators of transcription
TBST	TBS containing 0.1% Tween-20
TCHM	Traditional Chinese herbal medicine
TCM	Traditional Chinese medicine
T_H	T helper
TYK	Non-receptor Protein Tyrosine Kinase-2
VCP	Valosine containing protein
VEGF	Vascular endothelial growth factor

Chapter 1 Introduction

1.1 Malignant melanoma

Malignant melanoma (MM) is a resistant form of skin cancer that has poor prognosis for patients. Melanoma is among the top ten leading cancer types and the biological basis underlying the tumor-promoting characteristics of melanoma remains unclear, in part due to its heterogeneity[1]. Research has indicated that melanocytes have a low proliferation potential and are capable of preventing programmed cell death (PCD), which increases mutations' retention time and culminates in cutaneal melanoma formation, as well as leading to a low level of spontaneous apoptosis[2, 3]. The depression of apoptosis is associated with the initiation of metastasis in cutaneous melanoma. Metastatic melanoma cells can invade into the vascular or lymphatic system and adhere to organs like the brain, lung, liver and skin and continue proliferating, and over time the melanoma cells can change to vertical growth and initiate the mesenchymal transition. This aggressive characteristic of melanoma is a primary reason for its poor prognosis[4, 5].

1.1.1 Therapeutic targets for malignant melanoma

The Mitogen-activated protein kinase/extracellular-signal-regulated kinase (ERK) pathway (Ras/Raf/MEK), phosphoinositide 3-kinase pathway (PI3K) and microphthalmia-associated transcription factor pathways (MITF), among other pathways, are recognized to be important pathways for the progress of cell carcinogenesis[6]. These pathways have been targets for the discovery of antitumor drugs. For example, therapeutic drugs for melanoma have shown targeted the Ras–mitogen-activated protein kinase pathway (MAPK): sorafenib, GSK2141795B, and vemurafenib; the G protein alpha subunit q/G11α (GNAQ/GNA11) pathway: AZD6244; or the PI3K pathway: ZSTK474[7]. Examples of drugs being tested in clinical or pre-clinical trials for treating melanoma are shown in **Table 1.1**.

Table 1.1 Recent drugs in clinical or pre-clinical trials for targeted melanoma therapy.

Drug	Targets	Study phase	References
XL184	The receptor for hepatocyte growth factor (MET)/ Vascular endothelial growth factor (VEGF) receptor inhibitor	II	[8]
NCT01378377	MAPK/ERK kinases 1 and 2 (MEK1/2) inhibitor and Mammalian target of rapamycin (mTOR) inhibitor	I	[9]
GDC-0068	Akt murine thymoma viral oncogene (AKT) inhibitor	I	[10]
INCB024360	IDO1 inhibitor	I	[11]
AG311	HIF-1α inhibitor	Preclinical	[12]
BI 6727	Plk1 inhibitor	Preclinical	[13]

1.1.2 Clinical pharmacotherapy

Melanoma cell is a well know skin cancer with the characteristic of aggressive and chemotherapy resistant as well as poor prognosis especially in metastatic stage[3]. Metastasized melanoma patients suffer a low survival rate due to the lack of effective treatment options for melanoma. To date, no effective systemic therapy for malignant melanoma has yet been developed[14]. Current medication standard of care for melanoma treatment is interferon adjuvant therapy, which only has a modest success rate. The first FDA approved prescription for melanoma therapy, Dacarbazine, show low response rate and unsatisfied benefit for the patient with metastatic melanoma in clinical trial[15]. Other chemotherapies such as gangliosides, ipilimumab and bevacizumab, have been tested in melanoma but have not offered evidence of improved overall survival, and have therefore not yet been authorized for the treatment of cutaneous melanoma[16, 17]. Therefore, a new therapeutic strategy for melanoma is urgently needed[18, 19].

1.1.3 Drug resistance

The vast majority of chemotherapy drugs have not been effective at treating malignant melanoma. Drugs used for the treatment of melanoma, such as the alkylating agent dacarbazine (DTIC), show high chemoresistance and low response rates, which has been attributed to dysfunctional apoptosis. One of the major mechanisms of drug resistance is alterations in cell cycle and apoptosis regulation[20].Therefore, suppression of the pro-apoptotic pathway or activation of anti-apoptotic factors are thought to increase drug resistance in melanoma[21-23]. The mitochondrial pathway is regulated by the B-cell lymphoma 2 (Bcl-2) family which is a major apoptotic signal. Both the B-cell lymphoma-2-associated X protein (Bax) and B-cell lymphoma-extra large (Bcl-xL) are act to regulate cytochrome c release which contributes to apoptosome formation. This then activates caspase-3 to eventually induce PCD[20]. Meanwhile, the over-expression of Bcl-2 in melanoma is thought to prevent apoptosis induced by chemotherapeutic drugs[24, 25]. Most inhibitors targeting survival pathways such as B-Raf proto-oncogene, serine/threonine kinase (BRAF), Akt murine thymoma viral oncogene pathway and MAPK signaling have induced chemoresistance in melanoma[26, 27].In short, strategies that interfere with the cell proliferation and apoptosis have great potential for melanoma therapy[28]. Recent studies have demonstrated that the regulation of PCD could be a promising target for treating MM[29, 30]. Identifying additional molecular targets and understanding the mechanism of melanoma tumorigenesis could help develop new inhibitors for treating MM. Our group has hypothesized that the Janus kinase 2 (JAK2)/signal transducer and activator of transcription-3 (STAT3) pathway could be a potential target for promoting apoptosis in melanoma cells.

1.2 The JAK2/STAT3 pathway

The JAK/STAT signaling pathway is important for signal transduction from the cell membrane to nuclear genes[31]. Persistent activation of JAK2/STAT3 signaling pathways have been shown to trigger tumorigenesis, including in melanoma[31, 32]. The JAK2/STAT3 signal pathway plays an important function in promotion of

oncogenic and metastatic phenotypes in melanoma[33]. Therefore, JAK2/STAT3 signaling may be a promising target or the development of inhibitors to treat advanced melanoma.

1.2.1 Mechanism for JAK2 protein

Janus kinases (JAKs) are cytoplasmic protein kinases that are essential for cytokine-mediated signal transduction. JAK2 will be active in trans by other JAK kinases, witch response to the JAK-STAT signaling reactivation, a state of persistent JAK2 signaling[34]. Mechanically, JAK family proteins associate with a wide array of cytokine and hormone receptors, as well as receptors of growth factor which lack of intrinsic kinase activity. Such cytokine and growth factor play an important part in cell proliferation, cell differentiation and apoptosis of malignant cells[35]. Considering the pathogenic role that JAK2 plays in various malignancies, it has become the more extensively studied compared to the other JAKs[36, 37]. JAK2 is known to regulate tumorigenesis in melanoma. Activated JAK2 kinases can induce autophosphorylation of STAT3, leading to the overexpression of oncogenic genes[38]. JAK2 kinases can also modulate PCD through regulating the activities of Bcl-2 family, such as expression levels of Bcl-2 and Bcl-xL[35, 39, 40]. JAK2 can regulate downstream targets such as STAT3, putative G1 cyclin (cyclin D1), c-myc, Bcl-2 and Bax which are controllers of cell proliferation and apoptosis[41, 42]. Interestingly, inhibition of JAK2 increased overall survival in a mouse breast cancer model and reduced tumor seeding and metastasis[43, 44].Consequently, inhibiting JAK2 is a promising anticancer strategy for metastatic malignant melanoma that could be exploited for further therapeutic benefit.

1.2.2 Mechanism of JAK2/STAT3 signaling

STAT3, an essential part of the STAT family, and is persistently activated in a wide array of human tumors. A brief mechanism of STAT3-induced carcinogenesis is shown in **Fig.1.1**[45]. JAK2 specifically phosphorylates a conserved tyrosine residue nearby

the C-terminus of STAT3. After activation by JAK proteins, STAT3 itself can form homodimers, but can also form heterodimers with STAT1 or STAT5, and these dimers will subsequently enter the nucleus and bind to deoxyribonucleic acid (DNA)[46-48]. Animal studies have revealed that disruption of the STAT3 gene will result in embryonic lethality, unlike other members of the STAT family[49, 50]. STAT3 is an indispensable and pivotal mediator in cell survival and proliferation, angiogenesis, apoptosis as well as cell migration through regulating the expression of ML1 myeloid cell leukemia 1 (Mcl-1), Bcl-xL and Bcl-2, as well as the proliferation-associated proteins, including cyclin D1 and c-myc[51-53]. Inhibition of STAT3 activity not only induce cell apoptosis, but also amplify the therapy outcome in a syngeneic mouse melanoma model through the "bystander effect"[54-56]. Moreover, the phosphorylation level of STAT3 could be a potential biomarker for melanoma malignant progression[57].

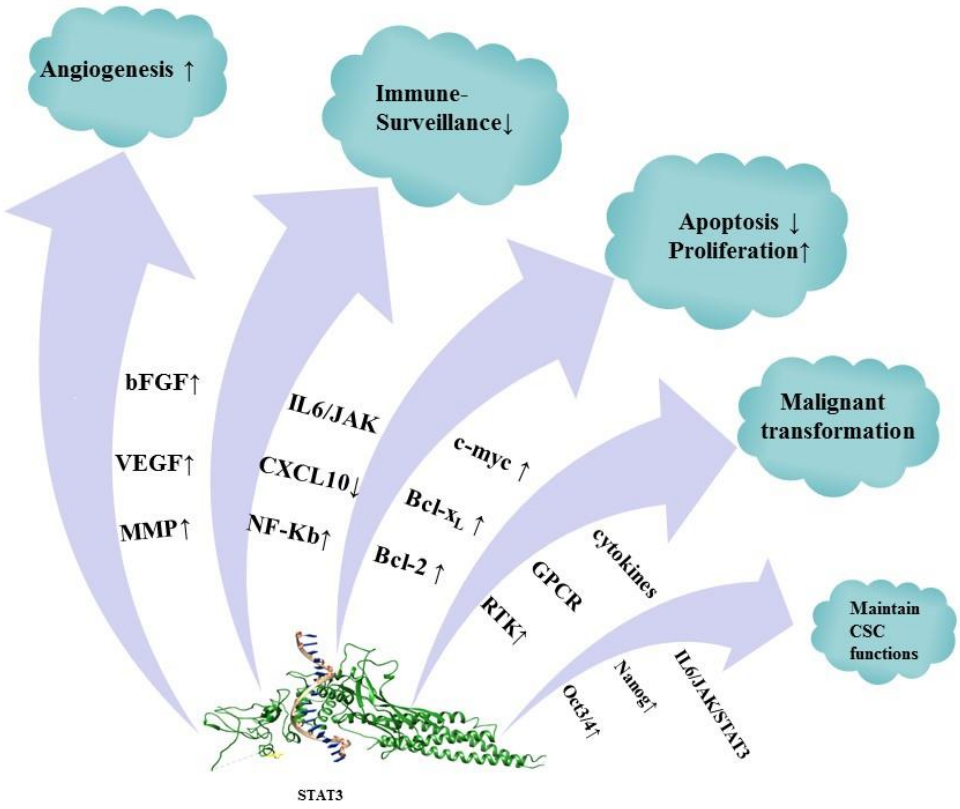


Fig. 1.1 Schematic drawing for STAT3-induced carcinogenesis[58]. Abnormal STAT3 regulates target genes that control malignant transformation, maintain cancer stem cells (CSC), inhibit apoptosis

and immunosurveillance, promote angiogenesis and enhance cell proliferation.

MMP; metalloproteinase, bFGF; basic fibroblast growth factor, IL6; interleukin 6, CXCL10; IFN-γ-inducible protein10, NF-κB; nuclear factor-kappa B, RTK; receptor tyrosine kinase, Oct 3/4; octamer-binding transcription factor 3 and 4.

1.2.3 JAK2/STAT3 signal pathway in melanoma

Melanoma is a cancer that maintains its own survival by modulating particular molecular pathways. Limitations of current therapies for MM have impelled researchers to explore the biology basis of melanoma and identify new targets for clinical therapy. The JAK2/STAT3 pathway, which plays important roles in cell proliferation and apoptosis, has been identified as therapeutic targets for melanoma[59, 60].

JAK2 is a critical regulator of constitutive and inducible STAT3 activation associated with diverse receptors[61-63]. The JAK2/STAT3 pathway is considered to be a central pathway that regulates cell proliferation and cell death. Cytokinesis, growth factors and hormones are the main activators of the pathway[64, 65]. JAK2 activation occurs through trans-phosphorylation of JAK2. Subsequently, the activated JAKs phosphorylate STAT3, which forms dimers through phosphate-SH2 domain interactions, and the STAT3 dimers enter the nucleus[66, 67]. STAT3 then modulates a wide variety of specific genes after nuclear translocation such as BCL-2, BCL-X, cyclin D1 (CCND1), MCL-1, IL-17, IL-23 and vascular endothelial growth factor (VEGF), which have anti-apoptosis, pro-proliferation, angiogenic as well as metastatic functions[68-72].

