Université de Montréal

ANALYSIS OF DNA METHYLATION STATUS OF p15 GENE IN LEUKEMIA

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Mémoire présenté à la Faculté des études supérieures

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Présenté par

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SUMMARY

Acute leukemia annually afflicts about 28,000 persons in the United States. Intensification of chemotherapy of acute myeloid leukemia has increased the response rate and survival duration, but only a limited number of patients are long-term survivors. New approaches should be done to improve the effectiveness and reduce the toxicity of chemotherapy.

One of the events that can happen during the development of leukemia is the inactivation of the genes that regulate growth. These genes block uncontrolled growth of white blood cells. Genetic and epigenetic mechanisms play role in the inactivation of tumor suppressor genes. One of these epigenetic changes is the aberrant methylation of CpG islands in the promoter region of the tumor suppressor genes that leads to silencing of their expression. Demethylation may restore the function of tumor suppressor genes and arrest the neoplasis.

5-Aza-2'-deoxyxytidine (5-AZA-CdR, Decitabine, 5AZA) is a potent inhibitor of DNA methylation. In clinical trials 5AZA was demonstrated to be an active antileukemic agent.

We investigated the *in vitro* antineoplastic activity of 5AZA on the human HL-60 and KG1a myeloid leukemic cells. We found that this agent is able to inhibit cell growth, DNA synthesis and clonogenic potential in these cell lines.

To prevent drug resistance in the therapy of leukemia one approach can be the combination of agents with different mechanisms of action. An interesting class of agents to use in combination with 5AZA is that of the histone deacetylase inhibitors. Histone acetylation facilitates the binding of transcription factors to the promoter region to activate gene transcription by conversion of chromatin from a compact to an open structure.

We investigated the *in vitro* antineoplastic activity of the histone deacetylase inhibitors, Tichostatin A (TSA) and depsipeptide FR901228 (Depsi), alone and in combination with 5AZA on the human HL-60 and KG1a myeloid leukemic cells. We found that these agents alone are able to inhibit cell growth, DNA synthesis and clonogenic potential in these cell lines. In combination these agents produced a greater inhibition of growth, DNA synthesis and colony formation than either agent alone in leukemic cells. It is interesting to note that the concentrations, which are used in these experiments, are in the range that can be used in the clinical trials.

Recent reports indicate that DNA methylation of the tumor suppressor gene, p15, is a common mechanism of gene suppression in leukemia. Base on these reports we investigated the expression of p15 tumor suppressor gene in Raji Burkitt's lymphoma cells. We found that p15 is not expressed in Raji cells. Following treatment with either 5AZA or TSA alone we detected the reactivation of p15 gene.

In conclusion this study supports future investigation on the use of 5AZA in combination with inhibitors of histone deacetylation in the therapy of leukemia.

RÉSUMÉ

La leucémie aiguë touche annuellement près de 28 000 personnes aux États-Unis. L'intensification de la chimiothérapie a augmenté le taux de réponse au traitement, cependant un nombre limité de patients présente un taux de survie prolongé. De nouvelles approches devraient être développées afin d'améliorer l'efficacité et réduire la toxicité du traitement.

Un des événements à survenir lors du développement de la leucémie est l'inactivation des gènes qui régulent la croissance cellulaire. Ces gènes, appelés suppresseurs de tumeurs, bloquent la croissance non-contrôlée des leucocytes. Des mécanismes génétiques et épigénétiques jouent un rôle dans l'inactivation des gènes suppresseurs de tumeurs. Un des mécanismes épigénétiques est la méthylation anormale des îlots CpG de la région promotrice de ces gènes ce qui mène à leur inactivation. La déméthylation pourrait rétablir la fonction des gènes suppresseurs de tumeurs et ainsi bloquer la néoplasie.

La 5-Aza-2'-désoxycytidine (5-AZA-CdR, Décitabine, 5AZA) est un puissant inhibiteur de la méthylation de l'ADN. Des essais cliniques ont démontré que cet agent actif a un effet anti-leucémique.

Nous avons étudié l'activité antinéoplasique de la 5AZA sur les lignées cellulaires humaines myéloïdes leucémiques HL-60 et KG1a. La 5AZA a inhibé la croissance de ces cellules de façon dose-dépendante et temps-dépendante. Au cours des essais d'inhibition de synthèse d'ADN, la thymidine radioactive a été ajoutée aux cellules après une exposition de 48 heures à la 5AZA afin d'évaluer les effets à long terme de cet agent. La 5AZA a inhibé la synthèse de l'ADN d'une manière dose-dépendante.

Afin d'empêcher la résistance aux médicaments dans la thérapie de la leucémie, nous croyons que la combinaison d'agents ayant des mécanismes d'action différents pourrait être une approche souhaitable. Les inhibiteurs de la déacétylase des histones constituent un groupe d'agents prometteurs pour la combinaison avec la 5AZA. L'acétylation des histones entraîne une modification de la chromatine d'un état compact à un état ouvert, ce qui facilite la liaison des facteurs de transcription dans la région promotrice et donc une activation de la transcription génique.

Récemment, une relation entre la méthylation de l'ADN et la déacétylation des histones a été rapportée. Cette relation implique un complexe formé de la protéine MeCP2 liant les cytosines méthylées de la région promotrice, ainsi que l'enzyme histone déacétylase. Il semblerait que MeCP2 prévienne la liaison des facteurs de transcription au promoteur alors que les histones déacétylés entraîne quant à eux une structure plus compacte de la chromatine empêchant la transcription.

Suite à ces observations, notre hypothèse de travail était que la combinaison d'inhibiteurs de la méthylation de l'ADN et de la déacétylation des histones puisse avoir une activité antinéoplasique augmentée sur les cellules leucémiques. Nous avons également étudié l'activité antinéoplasique *in vitro* de la Trichostatin A (TSA) et du Depsipeptide FR901228 (Depsi), deux puissants inhibiteurs de la déacétylase des histones, seuls et en combinaison avec la 5AZA sur les lignées cellulaires humaines myéloïdes leucémiques HL-60 et KG1a. Nous avons déterminé que ces agents lorsque utilisés seuls peuvent inhiber la croissance cellulaire et la synthèse d'ADN dans ces cellules. De plus, la combinaison de ces agents sur les cellules leucémiques, a produit une plus grande inhibition de croissance cellulaire et de synthèse d'ADN que chaque agent utilisé seul.

Afin d'expérimenter cliniquement ces agents thérapeutiques, l'évaluation des essais clonogéniques est plus pertinente que celle des essais de croissance et

d'inhibition de synthèse d'ADN. Les essais clonogéniques apportent des données sur la survie à long terme des cellules leucémiques en terme de potentiel de prolifération. Nous avons observé une interaction entre la 5AZA et le TSA ou le Depsi sur les cellules leucémiques HL-60. Il est intéressant de noter que les concentrations utilisées dans ces expériences correspondent à celles pouvant être employées dans les essais cliniques.

De récentes études indiquent que l'inactivation du gène suppresseur de tumeur p15 par la méthylation est un mécanisme fréquemment observé dans la leucémie. Nous avons donc analysé le statut de méthylation du gène p15 dans les cellules Raji du lymphome de Burkitt grâce à la méthode de PCR spécifique à la méthylation (MSP). Nos résultats ont démontré la présence de méthylation dans la région promotrice du gène p15 dans cette lignée cellulaire.

L'expression du gène p15 dans les cellules Raji a été étudiée par RT-PCR. Nous avons observé que p15 n'est pas exprimé dans ces cellules, cependant suite à un traitement à la 5AZA ou au TSA, nous avons détecté une réactivation du gène p15.

En conclusion, cette étude supporte des considérations futures quant à l'utilisation de la 5AZA en combinaison avec les inhibiteurs de la déacétylation des histones dans la thérapie de la leucémie.

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ABBREVIATIONS

AML	Acute myelocytic leukemia
ALL	Acute lymphoblastic leukemia
5-AZA-CdR	5-aza-2'-deoxycytidine
5-AZA-dCDP	5-AZA-2'-deoxycytidine –5'-diphosphate
5-AZA-dCMP	5-AZA-2'-deoxycytidine –5'-monophosphate
5-AZA-dCTP	5-AZA-2'-deoxycytidine –5'-triphosphate
5-AZA-dUMP	5-AZA-2'-deoxyuridine –5'-monophosphate
5-AZA-UdR	5-AZA-2'-uridine
bp	base pair
CML	Chronic myelocytic leukemia
CNS	Central nervous system
CoR	Corepressor
CR	Complete remission
CR deaminase	Cytidine deaminase
CSF	Colony stimulating factor
D/CDK4 and 6	Cyclin D-activated kinases 4 and 6
Depsi	Depsipeptide
DMTase	DNA methyltransferase
Dnmt	DNA methyl transferase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
INK	Inhibitor kinase
LOH	Loss of heterozygosity

5-MC	5-methylcytosine
MDBP1 and 2	Specific methylated DNA binding proteins
MDS	Myelodysplastic syndrome
MeCP1 and 2	methylated CpG binding proteins
MSP	Methylation specific PCR
Ms-SNUPE	Methylation sensitive single nucleotide primer extension
PCR	Polymerase chain reaction
RAR	Retinoic Acid Receptor
RT-PCR	Reverse transcriptase-polymerase chain reaction
SW1/SNF	Switching/sucrose nonfermenting
TSA	Trichostatin A

Dedicated to my dear parents,

kind husband Mostafa and my lovely son, Radbod

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PART1:

LITERATURE REVIEW

CHAPTER 1:

Leukemia

1.1 Epidemiology and etiology

Every year almost 28,000 new cases of acute leukemia are diagnosed in united State. Nearly 25% of the cases are children (Boring et al., 1992). Acute leukemia is the most common malignant disease of childhood. Acute lymphoblastic leukemia (ALL) is the most common form of leukemia in children. Acute myelogenous leukemia (AML) occurs more in adults and it's incidence increases with the age.

Radiation and chemicals such as Benzene and toluene and drugs are important leukemogenic factors in human. Investigators haven't found any relation between viruses and acute leukemia (Löwenberg et al., 1999).

1.2 Classification

The classification of acute leukemia is based on Morphologic identification of leukemic blasts in preparations of peripheral blood and bone marrow stained with Wright-Giemsa. The presence of more than 30 percent leukemic blasts in a bone marrow aspirate is required for a definitive diagnosis of acute leukemia.

The French-American-British cooperative Group classification of acute leukemia is based on:

- Cytochemistry
- Cytogenetic
- Quantitative bone marrow differential to differ subtypes of AML
- Immunologic markers that distinguish subsets of ALL
- Observation of Auer rods which their presence in the immature cells distinguishes AML from ALL.

1.3 Natural history

In patients with leukemia hematopoiesis is abnormal even before the proportion of blast cells in the marrow is apparently increased. The immature leukocyte progenitors gradually replace the normal bone marrow. Most patients Relapse, unless after chemotherapy remission lasts four or more years. Response to the therapy after relapse is weaker and duration of remission is much shorter (Löwenberg et al., 1999).

1.4 Diagnosis

Diagnosis of acute leukemia is based on series of symptoms, physical findings and laboratory studies.

-Fatigue, weakness, bruising, fever and weight loss are frequent symptoms. Headache, nausea, vomiting, blurred vision or cranial nerve dysfunction can be seen when the CNS is involved. Bone pain and abdominal fullness are more common in ALL.

-Pallor, petechiae, purpura are the more common physical findings in leukemia due to the presence of anemia and thrombocytopenia. Sternal tenderness, lymphadenopathy and hepatosplenomegaly are much more common in ALL than AML. Leukemic infiltrates in the optic fundus, bleeding, meningismus and gingival enlargement occur often in AML

-The laboratories studies are based on bone marrow findings. Blasts must be greater than 30% of the nucleated cells to establish the diagnosis. Cytogenetic abnormalities, hemogram, biochemical tests, radiologic studies, CSF examination and surveillance bacterial cultures are other tests that must be considered (Löwenberg et al., 1999).

1.5 Staging system

There is no staging system for acute leukemia. Complete remission (CR) is the paramount prognostic factor in all forms of acute leukemia. A CR is defined as a bone marrow with less than 5% blasts, normal counts of erythrocytes, granulocytes and platelets, resolution of organomegaly and return to normal performance status (Löwenberg et al., 1999).

1.6 Genetic alterations in leukemia

Mutation, chromosomal delition and translocation are the most common genetic alterations that cause leukemia. Also observed in leukemia is the loss of expression of some tumor suppressor genes. One of the mechanisms responsible for loss of their expression is DNA methylation (this event will be discussed in chapter 2). Tumor suppressor genes, which are methylated in different type of hematological malignancies, are listed in **table 1.1**.

Table 1.1 Leukemic cell lines with methylated tumor suppressor genes

Cell line	Gene	Reference
Burkitt's lymphoma		
Raji	p73 +/- P15 +/- , p16	Kawano et al (1999) Herman et al (1996)
AML		
HL-60	p 73 ???	Kawano et al (1999) Corn et al (1999)
KG1a	p73 ??? p16, p15	Kawano et al (1999) Corn et al (1999) Herman et al (1996)
U937	p73 +/-	Kawano et al (1999) Corn et al (1999)
T-ALL/LBL		
Molt4	p15	Aggerholm et al (1999)
Molt16	p73	Kawano et al (1999)
Jurcat	p73 +/-	Kawano et al (1999)
KOPT-K1	p73 +/-	Kawano et al (1999)
ED	p16	Nosaka et al (2000)
ATL-43T	p16	Nosaka et al (2000)
B-ALL		
BJAB	p16	Guo et al (2000)
DAUDI	p16, p15	Guo et al (2000)
NAMALVA	p16, p15	Guo et al (2000)
RAMOS	p16	Guo et al (2000)

Definitions :
+/- semi expressed

??? different results

1.7 Survival rates

For patients with AML who receive complete remission the median survival is 12 to 24 months. The first remission usually lasts for 10 to 12 months. Approximately,15 to 25 percent of the patients who achieve CR survive 5 or more years and many of them may be cured. Most relapses occur within three years.

In adolescents and adult patients with ALL the median duration of the first CR is about 12 to 18 months and the median survival is about 18 to 24 months. In childhood ALL over 75-80% of patients are cured (Löwenberg et al., 1999). New therapies should be investigated for patients who fail the current chemotherapy or show high-risks features, such as a very high white blood cell count, for whom conventional therapy is not very effective.

1.8 Treatment of leukemia

In patients with AML the current therapy for remission induction is an intensive chemotherapy nearly always to the point of severe bone marrow aplasia. A regimen of Cytarabine (cytosine arabinoside) and Idarubicin (4-demethoxydaunorubicin) is used and this combination therapy is repeated once or twice more if the blood and bone marrow is not clear of blast and if patient can tolerate such therapy. A relatively high doses of drugs are given shortly after the patients has achieved CR. Otherwise recurrence is inevitable. High dose cytarabine alone is commonly used.

Once remission is induced, further intensive treatment of patients with AML is essential to prevent relapse. Three options are available for younger patients: allogeneic bone marrow transplantation from HLA-matched related or unrelated donor, autologous bone marrow transplantation, or chemotherapy.

In ALL patients a combination of Vincristine with Prednisone in children produce CR in 85-90% of cases and in adults result in 45% CR. In adults the addition of an anthracycline with or without L-asparaginase increases the CR rate to 70-80% (Löwenberg et al., 1999).

CHAPTER 2:

DNA Methylation and leukemia

2.1 DNA methylation

DNA methylation is an epigenetic modification, which has important effects on the mammalian genome. It takes place after replication and has role in the control of gene expression. In mammalian cells, almost 3 to 5% of the cytosines residues in the genomic DNA are in the form of 5-methylcytosine (Erlish et al.,1982). Approximately 70% of the CpG residues in mammalian's genome are methylated (Bird, 1986). This sequence, when it's in high frequency is termed CpG islands. In normal cells CpG islands are protected from methylation by a mechanism, which is not yet known. DNA methylation has been shown to be essential for embryonic development (Kafri et al., 1992), where sequential activation and deactivation of genes are involved. Another aspect of methylation is inactivation of specific genes on the X chromosome (Riggs and Pfeifer, 1992). Methylation of cytosine has also role in genomic imprinting, in which specific genes are only expressed by the paternal or maternal chromosome (Falls et al., 1999). It has been proposed that DNA methylation can increase the rate of mutation (Rideout et al., 1990). It can also silence genes which control cellular proliferation and whose promoters are associated with CpG islands (Baylin et al., 1998). It has been shown for more than 15 years that the patterns of methylation in cancer cells have been changed relative to those of normal cells. Global hypomethylation (Goelz et al, 1985) is accompanied with hypermethlation in CpG islands in malignant tumors (Jones and Laird, 1999).

2.2 The Methylation Machinery

Methylation of cytosines is catalysed by DNA methyltransferase, using S-adenosyl-methionine as the methyl donor **(Figure. 2.1)**. Four DNA methyltransferases have been cloned from mammalian cells. The maintenance DNA methyltransferase (Dnmt 1) was cloned from mammalian cells by Bestor et al in 1988. Dnmt 1 uses hemimethylated

DNA as a preferential template (Bouchard and Momparler, 1983). This methylase maintain the same methylation pattern after DNA replication that is a characteristic of each type of differentiated cell (Tucker and Bestor, 1997). Dnmt 3a and Dnmt3b seem to act as de novo methylase since they can methylate both hemimethylated and unmethylated DNA with equal efficiency (Xie et al., 1999). Dnmt 2 was suggested to have the function of methylating integrated retroviral sequences (Yoder and Bestor, 1998). Okano et al in 1998 reported that Dnmt 2 was not required for maintenance methylation of viral DNA and de novo methylation. The role of all these DNA methyltransferases in tumorigenesis remains to be fully elucidated.

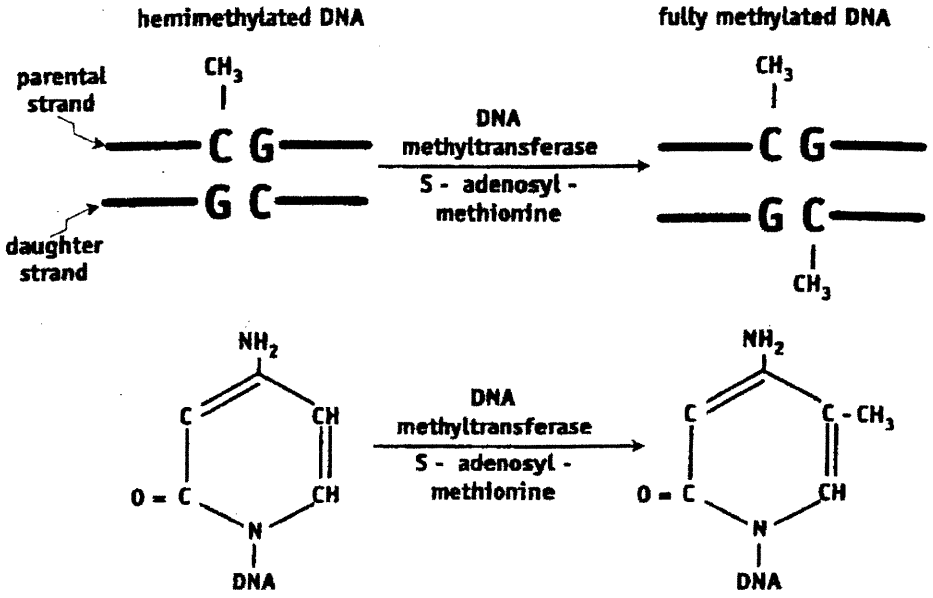


Figure 2.1 Representation of the DNA methylation reaction by DNA methyltransferase. After DNA replication, the daughter strand is methylated in order to maintain the methylation pattern characteristic of the cell (Top). S-adenosyl-methionine is the methyl donor. The structures of cytosine and 5-methylcytosine are shown (Bottom).

