Bioinformatics and Experimental Studies on the Selected Molecular Targets of Some Anticancer Drugs

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“Do the stuff that only you can do.

The urge, starting out, is to copy. And that is not a bad thing.

Most of us only find our own voices after we’ve sounded like a lot of other people. But the one thing that you have that nobody else has is you.

Your voice, your mind, your story, your vision”

(Unknown)

ABSTRACT

Cancer is one of the commonest causes of patient death in the clinic and a complex disease occurring in multiple organs per system, multiple systems per organ, or both, in the body. With increasing evidence that the interaction and network between genes and proteins play an important role in investigation of cancer molecular mechanisms to integrate systems biology, bioinformatics and computational science to improve diagnosis, therapies and prognosis of diseases. Cancer bioinformatics is a critical and important part of the systems clinical medicine in cancer and the core tool and approach to carry out the investigations of cancer in systems clinical medicine.

Cancer involves uncontrolled cell division and tissue invasiveness (metastasis) caused by a series of mutations in the genes of proteins that regulate the cell cycle. Proto-oncogenes are normal genes that promote cell growth and mitosis, whereas tumor suppressor genes repress cell growth. Proto-oncogenes can be mutated by carcinogens to become oncogenes that produce excessive levels of growth promoting proteins. Tumor suppressor gene products, typified by p53, is frequently transcription factors that suppress mitosis and cell growth to allow DNA repair. Activated p53 promotes cell cycle arrest to allow DNA repair and/or apoptosis to prevent the propagation of cells with serious DNA damage through the transactivation of its target genes implicated in the induction of cell cycle arrest and/or apoptosis. Poly (ADP-ribose) polymerase-1 (PARP1) is a chromatin-associated enzyme with key functions in the regulation of transcription, cell cycle, tumorigenesis, and cellular response to DNA damage. PARP1 is activated by DNA damage and has important roles in DNA base excision repair (BER), functioning as a nick sensor, recruiter, and modulator of key DNA repair molecules. Notably, p53 and PARP-1 are in transcriptional repressive complexes which is involved in epigenetic phenomenon of cancer.

This study is focusing on anti-cancer drugs using 5-aza-2'-deoxycytidine (5-Aza-dC). The 5-Aza-dC is FDA-approved widely used hypo-methylating anticancer drug. We conducted an analysis for new functions of 5-Aza-dC by applying bio-chemo-informatics approach. Induction of p53 by 5-Aza-dC was tested in vitro using cancer cells. While Bioinformatics analysis was used to predict protein target and its biological activity. It was predicted that 5-Aza-dC functions as a p53 inducer, radiosensitizer, and inhibitor of some enzymes and its protein target including MDM2, POLA1, POLB, and CXCR4 that are involved in the induction of DNA damage response and p53-HDM2-p21 signaling. In this study, we provide experimental evidence showing HDM2 is one of the targets of 5-AZA-

dC leading to activation of p53 pathway and growth arrest of cells. Furthermore, we found that the combinatorial treatment of 5-AZA-dC with three other drugs caused drug resistance. We discuss that 5-Aza-dC-induced senescence is a multi-module drug that controls cell proliferation phenotype.

Taken together our result proves novel evidence of bioinformatics and experimental analysis combination to support new insight for cancer therapeutic and to understand the specific molecular target even better.

Key words: 5-Aza-dC, anti-cancer, Bioinformatics.

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Abbreviations

PARP	: Poly (ADP-ribose) polymerase
PAR	: Poly(ADP-rybosyl)ation
DNA	: Deoxyribonucleic Acid
BER	: Base Excision Repair
5-Aza-dC	: 5-aza-2'-deoxycytidine
FDA	: The Food and Drug Administration
MDM2	: Mouse Double Minute 2
POLA1	: DNA Polymerase Alpha 1
POLB	: DNA Polymerase Beta
CXCR4	: C-X-C chemokine receptor type 4 (CXCR-4)
mRNA	: Messenger Ribonucleic acid
JNK	: c-Jun N-terminal kinases
SSB	: Single Strand Break DNA
Rb	: Retinoblastoma
PPIs	: Protein-Protein Interaction
CAPRI	: The Critical Assessment of Prediction of Interactions
RIN	: Residue Interaction Network
CFH	: Component Factor H
DDR	: DNA Damage Response
ATM	: Ataxia Telangiectasia Mutated
MAPK	: Mitogen Activated Protein Kinase
AIF	: Apoptosis Inducing Factor
ROS	: Reactive Oxygen Species
dNTP	: deoxyribonucleotides
UV	: Ultraviolet
IRs	: Ionizing Radiation
CPDs	: Cyclobutane Pyrimidine Dimers
HR	: Homologous Recombination
BER	: Base Excision Repair
NER	: Nucleotide Excision Repair
ARF	: ADP Ribosylation Factor

CDK	: Cyclin Dependent Kinase Complex
PI3K	: Phosphoinositide 3-kinase
CHK	: Checkpoint Kinase
miRNA	: micro RNA
CARF	: Collaborator ARF
BINGO	: A Biological Network Gene Ontology tool
PDB	: Protein Data Base
U2OS	: Human Osteosarcoma Cell Line
HeLa	: Human Cervical Cancer Cell Line
DMEM	: Dulbecco's Modified Eagle's Medium
PBS	: Phosphate Buffer Saline
DMSO	: Dimethyl sulfoxide
GPCR	: G protein-coupled receptor
5 FU	: 5 Flourouracil
FBS	: Fetal Bovine Serum
COX 1/IV	: Cytochrome C Oxidase Subunit 1/IV
siRNA	: small interfering RNA
shRNA	: small hairpin RNA
TBS	: Tris Buffer Saline
NAD+	: Nicotinamide
ATP	: Adenosine Tri Phosphate

Chapter I

Introduction

1. Introduction

1.1 Bioinformatics

Bioinformatics is simply defined as computational approach of molecular biology. This is the application of computer science and information to the field of biology and medicine. Bioinformatics is including how information is generated, transmitted, received, and interpreted in biology system [1]. The term of Bioinformatics for biologist is very huge including data analysis, data curator, database developer, statistician, software developer and many more. Need to be noted that appropriate software and web server selection is the key for computational biology. Here, Bioinformatics approach to support cancer therapeutics and many diseases will be explained including how to predict protein target, molecular modelling, docking analysis and interpretation data [2].

Protein–protein interactions (PPIs) play a central role in all biological processes. These processes result from the physical interaction of several protein molecules, thus forming the macromolecular assemblies that effectuate cellular function [3-6]. Many large-scale studies focusing on PPI have emerged in recent years, using graphs with nodes and edges to represent the protein components interacting with each other. Such binary representations capture a wealth of information but are inherently abstract and incomplete, since they contain no information as to time, place, or specificity. Such detailed information is indispensable for the guidance of mutagenesis studies or the design of inhibitor molecules. Protein–protein docking actually predates protein–ligand (small molecule) docking, that later extended to the interaction between macromolecules and small ligands [7]. The treatment of flexibility in the binding process is considerably easier with small molecules, even though a considerable computational cost is involved, and small molecule docking has become one of the most active research areas in computational drug discovery. Most if not all of present-day protein–protein docking algorithms have been developed in light of the critical assessment of prediction of interactions (CAPRI) experiment, which is a community-wide collaboration that has accelerated the development of computational protein docking methods [8-10]. CAPRI, which is modelled after critical assessment of protein structure prediction, organizes blind prediction trials; participants model their complex, and the models are assessed through comparison with an unknown crystal structure, made available to the assessors on a confidential basis and prior to publication. The early years have been essential for the development of docking algorithms, with the

incorporation of more elaborate scoring functions owing to efficient implementations of fast Fourier transform algorithms in docking as one of the key advancements. This also spawned the CAPRI [11].

The PPI interfaces have received increased attention during the past years. A prediction of the residues involved in the interaction interface may be used to guide the docking itself. Subsequent optimization of the interaction interface requires modification of side chain orientation; protein docking algorithms increasingly include flexibility treatments in their docking procedures, and more recent implementations favour the simultaneous docking of ensembles of unbound conformers [12]. Together, the reliable prediction of interface residues and the incorporation of global and local flexibility in the docking algorithms provide invaluable information to inform mutagenesis studies and to steer drug design applications. The scoring functions of docking programs have been successfully improved with additional descriptors based on residue interaction networks (RINs). RINs consist of networks generated from three-dimensional structures, where nodes correspond to residues and edges to detected interactions. RINs are small-world networks, and their topological analyses have been used in particular to study protein–protein interfaces107 and protein– ligand binding and to optimize scoring functions for the evaluation of docking poses. Using different approaches, it has been demonstrated that combining the network measures such as closeness centrality, between centrality, degree, or clustering coefficient with energy terms can improve the ranking in scoring functions [13].

Two different types of RINs, a hydrophobic one and a hydrophilic one, for each complex, and then calculated a network-based score considering the average degrees and clustering coefficients. They developed a scoring method that enhanced discrimination of the scoring method of RosettaDock112 by .10% on a subset of protein–protein docking benchmark 2.0.113 The RIN of each protein individually before docking and calculated four measures of the network, including closeness centrality. By integrating a score based on the closeness values into the pyDock scoring function, they improved by as much as 36% the top ten success rate on the protein–protein docking benchmark 4.0.115 Furthermore, residue centrality analyses which are based on the average shortest path length, can also be used on docked poses to evaluate the central residues located in the interface. These residues could subsequently be targeted for mutagenesis experiments or drug design [14].