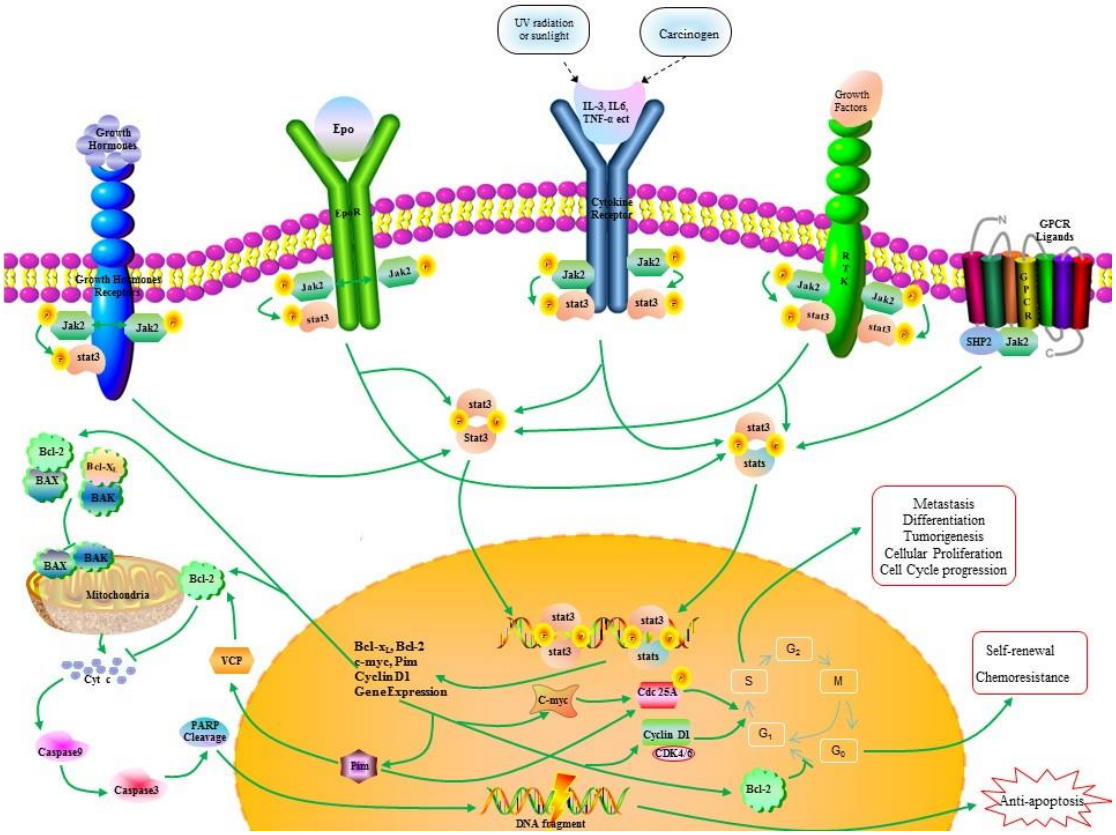


Fig. 1.2 The JAK2-STAT3 signaling pathway in melanoma. JAK2 can be activated by various receptors after the ligands bind to receptors on the cell surface. Subsequently, JAK2 activates STAT3, which can form homodimers or heterodimers via their SRC homology-2 (SH2) domains. STAT3 dimers eventually enter the nucleus and activate target genes such as anti-apoptotic Bcl-2 and Bcl-xL and polymorphic immunodominant molecule (pim) and cell cycle regulator cyclin D1. STATs; signal transducers and activators of transcriptions, Epo; erythropoietin, IL3/6; interleukin 3/6, IFN-γ; interferon gamma, RTK; receptor tyrosine kinases, SHP2; tyrosine phosphatase non-receptor type 11, Caspase 3/9; cysteinyl aspartate specific proteinases 3/9, cdc25A; cell division cycle 25A, VCP; valosine containing protein.

Main challenges for the clinical therapy of melanoma are therapy resistance and tumor relapse. Blocking pro-apoptotic pathways like JAK2/STAT3 signaling is a promising therapeutic strategy to overcome chemoresistance in melanoma[29]. Accordingly, mechanism for JAK2/STAT3 signal pathway induce apoptosis and cell proliferation is worth studying[73].

STAT3 can regulate apoptosis and the cell-cycle through controlling the genes expression. To determine the drug potency, the mechanism of JAK2/STAT3 and the function of downstream proteins should be clarified. In this signal pathway, Bcl-2 acts as an important anti-apoptotic protein that promotes caspase 3 inactivation and provides

a unique survival signal to allow oncogenes such as the myc proto-oncogene encoding a ubiquitous transcription factor (c-myc) to persist until being activated. In other words, inhibition of Bcl-2 expression will stabilize the mitochondrial membrane and up-regulate cytochrome c (cyt c) release, which leads to the activation of caspase 3 and poly(ADP-ribose) polymerase (PARP)[74-76]. Death protease caspase 3 played a central role in apoptotic program and trigger PARP protein cleave between Asp214 and Gly21[77, 78]. PARP served as signal for various cellular response to genotoxic damage[79]. Both were regarded as key mediators of mitochondrial apoptotic activity. The cleavage product contributes to the assessment of both caspase 3 and PARP protein activity. Measurement of the cleavage of caspase 3 and PARP provides an index for the evaluation of cell apoptosis status. caspase-3 is also important for the apoptotic program and an important influencing factor for the cleavage of PARP during cell death. PARP cleavage is known to have a positive effect on the onset of apoptosis[78]. Bcl-2 not only acts as the molecular switch in PCD, but also has an important function in the cancer cell proliferation, because of its antiproliferative effect. Bcl-2 and Bcl-xL could slow down cell cycle progression by facilitating G0/G1 arrest and prolong cell into G_1/S cell-cycle-transition[80-83]. The 'chain effect' described above is presented schematically in **Fig. 1.2**.

STAT3 inhibition will promote the expression of the pro-apoptotic gene Bax, which also up-regulates caspase-3 expression. Caspase-3 is generally accepted as a bio-marker for the induction of apoptosis[75, 84]. As heterodimerizing partner of Bcl-2 and Bcl-xL, Bax protein is important for the cell death pathway as well. Accumulation of Bax/Bak complexes on the outer membrane of mitochondria result in cyt c releasing. However, anti-apoptotic effectors Bcl-2 and Bcl-xL will have antagonistic effect on Bax/Bak oligomerization[85, 86]. The reason is that the conformation among Bax and Bcl-2 are very similar to each other **(Fig. 1.2**). In addition, Bcl-xL like Bcl-2 is an inhibitor of mitochondrial apoptosis[87-89]. As STAT3 downstream targets, Bcl-2 and Bax play crucial roles in apoptosis regulation. Bcl-2 is a pivotal regulator that prevents

cell death by competitively dimerizing with Bax, thus blocking the oligomerization of Bax in melanoma. The substantial repression of PCD is a characteristic hallmark of melanoma.

Activated STAT3 up-regulates c-myc expression to induce cell transformation[53]. c-myc, the Myc proto-oncogene, acts as a growth regulator to trigger cell proliferation, apoptosis and cellular transformation associated with metastasis invasiveness and poor prognosis in melanoma[90-92]. c-myc is also a key regulator towards G_1/S cell-cycle-transition as well as an inducer of cdc25A[92, 93]. At the same time, the STAT3-regulated gene pim associates with c-myc through enhancement of cdc25A phosphorylation and activation to regulate the transition from G_1 to S phase[94, 95]. Functionally, pim is likely participate in the anti-apoptosis signal mediated by STAT3 which could modify valosine containing protein (VCP) and lead to Bcl-2 induction, ultimately causing cell proliferation[96, 97].

Inhibition of cyclin-dependent kinases (Cdks), which consist of a kinase subunit and an activating subunit, can cause cell cycle arrest[98]. Cyclin-dependent kinases are well-known regulators of the cell cycle progression. CDK4 and CDK6 are fundamentally regulated by cyclin D1 at the G_0/G_1 phase[99, 100]. The D-type cyclin, cyclin D1 is one of the activating subunits of Cdks and acts as a growth factor sensor. The expression of cyclin D1 is more sensitive to extracellular cues than the position in the cycle[101, 102]. As the cell enters the cycle from the G_0 phase, cyclin D1 will assembly with CDK4 or CDK6. Then, the holoenzymes will promote progression through G1 and persist throughout the cycle. Decreasing cyclin D1 will inhibit the kinase activity and repress the cell cycle at the G_0/G_1 phase which will eventually lead to antiproliferation[102-104].

1.3 Significant for Type II inhibitors

AG490 is a tyrphostin compound regarded as the first reported JAK2 inhibitor, although it is not specific for JAK2[105]. Since then, many more inhibitors of JAk2 have been developed. Most of these are Type I inhibitors that mainly target the (Adenosine 5′-triphosphate) ATP binding site of JAK2 protein and stabilize it in an active confirmation, like ruxolitinib[106]. The active conformation of kinases is also known as the "DFG-in" (where DFG = Asp-Phe-Gly) conformation, in which the DFG phenylalanine packs into a hydrophobic region between the N- and C-lobes. In contrast, Type II inhibitors stabilize kinases in an inactive confirmation and can decrease activation loop phosphorylation[107]. In the inactive or "DFG-out" conformation, the phenylalanine residue rotates out of the pocket, which disrupts the orientation of the DFG aspartate and blocks the ATP binding site. Type II inhibitors can show higher kinase specificity relative to Type I inhibitors. This is because of the greater structural variability of the hydrophobic pocket in the DFG-out mode when compared to the ATP pocket[108, 109]. Hence, Type II JAK2 inhibitors represent a promising strategy for selectively inhibiting the JAK2 signal pathway. A detailed comparison of Type I/II kinase inhibitors can be found in **Table 1.2** and **Table 1.3**[108, 110].

Table 1.2 General comparisons between Type I / II inhibitors.

	Advantage	Disadvantage
Type I inhibitors	All kinases can adopt the DFG-in conformation.	Relatively low selectivity for ATP site.
	Inhibition of ATP binding.	Narrow binding region (only the ATP site).
	Less requirement for protein conformation.	Crowded patent space.
Type II inhibitors	Wider binding region (ATP site and allosteric site).	Only target kinases with a DFG-out conformation available.
	More chemical space to exploit (conformation among kinase in their inactive state)	
	Indirectly inhibit ATP binding.	Some of them require a specific conformation.
	High degree of selectivity	

Table 1.3 Conventional binding feature between Type I and Type II JAK2 inhibitors.

	Type I JAK2 inhibitors	Type II JAK2 inhibitors
Activation status	+	Inactive
Kinase binding site	Primarily bind in ATP site (in JAK2 active ‘‘DFG-in’’ conformation)	Usually bind in allosteric site (exploits an additional binding site that alter from DFG-in structure of JAK2 into a Type II-compatible conformation)
Selectivity	Usually low, but there is exception	Usually higher, the allosteric site could provide an additional handle for selectivity.
Conformation requirement	DFG-in conformation	DFG-out conformation
Inhibits ATP binding	Yes	Yes, indirectly

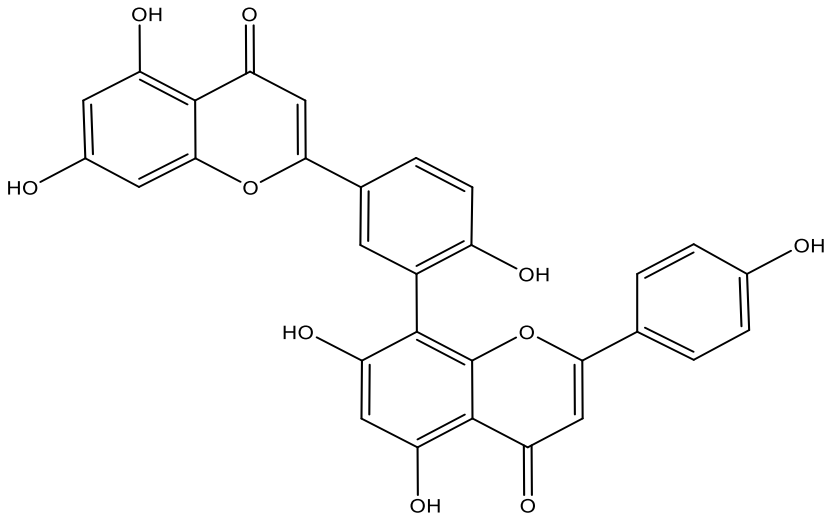
1.4 Structure-based discovery and optimization of Type II JAK2 inhibitors

Traditional Chinese herbal medicine (TCHM) is as an important part of classical traditional Chinese medicine (TCM), which have been utilized in China and other Asian countries for many centuries. From a pharmaceutical drug development viewpoint, TCHMs are a productive source of bioactive structures[111-114]. Flavonoids are widely distributed in the plant kingdom, including herbs, seeds, roots and tea, and have potential anti-inflammatory and anticancer effects[115, 116]. Amentoflavone is a biflavonoid that has been reported to trigger PCD in melanoma cell by decreasing Bcl-2 expression and inducing caspase 3 activation[117].