2.3 DNA Methylation and Regulation of Gene Expression

There are two mechanisms through which methylation of DNA can regulate gene expression. As the 5-methylcytosine sticks out into the major groove of the DNA helix (Tate and Bird, 1993), one possibility is that this modified cytosine interferes with the binding of transcription factors (Kass et al., 1997). The other possible mechanism can be the role of proteins that bind preferably to the methylated promoters therefore they prevent the binding of transcription factors to the target sequences. There are two classes of sequence specific methylated DNA binding proteins. MDBPs, including MDBP1 and MBDP2 (Huang et al., 1984; Jost and Hofsteenge, 1992) and MeCPs, which are MeCP1 (Meehan et al., 1989) and MeCP2 (Lewis et al., 1992). MeCP1 requires 12 CpGs for preferential binding to methylated DNA and it can repress transcription both *in vitro* and *in vivo* (Boyes and Bird, 1991). MeCP2 is an abundant chromosomal protein and requires only a single methylated CpG site for binding to DNA. It represses the *in vitro* transcription (Nan et al., 1997).

2.4 Tumor suppressor genes

Tumor suppressor genes normally suppress the potential of growth in the cells and they regulate the progression of the cell cycle. Loss of expression of tumor suppressor genes has an important role in Leukemogenesis. Mutation, deletion and methyaltion can cause less of function in tumor suppressor genes (Knudson, 1993).

2.5 Aberrant DNA Methylation of Cancer-Related Gene

Investigations have shown that both alleles of a tumor suppressor gene must be inactivated in order to suppress its biological function (Knudsen, 1993) and is referred to "two hit" hypothesis. Earlier studies showed that inactivation of a tumor suppressor gene

occurs by chromosomal deletion of a single allele (loss of heterozygosity; LOH) or by mutation.

The discovery that many tumor suppressor genes can also be inactivated by methylation of the CpG islands in the promoter region indicates that epigenetic events also have important role in tumorigenesis (Jones and Laird, 1999). The molecular mechanism responsible for the aberrant methylation of DNA during tumorigenesis is not still known. It is possible that DNA methyltransferase by mistake methylates CpG islands in the nascent strand of DNA without having a complimentary methylated CpG in the parental strand. Removal of CpG binding proteins that protect these sites from methylation can be another possibility for the aberrant methylation in tumorigenesis (Momparler & Bovenzi , 2000).

2.6 p15 Growth Suppressor Gene and leukemia

$p15^{INK4B}$ and $p16^{INK4A}$ are two neighboring genes at chromosome 9p21, the products of which specifically inhibit cyclin D/CDK4 and cyclin D/CDK6 complexes and, thereby, regulate the cell cycle negatively (Bartkova et al, 1997). The functional role of cyclin dependent kinases is to phosphoryalte specific proteins that program the cell to enter the S phase and undergo cell division (Hamel & Hanley-Hyde, 1997). Several studies suggest that inactivation of p15 and p16 genes are one of the most common genetic events in hematological malignancies (Batova et al, 1997; Herman et al, 1997; Ng et al, 1997). These genes play an important role in the pathogenesis of acute leukemia related to the p15/p16-Rb pathway (Guo et al, 2000). Both p15 and p16 genes are deleted in some cancer cell lines and primary tumors including acute lymphoblastic leukemia (Herbert et al, 1994; Achille et al 1995; Fizzotti et al 1995). Recent reports have also suggested that methylation of CpG islands that constitute the 5' end and promoter region of the p16 and p15 genes is a primary mechanism of inactivation of these genes in hematological malignancies (Batova et

al 1997; Herman et al, 1997; Ng et al, 1997). Unlike homozygous deletions, inactivation of these genes through promoter hypermethylation is selective, typically limited in solid tumors to only p16. However, in acute leukemias, selective hypermethylation of p15 was observed, suggesting that inactivation of p15 might be an important event in the development of hematological malignancies (Herman et al, 1996).

CHAPTER 3:

5-AZA-2'-DEOXYCYTIDINE

3.1. Introduction

5-Aza-2'-deoxycytidine (5AZA or Decitabine) is an analog of 2'-deoxycytidine.In this nucloside analog the carbon in the position 5 is replaced by a nitrogen .In **figure 3.1.** the chemical structures of 5AZA and 2'-deoxycytidine are shown. In 1964 Pliml and Sorm synthesized this analog for the first time, and in 1968 Sorm and Vesely showed the antileukemic activity of this agent in animal models.

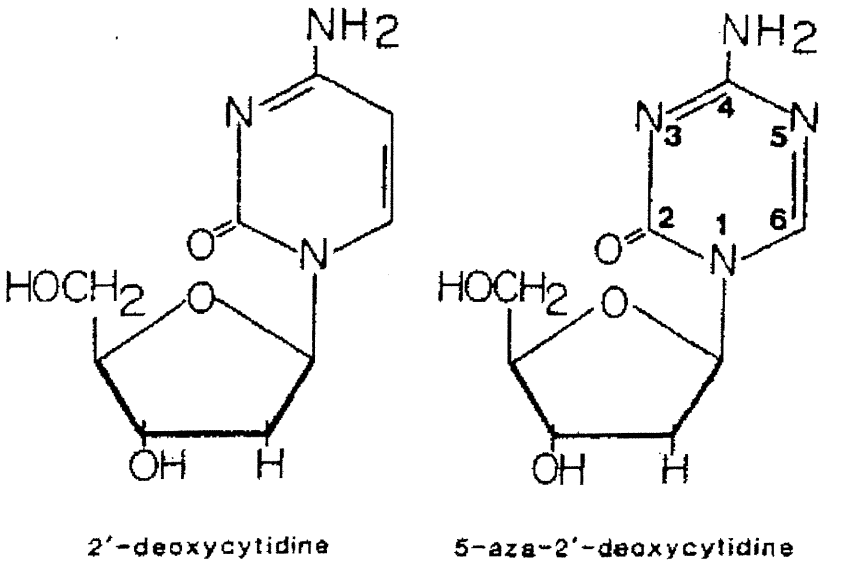


Figure 3.1 Molecular structure of 2'deoxycytidine and 5-aza-2'-deoxycytidine (5-AZA-CdR). The carbon in position 5 of the pyrimidine ring is replaced by an atom of nitrogen in 5-AZA-CdR. Once incorporated in the DNA the position 5 of the 5-AZA-cytosine can not be methylated

3.2 Metabolism

5AZA enters the cell by two mechanisms, transportation system for nucleosides and passive diffusion (Plagemann et al., 1978). This analog has to be phosphorylated to its triphosphate form to become an active inhibitor. The enzyme which is responsible for its phosphorylation to 5-AZA-dCMP is deoxycytidine kinase.5-AZA-dCMP is rapidly converted to 5-AZA-dCDP and 5-AZA-dCTP by other kinases, in the cells. 5AZA easily incorporates into the DNA and this process is catalyzed by DNA polymerase. The incorporated analog produces a potent inhibition of DNA methylation (Bouchard and Momparler 1983). 5-AZA-dCTP incorporates into the DNA with an affinity for DNA polymorase comparable to dCTP. In **figure 3.2** a schematic representation of metabolism of 5AZA is shown.

Two enzymes are responsible for the degradation of 5AZA; deoxycytidine-5'-monophosphate deaminase (dCMP deaminase) and cytidine deaminase (CR deaminase). dCMP deaminase catalyses the deamination of 5-AZA-dCMP to 5-AZA-dUMP (5-AZA-2'-deoxyuridine-5'-monophosphate). CR deaminase catalyzes the conversion of 5-AZA-CdR (5AZA) to 5-AZA-UdR (5-AZA-2'-uridine), which is an inactive metabolite (Chabot et al., 1983; Eliopoulos et al., 1998).Due to the high levels of CR deaminase in the human liver 5AZA has a short half-life (~15-20 min) in man (Momparler et al., 1997).

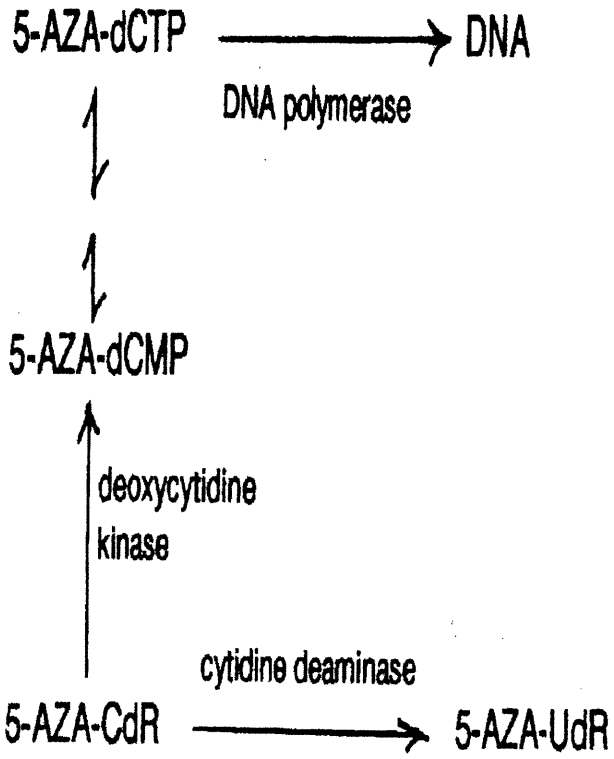


Figure 3.2 Metabolism of 5-AZA-CdR. As a nucleoside analog, 5-AZA-CdR is incorporated in the DNA by a DNA polymerase. The molecule needs to be phosphorylated to its triphosphate form: deoxycytidine kinase is the enzyme responsible for the phosphorylation to 5-AZA-dCMP, the rate limiting step. 5-AZA-dCMP is phosphorylated to 5-AZA-dCTP by other kinases. Cytidine deaminase is responsible for the deamination of 5-AZA-CdR to the inactive metabolite, 5-AZA-UdR.

3.3 Mechanism of action

5-AZA-CdR is an S phase specific agent and does not affect cells, which are in the other phases of the cell cycle (Momparler et al., 1984a). This is due to the fact that 5AZA needs to be incorporated into the DNA to exert its antitumor action.

5-AZA-dCTP by its incorporation into the DNA inhibits the methylation of cytosines, which is catalyzed by DMTase (bouchard and Momparler 1983; Creusot et al., 1982). Once incorporated in the DNA, 5-AZA-dCMP forms covalent adducts with DMTase from the cells (Jüttermann et al., 1994) resulting in demethylation of genomic DNA following treatment with 5AZA (Creusot et al., 1982, Michalowsky and Jones, 1987). 5-AZA-dCMP in the DNA because of the presence of a nitrogen on the position 5 of the cytosine ring that can not be methylated is not a good substrate for DMTase.

3.4 Toxicity

The main toxicity of 5AZA is myelosuppression (Rivard et al in 1981 and Momparler et al in 1986). In patients with leukemia who were treated with 5AZA observed a gradual decrease in the white blood cell count with the lowest count at about days 14 to 21 and recovery at about day 28. An approach to overcome the bone marrow toxicity following 5AZA is the use of gene therapy by inserting drug resistance gene, cytidine deaminase, into hematopoietic cells to protect them (Momparler et al., 1996).

3.5 Resistance to 5-AZA-CdR

Three mechanisms of resistance to 5AZA have been reported. Deficiency in deoxycytidine kinase, the enzyme that catalyzes the phosphorylation of 5AZA to 5-AZA-dCMP, is the major mechanism, which makes cells completely resistant to cytosine analogs (Momparler et al., 1982). Another mechanism of resistance to this deoxycytidine analog is

the increase in cytidine deaminase, the enzyme that deaminates 5-AZA-CdR to an inactive metabolite. Also, an increased pool of cellular dCTP induces the drug resistance by either feedback inhibition deoxycytidine kinase to reduce its activation by phosphorylation, or by competing with 5-AZA-dCTP for DNA polymerase to reduce its incorporation into DNA (reviewed by Momparler et al 1997).

3.6 5-AZA-CdR and cancer therapy

A large number of tumor suppressor genes involved in tumorogenesis have been silenced by DNA methylation, suggesting that inhibitors of this mechanism have potential to be used in cancer therapy.

5AZA is a potent inhibitor of DNA methylation. This nucleoside analog also has a novel mechanism of demethylation of DNA that is mentioned before in section 3.3 (Momparler & Bovenzi, 2000). In the mouse model 5AZA was reported to be a potent antileukemic agent and the extent of inhibition of DNA methylation produced by this analog correlated with its antineoplastic activity (Wilson et al., 1983). 5AZA inhibits DNA methylation in leukemic blasts from patients treated with this analog (Momparler et al 1984b). It can also induce complete remission in some patients with advanced AML (Rivard et al., 1981; Momparler et al., 1985; Richel et al., 1991) It is of interest to note that 5AZA therapy also produces positive responses in patients with CML in blastic crisis (Sacchi et al., 1999) and MDS, a preleukemic disease (Wijermans et al., 2000).

Several pilot clinical trials on the antitumor activity of 5AZA have been done. In studies on patients with head and neck cancer and with prostate cancer 5AZA showed minimal to moderate activity (Van Groeningen et al., 1986; Thibault et al., 1998). Interesting antitumor activity of this nucleoside analog was seen in a patient with stage IV non-small lung cancer, who survived more than 6 years (Momparler et al., 1997).

CHAPTER 4:

CHROMATIN STRUCTURE

AND HISTONE ACETYLATION

4.1 Chromatin structure

In eukaryotic cells, DNA is as an organized pattern of chromatin structures, resulting in compaction of nuclear DNA about 10,000 fold. Chromatin is organized in repeated structural units called nucleosomes (van Holde, 1988). Each nucleosome consists of about 146 bp of DNA wrapped around an octamer of proteins called histones. The core histones are H2A, H2B, H3 and H4 which are organized as an $(H3\text{-}H4)_2$ tetramer and two H2A-H2B that are located in each side of the tetramer **(Figure. 4.1)**. The DNA joining the nucleosomes is called linker DNA, and H1 or linker histone binds to this linker DNA. Variations in the length of linker DNA is important for the diversity of gene regulation (Spadafora et al., 1976).

Histones have defined spatial position within the nucleosome that makes interactions between histones and DNA very specific. Each histone has a similar folding pattern for each peptide chain that is based on a long α-helix, flanked on both sides by shorter helices that interact with DNA. 15-30 residues at the amino-termini in all of the histones are not structured and are referred to as tails. The terminal lysines of histones show an electrostatic interaction with the phosphodiester backbone of the DNA with minor non-polar interaction with the deoxyribose moiety (Arents and Moudrianakis, 1995).

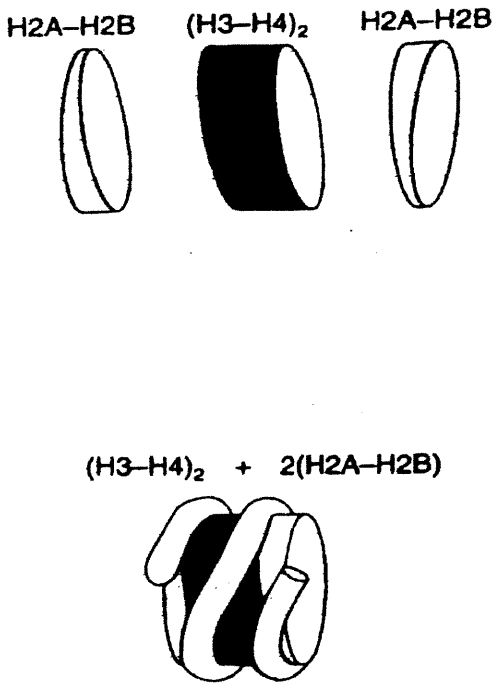


Figure 4.1 Spatial organization of histones and DNA in the nucleosome. The core histones are arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimmers positioned on each face of the tetramer. A 146 bp loop of DNA is wrapped around the core of histones.

4.2 Histones and gene expression

Histones are dynamic components of the machinery, which is responsible for the regulation of gene transcription. The structure of transcriptionally active chromatin differs from that of the compact DNA in its susceptibility to digestion to DNase and its salt solubility. In vitro studies on the compact chromatin fibers show that a decrease in ionic strength leads to a more relax conformation, which is more accessible to the binding proteins. Chromatin conformation changes in the cells are due to ATP-dependent chromatin remodeling involving ATP-dependent chromatin remodeling complexes and /or histone post-translational modifications. ATP-dependent chromatin remodeling involved ATP-dependent chromatin remodeling complexes and the most characterized one is SW1/SNF in yeasts (reviewed by Tyler and Kadonaga, 1999). The complexes use the energy derived ATP to promote nucleosome mobilization but the mechanism with which the histone-DNA bonds are changed is not clear.

Histone post-translational modifications include acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation (van-Holde, 1988). All these modification occur on the N-terminal tails, which protrude from the nucleosome core and are sensitive to protease. Histone modifications may alter chromatin structure by influencing histone-histone and histone-DNA interactions (Wolffe and Hayes, 1999). The most important post-translational modification of histones is acetylation, which is discussed below.

4.2.1 Histone acetylation and transcriptional regulation

The enzymes responsible for acetylation of the histones are called histone acetyltransferases (HATs). There are two types of HATs. The first class is the Cytoplasmic HATs which have role during nucleosome assembly and they are called B type HATs. The

second class of HATs or type A is located in the nucleus and is involved in the regulation of gene expression as explained below. The relative levels of histone acetylation are determined by the activities of both HATs and histone deacetylases (HDACs). Two classes of HDACs are known, based on their homology with the yeast HDACs; Rpd3 and Hda1 (Grozinger et al., 1999)

A great number of transcriptional coactivator complexes have been shown to contain HAT activity (Struhl and Moqtaderi, 1998). In vertebrates, many transcription factors have HAT activity. For example P300/CBP complex is a transcription factor that has intrinsic HAT activity and is able to bind transcription activators such as pCAF, which also have HAT activity. It is possible that pCAF and p300/CBP associate to acetylate chromatin in certain promoters.