The Interactome3D120 web service incorporates structural data into PPI networks to improve them with interface information. These structural data either come from experiments or are modelled through a comparative modelling pipeline. The value of this

tool by showing it allowed them to suggest a potential mechanism of action common to several disease-causing mutations. Indeed, they observed the mutations on structures of the complement cascade pathway involving, in particular, the complement component 3 (C3) and the component factor H (CFH) interaction [15]. Several disease-causing mutations were located at the interface of proteins, and these key elements could be targeted by drugs in order to stabilize the C3–CFH interface. Thus, with this type of network, it is possible to contextualize mutations related to different diseases involved in a pathway and draw potential links between them. It can help to better define the target to aim for and, hence, improve the drug design. Docking predictions could then be additionally integrated to these networks.

Furthermore, it was predicted that on average a drug binds to six different targets, including both the primary target and additional "off-targets". Following this idea, reverse docking can be performed, which consists in the screening of one single molecule against multiple receptors instead of screening multiple small molecules against several receptors. Homology modelling may be useful to enrich the screening, when experimental structures are not available. The building of structural PPI networks may then be used in drug design to predict the targets the drug may bind to, with their related potential adverse drug reactions. They can help to identify which proteins would be affected by a drug designed to disrupt a particular interface because they highlight the domains that are involved in PPI. These structural PPI networks can also be exploited for drug repositioning, considering the use of known approved drugs or the reconsideration of late-stage failures [16].

1.2 Cancer and cell senescence

Cellular senescence refers to the essentially irreversible arrest of cell proliferation (growth) that occurs when cells experience potentially oncogenic stress. The permanence of the senescence growth arrest enforces the idea that the senescence response evolved at least in part to suppress the development of cancer [17]. The senescence arrest is considered irreversible because no known physiological stimuli can stimulate senescent cells to re-enter the cell cycle. However, molecular biological manipulations, for example, the sequential inactivation of certain tumor suppressor genes, can cause senescent cells to proliferate. There may be as-yet-unrecognized physiological circumstances under which the senescence growth arrest is reversible [18]. Normal cells do not divide indefinitely due to a process

termed cellular or replicative senescence. Several lines of evidence suggest that replicative senescence evolved to protect higher eukaryotes, particularly mammals, from developing cancer. Senescent cells differ from their pre-senescent counterparts in three way: 1) they arrest growth and cannot be stimulated to re-enter the cell cycle by physiological mitogens; 2) they become resistant to apoptotic cell death; 3) they acquire altered differentiated functions. Replicative senescence occurs because, owing to the biochemistry of DNA replication, cells acquire one or more critically short telomere. The mechanism by which a short telomere induces the senescent phenotype is unknown. Recent findings suggest that certain types of DNA damage and inappropriate mitogenic signals can also cause cells to adopt a senescent phenotype. Thus, cells respond to a number of potentially oncogenic stimuli by adopting a senescent phenotype. These findings suggest that the senescence response is a fail-safe mechanism that protects cells from tumorigenic transformation [19].

Despite the protection from cancer conveyed by cellular senescence and other mechanisms that suppress tumorigenesis, the development of cancer is almost inevitable as mammalian organism's age. Certainly, aging predisposes cells to accumulate mutations, several of which are necessary before malignant transformation occurs, particularly in humans. However, many benign or relatively well-controlled tumors may also harbor many potentially oncogenic mutations, suggesting that the tissue microenvironment can suppress the expression of many malignant phenotypes. Although the idea remains controversial, cellular senescence has also been proposed to contribute to organismal aging [20]. Senescent cells have recently been shown to accumulate with age in human tissues. One possibility is that the tissue microenvironment is disrupted by the accumulation of dysfunctional senescent cells. Thus, mutation accumulation may synergize with the accumulation of senescent cells, leading to increasing risk for developing cancer that is a hallmark of mammalian aging.

Aging hallmarks can be divided into three categories: (1) primary, or the causes of age-associated damage; (2) antagonistic, or the responses to the damage; and (3) integrative, or the consequences of the responses and culprits of the aging phenotype. Senescence, a cellular response that limits the proliferation of aged or damaged cells belongs to the antagonistic class [21]. Although senescence plays physiological roles during normal development and it is needed for tissue homeostasis, senescence constitutes a stress response triggered by insults associated with aging such as genomic instability and telomere attrition, which are primary aging hallmarks themselves. There is also an intimate link between senescence and the other antagonistic hallmarks of aging. For example, senescent

cells display decreased mitophagy, resulting in an “old,” defective mitochondrial network that may contribute to metabolic dysfunction in age [22].

Regardless, the senescence arrest is stringent. It is established and maintained by at least two major tumor suppressor pathways the p53/p21 and p16INK4a/pRB pathways—and is now recognized as a formidable barrier to malignant tumorigenesis. Consistent with this view, cells undergo senescence in response to a host of potentially oncogenic stimuli [23]. Cellular senescence entails widespread changes in chromatin organization, including the formation of repressive heterochromatin at several loci that encode pro-proliferative genes. Perturbations to the epigenome can elicit a senescence response (Figure 1). For example, global chromatin relaxation (such as that caused by broad-acting histone deacetylase inhibitors) induces senescence, often by depressing the p16INK4a tumor suppressor, which promotes the formation of senescence-associated heterochromatin. Other inducers, for example, suboptimal c-MYC or p300 histone acetyltransferase activity, also appear to act by perturbing chromatin organization and inducing p16INK4a expression. Notably, p16INK4a, which is expressed by many senescent cells, is both a tumor suppressor and a biomarker of aging. Finally, under some circumstances, epigenomic perturbations can elicit a DDR in the absence of physical DNA damage. For example, histone deacetylase inhibitors activate the DDR protein ATM which initiates a DDR without DNA damage.

Chronic activation or overexpression of p53, pRB, p21, or p16INK4a is generally sufficient to induce a senescence growth arrest. The p53/p21 and p16INK4a/pRB pathways also regulate several although not always all other features of senescent cells. Genomic damage, including dysfunctional telomeres, activates the DDR, which engages the p53/p21 pathway [24-25]. This engagement is biphasic. The initial response is rapid (generally within minutes to an hour), robust, and transient (generally subsiding within 24–48 h), which is typical of the p53 response to many forms of DNA damage. However, if the damage is severe or irreparable enough to elicit a senescence response low-level p53 activation and p21 expression persist once the robust rapid phase declines. Persistent DDR signaling appears to initiate the senescence growth arrest (as opposed to a transient damage-induced growth arrest). Such signaling is also accompanied by the slow (occurring over days) activation of other signaling pathways, such as those governed by the stress-responsive p38MAPK and protein kinase C pathways, and increased reactive oxygen species, which also participate in signaling pathways. These pathways are initiated by poorly understood mechanisms. These additional signaling pathways, then, stimulate the

expression of p16INK4a, which, acting through pRB, ensures the essential irreversibility of the growth arrest [26].

1.3 Apoptosis in cancer

Apoptosis is a very tightly programmed cell death with distinct biochemical and genetic pathways that play a critical role in the development and homeostasis in normal tissues. It contributes to elimination of unnecessary and unwanted cells to maintain the healthy balance between cell survival and cell death in metazoan. It is critical to animal's especially long-lived mammals that must integrate multiple physiological as well as pathological death signals. Evidence indicates that insufficient apoptosis can manifest as cancer or autoimmunity, while accelerated cell death is evident in acute and chronic degenerative diseases, immunodeficiency, and infertility. Under many stressful conditions like precancerous lesions, activation of the DNA damage checkpoint pathway can serve to remove potentially harmful DNA-damaged cells via apoptosis induction to block carcinogenesis [27]. Thus, the apoptotic signals help to safeguard the genomic integrity while dysregulation of the apoptotic pathways may not only promote tumorigenesis but also render the cancer cell resistant to treatment. Thus, the evasi on of apoptosis is a prominent hallmark of cancer. Cancer cells are, in fact, harbouring alterations that result in impaired apoptotic signaling, which facilitates tumour development and metastasis [28].

Induction of apoptosis by chemotherapeutic agents, such as DNA-damaging agents, topoisomerase inhibitors, and antimitotic agents, is largely mediated by the mitochondrial apoptotic pathways (2), which in turn increase the permeability of outer mitochondrial membrane. This involves release of mitochondrial apoptotic components, such as cytochrome c, apoptosis-inducing factor (AIF), second mitochondrial-derived activator of caspase, endonuclease G, and Omi1/HtrA2. The released apoptotic proteins initiate caspase activation and trigger caspase-mediated apoptotic DNA fragmentation and eventually cell death [29]. On the other hand, mammalian cells in a certain circumstance can undergo caspase-independent apoptosis that is mediated by the disruption of the mitochondrial membrane potential and the translocation of AIF and endonuclease G to nucleus where they induce chromatin condensation and/or large-scale DNA fragmentation. It has been reported that AIF mediates cell death through a Caspase-independent pathway. Mitochondrial AIF

translocates to the nucleus on death stimuli and initiates nuclear condensation that leads to large-scale chromatin fragmentation followed by the cell death [30-31].

Apoptosis as a therapeutic goal offers advantages over non-apoptotic death mechanisms only if the therapeutic index or the availability of compounds that induce it is greater. In drug-curable malignancies, such as common pediatric leukemia and certain solid tumors, apoptosis is a prominent mechanism associated with the induction of tumor remission. In addition, the expression of apoptotic modulators within a tumor appears to correlate with its sensitivity to traditional cancer therapies. No strict correlation between the induction of apoptosis and a patient's long-term prognosis has emerged, perhaps in part because the ability to achieve initial remission alone does not adequately predict long-term outcome [32]

A drug that activates apoptosis might achieve a suitable therapeutic index in several ways. First, it might activate a death cascade via a drug target that is uniquely expressed in a cancer cell. Alternatively, it might be delivered to the target tissue in a manner that is selective for the cancer cell. These 2 strategies have proved frustratingly difficult to achieve in any broad clinical sense during the past decades. A third and perhaps more promising approach is to exploit a pathway that is activated by oncogenes, in order to provoke apoptosis selectively in cancer cells [33].