1.4.1 Identification of natural product scaffolds as Type II JAK2 inhibitor

The biflavonoid amentoflavone from the traditional Chinese herbal medicine Gingko biloba has been identified by our group to be a Type II JAK2 inhibitor through high-

throughput virtual screening[109]. The chemical structure of amentoflavone is shown in **Fig. 1.3**. Firstly, an inactive conformation of JAK2 designed for molecular docking-based screening was set up by using the deletion-of-loop Asp-Phe-Gly-in (DOLPHIN) protocol (**Fig. 1.4**)[118]. By using the DOLPHIN model, 150,000 natural products or natural product-like compounds from the Analyticon Discovery NATx and MEGabolite databases, the ZINC natural products database and the Hongcam natural products database were screened using molecular docking by the Internal coordinate mechanics (ICM) method. The ten compounds with the highest ICM scores were evaluated their ability to inhibit JAK2 phosphorylation in an ELISA assay. Amentoflavone showed dose-dependent inhibition of JAK2 enzyme activity with an estimated IC_{50} = 5 μM . This study suggested that amentoflavone was as a promising candidate for further optimization[109, 119].



(*S*)-8-(5-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)-2-hydroxyphenyl)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one

Fig. 1.3 The chemical structure of amentoflavone.

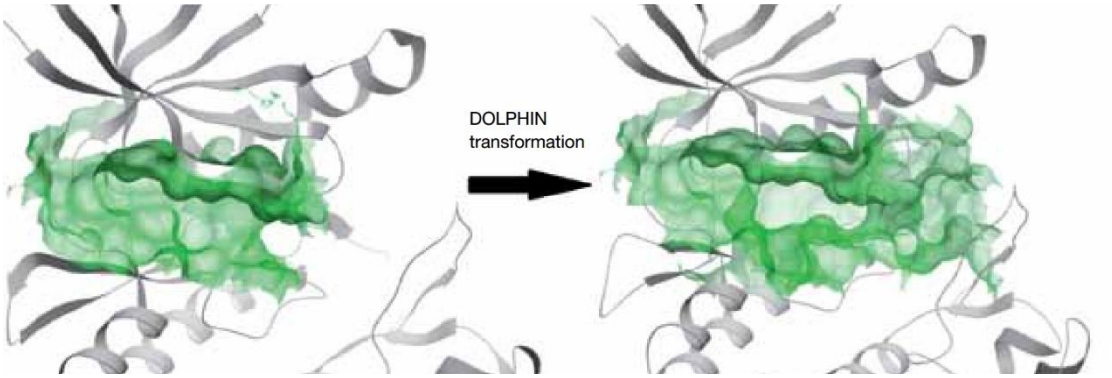
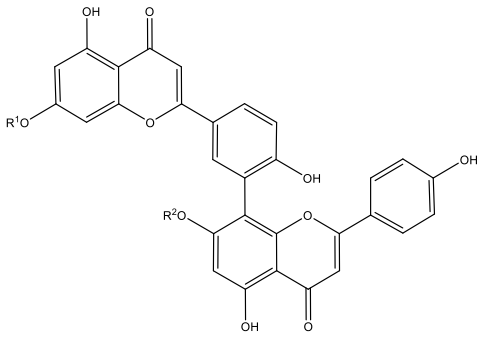


Fig. 1.4 The transformation from active conformation of JAK2 to inactive form of JAK2[119, 120]. According to the DOLPHIN model, the DFG motif and the next four residues in the sequence are removed, which converts the active conformation of JAK2 converted to an inactive form.

1.4.2 Optimization of natural product-like Type II JAK2 inhibitors

Amentoflavone could only partially occupy the large hydrophobic pocket that appeared in the DFG-out model of JAK2 in silico. Adding an additional aliphatic side chain to amentoflavone could allow the compound to better occupy the hydrophobic pocket. Thus, over 50 amentoflavone derivatives were designed and then docked against the DOLPHIN model of JAK2 in silico[109]. Eventually, eight top analogues with long alkyl chains were synthesised are shown in **Fig. 1.3** and **Table 1.4**[109, 119].

Table 1.4 Structures of amentoflavone analogous. Long aliphatic side chain process higher potential to extend deeper into the hydrophobic pocket.



Compound	R^1	R^2
Amentoflavone	H	H
1	H	$C_{16}H_{33}$
2	H	C_6H_{13}
3	H	$C_{13}H_{27}$
4	H	C_8H_{17}
5	C_6H_{13}	C_6H_{13}
6	C_8H_{17}	C_8H_{17}
7	H	$C_{10}H_{21}$
8	H	$C_{19}H_{39}$
9	H	Farnesyl

1.5 Hypothesis

In previous work, the ability of amentoflavone analogues to inhibit JAK2 autophosphorylation and suppress hepatitis C virus (HCV) activity was examined[109, 119, 121]. However, the application of amentoflavone derivatives to function against malignant melanoma has not been explored. Therefore, in the current work, we explored whether the Type II JAK2 inhibitors, i.e. the amentoflavone analogues, could show significant activity against human melanoma cells and induce apoptosis.

Chapter 2 Methods and Materials

2.1 Research methodology

Previous developed amentoflavone analogues were shown to be Type II JAK2 inhibitors. To investigate whether these natural products would have anticancer effects against melanoma and to study their mechanisms of action, a series of biological experiments were performed.

2.1.1 Determination of JAK2 inhibitor cytotoxicity

Cytotoxicity assay is a commonly used technique in molecular biology to evaluate the sensitivity of tumor cells towards inhibitors. The amentoflavone analogues will be tested against A375 cells and A2058 cells, which are chemotherapy-resistant human malignant melanoma cell lines. In addition, it will be important for the compounds to show low toxicity, including nephrotoxicity. The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays will be applied to cell viability determination. As the cells are killed or damaged by the cytotoxic analogues, there will be a reduction of formazan produced by the activity of mitochondrial dehydrogenases. From the assay, IC_{50} values of each compounds will be obtained. Normal human embryonic kidney cells 293 will also be treated with the amentoflavone analogues[122]. The IC_{50} values against the normal cells will allow us to determine the potential risk that the analogues might cause.

2.1.2 Inhibition of downstream protein expression

Western blotting, also known as immunoblotting, is a routine analytical technique in molecular cell biology to detect the presence of a particular protein. In this assay, specific antibodies are used against the target protein, which will generate a visible band whose thickness is related to the amount of protein present[123]. The use of Western

blotting will allow the detection of downstream proteins regulated by the JAK2/STAT3 pathway. Compounds that inhibit JAK2 would be expected to reduce the expression of downstream proteins regulated by the JAK2/STAT3 pathway.

2.1.3 Detection of apoptosis

Apoptosis is a normal phenomenon in cell life that modulates cell destruction or process of PCD to maintain tissue homeostasis and induce diverse biological and morphological variation[124, 125]. PCD is controlled by pro-apoptotic proteins known as Bax and Bak and anti-apoptotic proteins known as Bcl-2, Bcl-xL and Mcl-1[126]. Therefore, expression of Bcl-2 and Bax are critical biomarkers to evaluate the degree of apoptosis resistance in melanoma. Caspase-3 and PARP are classic effectors of DNA trauma that also function as biomarkers of apoptosis as well. Detection of these apoptotic protein markers can be achieved by Western blotting. The result can basically reflect the inhibitory effect toward cell apoptosis that compound **1** induced.

FITC Annexin V staining is a useful technique to determine the apoptosis status. By detecting whether the cell can be effectively stained, the stage of apoptosis in melanoma cells can be inferred. Fluorochrome-labeled Annexin V protein can selectively bind to phosphatidylserine (PS) that exists on the plasma membrane in healthy cells. Meanwhile, propidium iodide (PI) staining is used to measure DNA content and help distinguish the apoptotic and necrotic cells. Flow cytometry was used to measured cell apoptosis status. Thus, the ability of the test compounds that induce apoptosis in melanoma cells can be determined by these techniques.

2.1.4 Detection of cell proliferation

Malignant melanoma usually shows a high growth rate when it arrived at microinvasive phase. The carcinogenicity of cancer cell partially manifested in its influence on specific regulators that play a major role in G_1 phase progression. Cells will turn back

to G_0 phase or toward another division, once receive proper stimulation from extracellular signals[102]. During the $_{G1/}$S phase, DNA commence synthesis and cyclin D1 star induce cell growth. In the G_2 phase, cell continue to growth and prepare for mitosis. concurrently, c-myc, regarded as one of target gene of STAT3 could be found overexpression in melanoma. Inhibition of c-myc could effectively depress cell proliferation in melanoma. Detection the expression of c-myc and cyclin D1 proteins contribute to allude the drug potency and mechanism of signal cascade. As the functions of cyclin D1 and c-myc are more or less limited by their expression level, the detection of cyclin D1 functions could reflect the natural products' potency toward cell proliferation inhibition through identifying the distribution of cells cycle status.

2.1.5 Statistical analysis

All the presented data are expressed as means ± SD and the statistical significance comparisons were evaluated by one way ANOVA. $P < 0.05$ was considered statistically significant.

2.2 Materials

2.2.1 Reagents

The amentoflavone analogues, natural product-like compounds were identified by our group previously as Type II JAK2 inhibitors and were synthesized by Dr. Hui Yang and Dr. Wei Guo and from and Hong Kong Baptist University, respectively and University of Hong Kong. Compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/l.

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA).

JAK2 inhibitor NVP-BB-T594 was gift from Novartis Pharma AG (Basel, Switzerland). Anti-phospho-JAK2 with phosphorylation site at Y1007 and Y1008 , Anti-phospho-STAT3, anti-JAK2, anti-STAT, anti-Bcl-2, anti-Bax, anti-c-myc, anti-cyclin D1, anti-β-actin antibodies and the horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies against PARP and caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2.2 Cell culture

Normal human embryonic kidney cells 293, melanoma A2058 and A375 cells were preserved by our laboratory. They were routinely cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin (PS) and kept under humidified 5% CO2 mixed with 95% air and a temperature of 37 °C. The cells were regularly analyzed for the absence of mycoplasma contamination.

2.3 Methods

2.3.1 Cell viability assay

The MTT assay was used for measurement of the cell viability. Firstly, cells were seeded into a 96-well flat bottom microtiter plate at 5×103 cells per well in a fixed volume of 100 µl which help guaranteed 100% confluency of cells treatment. Then the plates were kept in the humidified cell incubator for 24 h for cell attachment. After incubation, gradient dilution of the analogues was performed to test the drug reaction. The compounds were mixed with the low FBS medium that the final concentration of DMSO will control in the range of 0 to 4% respectively. 72 h later, MTT solution (5 mg/ml) were dilute with DMEM medium in the ratio of 1:5 and 100 µL diluent were added to each well. Cells cultured in MTT solution (1mg/ml) were then incubate in for at least 4 h till the formation of cell-damaging formazan. After a while, the waste was

gently discarded and replace with 100 μl DMSO, in order to dissolved the formazan, for another 15min incubation. lastly, the color intensity was detected at 570 nm wavelength by the Thermo Scientific microplate reader. The IC_{50} values of the compounds (concentration required to decrease the absorbance by 50% when compared to the NVP-BB-T594) were determined by the dose-dependent manner of surviving cells. This assay was repeated three times independently.

2.3.2 Western blot analysis

A375 cells were seeded into a 6-well tissue culture plate with 6×105 cells per well in low FBS medium for 24h. Continuously, cells were treat with diverse concentration of compound **1** diluted in DMEM with 1% FBS for another 16h. After the drug treatment, cells were harvested then dissolved by using the radioimmunoprecipitation assay (RIPA) lysis buffer adding with protease inhibitor Cocktail (Cocktail) and Serine protease inhibitor (PMSF). Supernatant was collected after 20 min high speed centrifugation as the protein samples. Concentrations of protein samples were quantified by using BCA protein assay kit (ThermoFisher). 15μg total protein per samples were injected to 10% SDS-polyacrylamide gels and then resolved by electrophoresis. After gel electrophoresis, separated proteins were transferred to a PVDF membranes and incubated with blocking buffer, 5% nonfat milk diluted in TBS containing 0.05% Tween-20, at room temperature (RT) for 1 h. The next step was overnight probe with primary antibodies against the target protein at 4 °C. Followed by the incubation of secondary antibodies for 1h at room temperature, TBST washing is needed among each procedure additionally. Among each step, the bands should be washed 3-5 times with TBST. Chemiluminescence was applied to detect specific protein using the ECL western blotting substrate (ThermoFisher) under the X-ray exposure. Bio Rad chemiDoc XRS SRS system and Image Lab software (BioRad Hercules, CA) are powerful tool for visualization and data processing

2.3.3 Flow cytometric analysis of apoptosis

To determine the apoptosis, Annexin V Staining were commonly used and efficient tools to help. A375 cells were seeded into 6-well tissue culture plate at a density of 3×105 cells per well. After drug treatment for 16 h, cells were harvest and wash with phosphate buffered saline (PBS) at least three times to minimize the effect of impurity like trypsin. Cells were processed with regarded to the instructions of Annexin V–FITC Apoptosis Detection Kit (BD Biosciences). Apoptotic cells were measured by the BD Accuri C6 flow cytometer after staining. Data were analyzed by FlowJo X software.