These observations provided the evidence for regulatory role of the nucleosome in transcriptional regulation (Björklund et al., 1999). Highly acetylated chromatin is generally associated with actively transcribed DNA, whereas poorly acetylated chromatin is associated with inactive transcription.

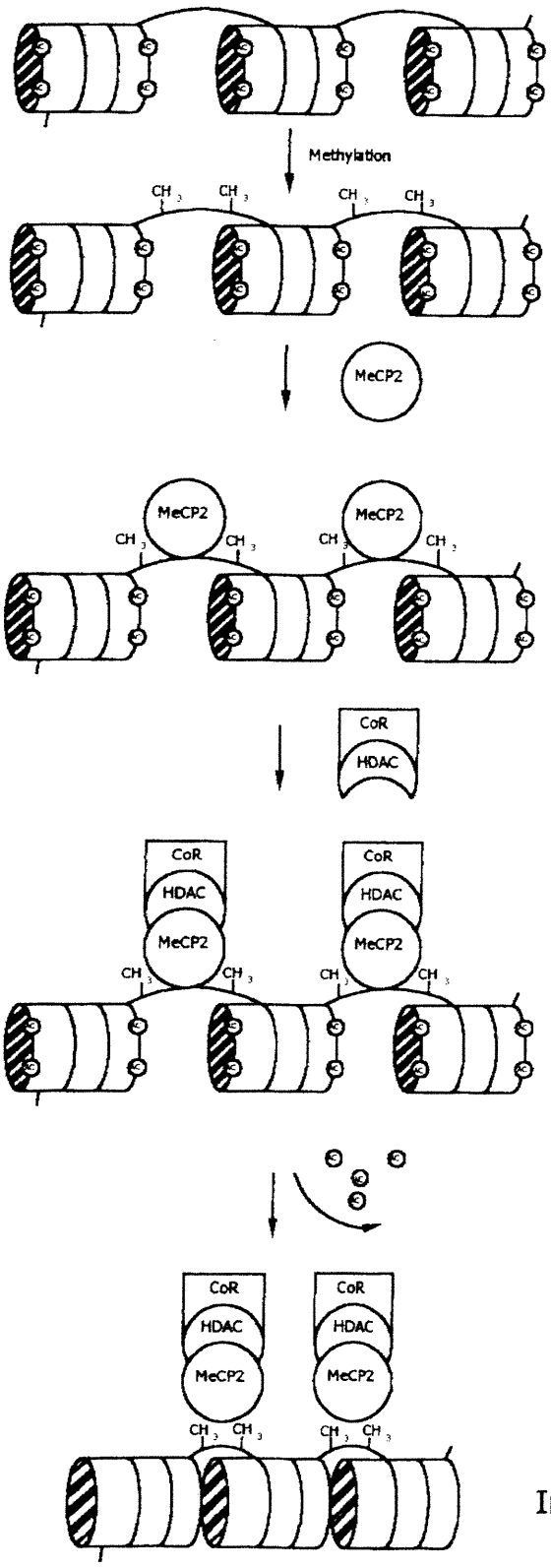
Acetylation of specific lysine residues in the histone N-terminal tails weakens the interaction between histone and DNA by reducing the positive charge on histones, and reducing its affinity for negatively charged phosphates of DNA (Allfrey et al., 1964). Deacetylation of the histones by restoring the positive charge favors the interaction between histone and DNA and leads to a more compact structure which is transcriptionally inactive. This phenomenon is very complex and many other factors have role in the transcriptional regulation.

4.3 The interaction between DNA methylation and histone acetylation

Recently investigators have found that there is an interaction between DNA methylation and histone acetylation to regulate the expression of specific genes. This

interaction involves methylation-binding proteins such as MeCP2. The mechanism involved for this interesting interaction is that the methylated cytosines in the promoter region bind MeCP2, which in turn recruits histone deacetylase to form a complex (Cameron et al., 1999; Nan et al., 1998; Jones et al., 1998) **(Figure. 4.2**). Apparently, MeCP2 prevents the binding of transcription factors to the promoter whereas deacetylated histones convert the chromatin to a more compact structure unfavorable for transcription.

Figure 4.2 Model for interaction between DNA methylation and histone deacetylation on silencing of gene expression. The methylated DNA binding protein (MeCP2) binds to 5-methylcytosines in the promoter region of target genes. MeCP2 recruits histone deacetylases (HDACs) and transcriptional corepressors (CoR) resulting in compaction of the chromatin and gene silencing.


Active transcription
Methylation
CH3
MeCP2
CoR
HDAC
Inactive transcription

4. 4 Histone deacetylase inhibitors

The role of acetylated histones has been elucidated by using the inhibitors of histone deacetylation.

4.4.1 Trichostatin A (TSA)

Tsuji et al in 1976 were the first to isolate TSA from *Streptomyces hygroscopicus,* an antifungal antibiotic. Its molecular structure is shown in **Figure. 4.3**. TSA can induce differentiation of leukemic cell lines (Yoshida et al., 1987). In addition TSA is a potent inhibitor of histone deacetylation (Yoshida et al., 1990). TSA can also induce differentiation, apoptosis and alteration of transcription (Yoshida et al., 1995). HDAC inhibitors induce G1cell cycle arrest and some morphological changes in different cell lines (Fallon and Cox 1979; Nakajima et al., 1998). Kim et al in 1999 reported that following treatment with TSA in different cell lines the level of p21WAF1 was up regulated. p21 gene encode for a protein, which acts as a cycline dependent kinase inhibitor. p16, which is another cyclin dependent kinase inhibitor, was also upregulated after treatment with TSA. Based on these results it seems that histone acetylation and deacetylation play role in the expression of p21WAF1 which in turn its over expression following TSA treatment is responsible for cell cycle arrest. The potent activity of HDAC inhibitors to induce cell cycle arrest and apoptosis makes them interesting candidates them to be used in cancer chemotherapy.

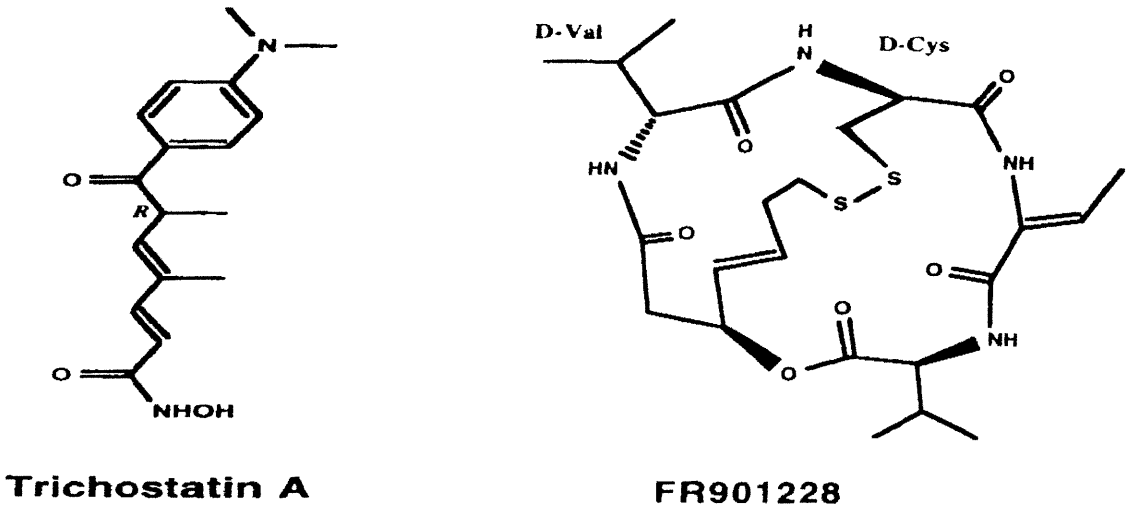


Figure 4.3 (A) Structure of Trichostatin A

(B) Structure of Depsipeptide (FR 901228)

4.4.2 Depsipeptide FR 901228

Depsipeptide (Depsi) is another inhibitor of HDAC, which has a structure unrelated to TSA.Depsi is a cyclic peptide and it was isolated for the first time from *Chromobacterium violaceum* strain WB968 by Ueda et al (1994). Its chemical structure is shown in **figure 4.3.** This compound has demonstrated potent cytotoxicity against human tumor cell lines and the in vivo efficacy against both human tumor and murine tumors. Depsi preferentially inhibits RNA synthesis and causes cell cycle arrest at G_0/G_1 without producing single strand breaks or cross- links (Ueda et al., 1994). It has shown selective in vitro activity against resistant B-cell chronic lymphoblastic leukemia (Byrd et al 1999). National Cancer Institute (U.S.A) has selected this compound for preclinical and early clinical development.

OBJECTIVES OF PRESENT WORK

1. To evaluate the in vitro antineoplastic activity of DNA methylation inhibitor, 5-AZA-CdR on human HL-60 and KG1a myeloid leukemic cells.

2. To evaluate the in vitro antineoplastic activity of histone deacetylation inhibitors, trichostatin A (TSA) or depsipeptide 901228 on human HL-60 and KG1a myeloid leukemic cells.

3. To evaluate the in vitro antineoplastic activity of 5-AZA-CdR in combination with TSA or depsipeptide on human HL-60 and KG1a myeloid leukemic cells.

4. To evaluate the methylation status of the promoter region of the tumor suppressor gene, p15, in Raji Burkitt's lymphoma Leukemic cells using the methylation specific PCR reaction (MSP).

5. To evaluate the activation of expression of p15 gene by 5-AZA-CdR and TSA alone on Raji Burkitt's lymphoma leukemic cells using the reverse transcriptase PCR (RT-PCR) reaction.

PART 2:

MATERIALS, METHODS AND RESULTS

CHAPTER 5:

MATERIALS AND METHODS

Materials

Cell Line

The human Raji Burkitt's lymphoma cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium (Life Technologies, Burlington, Ontario) containing 10% heat-inactivated fetal calf serum (Wisent, St. Bruno, Quebec) and kept in a 5% CO_2 incubator at 37°C.

Methods

5.1 Analysis of DNA Methylation of p15 gene by the Methylation-Specific PCR (MSP) Assay

MSP is a novel PCR assay that we can distinguish methylated from unmethylated DNA, which is treated with bisulfite using specific primers (Herman JG et al., 1996). With MSP assay we can rapidly analyze the methylation status of CpG islands comparing to genomic sequencing or Southern blot analysis. Another advantage of MSP is the small amount of DNA (~10 ng) required comparing with Southern blot analysis, which requires (~5-10μg) of DNA.

In the MSP method, genomic DNA is treated with bisulfite (the method is described later), which converts cytosine residues to uracil whereby 5-methylcytosine residues remain unchanged. The bisulfite treated DNA is amplified by PCR with specific primers for the promoter region of p15. During PCR due to the deamination of cytosine to uracil, thymine will be incorporated at these deamination sites whereas there is no change at the 5-methylcytosine residues, cytosine will be incorporated at these sites. Sequence analysis shows all the cytosines are converted to thymine (C to T) and all the 5-methylcytosines stays as cytosine (C stays C). The presence of cytosine in the sequence of bisulfite-treated and PCR amplified DNA will indicate the precise position of 5-methylcytosine.

We used Primers that Herman JG et al (1996) designed for the MSP assay to detect the methylation of the P15 gene (accession no. S75756). The sequences of the methylation-specific primers are: the sense top 5'-GCG TTC GTA TTT TGC GGT T-3' and the antisense bottom 5'-CGT ACA ATA ACC GAA CGA CCG A-3'. The sequences of the unmethylated -specific primers are: the sense top 5'-TGT GAT GTG TTT GTA TTT TGT GGT TT –3' and the antisense bottom 5'-CCA TAC AAT AAC CAA ACA ACC AA-3'. The methylation specific primers will amplify a 148 bp band when the p15 gene is methylated but not when in it is unmethylated. PCR was performed under the following conditions: 94°C for 15 min to allow the antibody to detach from the Taq polymerase and activate it; 39 cycles of 94°C for 1 min, 58°C for 30 sec 72°C for 30 sec followed by 1 cycle 72°C for 3min and 10°C for 10 min. The primers that can amplify the p15 gene when it's unmethylated will amplify a 154 bp band and the PCR was performed under the following conditions: 94°C for 15 min to allow the antibody to detach from the Taq polymerase and activate it; 34 cycles of 94°C for 1 min, 58°C for 30 sec 72°C for 30 sec followed by 1 cycle 72°C for 3min and 10°C for 10 min. All reactions contained 5-10 ng of bisulfite-converted DNA, 1 X PCR buffer, 10μM dNTP, 2.5μM primers, $MgCl_2$ 2.5mM, and 1.2 units of Qiagen HotStar Taq Polymerase in a total volume of 25μl. PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide and photographed.

5.1.1 Sodium bisulfite conversion of DNA

We used 2 μg of genomic DNA in 40 μl of water followed by denaturation of the DNA at 95°C for 5 min.

We made these solutions fresh: 10mM Hydroquinone

3.65 M Sodium Bisulfite pH5.0

3.0 NaOH

Then we added 2 µl of 6N NaOH (final concentration 0.3N) to a total volume of 42 µl. The samples were kept at 45°C for 20 min. We added to the solution 14 µl of 10mM Hydroquinone (final conc. 2.5 mM) to a total volume of 56 µl. 260 µl of 3.65 M Sodium bisulfite pH5 (final conc.3M) was added and the total volume was 316µl. Then we flushed it with Nitrogen gas and we placed it in a thermal cycler with this program:

Step 1	55°C	4h
2	90°C	2m this cycle was repeated 3 more times
3	20°C	10m
4	4°C	hold

The DNA was then purified with Prep-A-gene DNA purification Kit (Stratagene) as below: To the DNA solution we added 3 volumes of binding buffer (3 x 316 µl) and 5 µl of Prep-A-Gene DNA matrix (vortexed well before using). The mixture was placed it at 20°C for 20 min and we shaked it continuously to allow DNA to bind to matrix. Then it was placed on ice for 60 min and mixed every 5 min; centrifuged it at 5,000 rpm for 30 sec and the supernatant was discarded .We added 0.5 ml of 75% EtOH to wash pellet (3 times). All liquid was removed to the last drop and let the matrix dry (air dry or vacuum). We eluted DNA in 50 µl TE pH 7.5 at 60°C for 30 min and centrifuged 30 sec at the end we eluted in 50 µl of water at 60°C for 10 min and combine elutes. Next step is the disulphanation and precipitation of DNA:

We added to each sample (volume is approximately 90 µl) 4.75µl 6N NaOH to a final concentration of 0.3M. We incubated the mixture for 15 min at 40°C to desulphonate cytosine residues in DNA. We added to each sample: 105 µl 6M ammonium acetate pH 7 to a final concentration of 3 M (to naturalize NaOH), 1 µl of glycogen (20 mg/ml) and 500 µl of 100% EtOH (to precipitate DNA). It was precipitated at -20°C overnight. We centrifuged at max speed (14,000 rpm) for 20 min at 4°C and discarded the supernatant;

repeated wash, centrifuged at 14,000 rpm, 4°C for 10 min and discarded the supernatant. We let pellet dry (do not over dry). The DNA precipitate was dissolved in 70 µl of 10 mM Tris-HCl pH 8.5 and quantified by ultraviolet absorbance at 260nm.

5.2 Reverse Transcriptase PCR (RT-PCR) Analysis

In order to study the reactivation of p15 gene in Raji cells, we treated the cells with 5AZA(100-200 ng/ml) and also with TSA (2.5-5 ng/ml) alone for 48 hours. Total RNA was extracted from cells using the Rneasy mini kit (Qiagen). For cDNA synthesis, 500ng of total RNA were used in 20µl of reaction mixture using the RT Omniscript by Qiagen. The reaction mixture also contained 1xRT buffer (Qiagen), dNTPs 0.5mM each, random hexamers (1µM), RNase inhibitor (0.5µM) ± RT (4 units), The reaction was performed at 37°C for 1h, followed by 5 min at 93°C to inactivate the enzyme. The PCR amplification was performed using HotStar Taq Polymerase (Qiagen), using specific primers spanning different exons for p15 (accession no.L36844).The primers were: sense primer 5'-GGA TCC CAA CGG AGT CAA CC-3' (position 438-457) and antisense primer 5'-AGC ACC ACC AGC GTG TCC A-3' (position 602-620). Microglobulin housekeeping gene was amplified as an internal control. The PCR profile consisted of 95°C for 15 min to inactivate the antibody to bind to the Taq polymerase and activate the enzyme; 40 cycles of 94°C for 30 sec, 62°C for 1 min, 72°C for 1 min followed by 1 cycle of 72°C for 10 min. The reactions were performed in a programmable minicycler. Comparison of the mRNA expression of each gene was made during the exponential phase of DNA amplification. The PCR products were electrophoresed on 2% agarose gels and detected by ethidium bromide staining.

CHAPTER 6:

RESULTS

6.1 Analysis of DNA Methylation of P15 gene by the Methylation-Specific PCR (MSP) Assay

MSP was used to assess the methylation status of p15 gene in Raji cells. The unmethylation specific primers amplified a 154 bp band with Raji cells genomic DNA but not with white blood cells (WBC) genomic DNA **(Figure. 6.1.A).** The methylation specific primers amplified a 148 bp band with white blood cells genomic DNA but not with Raji cells genomic DNA **(figure. 6.1.B)**. These results show that p15 gene in Raji Burkitt's lymphoma cell is methylated.

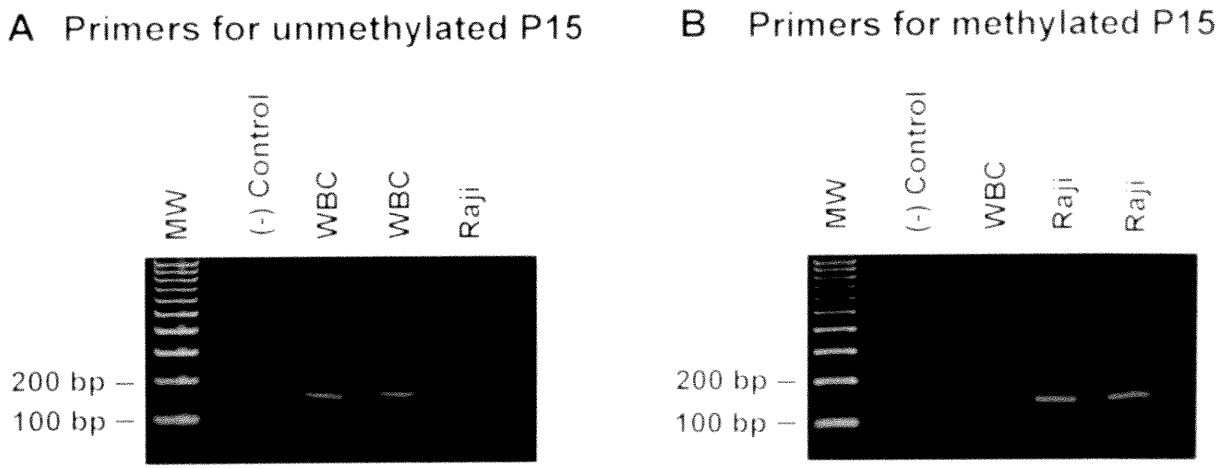


Figure 6.1. Methylation specific PCR (MSP) analysis of the promoter region of p15 gene in Raji Burkitt's lymphoma cell line. Genomic DNA was isolated from Raji leukemic cells. After treatment with bisulfite the DNA was used in the MSP assay with methylation-specific primers to amplify a 154bp DNA fragment (A) and unmethylation-specific primers (B) to amplify a 148 bp DNA fragment. Genomic DNA of WBC treated with bisulfite is used as a control.