1.3 DNA repair and therapeutic exploration

Living organisms have the crucial task to preserve their genome and faithfully transmit it across generations. Transmission of genetic information is constantly in a selective balance between the maintenance of genetic stability versus elimination of mutational change and loss of evolutionary potential. The DNA molecule is under the continuous attack of a multitude of endogenous and exogenous genotoxic insults and it has been estimated that every cell experiences up to 105 spontaneous or induced DNA lesions per day. Endogenous damage can result from DNA base lesions like hydrolysis (deamination, depurination, and depyrimidination) and alkylation (6-O-Methylguanine) or oxidation (8-oxoG) by intracellular ROS that can occur as by-products of mitochondrial respiration. Mutations can also arise during normal cellular metabolism for instance by erroneous incorporation of deoxyribonucleotides (dNTPs) during replication. Environmental sources of damage can be physical [e.g., ultraviolet (UV) light, ionizing radiations (IRs), and thermal disruption] or chemical (e.g., chemotherapeutic drugs,

industrial chemicals, and cigarette smoke) and their effects varies from the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs) following UV exposure, to the introduction of single and double DNA strand breaks upon IR treatment, or to inter- and intrastrand DNA crosslinks, which result from various chemotherapeutic drugs [34].

Poly (ADP-ribose) polymerase I (PARP1) is an abundant nuclear enzyme that catalyzes the formation of ADP-ribose polymers (PAR) on a host of protein substrates, including its own auto modification domain. Extensive research has revealed numerous PARP1 functions spanning a diverse array of nuclear processes, including DNA damage repair, chromatin remodeling, transcriptional regulation, telomere maintenance, and cell death. Owing primarily to the role of PARP1 (and the related but less abundant PARP2 and PARP3) in DNA damage repair, a number of PARP inhibitors are undergoing clinical development as anticancer agents with a focus on tumors with impaired homologous recombination (HR) capability and on combination regimens with DNA-damaging chemotherapy or radiation [35]. The function of PARP1 in DNA damage repair is complex and multifaceted, and includes contributions to multiple repair pathways including HR, base excision repair (BER), nucleotide excision repair (NER), and both classical and alternative nonhomologous end-joining. Preclinical studies have revealed combination activity of PARP inhibitors with DNA-alkylating agents, platinums, topoisomerase I inhibitors, and ionizing radiation. As the primary repair pathways for the lesions caused by each of these classes of agents differ, the mechanisms underlying their potentiation by PARP inhibitors is likely to be class specific.

Inhibition of DNA repair in cancer cells represents an attractive strategy for potentiating the cytotoxic effects of chemotherapy and radiation and therefore this has been a subject of scientific research for several decades. Of the known DNA repair inhibitors, Poly (ADP-ribose) polymerase inhibitors (PARPi) are furthest along in development and appear promising in a variety of cancer types, including breast and ovarian cancers. The first PARP enzyme was discovered over 40 years ago and PARP-1 is the most abundant and best-characterised member of the family of PARP enzymes. PARP-1 has a key role in the repair of single-strand breaks (SSBs), resulting from oxidative stress via the base excision repair/SSB repair (BER/SSBR) pathway [36].

1.4 Thesis structural and overall impact

This whole study provides a novel method to identify new molecular target either using FDA-approved drug or new chemical synthetically drug for cancer therapeutic using combination between bioinformatics and biochemical approaches.

Chapter 1 Introduction

In this chapter, an overview and background of this research is introduced including Bioinfomatics understanding, cancer and senescense, apoptosis in cancer, DNA repair and therapeutic exploration

Chapter 2 Induction of senescence in cancer cells by 5-Aza-2-deoxycytidine: Bioinformatics and experimental insights to its targets.

In this chapter, multimodule of 5-Aza-dC demethylating FDA-approved drug is explained very well by using combination of bioinfomatics and biochemical approach.

Chapter 3 Conclusions and future researches

In this chapter, the main results are summarized. In particular, the future research points were also directed.

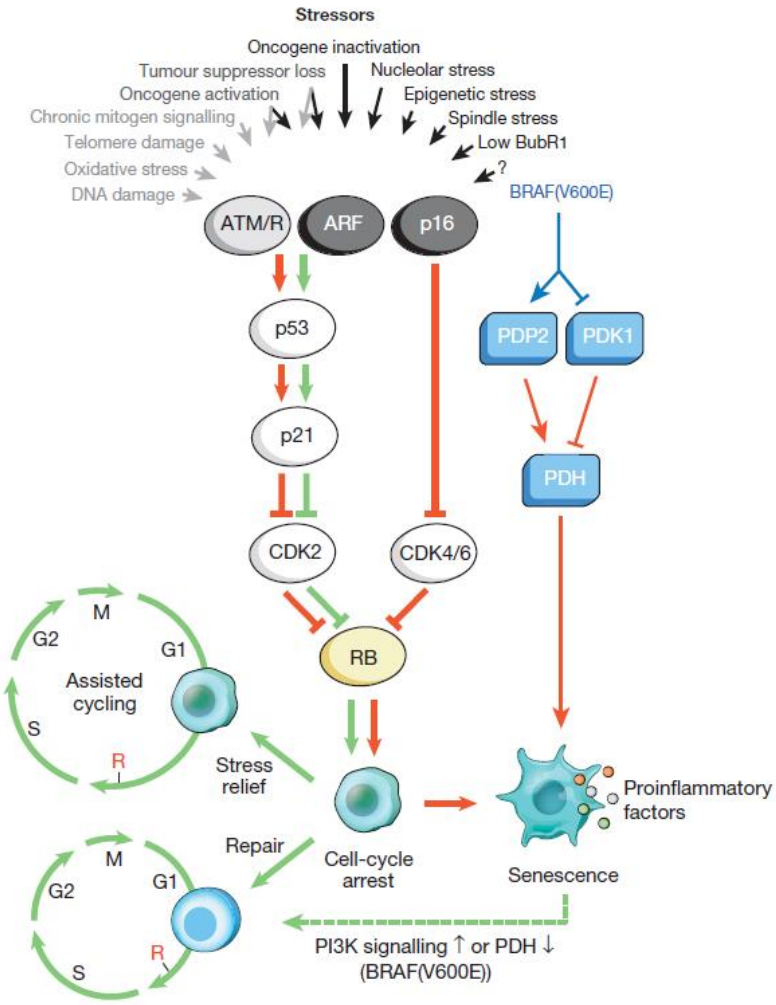

Fig 1.1 Stimuli and effector scheme in senescence cells

(Nature. 2014. 509: 439-446)

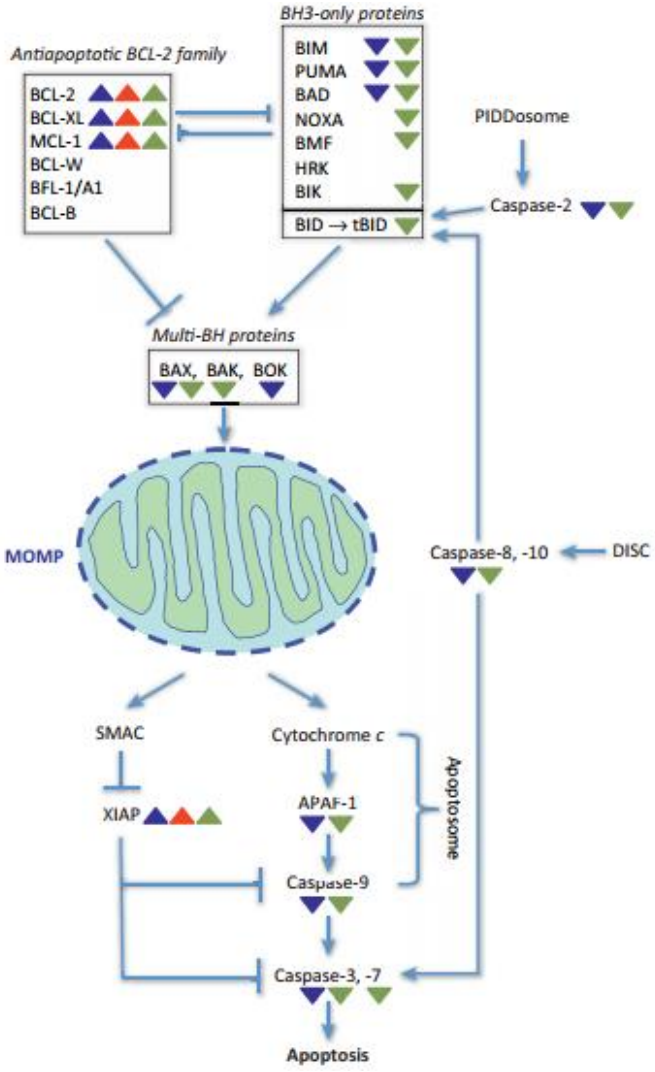

Fig 1. 2 An intrinsic pathway of apoptosis by activation of BH3-only protein

(Trend in Cell Biology. 2013. 23(12))