2.4 Result

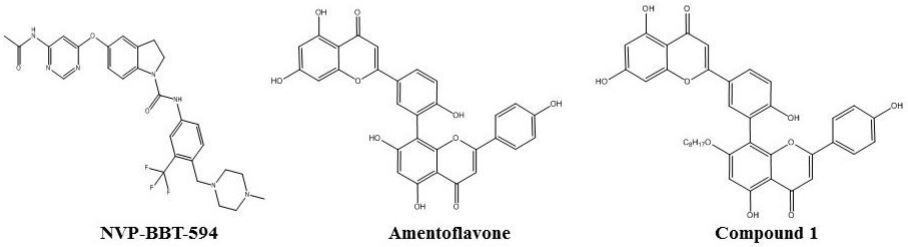
Compound **1–9** were potent analogues that design for the inhibition of JAK2 kinase as Type II inhibitors. The obviously different among them have been mainly reflected on the length of aliphatic side chain (**Table 1.4**). To evaluate the inhibition of this analogues against melanoma through JAK2/STAT3 signal pathway, we design series of bioassay.

2.4.1 Amentoflavone analogues inhibit melanoma cell viabilities

The cytotoxicity induced by the amentoflavone derivatives against metastatic malignant melanoma cells A375 and A2058 and Human embryonic kidney cells 293 (HEK293T) cells was determined by the cell viability assay. The result of this assays indicated that compound **1** exhibited inhibition effects on cell viability relative to the control compound NVP-BB-T594, and all the derivatives showed low cytotoxicity in the normal cell line HEK293T. NVP-BB-T594 is reported as a potent Type II inhibitor that could block JAK2 activity both in vitro and in vivo (**Fig.2.1(A)**)[127]. Interestingly, compound **1** was the most potent against melanoma cells A375, exhibiting an IC_{50} value of 2.31 μM in **Fig.2.1(B)** and was better than both amentoflavone and NVP-BB-T594, indicating that it had potential as a promising chemotherapeutic agent for melanoma. The result also shows that long aliphatic side chains are important for anticancer potency.

A

Compound	IC_{50} values (μM) A375 Melanoma cells	A2058 Melanoma cells	293T Human kidney cells
NVP-BBT594	7.35	5.31	>10
Amentoflavone	>10	>10	>10
1	2.31	6.94	>10



NVP-BBT-594 Amentoflavone Compound 1

B

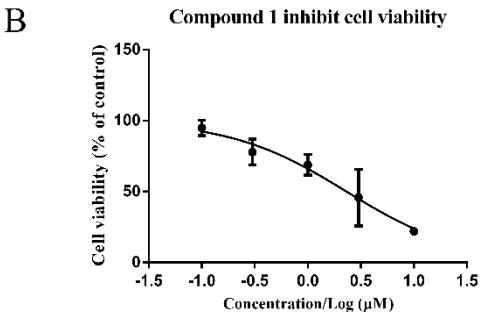


Fig. 2.1 Cell viabilities assay of amentoflavone analogues in melanoma cells and normal kidney cells. (A) The cell viability assay was conducted to assess the anticancer effect of the amentoflavone analogues against A375, A2058 melanoma cells and 293T normal kidney cells. The IC_{50} values were listed in **Appendices**. **(B)** Dose dependent manner of compound **1** in A375 melanoma cells. Cells were treated with gradient dilution of amentoflavone analogues for 72 h and the result was processed as percentage of the vehicle control.

2.4.2 Inhibition effect of compound 1 in human melanoma cells A375

In order to validate the mechanism of compound **1**, Western blotting was performed on treated A375 cells to assess the efficacy of the compound to block JAK2 autophosphorylation in vitro. Compound **1** blocked the activation of JAK2 kinase in a dosage-dependent fashion as shown in **Fig. 2.2** and **Fig. 2.3**, and the most potent inhibition was observed at a concentration of 3 μM. As expected, compound **1** can decrease JAK2 activation, with a concentration for 50% of maximal effect (EC_{50}) value of 0.16 μM.

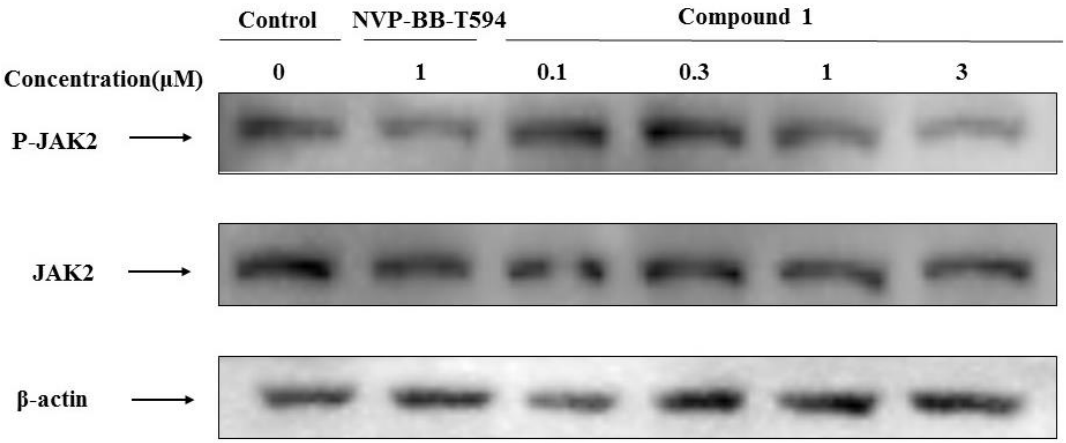


Fig. 2.2 Inhibitory effect of compound 1 against JAK2 phosphorylation. Human melanoma cells A375 were treated with compound **1** in a gradient concentration. JAK2 inhibition was increased as the concentration of compound **1** increased. The protein sample was electrophoresed by SDS-PAGE and probed with conjugated antibodies against phosphorylated JAK2 and total JAK2.

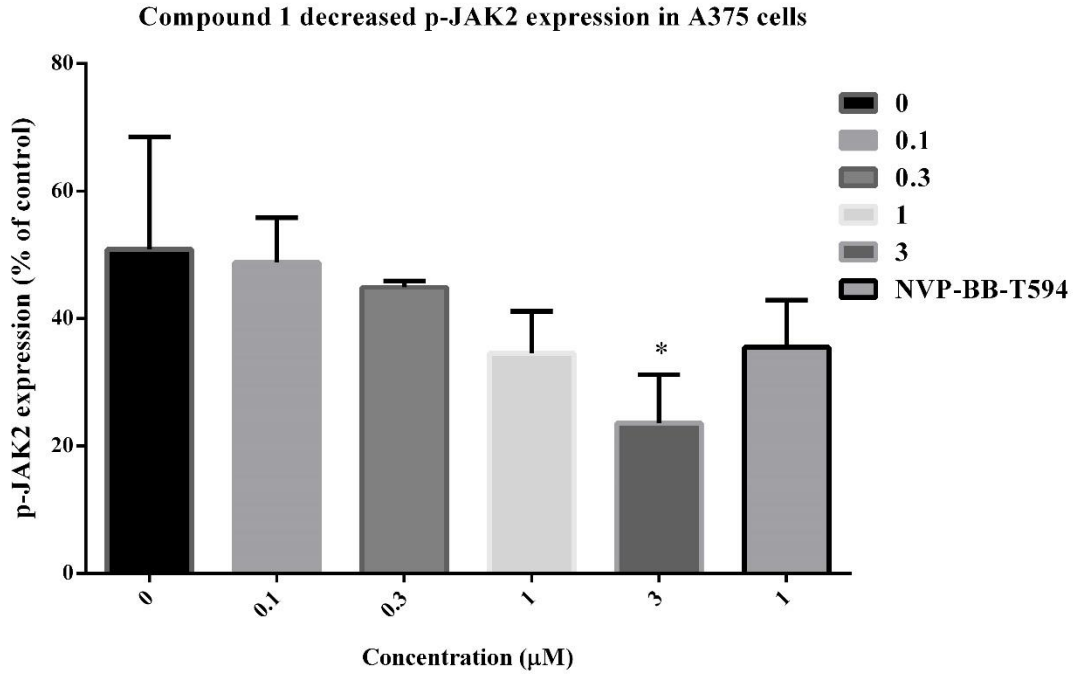


Fig. 2.3 Compound 1 inhibition against p-JAK2 expression in A375 cells. The data were extract from the result of Western blotting. Data were processed by GraphPad Prism 6.

As shown in **Fig. 2.3**, compound **1** shown dose dependent manner against p-JAK2 expression and had better inhibitory effect when compared with NVP-BB-T594. The most potent inhibitory was at the concentration of 3μM.

It is known that JAK2 activates its downstream protein, STAT3. The efficacy of compound **1** on STAT3 phosphorylation in treated A375 cells was also determined by Western blotting analysis. And western blotting analysis showed dose-dependent manner in expression of phosphorylate JAK2 previously.as the major target of the JAK2 kinase, expression level of phosphorylate STAT3 was inhibited by the compound **1** as well. Meanwhile, no significant changes in total STAT3 expression can be detected (**Fig. 2.4**).

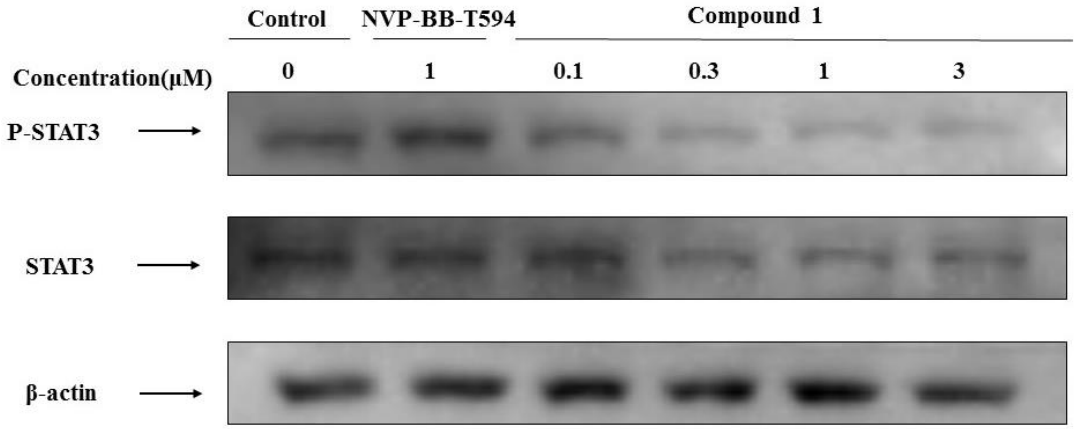


Fig. 2.4 Compound 1 positively regulate the JAK2 downstream protein STAT3 in A375 cells. A375 melanoma cells were treated with diverse concentrations of compound **1** for 16 h. The lysate sample was analyzed by SDS-PAGE and probed with conjugated antibodies against phosphorylated STAT3 and total STAT3.

The results revealed that depression of STAT3 activation could be partially attributed to the efficacy of compound **1**. Taken together, the analyzed result suggested that compound **1** was able to inhibit JAK2 activity including the downstream activity in melanoma cells.

2.4.3 Induction of apoptosis in melanoma cells

Disruption of apoptosis is a universal phenomenon occurring in malignant cancer cells, such as melanoma cells[32]. The DNA-binding activity of STAT3 will up-regulate several proteins' expression and Bcl-2 and Bax are one of them. Inhibition of JAK2/STAT3 signal are likely associate with the growth inhibitory effect by promoting apoptosis[128]. To measured, the effect of compound **1** in apoptosis inducement by interrupting STAT3 activation in melanoma cells western blot analysis was used. As representative regulators of apoptosis that are controlled by the JAK2/STAT3 pathway, the expression of pro-apoptotic factor Bax (EC_{50} = 0.07 μM) and anti-apoptotic factor Bcl-2 (EC_{50} = 0.15μM) were determined by immunoblotting in A375 cells treated with compound **1** (**Fig. 2.5** and **Fig. 2.6**).

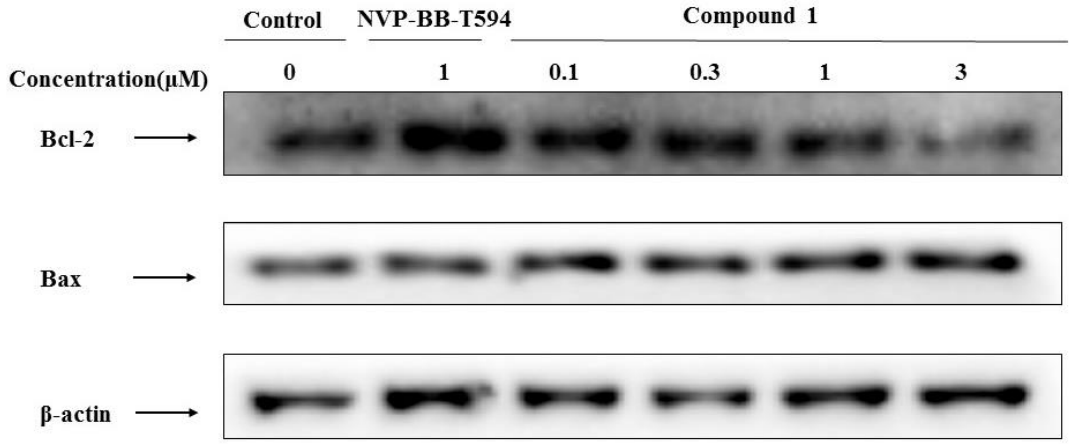


Fig. 2.5 Compound 1 induced apoptosis by down-regulated expression of Bcl-2 and up-regulated the expression of Bax through JAK2/STAT3 cascade. JAK2 was known as central mediator of STAT3. The Type II JAK2 inhibitor, compound **1**, effected on apoptosis in melanoma by blocking STAT3 signal and interrupting its DNA-binding activity.