6.2 Reverse Transcriptase PCR (RT-PCR) Analysis

To detect the expression of p15 in Raji leukemic cells we designed primers for RT-PCR. Raji cells don't express p15 gene. When we treated this cell line with TSA (2.5ng/ml) still we couldn't detect any expression of the p15 gene. Raji cells when treated with TSA (5ng/ml) we could see a band showing that p15 was weakly reactivated **(Figure. 6.2).** Treating this cell line with 5AZA (100 ng/ml) p15 gene was reactivated and a band in the size of 183 bp was detected. Using higher concentration of 5AZA (150 ng/ml) p15 gene we saw more expression and comparing with treated cells with 5AZA (200 ng/ml) we couldn't see any difference **(Figure. 6.3)** meaning that the amplification reaches a plateau. The expression of the microglobulin housekeeping gene is considered as the internal control.

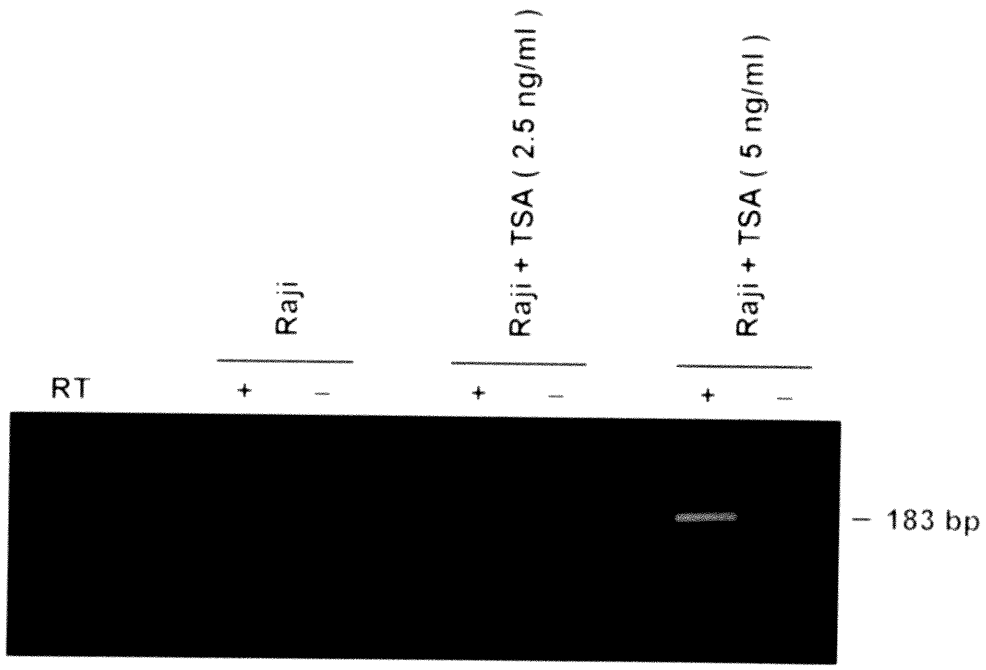


Figure 6.2. RT-PCR analysis of activation of expression of p15 by TSA. Total RNA of Raji cells was isolated after 48h. Raji cells were treated also with 2.5 ng/ml or5 ng/ml of TSA for 48h and PCR was performed using the cDNA with specific primers for p15 gene as described in methods. Another PCR with specific primers for microglobulin was performed as an internal control. The amplified DNA was separated on agarose gel and stained with ethidium bromide.

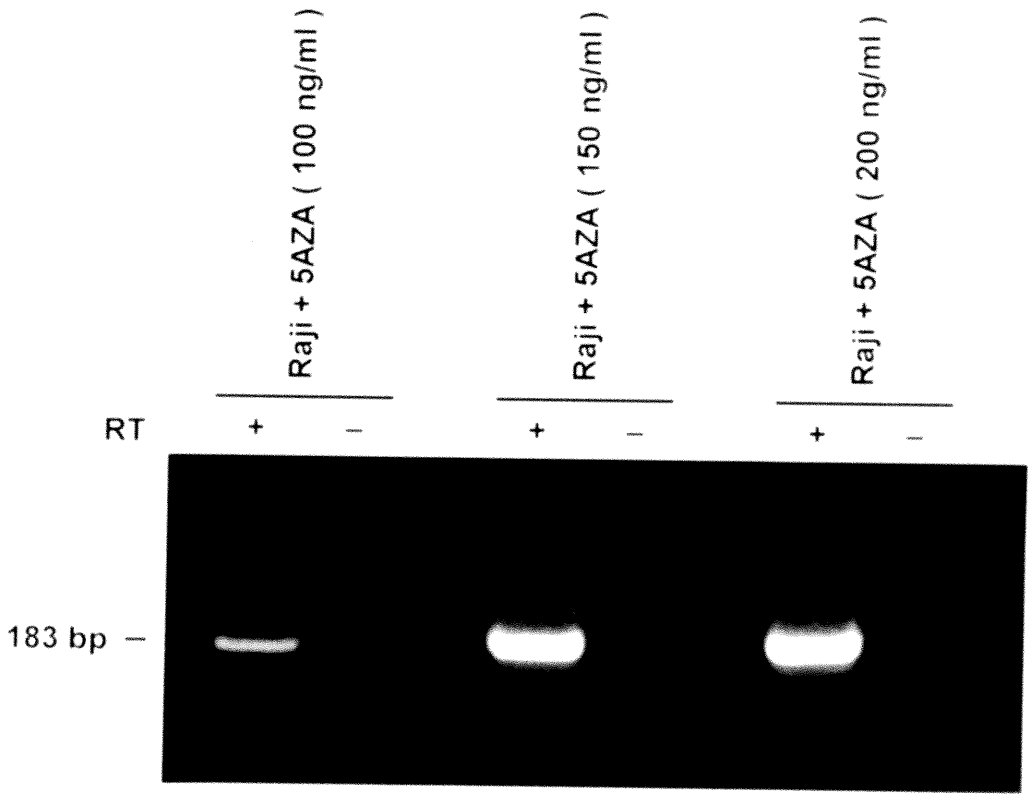


Figure 6.3. RT-PCR analysis of activation of expression of p15 by 5AZA. Total RNA of Raji cells treated with 5AZA (100-200ng/ml) for 48h was isolated. PCR was performed using the cDNA with specific primers for p15 gene as described in methods. Another PCR with specific primers for microglobulin was performed as an internal control. The amplified DNA was separated on agarose gel and stained with ethidium bromide.

PART 3:

PRESENTATION OF MANUSCRIPTS

CHAPTER 7:

Preclinical evaluation of antineoplastic activity of inhibitors of DNA methylation (5-AZA-2'-deoxycytidine and histone decetylation (Trichostatin A) in combination against myeloid leukemic cells

Preface

Based on the reports of Cameron et al (1999) and also Bovenzi & Momparler (2001) who observed a synergistic interaction between 5AZA-CdR (5AZA) and trichostatin A (TSA), a specific histone deacetylase inhibitor, on the reactivation of tumor suppressor genes, we investigated the antineoplastic effect of these two agents alone and in combination on two human myeloid leukemic cell lines HL-60 and KG1a.

Using growth inhibition, DNA synthesis assay and colony assay we found that both agents alone show a potent antineoplastic effect on these cells. The combination of these two agents resulted an inhibition of growth, DNA synthesis and colony formation that was more than using each drug alone. These results suggest that a combination of inhibitors of DNA methylation and histone deacetylation can be a good regimen to use for leukemic patients.

Preclinical evaluation of antineoplastic activity of inhibitors of DNA methylation (5-AZA-2'-deoxycytidine) and histone deacetylation (Trichostatin A) in combination against myeloid leukemic cells

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Running title: 5-AZA-CdR plus TSA therapy leukemia

Keywords: 5-AZA-2'-deoxycytidine, trichostatin A, DNA methylation, histone deacetylase, antineoplastic activity

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Submitted to Leukemia

ABSTRACT

Although current chemotherapy of acute myeloid leukemia can induce a high rate of remission, long-term survival rates remain unsatisfactory. New approaches should be sought to improve the effectiveness and reduce the toxicity of chemotherapy. During the development of leukemia, genes that suppress growth and induce differentiation can be silenced by aberrant DNA methylation and by changes in chromatin structure that involves histone deacetylation. It has been reported that a positive interaction between DNA methylation and histone deacetylation takes place to inhibit transcription. Based on this observation our working hypothesis was that a combination of inhibitors of these processes should produce an enhancement of their antineoplastic activity on leukemic cells. 5-AZA-2'-deoxycytidine (5AZA) is a potent inhibitor of DNA methylation, which can activate tumor suppressor genes that have been silenced by aberrant methylation. In clinical trials 5AZA was demonstrated to be an active antileukemic agent. The histone deacetylase inhibitor, Trichostatin A (TSA), was reported to induce in vitro differentiation and activate gene expression in leukemic cell lines. In this report we investigated the in vitro antineoplastic activity of 5AZA in combination with TSA on human HL-60 and KG1a myeloid leukemic cell lines. The results showed that the combination of 5AZA with TSA produced a greater inhibition of growth and DNA synthesis than either agent alone. In a clonogenic assay the combination of 5AZA and TSA showed a potent synergistic interaction to reduce colony formation by HL-60 leukemic cells. These results suggest that 5AZA used in combination with inhibitors of histone deacetylation may be an interesting chemotherapeutic regimen to investigate in patients with acute myeloid leukemia.

Introduction

One of the events that can occur during the development of leukemia is the inactivation of the genes that regulate growth and induce differentiation (1). The activation of these genes can have therapeutic implications (2). The silencing of tumor suppressor and genes that can induce differentiation can occur by an epigenetic mechanism, which involves aberrant methylation of cytosines in their promoter region (3, 4). These target genes can be reactivated by treatment with the inhibitor of DNA methylation, 5-aza-2'-deoxycytidine (5AZA) (5). 5AZA was demonstrated to induce the in vitro differentiation of leukemic cells (6, 7) and was reported to be an active antileukemic agent in man (8-10).

The novel mechanism of action of this cytosine analog has generated a renewed interest in its use in the therapy of neoplastic disease. However, the full chemotherapeutic potential of 5AZA may only be realized when it is used in combination with other agents that also activate gene expression and induce differentiation. An interesting class of agents to use in combination with 5AZA is that of the histone deacetylase inhibitors (11). Histone acetylation facilitates the binding of transcription factors to the promoter region to activate gene transcription by conversion of chromatin from a compact to an open structure (12, 13). Trichostatin A (TSA), a potent inhibitor of histone deacetylase (14), has been shown to activate the expression of genes that suppress the formation of leukemia (15). In addition, TSA can induce differentiation of leukemic cell lines (15, 16) suggesting that this class of agents may have potential for the therapy of leukemia.

In support of the association of inhibitors of DNA methylation and histone deacetylation is the observation that 5AZA in combination with TSA produces an additive activation of the tumor suppressor genes, p15 and p16 in neoplastic cells (17). The mechanism involved for

this interesting interaction is that the methylated cytosines in the promoter region bind MeCP2, a 5-methylcytosine-binding protein, which in turn recruits histone deacetylase to form a complex (17-19). Apparently, MeCP2 prevents the binding of transcription factors to the promoter whereas deacetylated histones convert the chromatin to a more compact structure unfavorable for transcription. TSA can activate some genes that are methylated, but not all methylated genes (17, 19, 20).

In this report we have investigated the in vitro antineoplastic activity of 5AZA in combination with TSA on human myeloid leukemic cell lines. We observed an interesting additive interaction between these agents, suggesting that this combination may have interesting potential in the chemotherapy of acute myeloid leukemia. Our laboratory has reported previously an additive antitumor interaction with 5AZA in combination with TSA on human breast carcinoma cells (20).

Materials and Methods

Materials

5AZA (Decitabine) was obtained from Pharmachemie (Haarlem, The Netherlands), dissolved in 0.45% NaCl containing 10 mM sodium phosphate pH 6.8 and stored at -70°C .TSA was obtained from Wako BioProducts, Richmond, Va, and was dissolved in absolute ethanol at concentration of (302 ng/ml $=10^{-6}$ M) protected from light and stored at -20°C. RPMI 1640 culture medium and fetal calf serum were obtained from Canadian Life Technologies, Burlington, Ontario.and WISENT, St. Bruno, Quebec, respectively.

Cell Culture

Human myeloid leukemic cell lines, HL-60 and KG1a, were obtained from ATCC, Manassas, Virginia .The cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum and kept in a 5% CO2 incubator at 37° C. The doubling times of HL-60 cells and KG1a cells were16-18 h and 27-30 h, respectively.

Inhibition of Growth

For the growth inhibition assay, cells in log phase were placed at a density of 1-2 x 10^4 cells/ml in 5 ml of medium. Different concentrations of 5AZA and TSA alone or in combination (simultaneously or sequentially) were added to the medium. Cell counts were performed at the indicated times using a model ZM Coulter Counter. The concentration that produces 50% inhibition of growth (IC50) was determined from the growth curves of the drug treated leukemic cell lines.

DNA Synthesis Assay

The rate of DNA synthesis was measured by the incorporation of radioactive thymidine into DNA. HL-60 ($5x10^3$ cells/ml) and KG1a cells (10^4 cells/ml) were suspended in 2 ml RPMI medium containing 10% fetal serum in 6-well (35 mm well diameter) dishes and incubated with different concentrations of corresponding drugs for 48 h (drugs were added simultaneously or sequentially, as indicated). At 48 h 0.5 μCi [^{3}H]thymidine (6.7 Ci/mmol, ICN, Irvin, CA) was added to each well and incubated for an additional 24 h. The cells were placed on GF/C glass fiber filters (2.4 cm diameter), washed with cold 0.9% NaCl, 5% cold trichloroacetic acid and ethanol. The filters containing the DNA were then dried,

placed in EcoLite scintillation liquid (ICN) and the radioactivity measured using Beckman LS 6000IC scintillation counter. The IC50 is defined as the concentration of drug that inhibits by 50% the DNA synthesis of the leukemic cell lines from the dose-response curve.

Clonogenic Assay

After drug treatment, the cells were centrifuged and suspended in drug free medium. An aliquot of 100 cells was placed in 2 ml of 1.8% soft agar medium containing 20% serum. After 20 days incubation at 37°C in 5% CO2 incubator, the number of colonies (>500 cells) was counted. The cloning efficiency of the control cells was in the range of 45-65%.

Statistical Analysis

Mean values were compared using the unpaired, single-tailed Student's t-test.

Results

Growth Inhibition

The effects of different concentrations of 5AZA or TSA alone on the growth of HL-60 and KG1a leukemic cells are shown in Figure 1. In order to estimate the effect of exposure time on drug action, the leukemic cell lines were treated with each drug for variable times between 24 and 96 h. Due to chemical instability (21), 5AZA was added to medium every 24 h. In both cell lines, 5AZA and TSA as single agents, inhibited cell growth in a dose and time-dependent manner. The IC50 values of 5AZA for both the HL-60 and KG1a leukemic cells for 72 and 96 h exposure were approximately 100 and 10 ng/ml, respectively. The IC50 value for 48h exposure of TSA for both HL-60 and KG1a cells was approximately 10

ng/ml. At the low drug concentration of 1 ng/ml both leukemic cell lines were more sensitive to 5AZA than TSA (Figure 1). At the higher drug concentrations of 10 and 100 ng/ml, TSA produced a much greater growth inhibition than 5AZA alone, especially at the shorter exposure times.

The effect of the combination of 5AZA and TSA on the growth inhibition of HL-60 and KG1a leukemic cells are shown in Figures 2 and 3. Two different schedules were used: For the sequential treatment the cells were exposed to 5AZA for 72 h with TSA being added to medium from 24 to 72 h; for the simultaneous treatment the cells were exposed to both drugs for 72 h. Using a sequential exposure on the HL-60 cells, 5AZA (10 ng/ml) and TSA (5 ng/ml) as single agents produced 29 and 33.9% growth inhibition, respectively, whereas the combination produced 63.7% inhibition (Figure 2A). For the simultaneous exposure at the same concentrations 5AZA and TSA alone produced 21.7 and 46.6% inhibition, respectively, whereas the combination produced 70% inhibition (Figure 2B).

The greater inhibition observed with TSA alone for the simultaneous exposure study was due to the longer exposure time (72 h) as compared to weaker inhibition observed in the sequential study where the exposure time for TSA was only 48 h. At the higher drug concentration of 5AZA (100 ng/ml), the drug combination also produced significantly greater inhibition than either drug alone.

In the experiments on the KG1a leukemic cells, using a sequential exposure 5AZA (1 ng/ml) and TSA (3 ng/ml) as single agents, produced 10 and 14.3 % growth inhibition, respectively, whereas the combination produced 33.3% inhibition (Figure 3A). For the simultaneous exposure at the same concentrations 5AZA and TSA produced 16.7 and

12.2% inhibition, respectively, whereas the combination produced 33.2% inhibition (Figure 3B). At the higher drug concentration of 5AZA (10ng/ml) , the combination produced significantly greater growth inhibition than either drug alone.

DNA Synthesis Inhibition

A second method to evaluate the antineoplastic action was used in which following a certain interval of drug exposure; radioactive thymidine was added to the cells to determine the rate of DNA synthesis. In these experiments, different concentrations of drugs were added at time 0, radioactive thymidine was added at 48 h and the rate of DNA synthesis determined at 72h. Due to its chemical instability, 5AZA was added every 24h.

In HL-60 and KG1a leukemic cell lines, 5AZA or TSA, alone, inhibited DNA synthesis in a dose dependent manner. The IC50 values of 5AZA for inhibition of DNA synthesis for a 72h exposure of 5AZA on HL-60 and KG1a cells were approximately 100 ng/ml and 1 ng/ml, respectively (Figure 4A and C). For TSA, the IC50 values for a 72h exposure of TSA on HL-60 and KG1a cells were approximately the same, 3 ng/ml (Figure 4B and D). TSA was a much more potent inhibitor than 5AZA for DNA synthesis for HL-60 cells, but not KG1a cells.