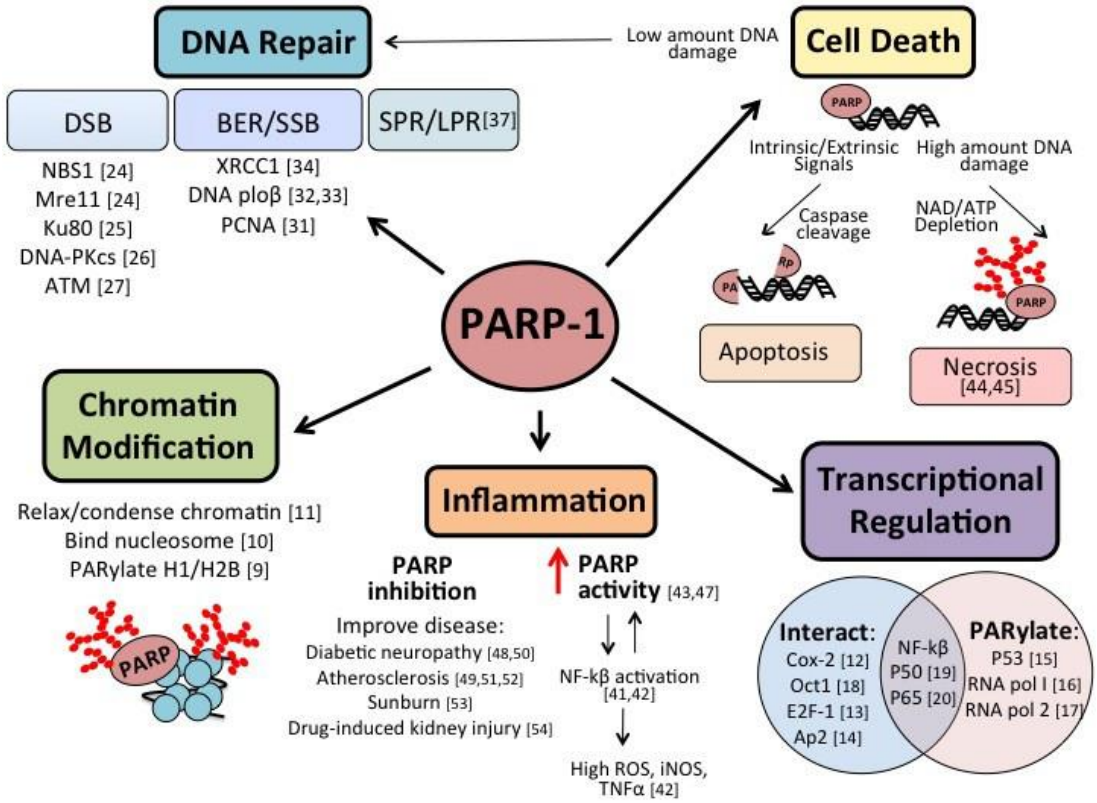

Fig 1.3 Schematic delineating the multifaceted nature of Poly (ADP) Ribose Polymerase (PARP): DNA repair, Chromatin Modification, Inflammation, Transcriptional Regulation, and Cell Death.

(Cancers. 2013. 5: 943-958)

Reference:

1. Sliwoski G., Kothiwale S., Meiler J., Lowe EW Jr. Computational methods in drug discovery. Pharmacol Rev. 2014;66(1):334–395.
2. Alvarez D.D, Sivignon A., Chalopin T. The Antiadhesive Strategy in Crohn's Disease: Orally Active Mannosides to Decolonize Pathogenic Escherichia coli from the Gut. Chembiochem. 2016;17(10):936–952.
3. Amin K.M., Anwar M.M., Kamel M.M., Kassem E.M., Syam Y.M., Elseginy S.A. 2013 Synthesis, cytotoxic evaluation and molecular docking study of novel quinazoline derivatives as PARP-1 inhibitors. Acta Pol Pharm. 70(5):833–849.
4. Sabbah D.A, Saada M, Khalaf R.A. 2015 Molecular modeling based approach, synthesis, and cytotoxic activity of novel benzoin derivatives targeting phosphoinostide 3-kinase (PI3Kalpha). Bioorg Med Chem Lett. 25(16):3120–3124.
5. Frederick R., Robert S., Charlier C. 2005. 3,6-disubstituted coumarins as mechanism-based inhibitors of thrombin and factor Xa. J Med Chem. 48(24):7592–7603.
6. Dong M.H., Chen H.F., Ren Y.J., Shao F.M. 2016. Molecular modeling studies, synthesis and biological evaluation of dabigatran analogues as thrombin inhibitors. Bioorg Med Chem. 24(2):73–84.
7. Mena-Ulecia K., Tiznado W., Caballero J. 2015. Study of the differential activity of thrombin inhibitors using docking, QSAR, molecular dynamics, and MM-GBSA. PLoS One. 10(11):e 0142774.
8. Vitoria M., Granich R., Gilks C.F. 2009. The global fight against HIV/ AIDS, tuberculosis, and malaria: current status and future perspectives. Am J Clin Pathol. 131(6):844–848. 55.
9. Obiol-Pardo C., Rubio-Martinez J., Imperial S. 2011. The methylerythritol phosphate (MEP) pathway for isoprenoid biosynthesis as a target for the development of new drugs against tuberculosis. Curr Med Chem. 18(9):1325–1338.
10. Guggisberg A.M., Amthor R.E., Odom A.R. 2014. Isoprenoid biosynthesis in Plasmodium falciparum. Eukaryot Cell. 13(11):1348–1359.
11. Takahashi S., Kuzuyama T., Watanabe H., Seto H. 1998. A 1-deoxy-Dxylulose 5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis. Proc Natl Acad Sci U S A. 95(17):9879–9884.

12. Jackson E.R., Dowd C.S. 2012. Inhibition of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr): a review of the synthesis and biological evaluation of recent inhibitors. Curr Top Med Chem. 12(7): 706–728.
13. Reuter K., Sanderbrand S., Jomaa H. 2002. Crystal structure of 1-deoxyD-xylulose-5-phosphate reductoisomerase, a crucial enzyme in the non-mevalonate pathway of isoprenoid biosynthesis. J Biol Chem. 277(7):5378–5384.
14. Singh N., Avery M.A., McCurdy C.R. 2007. Toward Mycobacterium tuberculosis DXR inhibitor design: homology modeling and molecular dynamics simulations. J Comput Aided Mol Des. 21(9):511–522.
15. Goble J.L., Adendorff M.R., de Beer T.A., Stephens L.L., Blatch G.L. 2010. The malarial drug target Plasmodium falciparum 1-deoxy-D-xylulose-5- phosphate reductoisomerase (PfDXR): development of a 3-D model for identification of novel, structural and functional features and for inhibitor screening. Protein Pept Lett. 17(1):109–120.
16. Andaloussi M, Henriksson L.M., Wieckowska A. 2011. Design, synthesis, and X-ray crystallographic studies of alpha-aryl substituted fosmidomycin analogues as inhibitors of Mycobacterium tuberculosis 1-deoxy-D-xylulose 5-phosphate reductoisomerase. J Med Chem. 54(14):4964–4976.
17. Dou Z., Ivanoz A., Adams P.D., Berger S.L. 2016. Mammalian autophagy degrades nuclear constituents in response to tumorigenic stress. Autophagy. 2:12(8):1416-7
18. Sancho S.C., Ouichi T. 2015. Cell Differentiation and Checkpoint. Int J Cancer Res Mol Mech. 1(2). Epub 2015 Jul 2.
19. Petrova N.V., Velichko A. K., Razin S. V., Kantidze O. L. 2016. Early S-phase cell hypersensitivity to heat stress. Cell Cycle.15(3): 337-44
20. Zhu Y., He W., Gao, X., Li, B., Mei, C., Xu, R., Chen, H. 2015. Resveratrol overcomes gefitinib resistance by increasing the intracellular gefitinib concentration and triggering apoptosis, autophagy and senescence in PC9/G NSCLC cells. Sci Rep. 5:17730.
21. Muñoz-Espín, D., Cañamero M., Maraver A., Gómez-López G., Contreras, J., Murillo-Cuesta S., Rodríguez-Baeza A., Varela-Nieto I., Ruberte, J., Collado M.,, Serrano M. 2013. Programmed cell senescence during mammalian embryonic development. Cell. 155:1104–1118
22. Muñoz-Espín, D., and M. Serrano. 2014. Cellular senescence: from physiology to pathology. Nat. Rev. Mol. Cell Biol. 15:482

23. Al-Khalaf H.H., Aboussekhra A. 2013. p16(INK4A) positively regulates p21(WAF1) expression by suppressing AUF1-dependent mRNA decay. PLoS One. 8(7): e70133.
24. Wani W.Y., Kandimalla R.J.L., Sharma D. R., Kaushal A., Ruban A., Sunkaria A., Vallamkondu J., Chiarugi, A., Reddy P.H., Gill, K. D. 2017. Cell cycle activation in p21 dependent pathway: An alternative mechanism of organophosphate induced dopaminergic neurodegeneration. Biochim Biophys Acta. 1863(7):1858-1866.
25. Kalra, R. S., Cheung, C. T., Chaudhary, A., Prakash, J., Kaul, S. C., Wadhwa, R. 2015. CARF (Collaborator of ARF) overexpression in p53-deficient cells promotes carcinogenesis. Mol Oncol. (9):1877-89.
26. Min E. Y., Kim I. H., Lee J., Kim E. Y., Choi Y. H., Nam T. J. 2014. The effects of fucodian on senescence are controlled by the p16INK4a-pRb and p14Arf-p53 pathways in hepatocellular carcinoma and hepatic cell lines. Int J Oncol. 45(1):47-56
27. Van Gurp M., Festjens N., van Loo G., Saelens X., Vandenabeele P. 2003. Mitochondrial intermembrane proteins in cell death. Biochem Biophys Res Commun. 304:487 – 97.
28. Ashkenazi A., Dixit V.M. 1998. Death receptors: signalling and modulation. Science. 281:1305– 1308.
29. Susin S.A, Lorenzo H.K, Zamzami N, et al. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. 397:441 – 6. 45.
30. Joza N, Susin SA, Daugas E, et al. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 2001;410: 549 – 54
31. Wiraswati H. L., Hangen E., Sanz A. B., Lam N. V., Reinhardt C., Sauvat A., Mogha A., Ortiz A., Kroemer G., Modjtahedi N. 2016. Apoptosis inducing factor (AIF) mediates lethal redox stress induced by menadione. Oncotarget. 7(47):76496-76507.
32. Rebecca S.Y.W. 2011. Apoptosis in cancer: from pathogenesis to treatment. Wong Journal of Experimental & Clinical Cancer Research. 30:87
33. Baig S., Seevansant I., Mohammad J., Mukheem A., Huri H.A., Kamarul T. 2016. Potential of apoptotic pathway-targeted cancer therapeutic research: Where do we stand? Cell Death and Disease. 7: e2058
34. Curtin N.J. 2005. PARP inhibitors for cancer therapy. Expert Rev Mol Med.7(4):1-20.
35. Bouchard V. J., Rouleau M., Poirier G.G. 2003. PARP-1, a determinant of cell survival in response to DNA damage. Exp Hematol. 31(6):446-54.