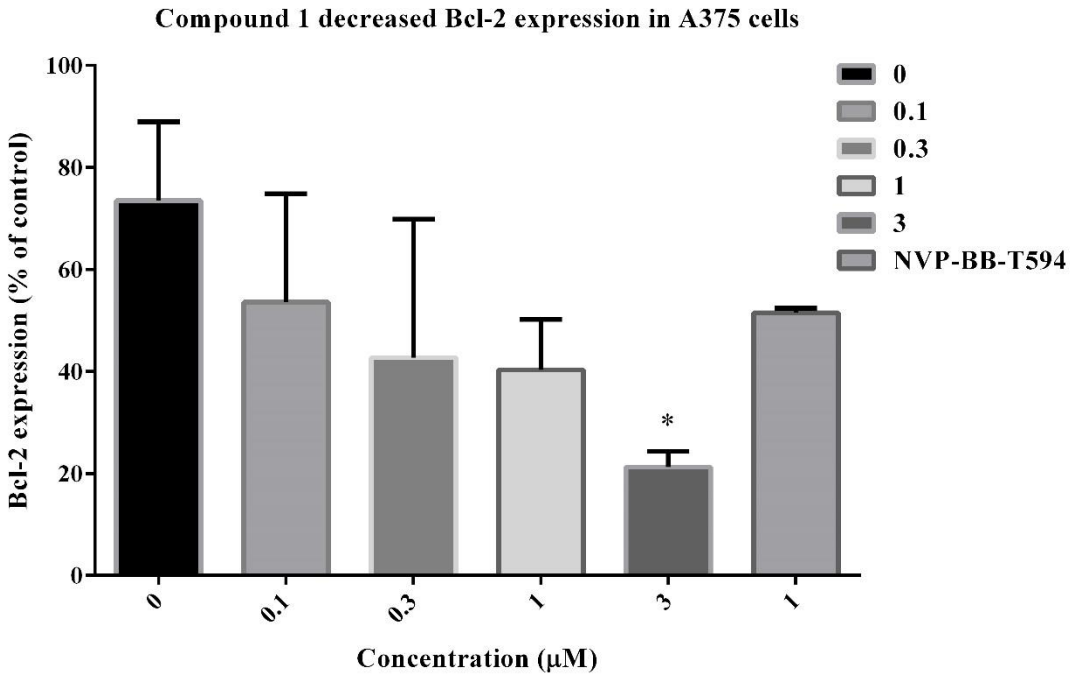


Fig. 2.6 Compound 1 inhibition against Bcl-2 expression in A375 cells. The data were extract from the result of Western blotting, described above. Data were processed by GraphPad Prism 6. The EC_{50} value of Bcl-2 equals to 0.15μM.

As shown in **Fig. 2.6**, the inhibitory effect of compound **1** against the anti-apoptotic Bcl-2 was better than the NVP-BB-T594. The most effective inhibition was at the concentration of 3μM.

Decreasing Bcl-2 expression associated with the increasing Bax expression illuminates a part of mechanism that how compound **1** induced melanoma apoptosis. The result demonstrated that depression of JAK2 and STAT3 activation by compound **1** associated with induction of apoptosis, the possible pathway can be found in **Fig. 1.2.**

Further tests were performed to validate the ability of compound **1** to induce apoptosis on the other hand. Caspase 3 and PARP proteins are associated with induction of apoptosis[129]. Inhibition of Bcl-2 by compound **1** depressed the releasing of cyt c from mitochondria, followed by increasing of apoptosis protein active caspase 3 and cleaved PARP, in **Fig. 1.2**. As the cleavage caspase 3 and PARP are normally occurs during early apoptosis, detection of their cleavage activity can elucidate mechanism of apoptosis in melanoma. As the caspase 3 and PARP are representative for the cell

apoptosis procedure, the evaluation of their expression can describe the ability of compound **1** against melanoma cells' apoptosis. Western blotting was applied to detect the expression of caspase 3, PARP proteins and their cleaved products (**Fig. 2.7** and **Fig. 2.8**).

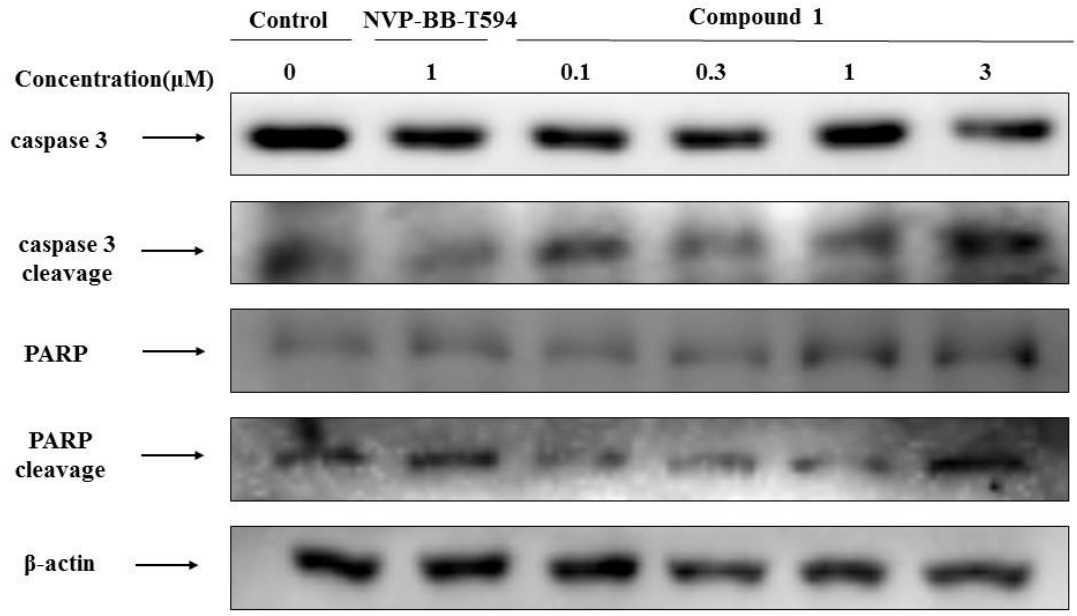


Fig. 2.7 Compound 1 induces apoptosis. The human melanoma cells were treated with compound **1** and then incubated for 16h. lysate samples were suspected to 10% SDS-PAGE and caspase 3 and PARP protein were separated by electrophoresis. The cleave product of caspase 3 and PARP were correlated with the concentration of compound **1**.

Protein immunoblot analysis of caspase 3 and PARP cleavage activity revealed that compound **1** induced cleavage generate that leaded to cell apoptosis through JAK2 inhibition. Compound **1** showed most potent inhibition at the concentration of 3μM, when inhibit the cleavage of caspase 3 and PARP. Meanwhile, the EC_{50} value of caspase 3 was 1.21μM.

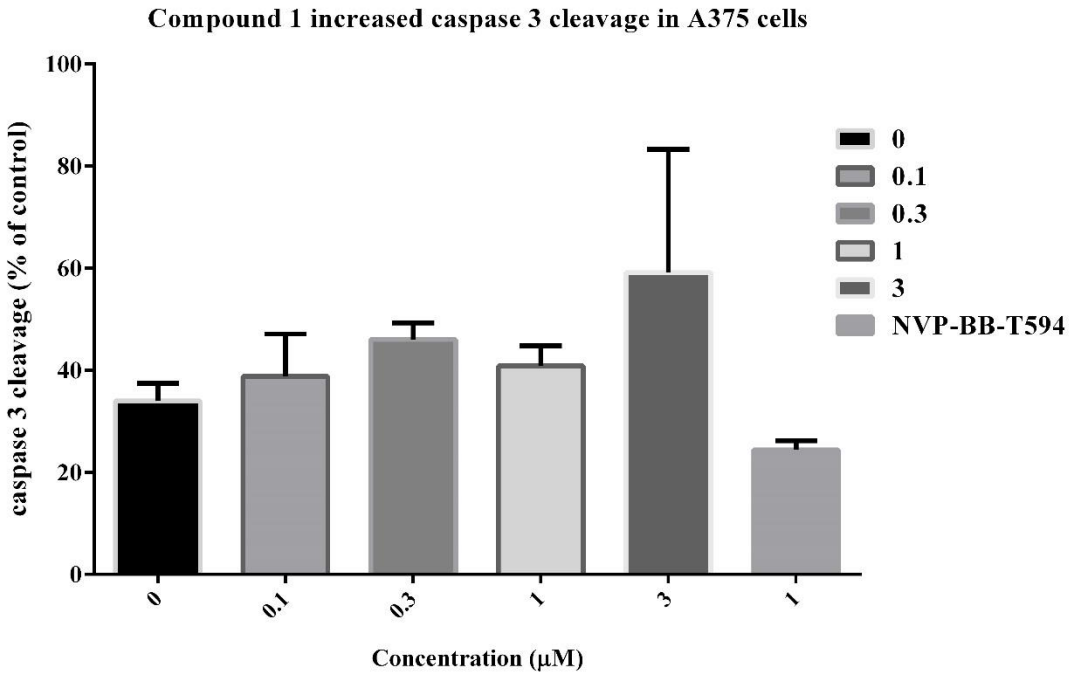


Fig. 2.8 Compound 1 influenced caspase 3 cleavage in A375 cells. The data were extract from the result of Western blotting, described in **Fig. 2.7**. Data were processed by GraphPad Prism 6. The EC_{50} value of cleaved caspase 3 equals to 1.21μM.

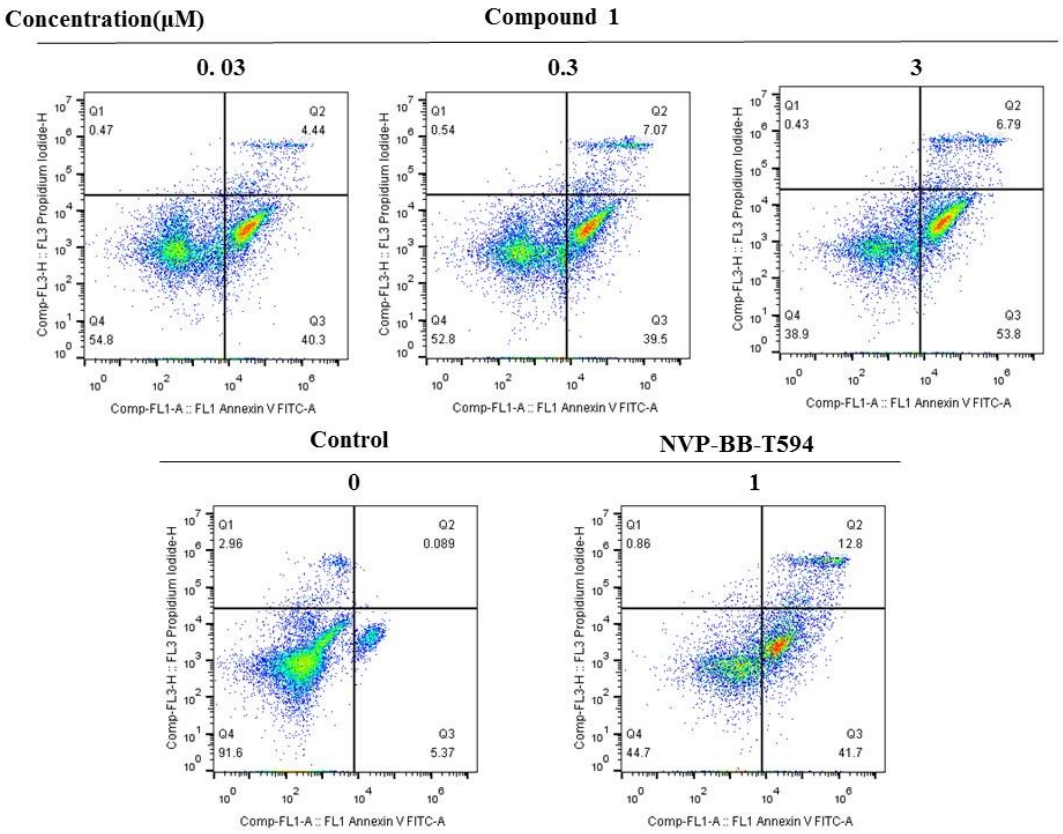


Fig. 2.9 Compound 1 induced apoptosis in A375 melanoma cells. Human melanoma cells A375 were treated with different concentration of compound **1** and incubated for 24h. Dead cell apoptosis assay were performed and analyzed by flow cytometry.