A sequential and simultaneous drug exposure was used to evaluate the DNA synthesis inhibition activity of 5AZA in combination with TSA on the myeloid leukemic cell lines. For the sequential drug exposure 5AZA was added at time 0 and TSA added at 24 h. For the HL-60 myeloid leukemic cells using the sequential drug exposure, 5AZA(100 ng/ml) or TSA (3 ng/ml) alone produced 38 and 19% reduction in DNA synthesis, respectively, whereas the combination produced a non-significant 45% inhibition as compared to 5AZA

alone (Figure 5A). For the simultaneous exposure 5AZA(100 ng/ml) or TSA (3 ng/ml) alone produced a 30.2 and 59.8% reduction in DNA synthesis, respectively, whereas the combination inhibited DNA synthesis by 75%, a significant difference as compared to either 5AZA or TSA alone (Figure 5B). As observed in the growth inhibition assay, TSA produced a greater inhibition of DNA synthesis with the simultaneous schedule, which was expected due to the longer drug exposure time (72 h) as compared to the sequential schedule (48 h).

In the assays on the KG1a myeloid leukemic cells, sequential treatment with 5AZA(1 ng/ml) or TSA (3 ng/ml) as single agents produced a 44.7 and 39% reduction in DNA synthesis, respectively, whereas the combination produced a non-significant 65.2% inhibition of DNA synthesis (Figure 6A). For the simultaneous exposure 5AZA(1 ng/ml) or TSA (3 ng/ml) alone produced a 35 and 46% reduction in DNA synthesis, respectively, whereas the combination inhibited significantly DNA synthesis by 69% as compared to either 5AZA or TSA alone (Figure 6B).

Inhibition of colony formation

The effect of 5AZA and TSA on colony formation by HL-60 leukemic cells was evaluated by clonogenic assay as shown in Figure 7. 5AZA alone for 72 h exposure produced a loss of clonogenicity of 64.8%. No loss of clonogenicity was detected after a 48 h exposure of TSA (Figure 7A) whereas a 72 h exposure produced a 15.3% loss of clonogenicity (Figure 7B). A sequential exposure of 5AZA in combination with TSA produced 80.3% loss of clonogenicity, a synergistic interaction as defined by Valierote and Lin (22). A 91.0% loss of clonogenicity was observed for the simultaneous exposure of 5AZA in combination with TSA, also a synergistic interaction.

Discussion

Intensification of chemotherapy of acute myeloid leukemia has increased the response rate and survival duration, but only a limited number of patients are long-term survivors (23, 24). It is important to find new effective drugs and approaches to treat this disease. A block in differentiation is one of the key events that occur during the development of AML (1) suggesting that differentiating agents have potential to treat this disease (2).

Several classes of agents have been demonstrated to induce the in vitro differentiation of myeloid leukemic cells (1, 2). The major limitation of differentiation agents is their limited potency to induce terminal differentiation in all the leukemic stem cells and the rapid development of resistance to these agents. The most well-know example is retinoic acid, which was shown as single agent to induce leukemic differentiation and complete remissions in patients with acute promyelocytic leukemia (25). However, continuous treatment with retinoic acid alone is not curative. In an interesting report on a patient with acute promyelocytic leukemia that showed drug resistance to retinoic acid, the addition of sodium phenylbutyrate, an inhibitor of histone deacetylase, produced a complete remission (25). Histone deacetylase inhibitors can activate genes by conversion of compact chromatin to a more open structure facilitating the binding of transcription factors (12, 13). The above report demonstrates that the effectiveness of leukemia therapy can be increased by combination of differentiating agents with different mechanisms of action.

Among the various classes of differentiating agents 5AZA is an interesting agent for combination therapy since it can induce in vitro differentiation of human myeloid leukemic cells (6, 7). This nucleoside analog also has a novel mechanism of demethylation of DNA,

which can lead to the activation of tumor suppressor genes that have been silenced by aberrant methylation (5). In the mouse model 5AZA was reported to be a potent antileukemic agent and the extent of inhibition of DNA methylation produced by this analog correlated with its antineoplastic activity (26). 5AZA can induce complete remission in some patients with advanced AML (8-10). It also inhibits DNA methylation in leukemic blasts from patients treated with this analog (27). It is of interest to note that 5AZA therapy also produces positive responses in patients with CML in blastic crisis (28) and MDS, a preleukemic disease (29).

Recent investigators have reported an interesting interaction between DNA methylation and histone deacetylation to regulate gene expression. Apparently, the 5-methylcytosine binding protein (MeCP2) binds to the methylated promoter and recruits histone deacetylase (HDAC) to repress transcription (18, 19). This process can be reversed by using inhibitors as shown by Cameron et al (17) who reported a synergistic interaction between 5AZA and TSA to reactivate silenced tumor suppressor genes in neoplastic cells.

This work provided the rationale to combine inhibitors of DNA methylation with inhibitors of HDAC to obtain an enhancement of their antineoplastic activity. In this regard, we observed an additive antineoplastic effect between 5AZA and TSA against human breast carcinoma cells (20). The activation of the tumor suppressor gene, retinoic acid receptor ß, appeared to be involved in this interaction (20). In accord with these findings is the report on lung carcinoma cells that 5AZA enhances apoptosis induced by inhibitors of histone deacetylation (30).

In this report we showed an enhancement of the in vitro antineoplastic activity of 5AZA and TSA on human myeloid leukemic cells. Both 5AZA and TSA as single agents produced a potent inhibition of growth of HL-60 and KG1a leukemic cells (Figure 1). The growth inhibition produced by these agents in combination was greater than that for either agent alone for both HL-60 and KG1a leukemic cells (Figures 2 and 3).

Similar results were obtained using a DNA synthesis assay. In this latter assay the radioactive thymidine was added after 48 h drug exposure to give a better assessment of the long term effects of the inhibitors. As single agents both 5AZA and TSA showed potent inhibition of DNA synthesis (Figure 4). For both leukemic cell lines, the combination produced a greater inhibition of DNA synthesis than either agent alone. It is interesting to note that the concentrations of 5AZA used in these in vitro studies are in the range of the plasma levels of this analog in clinical trials in both leukemia and lung cancer (8, 9, 31).

The clonogenic assay is more relevant for translation into clinical trial than growth and DNA synthesis assays since it provides data on the long-term survival of the leukemic cells in terms of their proliferative potential.Using two different schedules, sequential and simultaneous drug exposure, we observed a prominent synergistic interaction of the combination of 5AZA and TSA against the HL-60 leukemic cells (Figure 7). With both these schedules 5AZA in combination with TSA produced a greater antineoplastic effect than either agent alone. The optimal dose-schedule will be determined by future clinical trials on 5AZA in combination with inhibitors of histone deacetylase in patients with leukemia.

The molecular mechanism responsible for the enhancement of the antileukemic activity of 5AZA and TSA is not known. One hypothesis is that the combination of an inhibitor of DNA methylation with an inhibitor of histone deacetylation activates "growth suppressor" genes that were silenced by epigenetic events. It is interesting to note that 5AZA in combination with TSA was reported to produce a synergistic activation of the p15 tumor suppressor gene in KG1a leukemic cells (17). Further clarifications of the mechanisms responsible for the enhanced antileukemic activity of these agents may require the identification of other growth suppressor genes that are also silenced by epigenetic events in leukemia.

Since HDAC inhibitors have the potential to induce G1 arrest (11), it is possible that when used simultaneously they could antagonize the action of 5AZA, an S phase specific agent (32), by blocking the progression of leukemic cells into S phase. For this reason we investigated the antineoplastic activity of 5AZA and TSA using two schedules: sequential and simultaneous drug exposure.

Our data indicate that both schedules resulted in an enhancement of the antileukemic activity of 5AZA and TSA in combination. In clinical therapy the sequential schedule may have some advantages. 5AZA is a potent myelosuppressive drug taking about 5 to 6 weeks for adequate hematological recovery before a second cycle of therapy can be administered (9, 31). Significant progression of the leukemia can take place between cycles of 5AZA therapy. The interval between cycles of 5AZA therapy may be the optimal time to administer inhibitors of HDAC to prevent leukemic progression and to enhance the antineoplastic action of 5AZA. Since HDAC inhibitors do not produce significant hematopoietic toxicity (33), they should not interfere with the recovery from the

myelosuppression produced by 5AZA. Another approach that can be used to lessen the hematopoietic toxicity of 5AZA is to reduce the dose below its maximum tolerated dose. In combination with an inhibitor of histone deacetylation, the lower doses of 5AZA may still produce a potent antileukemic effect.

Although we have used TSA as a model-HDAC inhibitor, it is currently not available for clinical trials. Other inhibitors of HDAC have undergone some clinical investigation and have the potential to be used in combination with 5AZA. An example is sodium phenylbutyrate, which is a weak HDAC inhibitor and requires high plasma concentrations for clinical effectiveness (11). However, it is possible that interesting clinical responses be obtained with this agent in combination with 5AZA. Other potent HDAC inhibitors, such as Depsipeptide, showed significant antitumor activity in animal models (34), are under clinical investigation and may also have the potential to be very effective agents to use in combination with inhibitors of DNA methylation for the therapy of leukemia.

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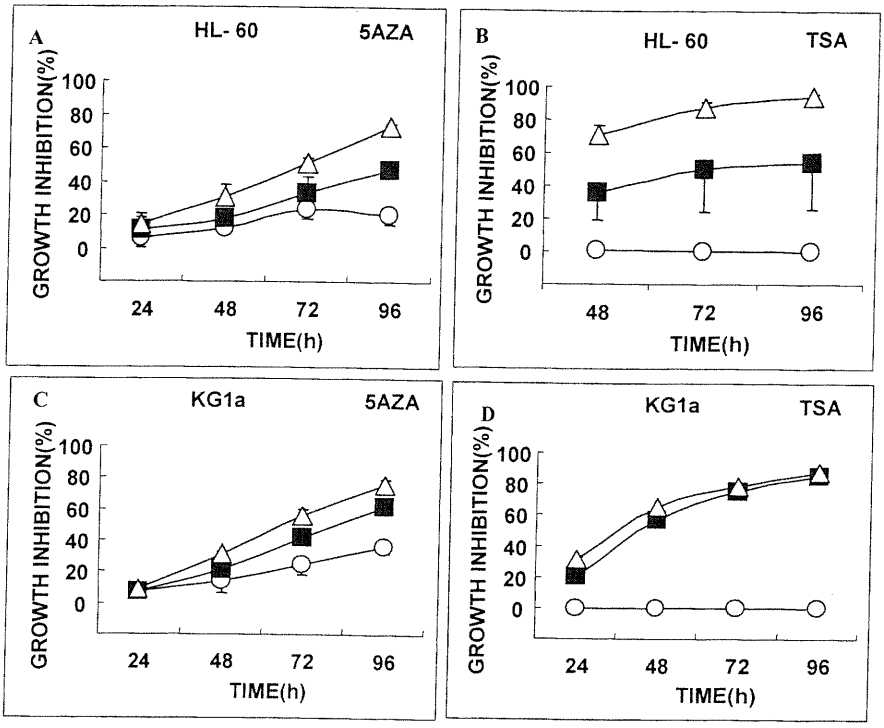

Fig.1. Effect of 5AZA or TSA on growth of HL-60 and KG1a myeloid leukemic cells for different times of drug exposure. Drug concentrations: (○)1 ng/ml; (■) 10 ng/ml; (△)100 ng/ml .

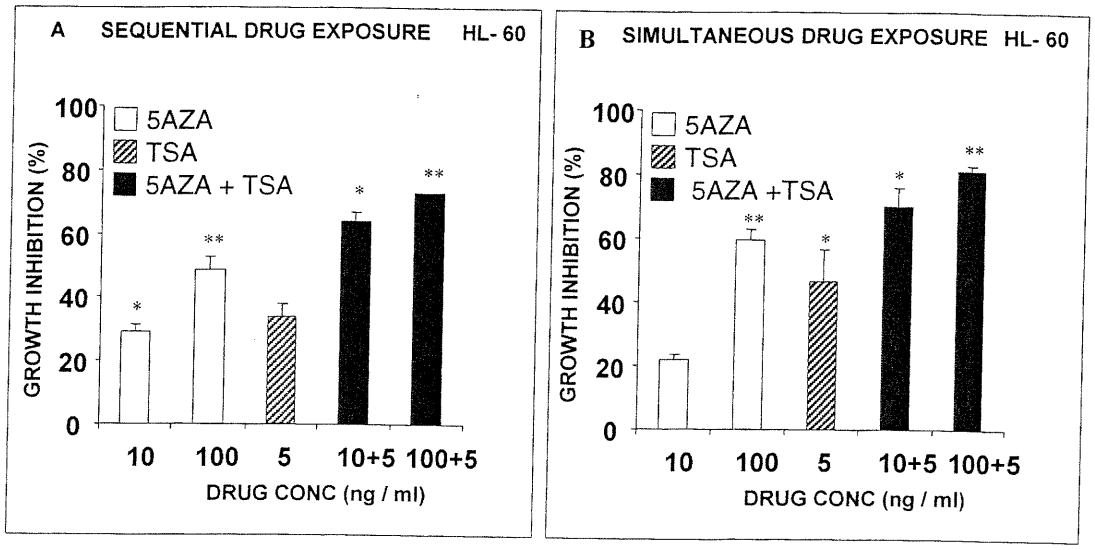

Fig.2. Effect of sequential and simultaneous exposure of 5AZA and TSA on growth inhibition in HL-60 myeloid leukemic cells. (A) 5AZA was added at time 0 and TSA added at 24h. (B) Both 5AZA and TSA were added at time 0. The growth inhibition was assessed after 72h drug treatment. Data shown are mean values ± S.D., n=3. Statistical analysis: A, *, $P<0.001$; **, $P<0.001$. B,*, $P<0.01$;**, $P=0.001$.

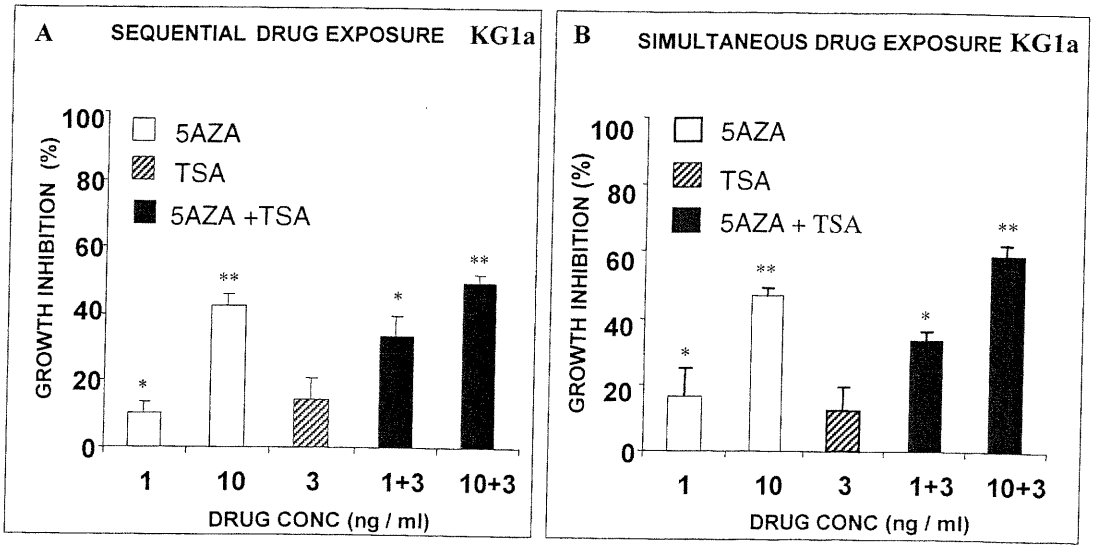

Fig.3. Effect of sequential and simultaneous exposure of 5AZA and TSA on growth inhibition in KG1a myeloid leukemic cells. (A) 5AZA was added at time 0 and TSA added at 24h. (B) Both 5AZA and TSA were added at time 0. The growth inhibition was assessed after 72h drug treatment. Data shown are mean values ± S.D., n=3. Statistical analysis: A, *, P=0.005; **, P<0.05. B,*, P<0.05;**, P<0.01.

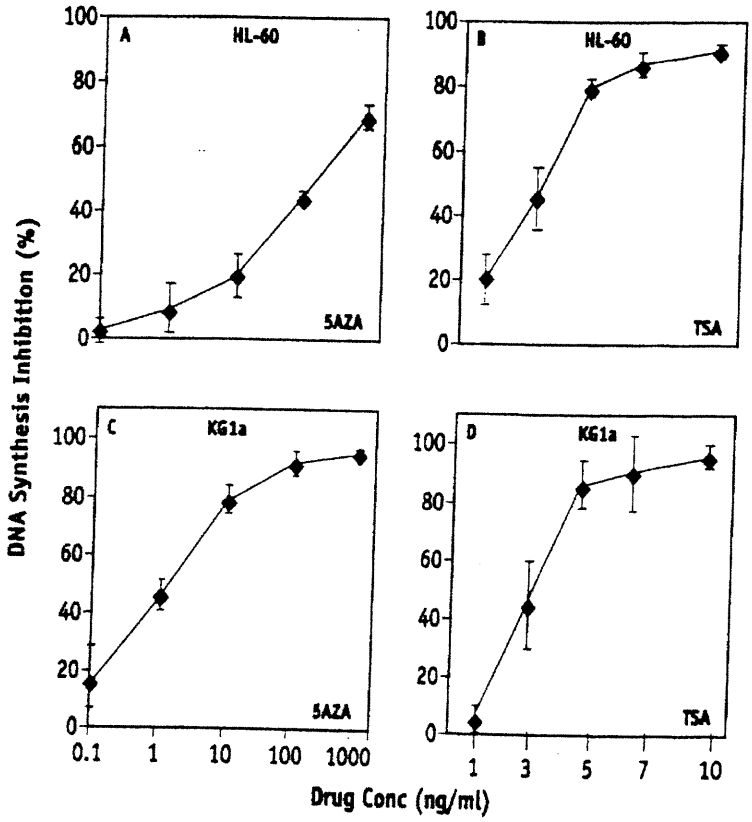

Fig.4. Effect of 5AZA or TSA on DNA synthesis of HL-60 and KG1a myeloid leukemic cells for different concentrations of drugs. Data shown are mean values ± S.D., n=3.