36. Bryant H. E., Schultz N., Thomas H. D., Parker K. M., Flower D., Lopez E., Kyle S., Meuth M., Curtin N.J., Helleday T. 2005. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. 2005 Apr 14;434(7035):913-7

Chapter 2

Induction of senescence in cancer cells by 5-Aza-2-deoxycytidine: Bioinformatics and experimental insights to its targets

2.1 Introduction

Cancer incidence is increasing rapidly worldwide and has prioritized anticancer drug discovery and development. In spite of remarkable progress in this field, only a small percent of drugs reach to clinical trials and even a smaller fraction of them develop to the drugs. This is because cancer is a diversive in its etiology and progression. Large databases on cancer genomics, transcriptomics and proteomic have established that although all cancers are accepted as a proliferative disease that resists apoptosis, they require multi-module treatment. All cancers escape controls on normal cell proliferation due to lack of one or more functional tumor suppressor proteins; p53 is inactivated in the vast majority of cancers. Besides the local surgery, radiotherapy and chemotherapy are the two mainstream treatments for cancer. Since the new drugs have to undergo lengthy process of approval for safety or side effects, we set out in search of multi-module functions of established cancer drugs so that they could be put into wider use in cancer therapeutics. Acetylsalicylic acid known as aspirin, commonly used to treat pain, fever, and inflammation, has been shown to prevent heart attacks, strokes, and blood clot formation. It has also been reported to possess anti-hypertension and anticancer activities. Such studies have initiated a new field of cross-functional research. An identification of new functionality of an existing drug is possible by Bio-Chem information technology. This approach has been widely used to analyze activities of a small molecule for the development of new drugs. DNA methylation is an important key of epigenetic modification to maintain genomic stability required in many biological steps including genomic imprinting, regulation of gene and chromatin remodeling. An abnormal pattern of DNA methylation has been reported in many tumor-derived cells leading to silencing of many tumor suppressor genes.

5-Aza-2- deoxycytidine (5-AZA-dC) is a FDA-approved widely used hypomethylating anticancer drug. It causes global demethylation of cytosine in the C-5 position of CpG rich promoters of tumor suppressor genes. Many studies have reported that 5-AZA-dC induces DNA damage response signal- ing through activation of hallmark proteins including ATM, ATR (ATM-Rad3-related), checkpoint kinase 1 (CHK1), BRCA1, NBS1, and RAD51. In an earlier study, we reported that 5-AZA-dC interacts with mortalin and Pex3P causing activation of growth arrest signaling by inhibition of these proteins that occurred independently of its demethylation effect. It has also been shown that demethylation causes activation of miRNAs, the noncoding regulators that have been implicated in wide cellular processes including differentiation, proliferation, and

carcinogenesis. Of note, hundred target genes can be silenced by a single miRNA and hence, miRNA deregulation has been closely and complexly related to the process of carcinogenesis. Recently, we recruited 5-AZA-dC-induced senescence in miR- induced loss-of-function screening and identified miRNA-335 as a methylation regulatory miR and its role in tumor suppression through targeting CARF, a regulator of p53-HDM2-p21 pathway, through mechanism independent of its demethylation effect. These reports have reflected the complexity of action of 5-AZA-dC. Carcinogenesis, tumor progression and metastasis have also been established as multifactorial phenom- ena involving loss and gain of function of tumor suppressor and oncogenes, respectively, by multiple and cross-talking pathways. It has been established that p53 is degraded by HDM2 by proteasomal degradation pathway and inactivated by binding to mortalin. Mortalin is widely distributed in the cytoplasm of normal cells, whereas the cancer cells show perinuclear localization. Human osteosarcoma cell lines when treated with 5-AZA-dC showed shift in mortalin staining pattern, from perinuclear to pancytoplasmic, and an upregulation and nuclear translocation of p53. Upregulation of HDM2 and mortalin has been reported in cancers that account for loss-of-function of p53 In agreement with these reports, drugs that abrogate p53-HDM2 and p53-mortalin interactions have been shown to cause growth arrest of cancer cells. In the present study, we conducted an analysis in search of new functions of 5-AZA-dC using bio-chemo-informatics approach. The data suggested that 5-AZA-dC, independent to its demethylation activity, is a potential inducer of p53-p21 pathway and inhibits the growth of cancer cells.

2.2 Material and Methods

2.2.1 Prediction protein target and bioactivity of 5-AZA-dC

The structure of 50-Aza-20-deoxycytidine (5-AZA-dC) was drawn and converted into a simplified molecular input line entry system (SMILES) by PubChem editor software [1]. SMILES structure was then used to predict the targeted protein and its bioactivity. The protein of targeted 5-AZA-dC was predicted by structural similarity toward the drug database by applying SuperPred method [2]. Meanwhile, the prediction of 5-AZA-dC bioactivity was made by comparing the active structure with the drug database by Molinspiration Cheminformatics 2016 and PASS online [3].

2.2.2 Protein network and biological process analyses

The predicted proteins showed binding capability with 5-AZA-dC are CXCR4, POLB, POLA1 and HDM2. These proteins were then analyzed for their interactions with other proteins (protein network) to determine their biological function in a cell. Protein network analysis was performed by Cytoscape software [4], based on the human protein in BIOGRID database [5]. Proteins that were interacting with the targeted proteins were analyzed for their biological function by using an algorithm of A Biological Network Gene Ontology tool (BINGO) [6].

2.2.3 Analysis molecular docking

The ligands used for docking analyses were 5-AZA-dC and POLB, POLA1, MDM2, CXCR4 protein inhibitors. SMILE structure of 5- AZA-dC was converted to the 3D structure in the format of Protein Data Bank (PDB) by Discovery Studio software ("Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 4.5, San Diego: Dassault Systèmes, 2015.," n.d.). The structure was then used as a ligand for docking. The structure of 5,6-dihydroxy-3- carboxyphenylester (BDBM50 293303; CHEMBL498103) Gallic acid as POLB/POLA1 inhibitor and Chalcone 4 as CXCR4 inhibitor were taken from BindingDB database [7]. Meanwhile, the structure of receptor protein was retrieved from protein data banks with access codes as follows 4oq3 (MDM2), 5bol (POLB), 4q5 v (POLA1), and 3oe6 (CXCR4). The proteins were then prepared by Discovery Studio software. Interactions between proteins (receptors) with the small molecule (ligand) were performed by Autodoc Vina integrated into the Pyrx software [8-9]. Docking was performed by imposing receptors as rigid molecules while the ligand molecule was set flexible and graded in the active site. Results of docking and bonding interactions between ligands and receptors were analyzed by Discovery Studio software.

2.2.4 Cell culture, cell growth inhibition analysis and immunostaining

Human osteosarcoma (U2OS) and breast carcinoma (MCF7) cells were obtained from Japanese Collection of Research Bioresources (JCRB, Japan). Cells were treated with 5-AZA-dC (10 mM) and maintained in DMEM (Dulbecco's Modified Eagle's Medium) (Life Technologies, Carlsbad, CA, USA)-supplemented with 10% fetal bovine serum and antibiotics at 5% CO_2 and 95% air in a humidified incubator for 72 h. Cells were harvested every day and counted by hemocytometer to examine the growth. Immuno-staining was performed on fixed (treated with pre-cooled methanol/acetone 1:1, vol/vol. for 5 min on ice)

cells grown on glass coverslips. The fixed cells were washed with cold phosphate-buffered saline (PBS) 3 times and permeabilized using 0.2% Triton X-100 in PBS for 10 min. The permeabilized cells were blocked with 2% bovine serum albumin (BSA) in PBS for 30 min and incubated with anti-MDM2 (HDM2-323) and p53 (FL-393) (Santa Cruz, CA, USA) antibodies followed by washing in 0.2% Triton X-100 in PBS 3 times, 10 min each, and incubation with Alexa 488- conjugated goat anti-mouse or Alexa-594-conjugated goat anti-rabbit antibodies (Molecular Probes, Invitrogen).They were then washed in 0.2% Triton X-100 in PBS 3 times, 10 min each. Nuclear staining was performed with Hoechst 33342 (Sigma) for 5 min in dark followed by three times washing. The cells were overlaid with a coverslip containing Fluoromount (Difco/BD, Franklin Lakes, and NJ) and observed under a Carl Zeiss microscope (Axiovert 200 M, Tokyo, Japan).