Flow cytometry analysis demonstrated that compound **1** can trigger cellular apoptosis. The result reveal that compound **1** induced melanoma cells' apoptosis mainly through inhibition of JAK2/STAT3 signal pathway.

In short, these results demonstrated that compound **1** showed obvious effects on inducing apoptosis, which indicates its potential to overcome the chemoresistance of malignant melanoma cells.

2.4.4 Interrupting the cell proliferation of melanoma cells

Senescence act as protective mechanism against unlimited proliferation in malignant tumor and the assemblage of cyclin D1 and CDK 4/6 was key regulator inducing DNA synthesis at G_1 phase[130, 131]. Interrupting the cell division by controlled the regulators that play a curtail role at sequential point throughout the cell cycle could be conducive to growth depression. The assemblage of cyclin D1 and CDK 4/6 played an important role in DNA synthesis at G_1 phase[132]. To investigate whether compound **1** will influence the cell cycle proliferation inhibition by decreasing the cyclin D1 expression level, a downstream protein of JAK2/STAT3 pathway, immunoblotting as performed (**Fig. 2.7)**.

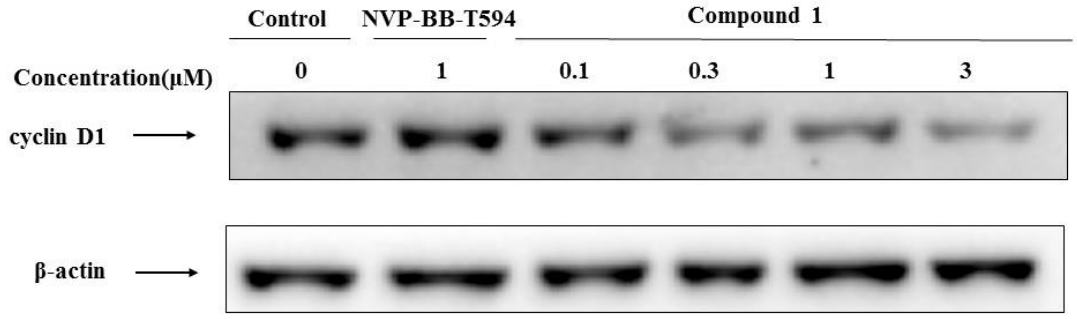


Fig. 2.10 Compound 1 decreased cyclin D1 expression. After 16h drug treatment, A375 cells were

harvest and injected to gel electrophoresis. Chemiluminescence was applied to determination of protein expression.

The result indicated that compound **1** could decrease the expression of cyclin D1(EC_{50}= 3.94 μM), which would act to enhance the growth-inhibitory effect in melanoma cells A375.

To further investigated whether compound **1** could suppress the production of the oncogene c-myc, Western blotting was performed in treated A375 cells. c-myc is a basic-helix-loop-helix/leucine zipper (bHLH/LZ) transcription factor that could regulate the G_1/S transition of the cell cycle, that is correlated to the cell proliferation[133, 134].

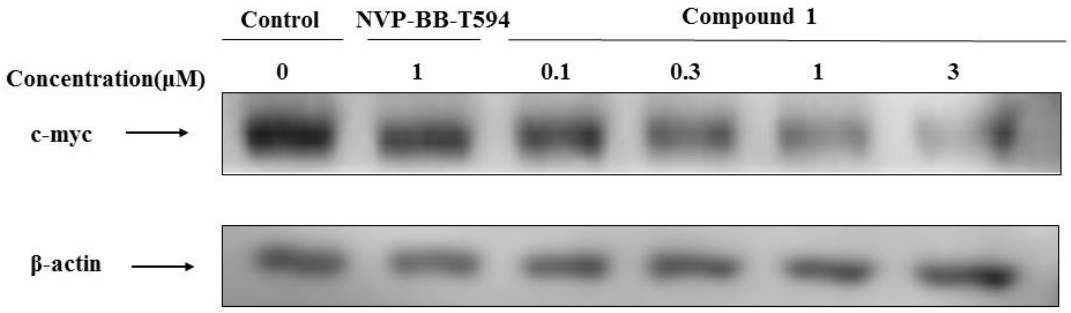


Fig. 2.11 Compound 1 decreased expression of c-myc in A375 cells. Human melanoma cells A375 were incubated with gradient concentration of compound **1**. Cells were harvested after drug treatment for16 h. Protein lysate were analyzed by electrophoresis.

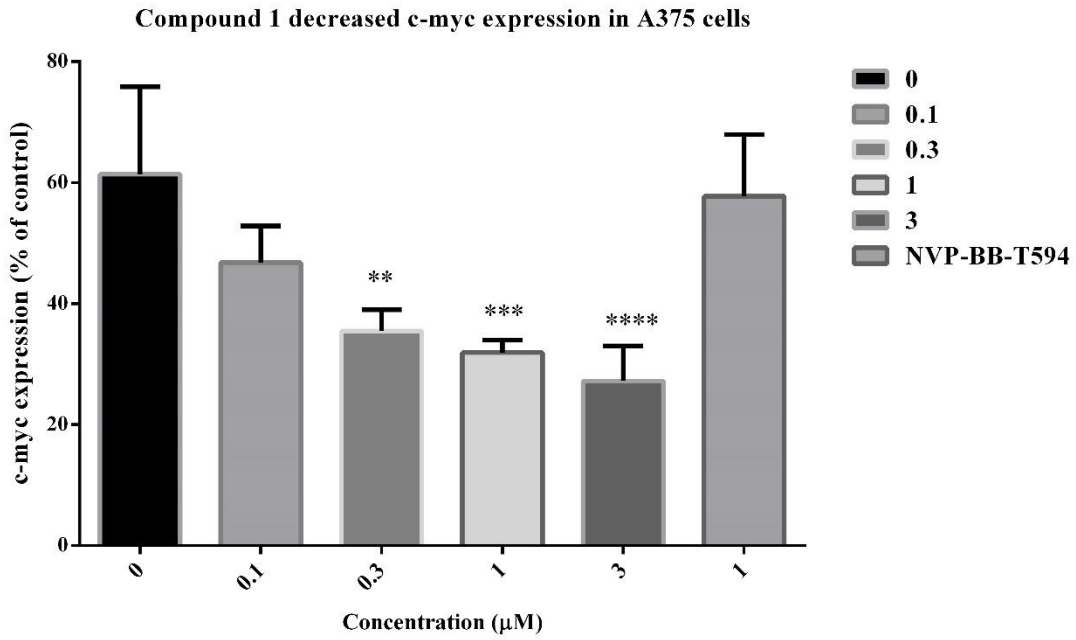


Fig. 2.12 Compound 1 inhibitory effect against c-myc. The data were extract from the result of Western blotting, described previously. Data were processed by GraphPad Prism 6. The EC_{50} value of cleaved c-myc equals to 0.04μM.

The Western blot analysis indicated that compound **1** could decrease the expression level of c-myc in a dose dependent fashion and the most effective concentration wad 3μM. Compound **1** is likely to inhibit cell growth in A375 by depressed the c-my functional activity, such as depression of the G_1/S transition of the cell cycle. At the same time, the decrease in c-myc expression induced by 1 will contribute to inhibition of cell proliferation.

Chapter 3 Conclusions

For many years, the search for an effective therapy for melanoma has been fruitless. Currently, mainstream treatment of malignant melanoma relies on surgery or chemotherapy against certain pathways, such as BARF or MAPK/ERK kinases, however, prognosis remains poor. Novel strategies are therefore needed for melanoma therapy.

In this study, we showed that blocking of the JAK2/STAT3 signaling pathway by compound **1** was associated with the growth inhibition of human malignant melanoma cells A375, through both apoptosis induction and cell proliferation inhibition. Compound **1** resulted clearly inhibited melanoma cell viability with the IC_{50} values of 2.31 μM, while the IC_{50} values of NVP-BB-T594 was 4.34 μM in vitro. Downstream proteins of the JAK2/STAT3 were also affected by compound **1**, as analyzed by Western blotting. The result demonstrated that compound **1** showed most potent inhibition in western blot at the concentration of 3 μM and selective to A375 cell line. Furthermore, compound **1** lowered the expression levels of the anti-apoptotic protein Bcl-2 and increased the expression levels of the pro-apoptotic protein Bax, which were consist with the result of Annexin V–FITC apoptosis detection assay demonstrated that compound **1** contributed to promoting apoptosis in melanoma cells and better than the control group. The inhibition of cyclin D1 and c-myc expression suggested that compound **1** suppressed proliferation by regulation of G_1 to S phase.

Importantly, our work has uncovered the mechanisms by which compound **1** exerts its anti-proliferative effects in melanoma cells. This study is the first to investigate the anti-tumor ability of amentoflavone analogues in melanoma cells, and validate the use of virtual screening to identify Type II JAK2 inhibitors as anticancer leads.

References

[1]. Siegel, R.L., K.D. Miller, and A. Jemal, Cancer Statistics, 2017. A Cancer Journal for Clinicians, 2017. Vol. 67(1): 7-30.

[2]. Day, C.-P., et al., Mouse models of UV-induced melanoma: genetics, pathology, and clinical relevance. *Laboratory Investigation*, 2017.

[3]. Gray-Schopfer, V., C. Wellbrock, and R. Marais, Melanoma biology and new targeted therapy. *Nature*, 2007. Vol. 445(7130): 851-857.

[4]. Murakami, T., A.R. Cardones, and S.T. Hwang, Chemokine receptors and melanoma metastasis. *Journal of Dermatological Science*, 2004. Vol. 36(2): 71-78.

[5]. Ziman, M., *Circulating Melanoma Cells*. 2011: INTECH Open Access Publisher.

[6]. Yajima, I., et al., RAS/RAF/MEK/ERK and PI3K/PTEN/AKT signaling in malignant melanoma progression and therapy. *Dermatology Research and Practice*, 2011. Vol. 2012.

[7]. Margolin, K., et al., CCI‐779 in metastatic melanoma. *Cancer*, 2005. Vol. 104(5): 1045-1048.

[8]. Daud, A., et al., Phase II randomised discontinuation trial of the MET/VEGF receptor inhibitor cabozantinib in metastatic melanoma. *British Journal of Cancer*, 2017.

[9]. Mita, M., et al., Phase I trial of MEK 1/2 inhibitor pimasertib combined with mTOR inhibitor temsirolimus in patients with advanced solid tumors. *Investigational New Drugs*, 2017. 1-11.

[10]. Saura, C., et al., A First-in-Human Phase I Study of the ATP-Competitive AKT Inhibitor Ipatasertib Demonstrates Robust and Safe Targeting of AKT in Patients with Solid Tumors. *Cancer Discovery*, 2017. Vol. 7(1): 102-113.

[11]. Beatty, G.L., et al., First-in-Human Phase 1 Study of the Oral Inhibitor of Indoleamine 2, 3-dioxygenase-1 Epacadostat (INCB024360) in Patients With Advanced Solid Malignancies. *Clinical Cancer Research*, 2017. clincanres. 2272.2016.

[12]. Bastian, A., et al., AG311, a small molecule inhibitor of complex I and hypoxia-induced HIF-1α stabilization. *Cancer Letters*, 2017. Vol. 388: 149-157.

[13]. Cholewa, B.D., et al., Small molecule inhibition of polo-like kinase 1 by volasertib (BI 6727) causes significant melanoma growth delay and regression in vivo. *Cancer Letters*, 2017. Vol. 385: 179-187.

[14]. Fang, L., A.S. Lonsdorf, and S.T. Hwang, Immunotherapy for advanced melanoma. *Journal of Investigative Dermatology*, 2008. Vol. 128(11): 2596-2605.

[15]. Chapman, P.B., et al., Phase III multicenter randomized trial of the Dartmouth regimen versus dacarbazine in patients with metastatic melanoma. *Journal of Clinical Oncology*, 1999. Vol. 17(9): 2745-2745.

[16]. Guillot, B., et al., French updated recommendations in Stage I to III melanoma treatment and management. *Journal of the European Academy of Dermatology and Venereology*, 2017.

[17]. Pandiaraja, J., An update in the management of malignant melanoma. *Journal of Surgical Dermatology*, 2017. Vol. 2(1).

[18]. Menzies, A.M. and G.V. Long, New combinations and immunotherapies for melanoma: latest evidence and clinical utility. *Therapeutic Advances in Medical Oncology*, 2013. Vol. 5(5): 278-285.

[19]. Gupta, S. and H. Tsao, *Epidemiology of Melanoma*, in *Pathology and Epidemiology of Cancer*. 2017, Springer. 591-611.

[20]. La Porta, C.A., Drug resistance in melanoma: new perspectives. *Current Medicinal Chemistry*, 2007. Vol. 14(4): 387-391.