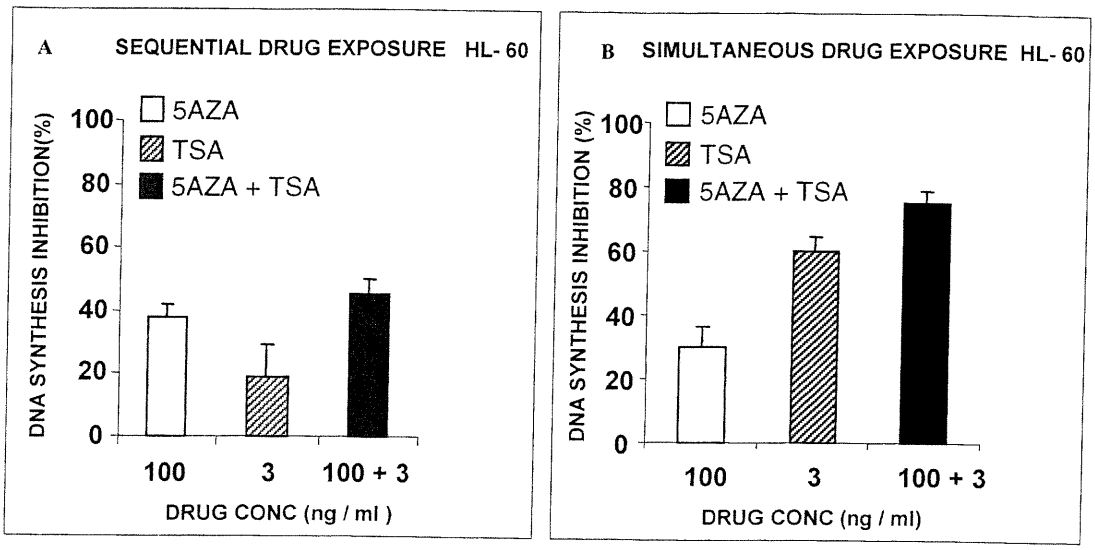

Fig.5. Effect of sequential and simultaneous exposure of 5AZA and TSA on DNA synthesis in HL-60 myeloid leukemic cells. (A) 5AZA was added at time 0 and TSA added at 24h. (B) Both 5AZA and TSA were added at time 0. DNA synthesis for both experiments was assessed after 72h drug treatment. Data shown are mean values ± S.D., n=3. Statistical analysis: A, 5AZA *versus* 5AZA + TSA, P=not significant; TSA *versus* 5AZA + TSA, $P<0.05$. B, 5AZA *versus* 5AZA + TSA, $P<0.001$; TSA *versus* 5AZA + TSA, $P<0.05$.

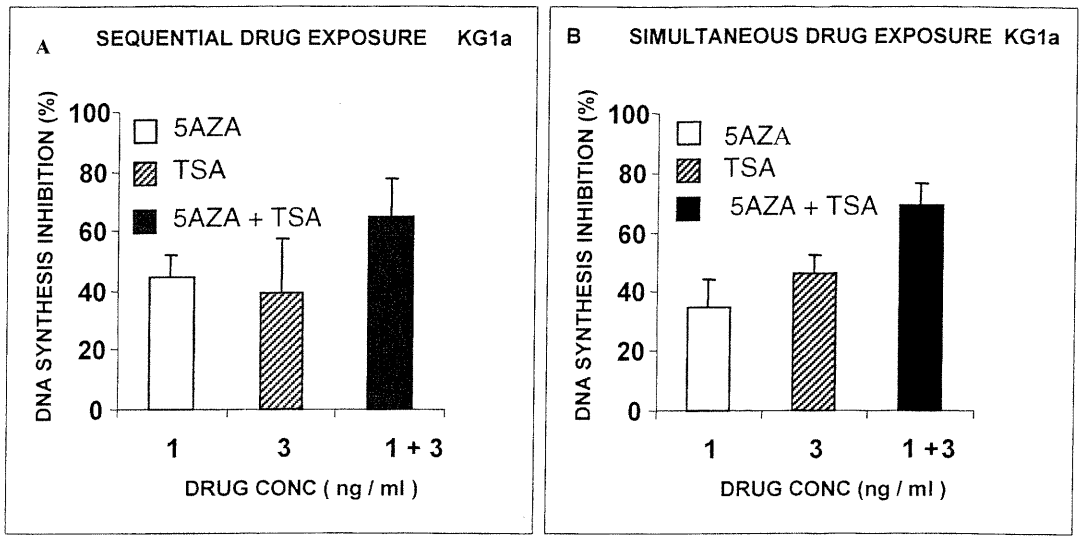

Fig.6. Effect of sequential and simultaneous exposure of 5AZA and TSA on DNA synthesis in KG1a myeloid leukemic cells. (A) 5AZA was added at time 0 and TSA added at 24h. (B) Both 5AZA and TSA were added at time 0. DNA synthesis for both experiments was assessed after 72h drug treatment. Data shown are mean values ± S.D., n=3. Statistical analysis: A, 5AZA *versus* 5AZA + TSA, P=not significant; TSA *versus* 5AZA + TSA, P=not significant. B, 5AZA *versus* 5AZA + TSA, $P<0.01$; TSA *versus* 5AZA + TSA, $P<0.05$.

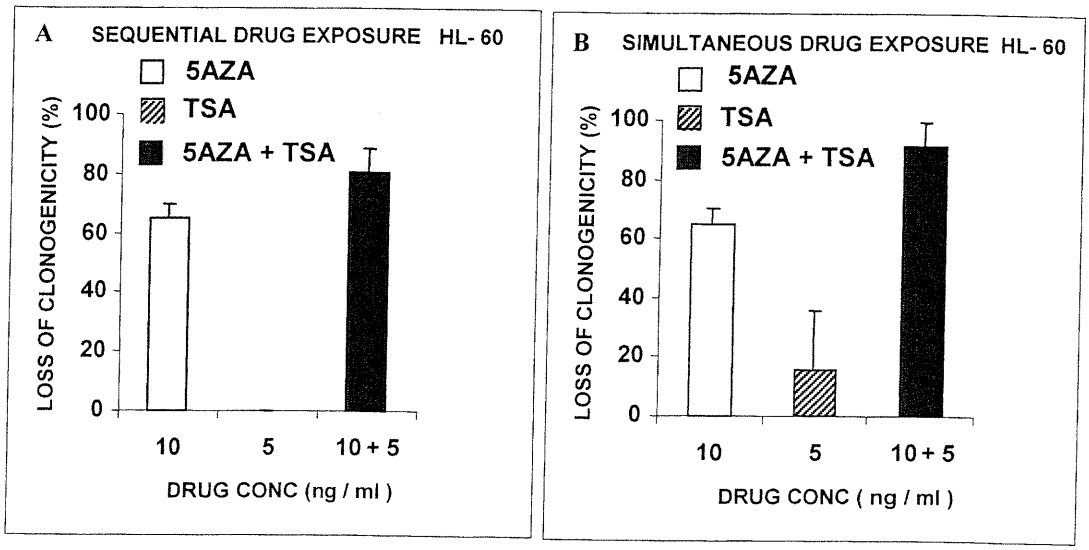

Fig.7. Effect of sequential and simultaneous exposure of 5AZA and TSA on loss of clonogenicity of HL-60 myeloid leukemic cells. (A) 5AZA was added at time 0 and TSA added at 24h. (B) Both 5AZA and TSA were added at time 0.The drugs were removed at 72h and colony formation determined on day 20. Data shown are mean values ± S.D., n=3. Statistical analysis: A, 5AZA *versus* 5AZA + TSA, $P<0.05$; TSA *versus* 5AZA + TSA, $P<0.001$; B, 5AZA *versus* 5AZA + TSA, $P=0.01$; TSA *versus* 5AZA + TSA, $P<0.01$.

CHAPTER 8:

Antineoplastic action of 5-aza-2'- deoxycytidine and depsipeptide (FR 901228) in combination against human myeloid Leukemic cells.

Preface

Previously, we observed an enhancement of the antineoplastic effect of 5-AZA-CdR by trichostatin A (TSA) on human breast cancer cells (Bovenzi & Momparler, 2001) and on human myeloid leukemic cells (Shaker et al, paper submitted). In this work we investigated another histone deacetylase inhibitor, depsipeptide (depsi), which was reported to be a potent antineoplastic agent.

We investigated the antineoplastic effect of 5-AZA-CdR and depsipeptide alone and in combination on human HL-60 and KG1a myeloid leukemic cells. Using inhibition of growth, DNA synthesis assay and colony assay each agent shows a potent antineoplastic effect on these cell lines. Combination of these two agents shows an interesting interaction of these two agents on leukemic cells. We conclude that combination of 5AZA with depsi have a potential to be used in clinical trials with leukemic patients.

Antineoplastic action of 5-aza-2'-deoxycytidine and depsipeptide (FR901228) in combination against human myeloid leukemic cells

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Running title: 5-AZA-CdR plus depsipeptide therapy leukemia

Keywords: 5-AZA-2'-deoxycytidine, Depsipeptide (FR 901228), DNA methylation, histone deacetylase, antineoplastic activity

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Be submitted to Clinical Cancer Research

Abstract

Despite improvements in the chemotherapy of acute myeloid leukemia (AML) during the past decades, less than half of the patients survive. New approaches for therapy of this disease should be explored. Silencing of regulatory genes has a key role in the development of leukemia. The interaction between two epigenetic mechanisms, DNA methylation and histone deacetylation, is reported to be involve in the inhibition of the transcription of these genes. 5-Aza-2'-deoxycytidine (5AZA), a potent inhibitor of DNA methylation, which can lead to gene activation, is an active antileukemic agent in man. However, the full chemotherapeutic potential of 5AZA may be only realized when it is used in combination with other agents. An interesting class of agents to use in combination with 5AZA is the histone deacetylase inhibitors. Depsipeptide (FR901228), a potent antitumor antibiotic and a histone deacetylase inhibitor (HDI), is currently under clinical investigations. HDI can also activate silent genes by modification of chromatin structure. Based on these reports we hypothesized that depsipeptide in combination with 5AZA can enhance its antineoplastic activity on myeloid leukemic cells. The combined treatment of depsipeptide and 5AZA produced greater inhibition of growth, inhibition of DNA synthesis and reduction in colony formation on myeloid leukemic cell lines than either agent alone. This interesting interaction between 5AZA and depsipeptide provides a rational for the use of the inhibitors of DNA methylation and histone deacetylation in combination for the chemotherapy of acute leukemia.

Introduction

Advances in therapeutic approaches over the past 30 years have markedly increased the five-year survival rates in patients with acute myeloid leukemia (AML). However, despite these improvements the survival rate of most AML patients is only 40% (Löwenberg B, 1999). New approaches should be sought to improve the effectiveness of therapy. Recent developments in the molecular biology of leukemia can provide interesting avenues for investigation of novel therapies.

Inactivation of the tumor suppressor genes is one of the important events in the development of hematological malignancies (Drexler HG, 1998). Different mechanisms are involved in the silencing of these genes. Methylation of the cytosines in promoter region of tumor suppressor genes is one of the important epigenetic mechanisms, which can be responsible for their loss of expression (Jones PA & Laird PW, 1999; Baylin SB et al., 2000). This process can be reversed by 5AZA, which is a potent inhibitor of DNA methylation and can induce the differentiation of neoplastic cells (Razin A & Riggs AD, 1980; Jones PA & Taylor SM, 1980) including a human leukemic cell line (Momparler RL et al., 1985) and leukemic blasts from patients (Pinto A et al, 1984). In clinical trials 5AZA has demonstrated to be an active antileukemic agent (Rivard GE et al., 1981; Momparler et al., 1985; Richel et al., 1991; Sacchi S et al 1999).

Another mechanism, which affects regulation of the transcription of tumor suppressor genes, is the acetylation of the histones. In general, histone acetylation is associated with active transcription whereas histone deacetylation is associated with inactive transcription (Wolffe AP, 1996; Archer SY, 1999). Depsipeptide (Depsi) is a potent antitumor antibiotic and histone deacetylase inhibitor (Nakajima H et al., 1998). This antibiotic has shown selective in vitro activity against resistant B-cell chronic

lymphoblastic leukemia (Byrd JC et al 1999). Other inhibitors of histone deacetylation (Trishostatin A, TSA) have also shown interesting antileukemic activity. TSA has been shown to activate the expression of genes that suppress the formation of leukemia (Kosugi H et al., 1999). In addition, TSA can induce differentiation of the leukemic cell lines (Kosugi H et al., 1999; Wang J et al., 1999).

Recently, DNA methylation and histone deacetylation have been shown to be linked through the family of 5-methylcytosine binding proteins (Magdinier F & Wolffe AP, 2001). Apparently, these proteins bind to the methylated promoter and recruit histone deacetylase leading to gene silencing by interference with the binding of transcription factors and conversion of the chromatin to a more compact configuration by deacetylation of lysines in histones. The interaction between these events is illustrated by the synergistic interaction of 5AZA and TSA to activate the tumor suppressor genes, p16 and p15 (Cameron EE et al. 1999). This interaction can have therapeutic implications. 5AZA was reported to enhance the apoptosis induced by HDAC inhibitors in lung cancer cells (Zhu WG et al 2001). In addition, we observed an additive antineoplastic effect between 5AZA and TSA against human breast carcinoma cells (Bovenzi V & Momparler RL in press).

Based on these results, we performed experiments in human myeloid leukemic cells, KG1a and HL-60 to investigate the antineoplastic interaction between inhibitors of DNA methyaltion and HDAC inhibitors. Both cell lines were treated with 5AZA or depsi alone or in combination. The results show that depsi in combination can enhance the effect of 5AZA on inhibition of growth, colony formation and DNA synthesis on these leukemic cell lines. These data suggest that a combination of 5AZA and depsipeptide may have the potential to be an effective therapeutic regimen to treat patients with AML.

Materials and Methods

Materials. 5AZA (Decitabine) was obtained from Pharmachemie (Haarlem, The Netherlands), dissolved in 0.45% NaCl containing 10 mM sodium phosphate pH 6.8 and stored at -70°C. Depsipeptide (FR901228) was obtained from Fujisawa pharmaceutical co., (Osaka, Japan) and stored at -20°C. RPMI 1640 culture medium and fetal calf serum were obtained from Canadian Life Technologies, Burlington, Ontario and WISENT, St. Bruno, Quebec, respectively.

Cell Culture. Human myeloid leukemic cell lines, HL-60 and KG1a, were obtained from ATCC, Manassas, Virginia .The cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum and kept in a 5% CO2 incubator at 37° C. The doubling times of HL-60 cells and KG1a cells were16-18 h and 27-30 h, respectively.

Cell growth inhibition assay. For the growth inhibition assay, cells in log phase were placed at a density of 1-2 x 10^4 cells/ml in 5 ml of medium. Different concentrations of 5AZA and depsi alone or in combination (simultaneously or sequentially) were added to the medium. Cell counts were performed at the indicated times using a model ZM Coulter Counter. The concentration that produces 50% inhibition of growth (IC50) were determined by approximation from the growth curves of the drug treated leukemic cell lines.

DNA Synthesis Assay. The rate of DNA synthesis was measured by the incorporation of radioactive thymidine into DNA. HL-60 ($5x10^3$ cells/ml) and KG1a cells (10^4 cells/ml) were suspended in 2 ml RPMI medium containing 10% fetal serum in 6-well (35 mm well diameter) dishes and incubated with different concentrations of corresponding drugs for 48

h (drugs were added simultaneously). At 48 h 0.5 μCi [^{3}H]thymidine (6.7 Ci/mmol, ICN, Irvin, CA) was added to each well and incubated for an additional 24 h. The cells were placed on GF/C glass fiber filters (2.4 cm diameter), washed with cold 0.9% NaCl, 5% cold trichloroacetic acid and ethanol. The filters containing the DNA were then dried, placed in EcoLite scintillation liquid (ICN) and the radioactivity measured using Beckman LS 6000IC scintillation counter. The IC50 were determined by approximating the concentration of drug that inhibits by 50% the DNA synthesis of the leukemic cell lines from the dose-response curve.

Clonogenic Assay. After drug treatment, the cells were centrifuged and suspended in drug free medium. An aliquot of 100 cells was placed in 2 ml of 1.8% soft agar medium containing 20% serum. After incubation for 15 days at 37°C in 5% CO2 incubator, the number of colonies (>500 cells) was counted. The cloning efficiency of the control cells was in the range 45-65%.

Results

Cell growth inhibition. The effects of different concentrations of 5AZA or depsi alone on the growth of HL-60 and KG1a leukemic cells are shown in Figure 1. In order to estimate the effect of exposure time on drug action, the leukemic cell lines were treated with each drug for variable times between 24 and 96 h. Due to chemical instability (Lin KT et al., 1981), 5AZA was added to medium every 24 h. In both cell lines, 5AZA and depsi as single agents, inhibited cell growth in a dose and time-dependent manner. The IC50 values of 5AZA for both the HL-60 and KG1a leukemic cells for 72 and 96 h exposure were approximately 100 and 10 ng/ml, respectively. The IC50 value for 24h exposure of depsi

for HL-60 cells was approximately 10 ng/ml and for 48h exposure for KG1a cells was approximately 100 ng/ml. At the low drug concentration of 1 ng/ml KG1a was more sensitive to 5AZA than depsi (Figure 1) and HL-60 was more sensitive to depsi. At the higher drug concentration of 100 ng/ml, depsi produced a much greater growth inhibition than 5AZA, especially at the shorter exposure time.

The effect of the combination of 5AZA and depsi on the growth inhibition of HL-60 and KG1a leukemic cells are shown in Figure 2. Two different schedules were used: For the sequential treatment the cells were exposed to 5AZA for 72 h with depsi being added to medium from 24 to 72 h; for the simultaneous treatment the cells were exposed to both drugs for 72 h.

Using a sequential exposure on the HL-60 cells, 5AZA (10 ng/ml) and depsi (1 ng/ml) as single agents produced 29.17% and 32.92% Growth inhibition, respectively, whereas the combination produced 57.17% inhibition (Figure 2A). For the simultaneous exposure at the same concentrations 5AZA and depsi alone produced 23.38 and 54.71% inhibition, respectively, whereas the combination produced 66.75 % inhibition, which was not significant (Figure 2B).

The greater inhibition observed with depsi alone for the simultaneous exposure study was due to the longer exposure time (72 h) as compared to weaker inhibition observed in the sequential study where the exposure time for depsi was only 48 h. At the higher drug concentration of 5AZA (100 ng/ml), the drug combination produced significantly greater inhibition than either drug alone.

In the experiments on the KG1a leukemic cells, using a sequential exposure 5AZA (1 ng/ml) and depsi (15 ng/ml) as single agents, produced 12 and 37.33 % growth inhibition, respectively, whereas the combination produced 52.67% inhibition (Figure 2C). For the simultaneous exposure at the same concentrations 5AZA and depsi produced 19.5 and 56 % inhibition, respectively, whereas the combination produced 65.17% inhibition (Figure 2D). At the higher drug concentration of 5AZA (10 ng/ml), the combination produced significantly greater growth inhibition than either drug alone.

DNA Synthesis Inhibition. A second method to evaluate the antineoplastic action was used in which following a certain interval of drug exposure; radioactive thymidine was added to the cells to determine the rate of DNA synthesis. In these experiments, different concentrations of drugs were added at time 0, radioactive thymidine was added at 48 h and the rate of DNA synthesis determined at 72h. Due to its chemical instability, 5AZA was added every 24h.

In HL-60 and KG1a leukemic cell lines, 5AZA or depsi, alone, inhibited DNA synthesis in a dose dependent manner. The IC50 values of 5AZA for inhibition of DNA synthesis for a 72h exposure of 5AZA on HL-60 and KG1a cells were approximately 100 ng/ml and 1 ng/ml, respectively (Figure 3A and C). For depsi, the IC50 value for a 72h exposure of depsi on HL-60 cells was approximately 1 ng/ml (Figure 3B). The IC50 value for a 72h exposure of depsi on KG1a cells was approximately 6 ng/ml (Figure 3D). Depsi was a much more potent inhibitor than 5AZA for DNA synthesis for HL-60 cells, but not KG1a cells.