2.2.5 Cell viability in response to drugs

U2OS cells were seeded in 96-well plate for 24 h. At about 65– 70% confluency, 10 mM 5-AZA-dC treatment was given for the next 24 h followed by 5-Flourourasil (20, 30 and 40 mM), Nacodazole (120, 160 and 200 mM), and Curcumin (10, 30 and 50 mM) treatment for 24 h. DMSO in equal volume was prepared as a control. Viability of cells was measured following manufacturer's instructions (Life Technologies, Carlsbad, CA, USA) and as described earlier [10].

2.3 Results and Discussion

I examined the possibility of new functions of 5-AZA-dC (the structure is shown in Fig. 1A) by bio-chemo-informatics analyses and predicted a new mechanism of its anticancer activity. It was predicted that 5-AZA-dC serves as an inhibitor of CXCR4, HDM2, POLB, and POLA1 resulting in up-regulation of p53 pathway, growth arrest or apoptosis (Fig. 1B-E and 2B). Moreover, it was predicted that 5-AZA-dC might induce p53 by disrupting p53-HDM2 binding complex. The docking analysis showed that 5-AZA-dC could bind to CXCR4, HDM2, POLB and POLA1 at its active site, where the known inhibitors bind (Fig. 2A). Superimpose showed that 5-AZA-dC occupied the same position as a known inhibitor bound to proteins (Fig. 2A, bottom panel). The 5-AZA-dC binds to the binding site of 6-dihydroxy-3- carboxyphenylester, gallic acid and chalcone on PASS online analysis predicted that 5-AZA-dC may target several proteins involved in DNA damage and p53 tumor suppressor pathway (Fig. 1B). SuperPred analysis result suggested that it binds

to POLA1, POLB, and CXCR4 (Fig. 1C). Molinspiration analysis predicted its inhibitory action on several enzymes, and as GPCR ligand (Fig. 1D).

Of note, SuperPred analysis showed that 5-AZA-dC could target CXCR4 protein (Fig. 1C) in line with the predicted outcomes of molinspiration i.e. the drug has an activity as GPCR ligand (Fig. 1D). G protein-coupled receptor (GPCR) is an orphan protein that has recently been focused in the pharmaceutical industry as a novel pro-apoptosis protein [11-14]. Together with these analyses, in order to depict the role of 5-AZA-dC in cell physiology, I examined the interacting protein patterns of predicted protein targets of 5-AZA-dC. The Interactome analysis by BINGO revealed that the targets of 5-AZA-dC were involved in various mechanisms including apoptosis, DNA damage response, and p53/p21 signaling (Fig. 1E), and endorsed its function as an anticancer drug. Furthermore, CXCR4, POLA1, POLB, and HDM2 regulates p53-p21 pathway, apoptosis and growth arrest. I also examined the potential of 5-AZA-dC as HDM2 inhibitor leading to activation of p53 in cell-based assays. Fig. 3A shows the HDM2 and p53 leading to degradation of p53 via proteasome. 5-dAZA-dC-treated cells in a well were examined for senescence-associated β-galactoside staining As shown in Fig. 3B, the treated cells showed intense β-galactoside staining suggesting the induction of senescence. Co-immuno-staining using anti-HDM2 and anti-p53 antibodies showed downregulation of HDM2 and upregulation of p53 in 5-AZA-dC-treated cells (Fig. 3C). The latter was also endorsed by Western blotting with anti-p53 antibody (Fig. 3D) and p53 dependent reporter assays (Fig. 3E). Since cancer chemotherapy often involves a combination of drugs, we next examined if 5-AZA-dC could potentiate the effect of other three (5FU, Nocodazole, and Curcumin) anticancer drugs. To my surprise, I found that the combination treatment did not result in any accumulative/ synergistic effect rather caused minor resistance to all the three drugs used (Fig. 4 A-C).

In the present report, I show that 5-AZA-dC targets POLA1, POLB, CXCR4 and HDM2 proteins. POLA1 and POLB play crucial functions in base excision repair (BER) in response to genotoxic and cytotoxic stresses. HDM2 is an established antagonist of tumor suppressor p53 protein. This complex abolishes the activation of p53 as a tumor suppressor (Fig. 3A). As expected, HDM2 targeting caused activation of p53 protein (key transcriptional regulator of stress response, including DNA damage). Together, POLA1, POLB and p53 proteins are predicted to be involved in cell response to 5- AZA-dC. Furthermore, GPCR protein, an important player of cell proliferation and apoptosis was identified as a 5-AZA-dC target.

Blocking of GPCR receptor has been shown to inhibit cell division and metastasis resulting in growth arrest in cancer cells. CXCR4 protein is a member of GCPR involved in cancer pathways (KEGG: hsa05200) [15]. It regulates the intracellular calcium ion levels and enhances MAPK1/MAPK3 activation (Uniprot: P61073) (Magrane and UniProt Consortium, 2011). Blocking of CXCR4 protein induces cell death or growth arrest and hence has been proposed as a target for cancer drug development [16-18]. My study suggested that demethylation-independent targeting of proteins by 5-AZA-dC might also be a promising strategy for cancer therapy. Through bio-chemo-informatics approach, I identified a new function of 5-AZA-dC as a p53 inducer, mediated by inhibition of HDM2, an established antagonist of p53. Besides having the ability to demethylate the genome, 5-AZA-dC was also predicted to inhibit mortalin [19]. I report that an increase in the level of p53 in 5-AZA-dC-treated cells is mediated by down-regulation of HDM2. Besides inhibiting MDM2 and mortalin, 5- AZA-dC was also able to inhibit DNA polymerases and DNA repair signaling. Cancer cells tend to have a high metabolism, and release free radicals that can cause DNA damage [20]. Previous research has indicated that several noncoding tumor suppressors (miRNAs) are regulated by p53 [21-23]. Interestingly, I investigated new regulatory targets by loss-of-function miRNA screening and found induction of miRNA-335 that targeted CARF (collaborator ARF) and several cell cycle regulatory proteins leading to growth suppression in cancer cells. Transfection of miRNA-335 to cells prior to 5-AZA-dC treatment showed down-regulation of CARF, p53, and p16INK4A expressions. In contrast, MDM2 and p21WAF1 remained high that led to cell cycle arrest [24]. Adequate expression of CARF itself is necessary for cell survival mechanism while overexpression abolished p53 protein expression [25].

Taken together, 5-AZA-dC, an established demethylating drug leads to growth arrest of cancer cells through mechanisms involving direct targeting of proteins leading to activation of p53 pathway. Combination of more than one anticancer agents is often used to control the progress and metastasis of cancers. The cocktail of anticancer drugs is thought to be more powerful than any single drug administration [26-27]. I performed drug combination by sensitizing cells by 5-AZA-dC for 24 h followed by addition of three anticancer drugs (Nacodazole, 5FU and Curcu-min) independently. Surprisingly, the data showed that prior treatment of 5AZA-dC caused resistance to the three drugs. The possible interpretation of this effect could be that these drugs work on proliferating cells, growth arrest caused by prior 5-AZA-dC treatment resulted in subsequent drug resistance. This

finding suggested that the combination of 5-AZA-dC with other anticancer drugs should be selected very carefully so as not to yield drug resistance phenotype.

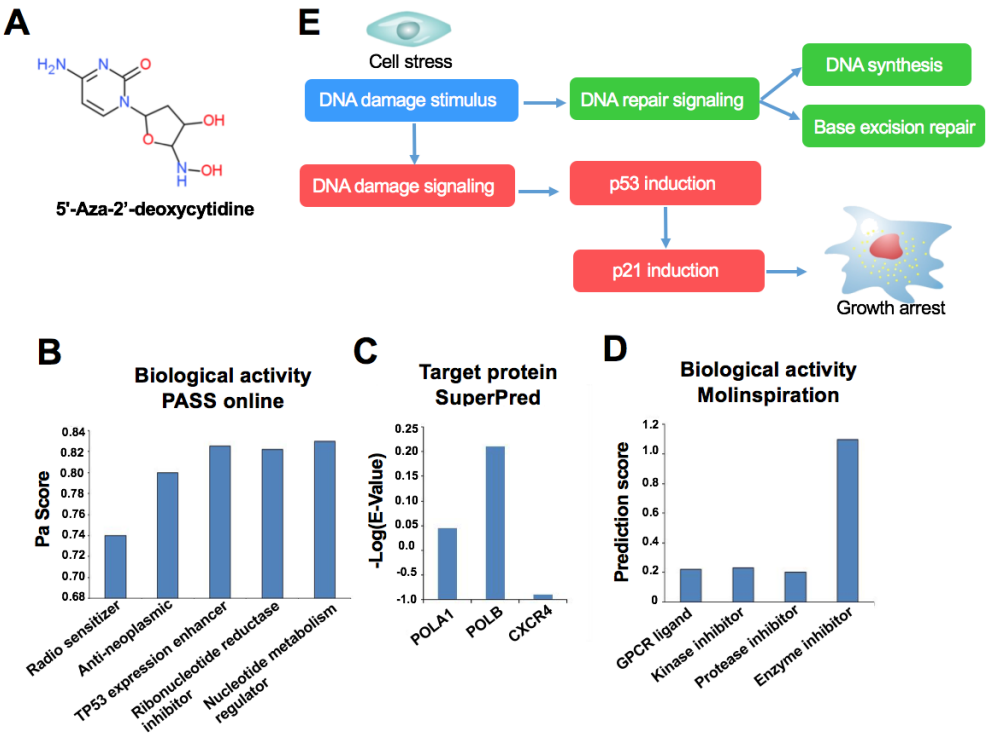

Fig 2.1 5-AZA-dC (A) that has a role in upregulation of p53 (B) was predicted to bind to DNA polymerase (C) and inhibit the activity of several enzymes (D). The drug induces apoptosis and growth arrest through several pathways especially DNA damage response through p53 signalling pathway (E).