[21]. Grossman, D. and D.C. Altieri, Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. *Cancer and Metastasis Reviews*, 2001. Vol. 20(1): 3-11.
[22]. Soengas, M.S. and S.W. Lowe, Apoptosis and melanoma chemoresistance. *Oncogene*, 2003. Vol. 22(20): 3138-3151.
[23]. Cao, H.-H., et al., Quercetin exerts anti-melanoma activities and inhibits STAT3 signaling. *Biochemical Pharmacology*, 2014. Vol. 87(3): 424-434.
[24]. Miyashita, T. and J.C. Reed, Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood*, 1993. Vol. 81(1): 151-157.
[25]. Decaudin, D., et al., Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. *Cancer Research*, 1997. Vol. 57(1): 62-67.
[26]. Sullivan, R.J. and K.T. Flaherty, Resistance to BRAF-targeted therapy in melanoma. *European Journal of Cancer*, 2013. Vol. 49(6): 1297-1304.
[27]. Sinnberg, T., et al., Inhibition of PI3K-AKT-mTOR signaling sensitizes melanoma cells to cisplatin and temozolomide. *Journal of Investigative Dermatology*, 2009. Vol. 129(6): 1500-1515.
[28]. Chen, K.G., et al., Involvement of ABC transporters in melanogenesis and the development of multidrug resistance of melanoma. *Pigment Cell & Melanoma Research*, 2009. Vol. 22(6): 740-749.
[29]. Eberle, J. and L.F. Fecker, *Regulation of Apoptosis in Melanoma Cells: Critical Targets for Therapeutic Strategies*, in *Melanoma Development*. 2017, Springer. 271-287.
[30]. Baguley, B.C., Multiple drug resistance mechanisms in cancer. *Molecular Biotechnology*, 2010. Vol. 46(3): 308-316.
[31]. Schindler, C. and J. Darnell Jr, Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annual Review of Biochemistry*, 1995. Vol. 64(1): 621-652.
[32]. Hedvat, M., et al., The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. *Cancer Cell*, 2009. Vol. 16(6): 487-497.
[33]. Bill, M.A., et al., Structurally modified curcumin analogs inhibit STAT3 phosphorylation and promote apoptosis of human renal cell carcinoma and melanoma cell lines. *PloS One*, 2012. Vol. 7(8): e40724.
[34]. Koppikar, P., et al., Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. *Nature*, 2012. Vol. 489(7414): 155-159.
[35]. Verma, A., et al., Jak family of kinases in cancer. *Cancer and Metastasis Reviews*, 2003. Vol. 22(4): 423-434.
[36]. James, C., et al., A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*, 2005. Vol. 434(7037): 1144-1148.
[37]. Baxter, E.J., et al., Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *The Lancet*, 2005. Vol. 365(9464): 1054-1061.
[38]. Marotta, L.L., et al., The JAK2/STAT3 signaling pathway is required for growth of CD44+ CD24– stem cell–like breast cancer cells in human tumors. *The Journal of Clinical Investigation*, 2011. Vol. 121(7): 2723-2735.
[39]. Sakai, I. and A.S. Kraft, The kinase domain of Jak2 mediates induction of bcl-2 and delays cell death in hematopoietic cells. *Journal of Biological Chemistry*, 1997. Vol. 272(19): 12350-12358.
[40]. Meyer, S.C. and R.L. Levine, Molecular pathways: molecular basis for sensitivity and resistance to JAK kinase inhibitors. *Clinical Cancer Research*, 2014. Vol. 20(8): 2051-2059.
[41]. Fukada, T., et al., STAT3 orchestrates contradictory signals in cytokine‐induced G1 to S cell‐cycle transition. *The EMBO Journal*, 1998. Vol. 17(22): 6670-6677.

[42]. Fukada, T., et al., Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity*, 1996. Vol. 5(5): 449-460.
[43]. Britschgi, A., et al., JAK2/STAT5 inhibition circumvents resistance to PI3K/mTOR blockade: a rationale for cotargeting these pathways in metastatic breast cancer. *Cancer Cell*, 2012. Vol. 22(6): 796-811.
[44]. Abubaker, K., et al., Inhibition of the JAK2/STAT3 pathway in ovarian cancer results in the loss of cancer stem cell-like characteristics and a reduced tumor burden. *BMC Cancer*, 2014. Vol. 14(1): 317.
[45]. Xiong, A., et al., Transcription factor STAT3 as a novel molecular target for cancer prevention. *Cancers*, 2014. Vol. 6(2): 926-957.
[46]. Heim, M.H., et al., Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. *Science*, 1995. Vol. 267(5202): 1347.
[47]. Bromberg, J., Stat proteins and oncogenesis. *The Journal of Clinical Investigation*, 2002. Vol. 109(9): 1139-1142.
[48]. Levy, D.E. and J. Darnell, Stats: transcriptional control and biological impact. *Nature reviews Molecular Cell Biology*, 2002. Vol. 3(9): 651-662.
[49]. Takeda, K., et al., Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proceedings of the National Academy of Sciences*, 1997. Vol. 94(8): 3801-3804.
[50]. Shen, Y., et al., Essential role of STAT3 in postnatal survival and growth revealed by mice lacking STAT3 serine 727 phosphorylation. *Molecular and Cellular Biology*, 2004. Vol. 24(1): 407-419.
[51]. Turkson, J., et al., Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. *Journal of Biological Chemistry*, 2001. Vol. 276(48): 45443-45455.
[52]. Bowman, T., et al., STATs in oncogenesis. *Oncogene*, 2000. Vol. 19(21): 2474.
[53]. Bowman, T., et al., Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proceedings of the National Academy of Sciences*, 2001. Vol. 98(13): 7319-7324.
[54]. Liu, L., et al., A novel 7-bromoindirubin with potent anticancer activity suppresses survival of human melanoma cells associated with inhibition of STAT3 and Akt signaling. *Cancer Biology & Therapy*, 2012. Vol. 13(13): 1255-1261.
[55]. Kortylewski, M., R. Jove, and H. Yu, Targeting STAT3 affects melanoma on multiple fronts. *Cancer and Metastasis Reviews*, 2005. Vol. 24(2): 315-327.
[56]. Harada, D., N. Takigawa, and K. Kiura, The role of STAT3 in non-small cell lung cancer. *Cancers*, 2014. Vol. 6(2): 708-722.
[57]. Wang, W., et al., STAT3 as a Biomarker of Progression in Atypical Nevi of Patients with Melanoma: Dose–Response Effects of Systemic IFNα Therapy. *Journal of Investigative Dermatology*, 2008. Vol. 128(8): 1997-2002.
[58]. Yuan, Z.-l., et al., Stat3 dimerization regulated by reversible acetylation of a single lysine residue. *Science*, 2005. Vol. 307(5707): 269-273.
[59]. Ananthakrishnan, R., et al., JAK-STAT pathway in cardiac ischemic stress. *Vascular Pharmacology*, 2005. Vol. 43(5): 353-356.
[60]. Abdel-Malek, Z.A., V.B. Swope, and A. Indra, *Revisiting Epidermal Melanocytes: Regulation of Their Survival, Proliferation, and Function in Human Skin*, in *Melanoma Development*. 2017, Springer. 7-38.
[61]. Argetsinger, L.S., et al., Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell*, 1993. Vol. 74(2): 237-244.

[62]. Parganas, E., et al., Jak2 is essential for signaling through a variety of cytokine receptors. *Cell*, 1998. Vol. 93(3): 385-395.
[63]. Witthuhn, B.A., et al., JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell*, 1993. Vol. 74(2): 227-236.
[64]. Ritter, S.L. and R.A. Hall, Fine-tuning of GPCR activity by receptor-interacting proteins. *Nature reviews Molecular Cell Biology*, 2009. Vol. 10(12): 819-830.
[65]. Smit, L.S., et al., The role of the growth hormone (GH) receptor and JAK1 and JAK2 kinases in the activation of Stats 1, 3, and 5 by GH. *Molecular Endocrinology*, 1996. Vol. 10(5): 519-533.
[66]. Marrero, M.B., Introduction to JAK/STAT signaling and the vasculature. *Vascular Pharmacology*, 2005. Vol. 43(5): 307-309.
[67]. Gao, B., Cytokines, STATs and liver disease. *Cell Mol Immunol*, 2005. Vol. 2(2): 92-100.
[68]. Heinrich, P.C., et al., Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochemical Journal*, 2003. Vol. 374(1): 1-20.
[69]. Heinrich, P.C., et al., Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochemical Journal*, 1998. Vol. 334(2): 297-314.
[70]. Yu, H., D. Pardoll, and R. Jove, STATs in cancer inflammation and immunity: a leading role for STAT3. *Nature Reviews Cancer*, 2009. Vol. 9(11): 798-809.
[71]. Plettenberg, A., et al., Human melanocytes and melanoma cells constitutively express the Bcl-2 proto-oncogene in situ and in cell culture. *The American Journal of Pathology*, 1995. Vol. 146(3): 651.
[72]. Guenterberg, K.D., et al., Interleukin-29 binds to melanoma cells inducing Jak-STAT signal transduction and apoptosis. *Molecular Cancer Therapeutics*, 2010. Vol. 9(2): 510-520.
[73]. Tian, Y., et al., Spirooxindole derivative SOID-8 induces apoptosis associated with inhibition of JAK2/STAT3 signaling in melanoma cells. *PloS One*, 2012. Vol. 7(11): e49306.
[74]. Weng, C., et al., Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. *Journal of Biological Chemistry*, 2005. Vol. 280(11): 10491-10500.
[75]. Chen, M., et al., The Anticancer Properties of Herba Epimedii and Its Main Bioactive Componentsicariin and Icariside II. *Nutrients*, 2016. Vol. 8(9): 563.
[76]. Vaux, D., S. Cory, and J. Adams, Bcl-2 and cell survival. *Nature*, 1988. Vol. 335: 440-2.
[77]. Nicholson, D.W., et al., Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, 1995. Vol. 376(6535): 37.
[78]. Boulares, A.H., et al., Role of poly (ADP-ribose) polymerase (PARP) cleavage in apoptosis Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. *Journal of Biological Chemistry*, 1999. Vol. 274(33): 22932-22940.
[79]. Le Rhun, Y., J. Kirkland, and G.M. Shah, Cellular responses to DNA damage in the absence of poly (ADP-ribose) polymerase. *Biochemical and Biophysical Research Communications*, 1998. Vol. 245(1): 1-10.
[80]. Bonnefoy-Berard, N., et al., Control of proliferation by Bcl-2 family members. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 2004. Vol. 1644(2): 159-168.
[81]. He, K., et al., Resistance of leukemia KG1a cells with positive N-cadherin in phase G (0) against killing activity of VP16. *Zhongguo shi yan xue ye xue za zhi/Zhongguo bing li sheng li xue hui= Journal of experimental hematology/Chinese Association of Pathophysiology*, 2011. Vol. 19(5): 1102-1106.
[82]. Pichichero, E., et al., Acacia honey and chrysin reduce proliferation of melanoma cells through alterations in cell cycle progression. *International Journal of Oncology*, 2010. Vol. 37(4): 973.

[83]. Zinkel, S., A. Gross, and E. Yang, BCL2 family in DNA damage and cell cycle control. *Cell Death & Differentiation*, 2006. Vol. 13(8): 1351-1359.
[84]. Pourgholami, M.H., et al., Minocycline inhibits malignant ascites of ovarian cancer through targeting multiple signaling pathways. *Gynecologic Oncology*, 2013. Vol. 129(1): 113-119.
[85]. Emily, H.-Y.C., et al., BCL-2, BCL-X L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Molecular Cell*, 2001. Vol. 8(3): 705-711.
[86]. Correia, C., et al., Emerging understanding of Bcl-2 biology: Implications for neoplastic progression and treatment. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 2015. Vol. 1853(7): 1658-1671.
[87]. Yang, E. and S. Korsmeyer, Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood*, 1996. Vol. 88(2): 386-401.
[88]. Dai, H., X.W. Meng, and S.H. Kaufmann, BCL2 Family, Mitochondrial Apoptosis, and Beyond. *Cancer Translational Medicine*, 2016. Vol. 2(1): 7.
[89]. Nikolaou, V.A., et al., Melanoma: new insights and new therapies. *Journal of Investigative Dermatology*, 2012. Vol. 132(3): 854-863.
[90]. Hoffman, B. and D. Liebermann, Apoptotic signaling by c-MYC. *Oncogene*, 2008. Vol. 27(50): 6462-6472.
[91]. Evan, G. and T. Littlewood, A matter of life and cell death. *Science*, 1998. Vol. 281(5381): 1317-1322.
[92]. Henriksson, M. and B. Lüscher, Proteins of the Myc network: essential regulators of cell growth and differentiation. *Advances In Cancer Research*, 1996. Vol. 68: 109-182.
[93]. Galaktionov, K., X. Chen, and D. Beach, Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature*, 1996. Vol. 382(6591): 511.
[94]. Hoffmann, I., G. Draetta, and E. Karsenti, Activation of the phosphatase activity of human cdc25A by a cdk2-cyclin E dependent phosphorylation at the G1/S transition. *The EMBO Journal*, 1994. Vol. 13(18): 4302.
[95]. Jinno, S., et al., Cdc25A is a novel phosphatase functioning early in the cell cycle. *The EMBO Journal*, 1994. Vol. 13(7): 1549.
[96]. Mochizuki, T., et al., Physical and functional interactions between pim-1 kinase and cdc25a phosphatase implications for the pim-1-mediated activation of the c-myc signaling pathway. *Journal of Biological Chemistry*, 1999. Vol. 274(26): 18659-18666.
[97]. Hirano, T., K. Ishihara, and M. Hibi, Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene*, 2000. Vol. 19(21): 2548.
[98]. Lavarone, A. and J. Massagué, Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature*, 1997. Vol. 387(6631): 417.
[99]. Elledge, S.J., Cell cycle checkpoints: preventing an identity crisis. *Science*, 1996. Vol. 274(5293): 1664.
[100]. Morgan, D.O., Principles of CDK regulation. *Nature*, 1995. Vol. 374(6518): 131.
[101]. Lin, X., et al., C-myc overexpression drives melanoma metastasis by promoting vasculogenic mimicry via c-myc/snail/Bax signaling. *Journal of Molecular Medicine*, 2017. Vol. 95(1): 53-67.
[102]. Sherr, C.J., Mammalian G1 cyclins. *Cell*, 1993. Vol. 73(6): 1059-1065.
[103]. Sherr, C.J., Cancer cell cycles. *Science*, 1996. Vol. 274(5293): 1672.