For the simultaneous exposure 5AZA(100 ng/ml) or depsi (1 ng/ml) alone produced a 23.38 and 34% reduction in DNA synthesis, respectively, whereas the combination inhibited

DNA synthesis by 65.75, a significant difference as compared to either 5AZA or depsi alone (Figure 4A).

In the assays on the KG1a myeloid leukemic cells, for the simultaneous exposure 5AZA(1 ng/ml) or depsi (3 ng/ml) alone produced a 43.5 and 27.33% reduction in DNA synthesis, respectively, whereas the combination inhibited significantly DNA synthesis by 58.67% as compared to either 5AZA or depsi alone (Figure 4B).

Inhibition of colony formation. The effect of 5AZA and depsi on colony formation by HL-60 leukemic cells was evaluated by clonogenic assay as shown in Figure 5. 5AZA alone for 72 h exposure produced a loss of clonogenicity of 51.3%. 37% loss of clonogenicity was detected for a 48 h exposure of depsi (Figure 5A) whereas a 72 h exposure produced a 80% loss of clonogenicity (Figure 5B). A sequential exposure of 5AZA in combination with depsi produced 99.67% loss of clonogenicity, a synergistic interaction as defined by Valierote and Lin (1975). A 99.3% loss of clonogenicity was observed for the simultaneous exposure of 5AZA in combination with depsi that comparing with depsi was not significant.

Discussion

Recent reports showed interesting interaction between inhibitors of DNA methylation and histone deacetylation. Cameron EE et al., (1999) reported that 5AZA in combination with TSA produced a synergistic activation of the p15 tumor suppressor gene in KG1a myeloid leukemic cells. Based on this observation our working hypothesis was that there should be an additive or synergistic antineoplastic action between these agents on leukemic cells.

For this investigation we chose depsi, which is a more potent inhibitor HDAC than TSA. In addition, it has been reported that the antiproliferative effects of depsi on neoplastic cells are much greater than TSA (Nakajima H et al., 1998). Of interest is the report that depsi showed remarkable antitumor activity of depsi against xenografted human solid tumors (Ueda, H.et al 1994).

We also selected 5AZA for this study since it has the novel mechanism of demethylation of DNA, which can lead to the activation of tumor suppressor genes that have been silenced by aberrant methylation (Momparler RL & Bovenzi V, 2000). In phase I clinical trials on 5AZA showed that it was an active antileukemic agent (Rivard GE, Momparler RL et al.1981; Momparler RL, et al., 1985; Kantarjian HM et al., 1997). However, the full chemotherapeutic potential of 5AZA may be only realized when it is used in combination with other agents that also activate gene expression and induce differentiation.

In this present study our data show an enhancement of the in vitro antineoplastic activity of 5AZA and depsi when used in combination on human HL-60 and KG1a myeloid leukemic cells. Both 5AZA and depsi as single agents produced a potent inhibition of growth and DNA synthesis of both cell lines (Figures.1 and 3). The growth inhibition produced by these agents in combination was greater than either agent alone for both HL-60 and KG1a leukemic cells (Figure 2). For both leukemic cell lines, for a simultaneous drug exposure the combination produced a significant greater inhibition of DNA synthesis than either agent alone (Figure 4). The results obtained with these agents on DNA synthesis using the sequential schedule showed large variations and are not presented.

A colony-forming assay was also used to evaluate the antileukemic activity of 5AZA in combination with depsi. This assay provides data on the long-term proliferative potential of the leukemic cells after drug treatment, which is very relevant for translation into clinical trials. We used two different schedules, sequential and simultaneous, for the drug exposure. We observed a significant synergistic interaction of the combination of 5AZA and depsi against the HL-60 leukemic cells with the sequential drug (Figure 5). For the simultaneous schedule there was no significant difference between the drug combinations as compared to depsi (1 ng/ml) alone. A possible explanation for this is that the concentration of depsi used is on the steep part of the dose-response curve (Figure 3) and that as a single agent it produced about 80% loss of clonogenicity which may be too large to observe a significant interaction with 5AZA. It is of interest to note that the concentrations of 5AZA used in these in vitro studies are in the range of the plasma levels of this analog in clinical trials in both leukemia and lung cancer (Rivard GE et al., 1981; Momparler RL et al., 1985; Momparler RL et al., 1997). The dose-schedule chosen for clinical trials on this combination should take into account that depsi can cause cell cycle arrest at G1 and G2/M (Nakajima H et al., 1998).

Our data are in accord with previous reports, which showed that other inhibitors of histone deacetylation enhanced the antineoplastic action of 5AZA. Previously our laboratory reported an additive antineoplastic effect between 5AZA and TSA against human breast carcinoma cells (Bovenzi V & Momparler RL in press). An enhancement of induction of apoptosis on lung carcinoma cells was observed when depsi was used in combination with 5AZA (Zhu WG et al., 2001).

The molecular mechanism involved in the interaction between inhibitors of DNA methylation and histone deacetylation with respect to its antileukemic action remains to be identified. It could involve the synergistic activation of a tumor suppressor gene as reported by Cameron et al for p15 activation in leukemic cells and/or activation of many other growth-regulatory genes that were silenced by aberrant methylation. Future identification of the role of these growth-regulatory genes in leukemia and the effects of epigenetic changes on their expression will clarify the antineoplastic action of this drug combination. Based on our results, the combination of 5AZA with depsi may be an interesting therapeutic regimen to investigate in patients with advanced leukemia.

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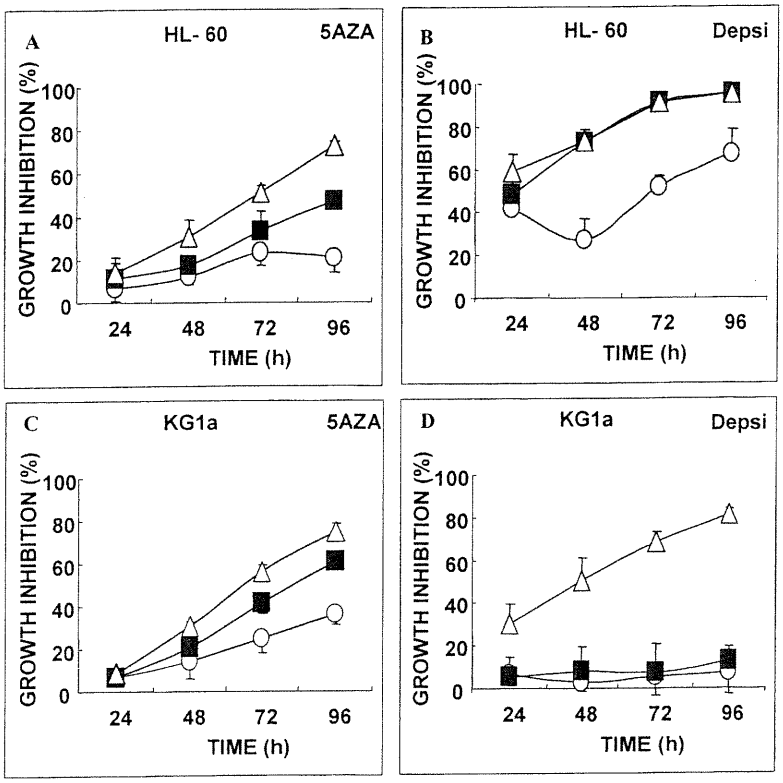

Fig.1.Effect of 5AZA and Depsi on growth inhibition in HL-60 and KG1a human myeloid leukemic cells in different times of drug exposure.(○)1 ng/ml, (■)10 ng/ml, (△)100 ng/ml.

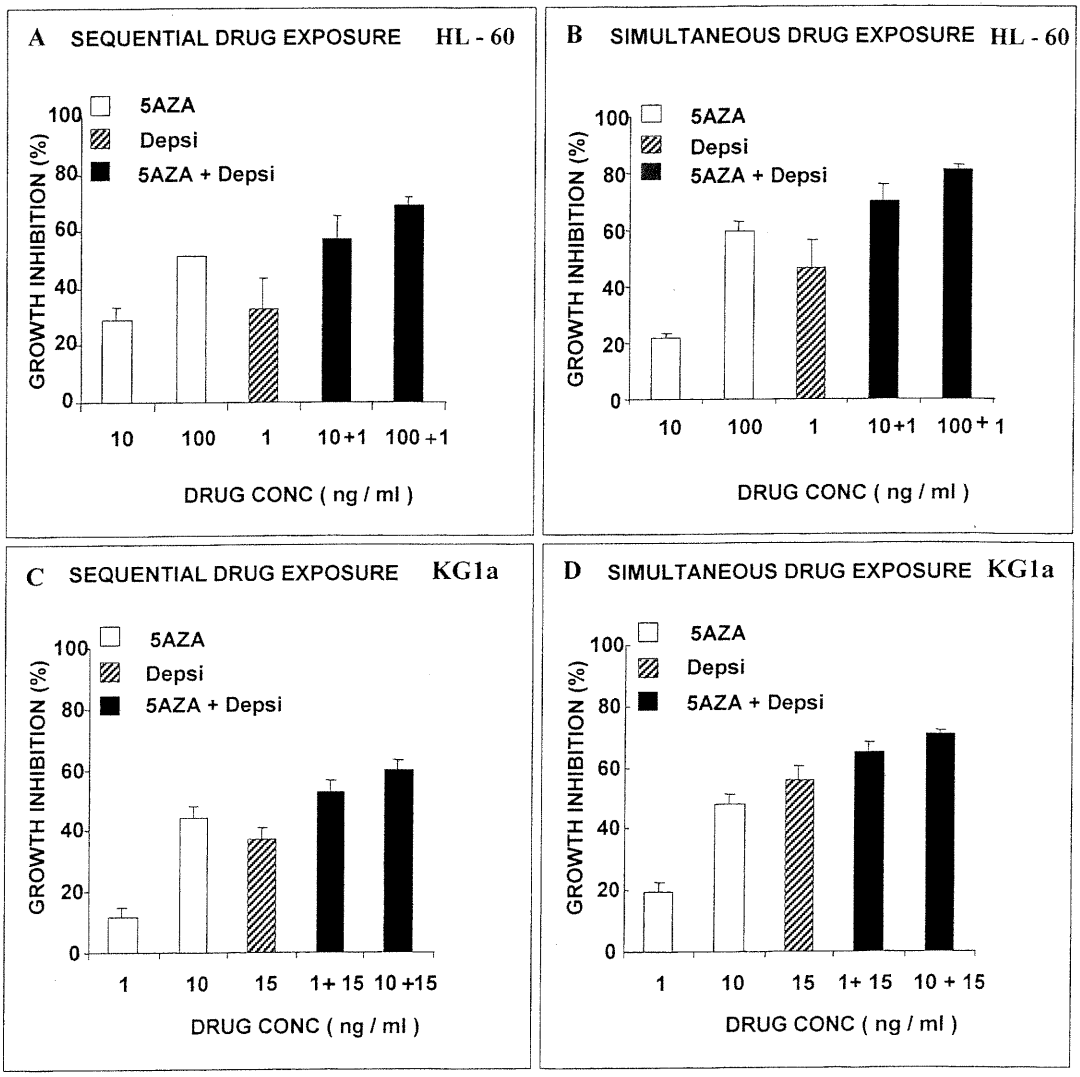

Fig.2. Effect of 5AZA and Depsi alone and in combination on growth inhibition in HL-60 and KG1a myeloid leukemic cells. (A,C) 5AZA was added at time 0 and Depsi was added sequentially after 24hs. (B,D) Both drugs were added simultaneously at time 0. The growth inhibition was assessed after 72h drug treatment. Data shown are mean values ± S.D., n=3. Statistical analysis: A, Depsi (1) *versus* 5AZA (10)+Depsi (1), $P<0.01$; 5AZA (100) *versus* 5AZA (100)+Depsi (1), $P<0.001$. B,Depsi (1) versus 5AZA (10)+Depsi (1), P=not significant; 5AZA (100) *versus* 5AZA(100)+Depsi (1), $P<0.005$. C, Depsi (15) *versus* 5AZA (1)+Depsi (15), $P<0.01$; 5AZA (10) *versus* 5AZA (10)+Depsi (15), $P<0.005$. D, Depsi (15) (15), P=0.005.

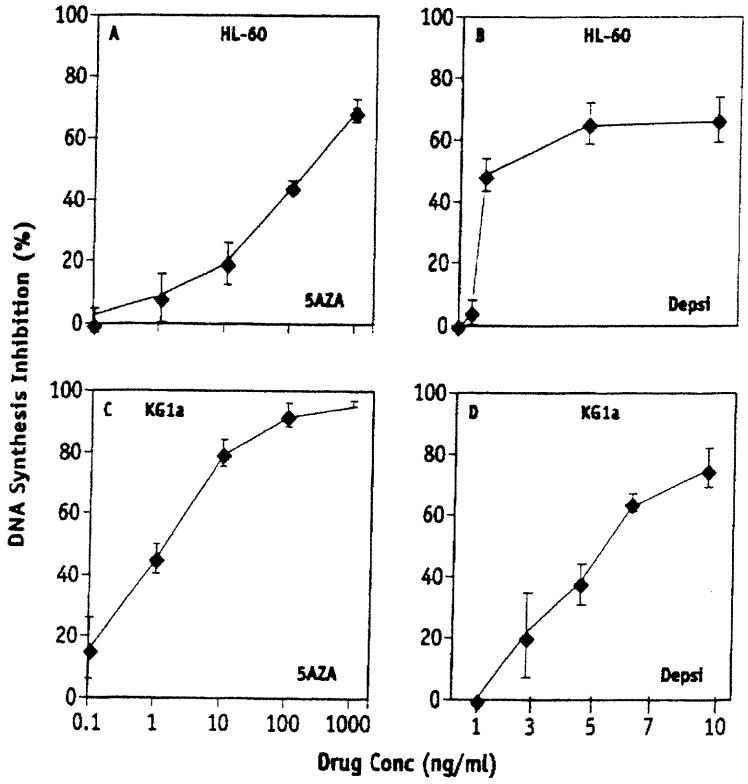

Fig.3. Effect of 5AZA or Depsi on DNA synthesis of HL-60 and KG1a myeloid leukemic cells for different concentrations of drugs. Data shown are mean values ± S.D., n=3.

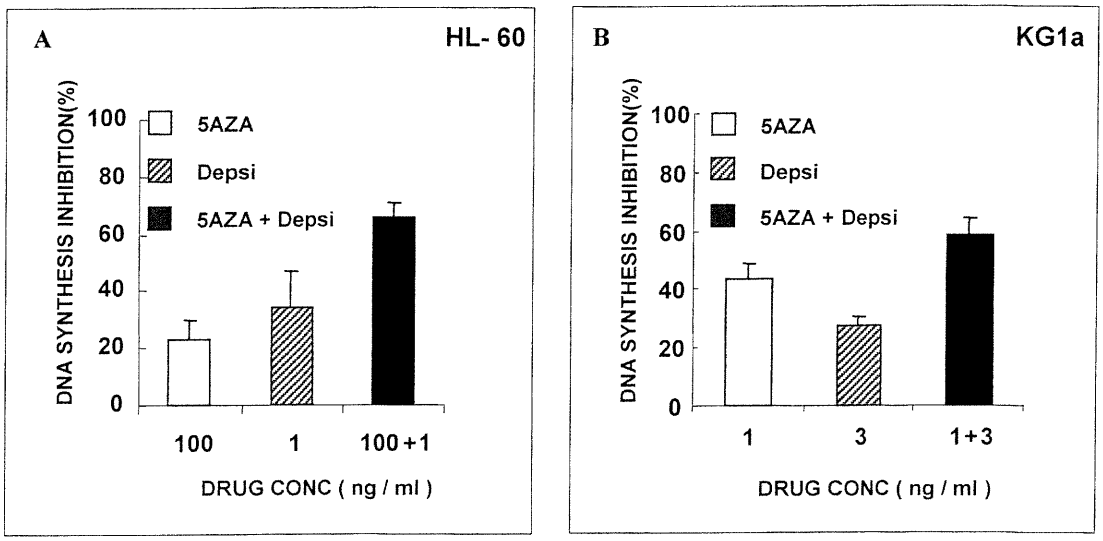

Fig.4. Effect of 5AZAR and Depsi alone and in combination on DNA synthesis in HL-60 and KG1a myeloid leukemic cells.Both drugs were added simultaneously at time 0.DNA synthesis for both experiments was assessed after 72h drug treatment. Data shown are mean values ± S.D., n=3. Statistical analysis: A, 5AZA *versus* 5AZA + Depsi, $P<0.001$; Depsi *versus* 5AZA + Depsi, $P<0.005$. B, 5AZA *versus* 5AZA + Depsi, $P<0.05$; Depsi *versus* 5AZA + Depsi, $P=0.001$.

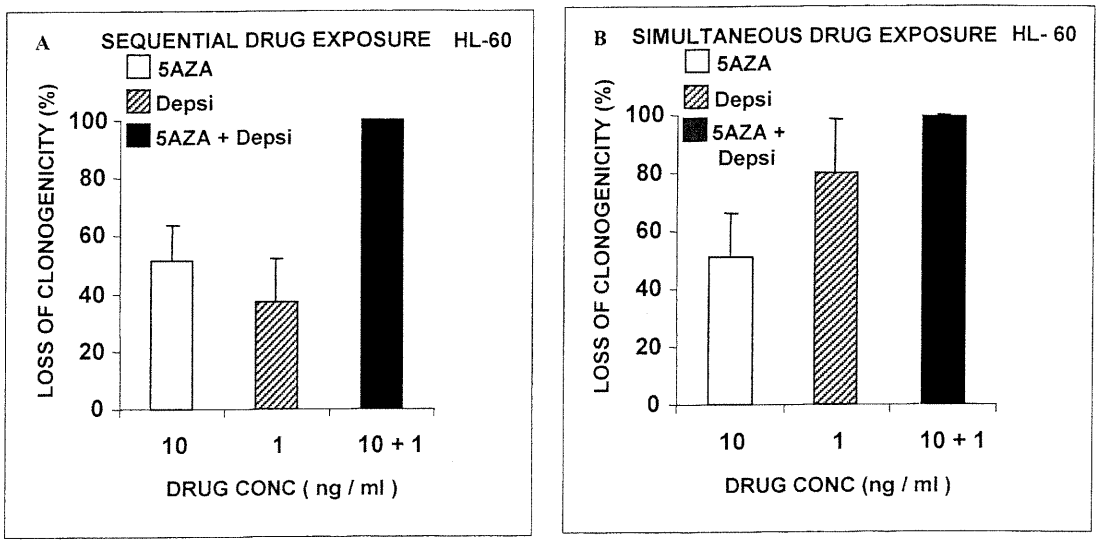

Fig.5. Effect of 5AZA and depsi alone and in combination on loss of clonogenicity in HL-60 myeloid leukemic cells.(A) 5AZA was added at time 0 and Depsi was added sequentially after 24hs.(B) Both drugs were added simultaneously at time 0.The clonogenicity assay was done after 72hs treatment.Data shown are mean values ± S.D., n=3. Statistical analysis:A, 5AZA *versus* 5AZA + Depsi, P=0.005 ; Depsi *versus* 5AZA + Depsi, P<0.005 ; B, 5AZA *versus* 5AZA + Depsi, P<0.05; Depsi *versus* 5AZA + Depsi, P=not significant.