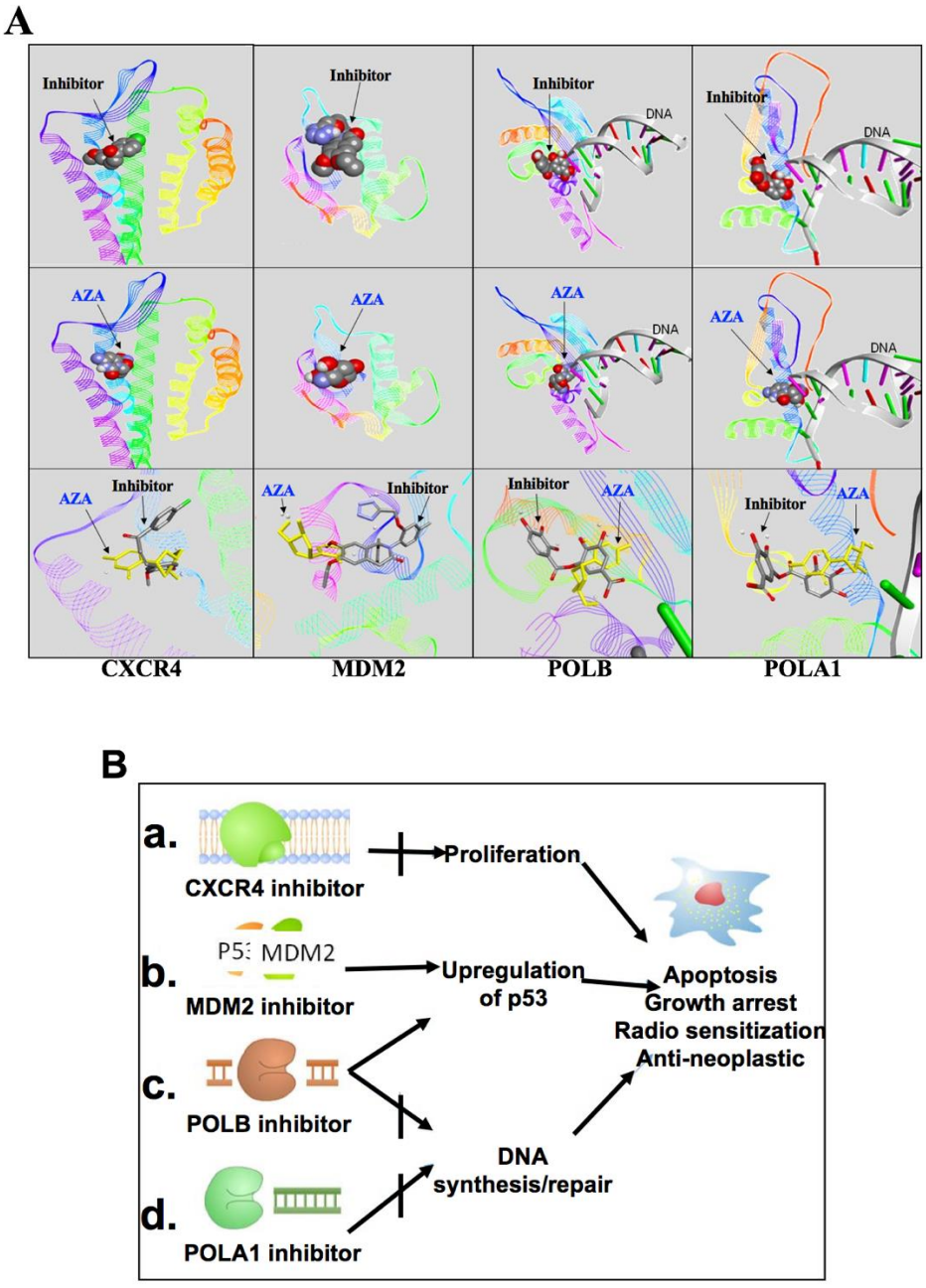

Fig 2.2 5-AZA-dC binds to the active sites of CXCR4, MDM2, POLB and POLA1. The bindings of 5-AZA-dC to these proteins share similar pattern and position with their inhibitor (A). The data suggested that 5-AZA-dC potentially block the activity of MDM2, CXCR4 POLB, and POLA1 (B).

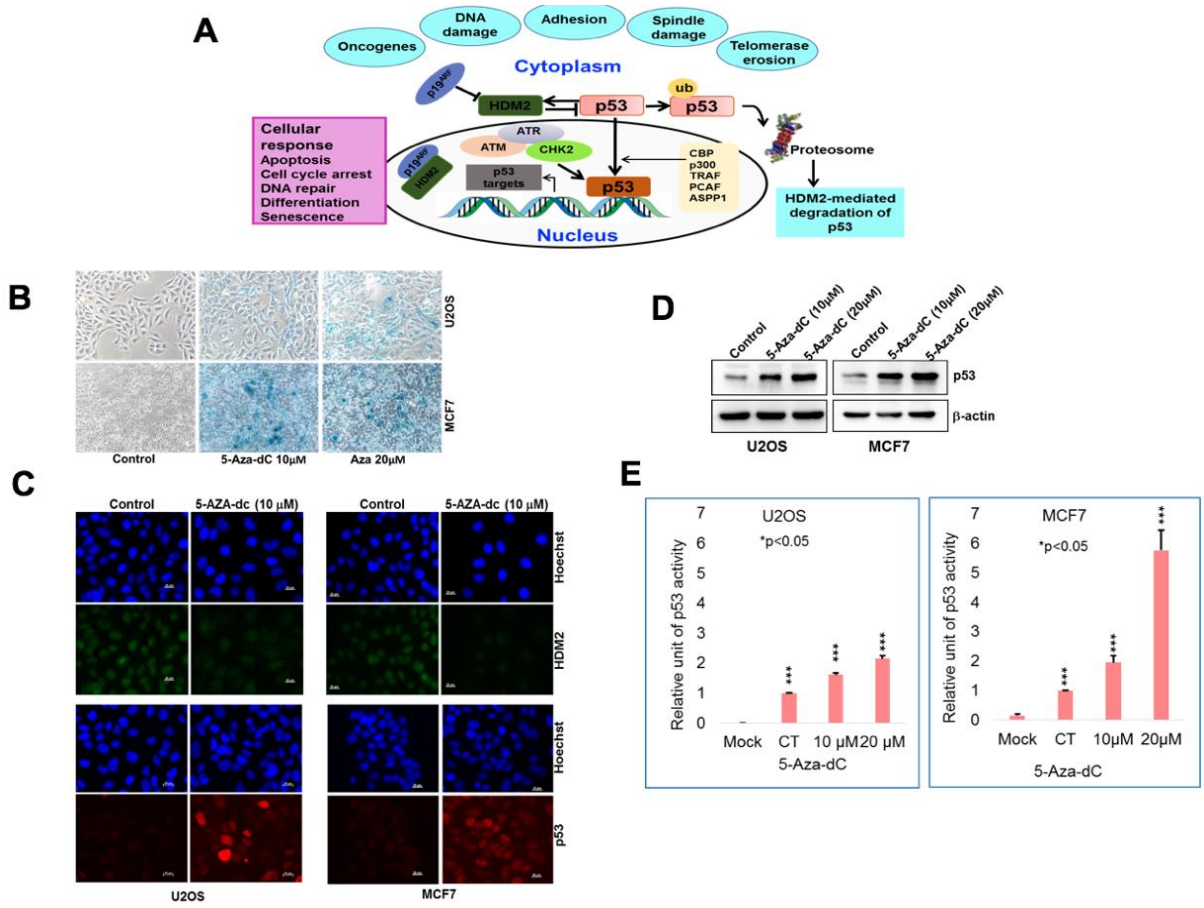

Fig 2.3 Schematic drawing of HDM2-p53 pathway (A). β-galactoside staining indicates cells senescence (B). Immunostaining showing up-regulation of p53 protein induced in response of 5-AZA-dC treatment in U2OS and MCF cell lines (C). Western blotting of p53 (D). Induction of p53 showed by p53-dependent reporter assay (E).

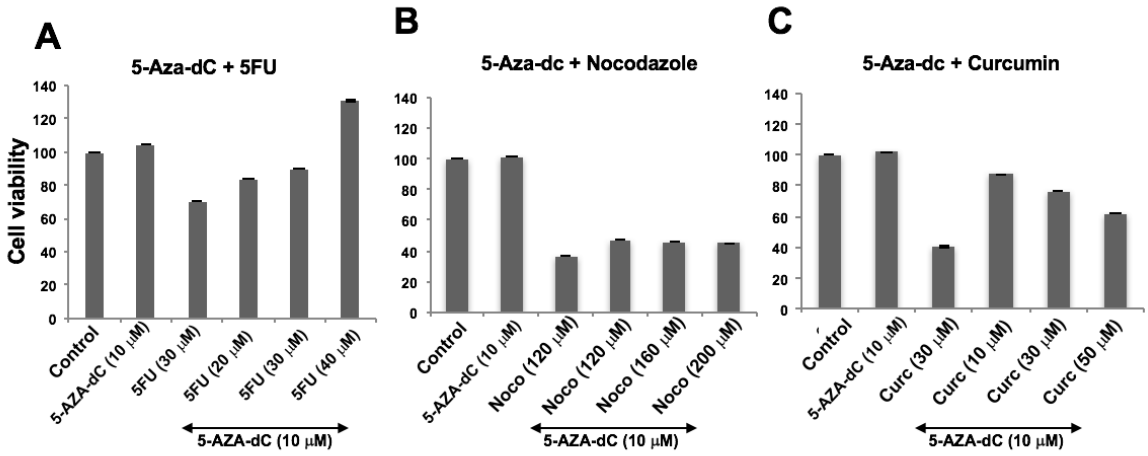

Fig 2.4 5-AZA-dC caused resistance to anticancer drugs (5-Fluorourasil (5FU), Nocodazole and Curcumin)