[104]. Vermeulen, K., D.R. Van Bockstaele, and Z.N. Berneman, The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Proliferation*, 2003. Vol. 36(3): 131-149.
[105]. Kiss, R., P.P. Sayeski, and G.M. Keserű, Recent developments on JAK2 inhibitors: a patent review. *Expert Opinion on Therapeutic Patents*, 2010. Vol. 20(4): 471-495.
[106]. Weigert, O., et al., Genetic resistance to JAK2 enzymatic inhibitors is overcome by HSP90 inhibition. *Journal of Experimental Medicine*, 2012. Vol. 209(2): 259-273.
[107]. Andraos, R., et al., Modulation of activation-loop phosphorylation by JAK inhibitors is binding mode dependent. *Cancer Discovery*, 2012. Vol. 2(6): 512-523.
[108]. Liu, Y. and N.S. Gray, Rational design of inhibitors that bind to inactive kinase conformations. *Nature Chemical Biology*, 2006. Vol. 2(7): 358-364.
[109]. Ma, D.-L., et al., Virtual screening and optimization of Type II inhibitors of JAK2 from a natural product library. *Chemical Communications*, 2014. Vol. 50(90): 13885-13888.
[110]. Schindler, T., et al., Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science*, 2000. Vol. 289(5486): 1938-1942.
[111]. Li, T. and T. Peng, Traditional Chinese herbal medicine as a source of molecules with antiviral activity. *Antiviral Research*, 2013. Vol. 97(1): 1-9.
[112]. Lee, K.-H., Research and future trends in the pharmaceutical development of medicinal herbs from Chinese medicine. *Public Health Nutrition*, 2000. Vol. 3(4a): 515-522.
[113]. Harvey, A.L., Natural products in drug discovery. *Drug Discovery Today*, 2008. Vol. 13(19): 894-901.
[114]. Koehn, F.E. and G.T. Carter, The evolving role of natural products in drug discovery. *Nature reviews Drug discovery*, 2005. Vol. 4(3): 206-220.
[115]. Middleton, E., C. Kandaswami, and T.C. Theoharides, The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews*, 2000. Vol. 52(4): 673-751.
[116]. Kim, H.K., et al., Amentoflavone, a plant biflavone: a new potential anti-inflammatory agent. *Archives of Pharmacal Research*, 1998. Vol. 21(4): 406-410.
[117]. Guruvayoorappan, C. and G. Kuttan, Amentoflavone stimulates apoptosis in B16F-10 melanoma cells by regulating bcl-2, p53 as well as caspase-3 genes and regulates the nitric oxide as well as proinflammatory cytokine production in B16F-10 melanoma cells, tumor associated macrophages and peritoneal macrophages. *Journal of Experimental Therapeutics & Oncology*, 2008. Vol. 7(3).
[118]. Kufareva, I. and R. Abagyan, Type-II kinase inhibitor docking, screening, and profiling using modified structures of active kinase states. *Journal of Medicinal Chemistry*, 2008. Vol. 51(24): 7921-7932.
[119]. Ma, E., et al., Structure-based discovery and development of natural products as Type II JAK2 inhibitors for the treatment of hepatitis C. *Hong Kong Medical Journal*, 2016. Vol. 22(3 Supplement 4).
[120]. Kufareva, I. and R. Abagyan, Type-II kinase inhibitor docking, screening, and profiling using modified structures of active kinase states. *Journal of Medicinal Chemistry*, 2008. Vol. 51(24): 7921.
[121]. Ma, D.-L., D.S.-H. Chan, and C.-H. Leung, Drug repositioning by structure-based virtual screening. *Chemical Society Reviews*, 2013. Vol. 42(5): 2130-2141.
[122]. Mueller, H., M.U. Kassack, and M. Wiese, Comparison of the usefulness of the MTT, ATP, and calcein assays to predict the potency of cytotoxic agents in various human cancer cell lines. *Journal of Biomolecular Screening*, 2004. Vol. 9(6): 506-515.

[123]. Mahmood, T. and P.-C. Yang, Western blot: technique, theory, and trouble shooting. *North AmericanJournal of Medical Sciences*, 2012. Vol. 4(9): 429.
[124]. Enos, R.T., et al., Influence of dietary saturated fat content on adiposity, macrophage behavior, inflammation, and metabolism: composition matters. *Journal of Lipid Research*, 2013. Vol. 54(1): 152-163.
[125]. Tsao, C.-H., et al., Interleukin-4 regulates lipid metabolism by inhibiting adipogenesis and promoting lipolysis. *Journal of Lipid Research*, 2014. Vol. 55(3): 385-397.
[126]. Wang, X., The expanding role of mitochondria in apoptosis. *Genes & Development*, 2001. Vol. 15(22): 2922-2933.
[127]. Wu, S.-C., et al., Activity of the type II JAK2 inhibitor CHZ868 in B cell acute lymphoblastic leukemia. *Cancer Cell*, 2015. Vol. 28(1): 29-41.
[128]. Niu, G., et al., Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. *Oncogene*, 2002. Vol. 21(46): 7001.
[129]. Decker, P., D. Isenberg, and S. Muller, Inhibition of caspase-3-mediated poly (ADP-ribose) polymerase (PARP) apoptotic cleavage by human PARP autoantibodies and effect on cells undergoing apoptosis. *Journal of Biological Chemistry*, 2000. Vol. 275(12): 9043-9046.
[130]. Wynford‐Thomas, D., Cellular senescence and cancer. *The Journal of Pathology*, 1999. Vol. 187(1): 100-111.
[131]. Michaloglou, C., et al., BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*, 2005. Vol. 436(7051): 720-724.
[132]. Ranade, K., et al., Mutations associated with familial melanoma impair p16INK4 function. *Nature Genetics*, 1995. Vol. 10(1): 114-116.
[133]. Adhikary, S. and M. Eilers, Transcriptional regulation and transformation by Myc proteins. *Nature reviews Molecular Cell Biology*, 2005. Vol. 6(8): 635-645.
[134]. Nilsson, J.A. and J.L. Cleveland, Myc pathways provoking cell suicide and cancer. *Oncogene*, 2003. Vol. 22(56): 9007-9021.
[135]. Yang, H., Structure-based discovery and development of c-myc down-regulators and JAK2 inhibitors. 2013.

Appendices

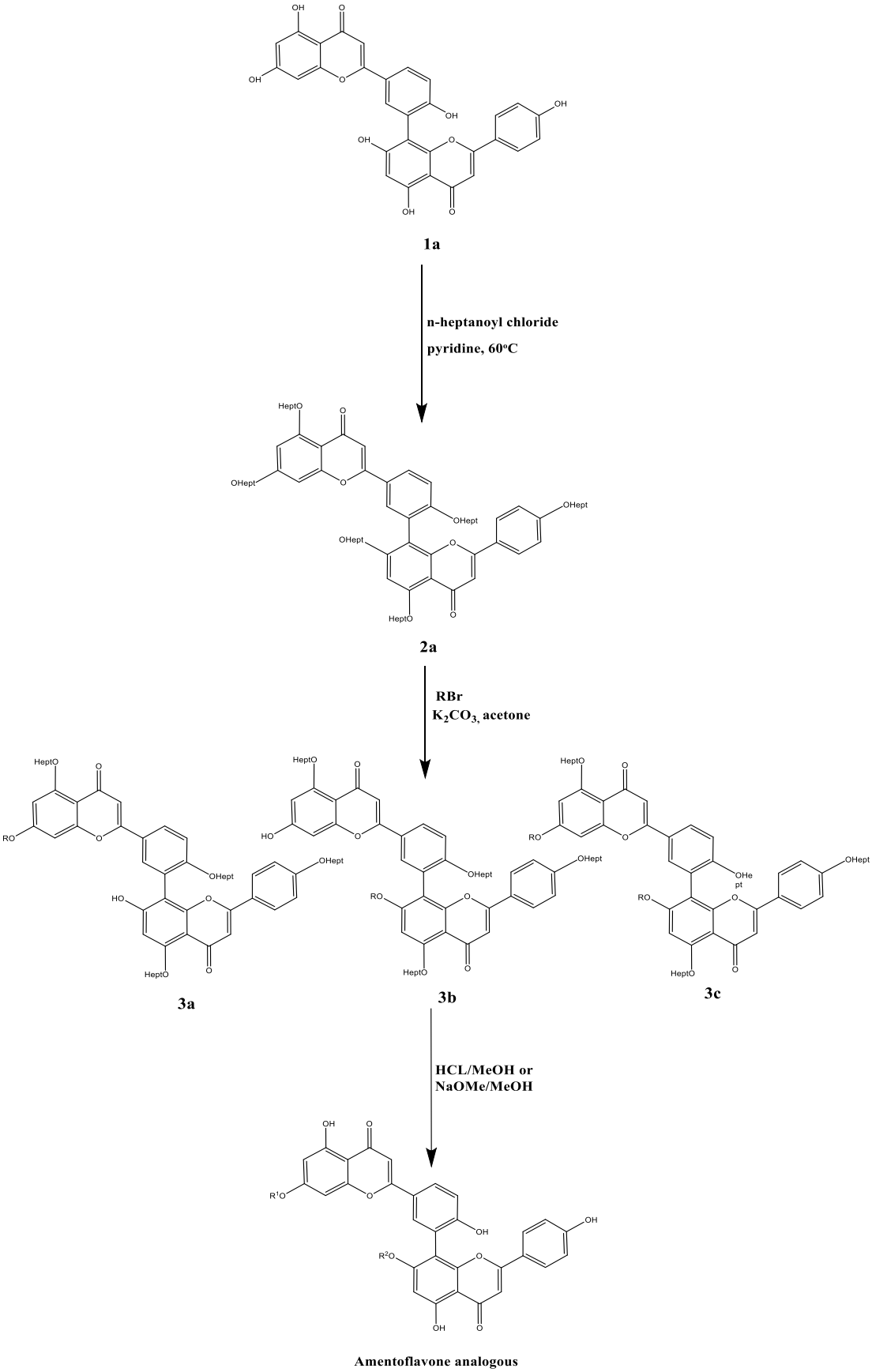

1a
n-heptanoyl chloride
pyridine, 60°C
2a
RBr
K2CO3, acetone
3a
3b
3c
HCL/MeOH or
NaOMe/MeOH
Amentoflavone analogous

Fig. S1 The synthetic procedure for amentoflavone analogous. Amentoflavone 1a was treated with an excess of heptanoyl chloride in pyridine at 60 °C overnight. The heptyl protecting group was selected to deal with the poor solubility problem. This procedure could afford enough peracylated derivative 2a. Treating the biflavone 2a that are fully protected with alkyl bromide in acetone with potassium carbonate as a base will selectively get three alkylated products that hydrolysis and alkylation at the C7 and C7″ phenolic positions, which are the 7-monoalkylated derivatives 3a, 7″-monoalkylated derivatives 3b and the 7,7″-dialkylated derivatives 3c. The reaction of 2a with farnesyl bromide proceeded in mil conditions to generate enough amounts of the mono-alkylated products. Because 3a-c were very similar in polarity and hard to tell each other, even using the silica gel chromatography, without further purification, 3a-c were directly subjected to the next step as mixture that hydrolyzed under acidic conditions using 3M HCl/MeOH for the deprotection of the heptanoyl groups. For the synthesis of 8, deprotection was induced under basic conditions. The end products were isolated and purified by repeated preparative TLC or HPLC[109, 135].

IC_{50} values (μM)

Compound	A375 Melanoma cells	A2058 Melanoma cells	293T Human normal
NVP-BBT594	4.34	5.31	>10
Amentoflavone	>10	>10	
1	2.31	5.32	
2	>10	>10	
3	>10	>10	
4	>10	>10	
5	>10	>10	
6	>10	>10	
7	>10	>10	
8	>10	>10	

Fig. S2 Different IC_{50} values of Amentoflavone analogues. Cytotoxicity of NVP-BB-T594, Amentoflavone and its' analogues against various human cell lines represented as IC_{50} value determined by MTT assay

Publications

Wu K-J, Huang J-M, et al. A natural product-like JAK2/STAT3 inhibitor induces apoptosis of malignant melanoma cells. PLOS ONE, 2017. Vol. 12(6): e0177123.