PART 4:

DISCUSSION

CHAPTER 9:

DISCUSSION

Despite all the improvement, in the chemotherapy of leukemia, less than 50% of the patients with this disease are cured (Löwenberg et al., 1999; Flasshove et al., 2000). New drugs should be sought that are more effective to treat this disease.

Acute leukemias occur when immature hematopoietic cells are blocked from undergoing terminal differentiation and continue to proliferate in an unlimited manner (Olsson et al., 1996; Grimwade & Solomon, 1997). One of the early events that can occur during the development of leukemia is the inactivation of the tumor suppressor genes. The discovery that tumor suppressor genes are inactivated by aberrant methylation of CpG islands in their promoter regions indicates that epigenetic mechanisms can play an important role in neoplasis. Alterations in the global levels and regional changes in the pattern of DNA methylation are among the earliest and most frequent known events to occur in human cancer (Baylin et al., 1998).

p15 gene codes for a cyclin-dependent kinase inhibitor. The role of cyclin-dependent kinases is to phosphorylate specific proteins that program the cell to enter the S phase and undergo cell division (Hamel & Hanley-Hyde, 1997). Recent reports indicate that DNA methylation of the tumor suppressor gene p15 is a common mechanism of gene suppression in leukemia. Herman et al (1996) reported that p15 gene showed promoter hypermethylation in 86% patients with AML and 50% patients with ALL. These investigators also observed that 5AZA, an inhibitor of DNA methylation, could activate the expression of p15 in leukemic cell lines.

The demethylation induced by 5AZA can result in gene activation and induction of differentiation of neoplastic cells (Razin & Riggs, 1980; Jones & Taylor,

1980). In phase I clinical trials in both childhood and adult leukemia 5AZA was demonstrated to be an active antileukemic agent (Rivard et al., 1981; Momparler et al., 1986). Hematopoietic toxicity, especially granulocytopenia, is the major side effect produced by 5AZA (Momparler et al., 1986). Combination of 5AZA with other agents that also induce the differentiation could lead to more effective therapy without increasing drug toxicity.

Recently investigators have shown that there is a synergistic interaction between promoter hypermethylation and histone deacetylation in the repression of gene expression. Methyl-CpG binding proteins, such as MeCP2, attract histone deacetylases and make a complex with co-repressors that change the chromosome structure to a compact form which is not accessible to transcription factors (Nan et al, 1997; Jones et al, 1998; Cameron et al, 1999). According to recent studies, histone deacetylase inhibitors is an appropriate class of agents to be combined with 5AZA in order to activate tumor suppressor genes, such as p15 that are repressed in leukemia. HDIs also induce terminal cellular differentiation

In this regard, our laboratory observed an additive antineoplastic effect between 5AZA and TSA against human breast carcinoma cells (Bovenzi & Momparler 2001). The activation of the tumor suppressor gene, retinoic acid receptor ß, appeared to be involved in this interaction. In accord with these findings is the report on lung carcinoma cells that 5AZA enhances apoptosis induced by inhibitors of histone deacetylation (Zhu et al., 2001).

In this work we showed an enhancement of the *in vitro* antineoplastic activity of 5AZA on human myeloid leukemic cells by addition of the histone deacetylase inhibitors, TSA or depsi. 5AZA, TSA and depsi as single agents produced potent inhibition of growth of HL-60 and KG1a leukemic cells **(Figures 7.1 and 8.1)**. The growth inhibition produced by the combination of 5AZA with TSA **(figures 7.2 and 7.3)** or 5AZA with depsi **(figure 8.2)** was greater than that for either agent alone for both HL-60 and KG1a leukemic cells.

Similar results were obtained using a DNA synthesis assay. In this latter assay the radioactive thymidine was added after 48 h drug exposure to give a better assessment of the long- term effects of the inhibitors. As single agents 5AZA, TSA and depsi showed potent inhibition of DNA synthesis **(figures 7.4 and 8.3)**. For both leukemic cell lines, the combination of 5AZA with either TSA **(figures 7.5 and 7.6)** or depsi **(figure 8.4)** produced a greater inhibition of DNA synthesis than either agent alone. It should be pointed out that the concentrations of 5AZA used in these in vitro studies are in the range of the plasma levels of this analog used in clinical trials with responses in both leukemia and lung cancer patients (Rivard et al., 1981; Momparler et al., 1985; Momparler et al., 1997). The scale ng/ml for the concentration of the drugs was chosen based on the fact that in pharmacokinetic in patients the plasma concentration of chemotherapeutic agents is in this scale (ng/ml).

The clonogenic assay is more relevant for translation into clinical trial than growth and DNA synthesis assays since it provides data on the long-term survival of the leukemic cells in terms of their proliferative potential. We observed an interesting

interaction of the combination of 5AZA and TSA **(figure 7.7)** or depsi **(figure 8.5)** to reduce colony formation by the HL-60 leukemic cells (see photographs in the appendix).

We used two different schedules for the drug exposure to have an idea which would be more effective for future clinical trials. Simultaneous exposure of the cells to both inhibitors of methylation and histone deacetylation or sequential exposure to these agents. With both these schedules 5AZA in combination with inhibitors of HDAC produced a greater antineoplastic effect than either agent alone **(figures 7.2-3, 7.5-7 and 8.2, 8.4-5)**. However, The optimal dose-schedule will be determined by future clinical trials on 5AZA in combination with inhibitors of histone deacetylase in patients with leukemia.

For discussing the interaction of anticancer agents at the cellular level, we have to define the interactions in a quantitative manner. The terms additive and synergistic are used for the interaction of the agents in this work. If the cell survival fraction (SF) for the combination of the two agents were equal to the multipliclation of cell survival fraction of each agent alone, the effect would be termed additive. When the agents together results in surviving fraction of less than multiplication of cell survival fraction of each agent the effect woul be termed synergistic (Valeriote & Lin, 1975)

$SF_{(A+B)} = SF_{(A)} \times SF_{(B)}$ Additive

$SF_{(A+B)} < SF_{(A)} \times SF_{(B)}$ Synergistic

A PCR assay (MSP) was developed to evaluate the methylation status of genes by using specific primers that can amplify DNA sequences containing 5-methylcytosine (Herman et al., 1996). Using MSP assay we showed that promoter of p15 gene in Raji Burkitt's lymphoma cells is completely methylated **(figure 6.1)**. This method

can be used as a rapid and precise assay to evaluate the methylation status of different tumor suppressor genes and to monitor therapy in patients with leukemia.

In the RT-PCR assay loss of expression of the p15 gene in Raji leukemic cells was shown. We detected the reactivation of this gene by treating these cells with 5AZA **(figure 6.3)**. This event was probably due to the demethylation of the promoter region in p15 gene by this analog. Also, we detected the reactivation of the p15 gene with the low concentration of 5 ng/ml of TSA in the same cell line **(figure 6.2)**. This latter event is probably due to the effect of TSA on the chromatin structure making it more favorable for transcription.

In conclusion, the observations of a significant interaction of 5AZA with TSA or depsi on the growth inhibition, inhibition of DNA synthesis and on loss of colony formation suggests that: the combination of inhibitors of DNA methylation and HDAC may show potential in the therapy of acute leukemia. The rational of this therapy is to activate the key genes that induce irreversible terminal differentiation so that the neoplastic cells lose completely their proliferative potential.

It appears that methylated cytosines, methylation binding proteins, histone deacetylase and co repressors act as different levels for silencing of the cancer related genes. It remains to be elucidated which gene has the dominant role in the repression of the transcription for different genes. However each gene can be a target for reactivation by inhibitors of DNA methylation and histone deacetylation. Also, it is possible that a threshold number of genes must be reactivated for the hematopoietic cells to undergo terminal differentiation. Using different agents with different mechanisms of action for the

activation of gene expression can increase the probability to induce terminal differentiation in all the leukemic cells.

Additional studies have to be done in order to completely elucidate the molecular mechanism of this drug interaction including:

- Quantitative RT-PCR on human lymphoid and myeloid leukemic cell lines such as Raji and KG1a (Herman et al., 1996), in which p15 gene is silenced by DNA methylation can help us to determine if an additive or synergistic interaction occurs for the activation of this gene by combination of inhibitors of DNA methylation and HDAC. In this latter assay HL-60 can be used as a positive control (Herman et al., 1996).

- Quantitating the demethylation of 5-methylcytosine residues in the promoter region of the p15 gene in human leukemic cells by different concentrations of 5AZA using the methylation-sensitive nucleotide extension reaction (Bovenzi & Momparler, 2000) and to determining if histone deacetylase inhibitors affect this process.

- MSP assays on leukemic cells treated with histone deacetylase inhibitors also can show us if these agents have any role in the demethylation of p15 gene.

- Many tumor suppressor genes have been reported to be inactivated by methylation in different kinds of leukemia **(table 1.1)**. Investigation of the effect of these two classes of agents on the other genes can provide useful information for the later clinical trials in patients.

-*In vivo* antineoplastic activity of 5AZA in combination with inhibitors of histone deacetylation can be determined in mouse model. Positive in vivo data will support the evaluation of this form of differentiation therapy in future clinical trials in patients with acute leukemia.

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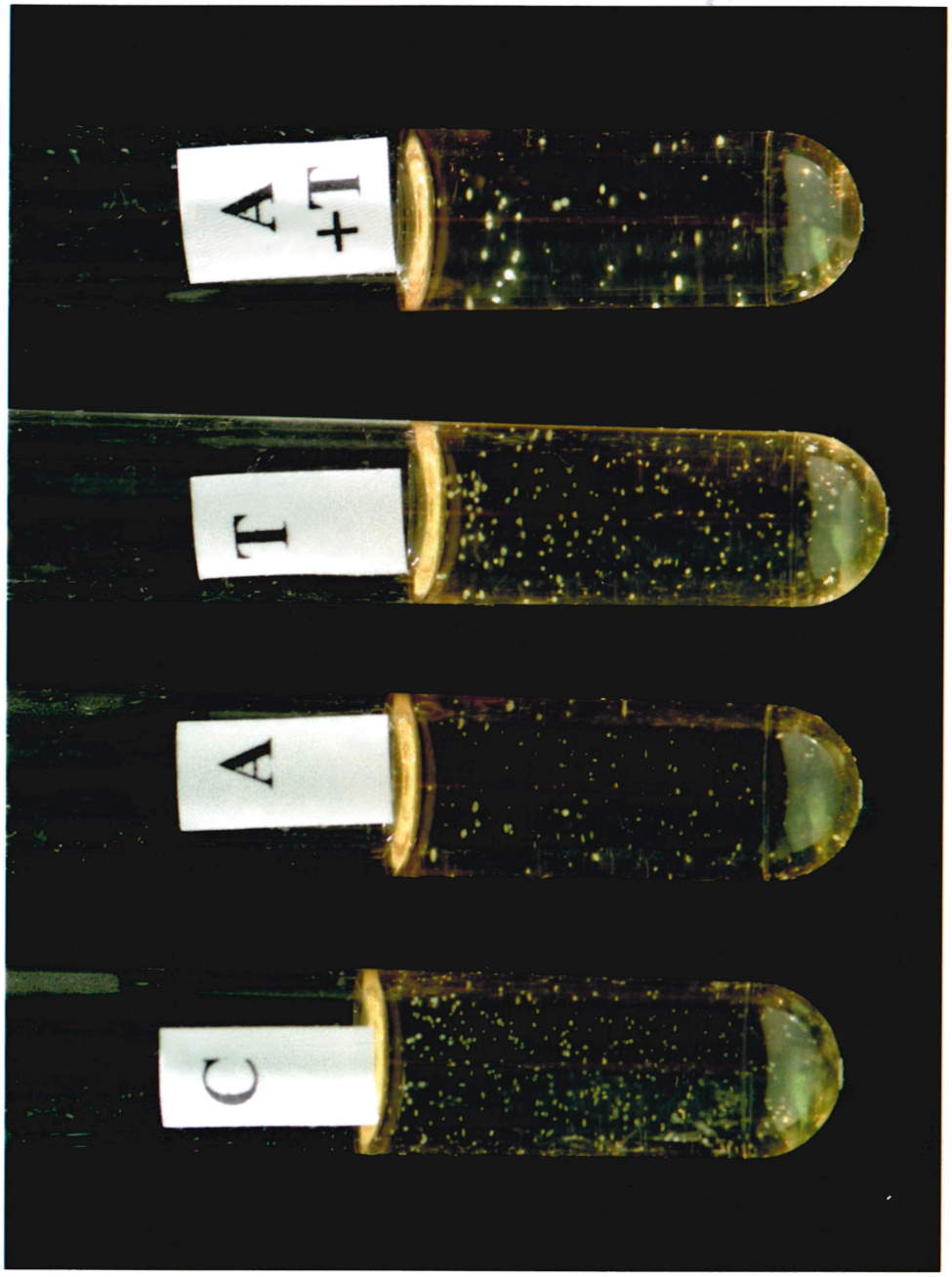
Appendix I

The effect of 5AZA, TSA alone and in combination on HL-60 myeloid leukemic cells. Cells were treated with 5AZA or TSA for 72h. For the combination cells were treated with both drugs at time 0. After drug treatment, the cells were suspended in drug free medium. An aliquot of 200 cells was placed in 2 ml of 1.8% soft agar medium containing 20% serum. After incubation for 20 days at 37°C in 5% CO2 incubator, the number of colonies (>500 cells) was counted. The cloning efficiency of the control cells was in the range 45-65%.5AZA and TSA alone for 72 h exposure produced a loss of clonogenicity of 64.8% and 15.3%,respectively. The combination produced 91% loss of clonogenicity, a synergistic interaction.


A +T
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A
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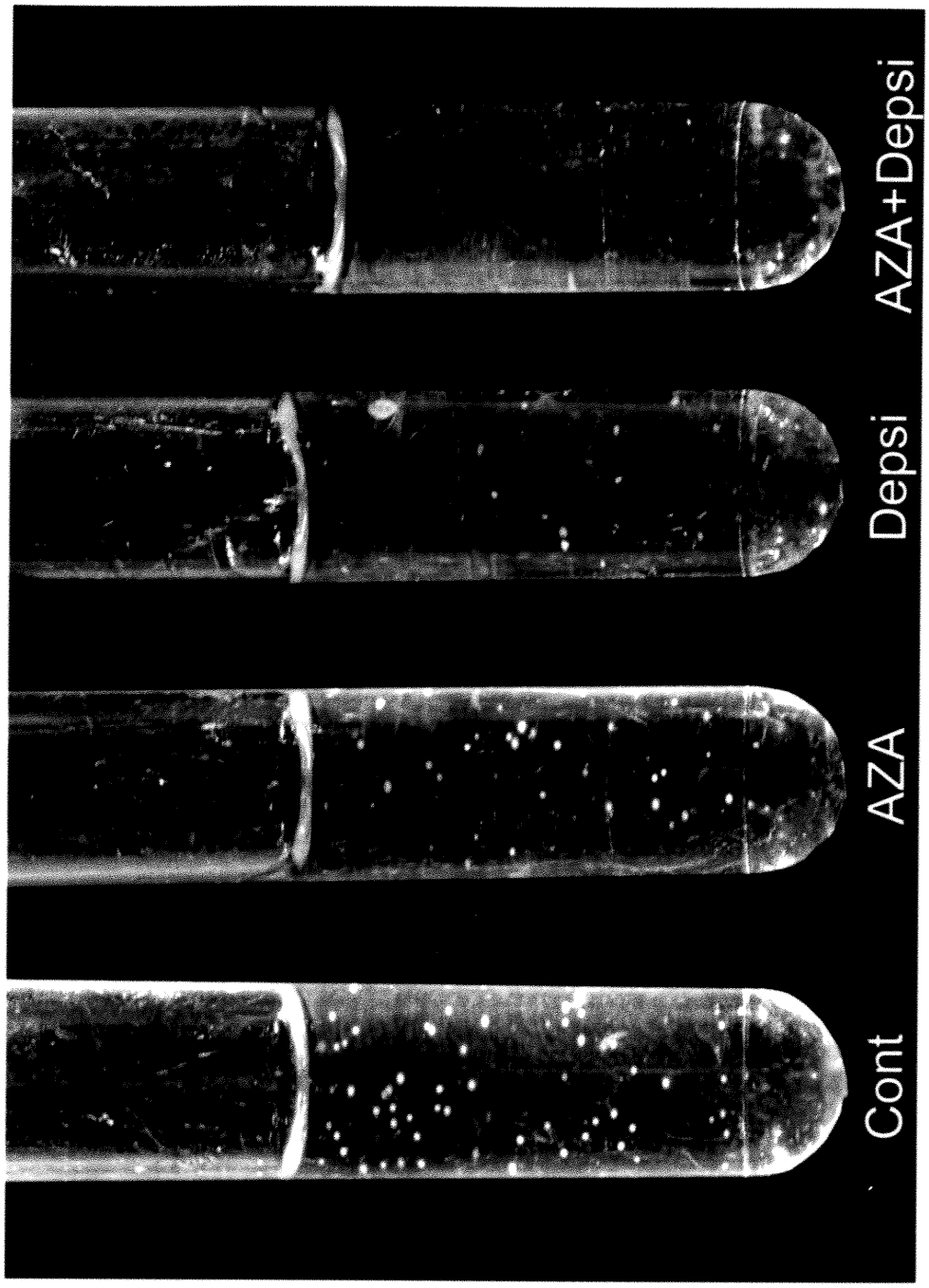
Appendix II

The effect of 5AZA, Depsi alone and in combination on HL-60 myeloid leukemic cells. Cells were treated with 5AZA for 72h and with Depsi for 48h. For the combination cells were treated with 5AZA at time 0 and Depsi was added at 24h After drug treatment, the cells suspended in drug free medium. An aliquot of 200 cells was placed in 2 ml of 1.8% soft agar medium containing 20% serum. After incubation for 15 days at 37°C in 5% CO2 incubator, the number of colonies (>500 cells) was counted. The cloning efficiency of the control cells was in the range 45-65%.5AZA alone for 72 h exposure produced a loss of clonogenicity of 51.3%. A 37% loss of clonogenicity was detected for a 48 h exposure of depsi alone. The combination produced 99.67% loss of clonogenicity, a synergistic interaction.


Cont
AZA
Depsi
AZA+Depsi