Reference:

1. Cheng T., Pan Y., Hao M., Wang Y., Bryant S. H. 2014. PubChem applications in drug discovery: a bibliometric analysis. Drug Discov Today 19. 1751-1756.
2. Nickel J., Gohlke B.O., Erehman J., Banerjee P., Rong W.W., Goede A., Dunkel M., Preissner R., 2014. SuperPred: update on drug classification and target prediction. Nucleic Acids Res, 42(Web Server issue). W26-31.
3. Filimonov D.A., Lagunin A.A., Gloriozova T.A., Rudik A.V., Druzhilovskii D.S., Pogodin P.V., Poroikov V.V. 2014. Prediction of the biological activity spectra of organic compounds using the Pass Online Web Resource. Chem Heterocycl Comp 50. 444-457.
4. Shannon P., Markiel A., Ozier O., Baliga N.S., Wang J.T., Ramag, D., Amin N., Schwikowski B., Ideker T. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13. 2498-2504.
5. Stark C., Breitkreutz B.J., Reguly T., Boucher L., Breitkreutz A., Tyers M. 2006. BioGRID: a general repository for interaction datasets. Nucleic Acids Res. 34. D535-539.
6. Maere S., Heymans K., Kuiper M. 2005. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics 21. 3448-3449.
7. Liu T., Lin Y., Wen X., Jorissen R.N., Gilson M.K. 2007. BindingDB: a web-accessible database of experimentally determined protein-ligand binding affinities. Nucleic Acids Res. 35. D198-201.
8. Trott O., Olson A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 31. 455-461.
9. Dallakyan S., Olson A.J., 2015. Small-molecule library screening by docking with PyRx. Methods Mol Biol. 1263. 243-250.
10. Nigam N., Grover A., Goya, S., Katiyar S.P., Bhargava P., Wang P.C., Sundar D., Kaul S.C., Wadhwa R. 2015. Targeting Mortalin by Embelin Causes

Activation of Tumor Suppressor p53 and Deactivation of Metastatic Signaling in Human Breast Cancer Cells. PLoS One. 10. e0138192.

11. Howard A.D., McAllister G., Feighner S.D., Liu Q., Nargund R.P., Van der Ploeg L.H., Patchett A.A. 2001. Orphan G-protein-coupled receptors and natural ligand discovery. Trends Pharmacol Sci. 22. 132-140.
12. Kobilka B. K. 2007. G protein coupled receptor structure and activation. Biochim Biophys Acta. 1768. 794-807.
13. Bockaert J. 2012. G-protein coupled receptors. Nobel Prize 2012 for chemistry to Robert J. Lefkowitz and Brian Kobilka. Med Sci (Paris) 28. 1133-1137.
14. Tosolini M., Pont F., Betous D., Ravet E., Ligat L., Lopez F., Poupot M., Poirot M., Perouzel E., Tiraby G., Verhoeyen E., Fournie J.J. 2015. Human monocyte recognition of adenosine-based cyclic dinucleo-tides unveils the A2a Galphas protein-coupled receptor tonic inhibition of mitochondrially induced cell death. Mol Cell Biol. 35. 479-495.
15. Kanehisa M., Kanehisa M., Goto S., Sato Y., Furumichi M., Tanabe M. 2012. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res 40 (Database issue): D109-114.
16. Narumi T., Tanaka T., Hashimoto C., Nomura W., Aikawa H., Sohma A., Itotani K., Kawamata M., Murakami T., Yamamoto N., Tamamura H. 2012. Pharmacophore-based small molecule CXCR4 ligands. Bioorganic Medicinal Chem Lett. 22. 4169-4172.
17. Fontanella R., Pelagalli A., Nardelli A., D'Alterio C., Ierano C., Cerchia L., Lucarelli E., Scala S., Zannetti A. 2016. A novel antagonist of CXCR4 prevents bone marrow-derived mesenchymal stem cell-mediated osteosarcoma and hepatocellular carcinoma cell migration and invasion. Cancer Lett. 370. 100-107.
18. Jiang K., Li J., Yin J., Ma Q., Yan B., Zhang X., Wang L., Wang L., Liu T., Zhang Y., Fan Q., Yang A., Qiu X., Ma B. 2015. Targeted delivery of CXCR4-siRNA by scFv for HER2+ breast cancer therapy. Biomaterials. 59. 77-87.
19. Widodo N., Deocaris C.C., Kaur K., Hasan K., Yaguchi T., Yamasaki K., Sugihara T., Ishii T., Wadhwa R., Kaul S.C. 2007. Stress chaperones, mortalin, and pex19p mediate 5-aza-2' deoxycytidine-induced senescence of cancer cells

by DNA methylation-independent pathway. J Gerontol A Biol Sci Med Sci. 62. 246-255

20. Dizdaroglu M. 2015. Oxidatively induced DNA damage and its repair in cancer. Mutat Res Rev. 763. 212-245.
21. Tazawa, H., Tsuchiya, N., Izumiya, M., Nakagama, H., 2007. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. Proc Natl Acad Sci USA, 104, 15472-15477.
22. Noonan, E.J., Place, R.F., Basak, S., Pookot, D., Li, L.C., 2010. miR-449a causes Rb-dependent cell cycle arrest and senescence in prostate cancer cells. Oncotarget, 1, 349-358.
23. Xing, Z., Li, D., Yang, L., Xi, Y., Su, X., 2014. MicroRNAs and anticancer drugs. Acta Biochim Biophys Sin (Shanghai), 46, 233-239.
24. Yu, Y., Gao, R., Kaul, Z., Li, L., Kato, Y., Zhang, Z., Groden, J., Kaul, S.C., Wadhwa, R., 2016. Loss-of-function screening to identify miRNAs involved in senescence: tumor suppressor activity of miRNA-335 and its new target CARF. Sci Rep, 6, 30185.
25. Kalra, R.S., Cheung, C.T., Chaudhary, A., Prakash, J., Kaul, S.C., Wadhwa, R., 2015. CARF (Collaborator of ARF) overexpression in p53-deficient cells promotes carcinogenesis. Mol Oncol, 9, 1877-1889.
26. Lu, W.J. Lee, N. P., Kaul, S.C., Lan, F., Poon, R.T., Wadhwa, R., Luk, J.M., 2011. Mortalin–p53 interaction in cancer cells is stress dependent and constitutes a selective target for cancer therapy. Cell Death Different, 18, 1046-1056.
27. Lu, D-Y., Lu, T-R., Cao, S., 2013. Drug combinations in cancer treatments. Clin Exp Pharmacol, 3, 4.
28. Wadhwa, R., Takano, S., Kaur, K., Deocaris, C.C., Pereira-Smith, O.M., Reddel, R.R., Kaul, S.C., 2006. Upregulation of mortalin/mthsp70/ Grp75 contributes to human carcinogenesis. Int J Cancer, 118, 2973-2980.
29. Wang, B., 2016. MicroRNA-153 regulates NRF2 expression and is associated with breast carcinogenesis. Clin Lab, 62, 39-47.

30. Wang, L.Q., Wong, K.Y., Rosen, A., Chim, C.S., 2015. Epigenetic silencing of tumor suppressor miR-3151 contributes to Chinese chronic lymphocytic leukemia by constitutive activation of MADD/ERK and PIK3R2/AKT signaling pathways. Oncotarget, 6, 44422-44436.
31. Wang, X., Teng, Y., Liu, Q., 2014. Demethylation of the miR-146a promoter by 5-Aza-2'-deoxycytidine correlates with delayed progression of castration-resistant prostate cancer. BMC Cancer, 14, 308.
32. Welsh, R.C., Roe, M.T., Steg, P.G., James, S., Povsic, J., Bode, C., Gibson, C.M., Ohman, E.M., 2016. A critical reappraisal of aspirin for secondary prevention in patients with ischemic heart disease. Am Heart J, 181, 92-100.
33. Yiannakopoulou, E., 2014. Targeting epigenetic mechanisms and microRNAs by aspirin and other non-steroidal anti-inflammatory agents--implications for cancer treatment and chemoprevention. Cell Oncol (Dordr), 37, 167-178.
34. Yu, F., Lu, Z., Chen, B., Wu, X., Dong, P., Zheng, J., 2015. Salvianolic acid B-induced microRNA-152 inhibits liver fibrosis by attenuating DNMT1-mediated Patched1 methylation. J Cell Mol Med, 19, 2617-2632.
35. Zong, M., Fan, D.D., Lin, S., Song, Y.P., Wang, Z.Y., Ma, X.L., Qiu, W.H., Bai, Y.H., Li, L., Li, S., 2016. Anti-cancer activity and potential mechanism of a novel aspirin derivative. Eur J Pharmacol, 791, 137-146.

Chapter 3

Conclusions and future research

4.1 Conclusion

In this thesis I used two anticancer drugs, (i) FDA-approved drug (5-Aza-dC) which shown cause demethylating in cancer cells and. By using Bioinformatics and Biochemical combination approaches that helps us to understand better new module of 5-Aza-dC.

Independently from work on 5-Aza-dC we conclude that:

1. 5-Aza-dC is targeting some enzyme involved in apoptosis including POLA1, POLB and CXCR4
2. Activates p53 protein expression
3. Combination with others anticancer drugs caused cells resistant with treatment comparing each treatment alone

4.1 Future work

For 5-Aza-dC, more deeply observations need to be done regarding its effects on POLA1, POLB, and CXCR4 and *in vivo* experiment.