Methylation of Gene Promoters in Leukemogenesis

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Methylcytosine, "the 5th base"

Who you are depends not only on your heritage but also on what you have done with it. This holds true for cells in the human body as well: despite sharing essentially identical genomes, they are phenotypically different due to distinct expression profiles. The transcription patterns determine the unique biochemical content of a particular cell lineage and, therefore, its differentiation type and adaptation to a particular function. These stable expression profiles are clonally perpetuated in the so-called epigenetic mode of inheritance, which does not involve alterations of the nucleotide sequences. DNA methylation is a major molecular mechanism underlying the epigenetic phenomena.

In mammalian cells, most of the cytosine residues followed by guanosine are modified by the addition of a methyl group. Normally under-represented in the genome, the CpG dinucleotides ("p" stands for a phosphate group) are clustered in stretches of 300–3,000 base pairs termed "CpG islands" [Figure 1]. These are usually associated with the 5' regions of expressed genes (about 50% of all promoters) and typically remain methylation-free. Hypermethylation of dense CpG islands is tightly correlated with transcriptional inactivation of the corresponding promoters. At the molecular level, this substitution of cytosine with methylcytosine affects DNA-protein interactions both directly and by alteration of chromatin packaging. These effects are mediated to a large extent by specific methyl-CpG binding proteins capable of recruiting histone deacetylases. The latter, in turn, convert open and transcriptionally active euchromatic DNA into condensed, inert heterochromatin. Once such a gene silencing takes place, it is essentially irreversible in a normal differentiated cell and its progeny. Of interest, this is currently a major obstacle in cloning mammals from somatic nuclei.

The proposed roles for DNA methylation in mammals include regulation of gene expression, maintenance of genome stability and chromatin structure, mediation of gene imprinting, and inactivation of X chromosome, transposons and foreign DNA [1]. Accordingly, adequate functioning of the methylation machinery is deemed

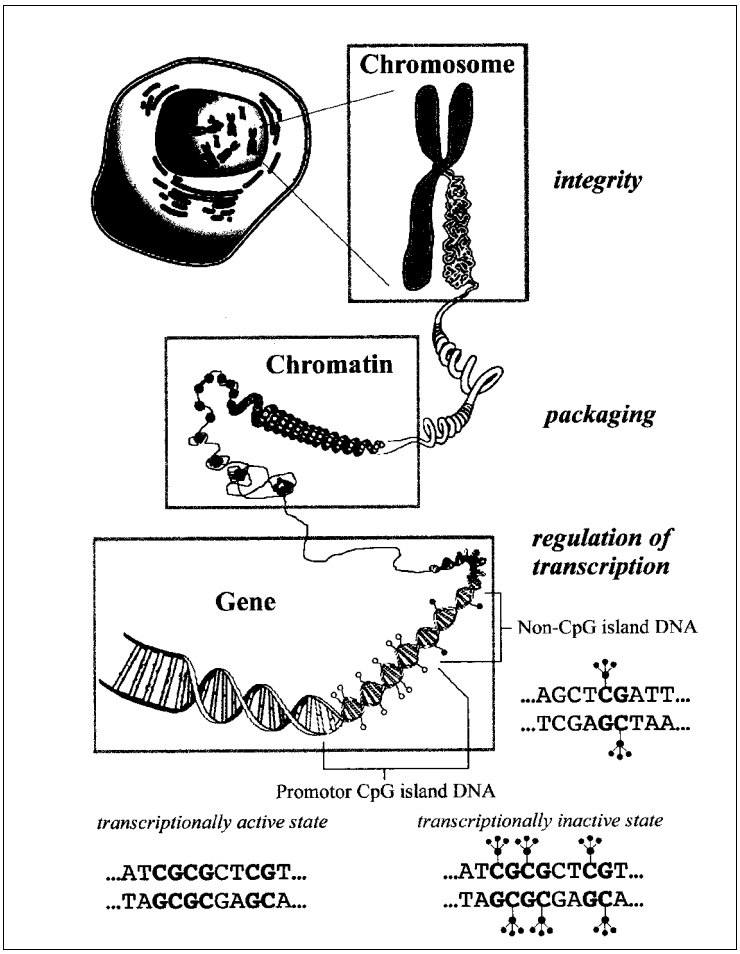

Figure 1. The principal roles played by DNA methylation at different levels of organization of the genetic material.
Normal chromosomal structure is based on appropriate arrangement of highly condensed heterochromatin domains and relatively loose areas of euchromatin. The pattern of DNA packaging is determined by acetylation of the core proteins, histones. In turn, this modification of the chromatin scaffold is regulated by recognition of methylated CpG dinucleotides. Besides its impact on gene accessibility, methylation of cytosines also directly affects the interaction of the DNA-binding proteins with the major groove of DNA. In particular, methylation of promoter CpG islands modulates expression of the corresponding genes.
(Modified from www.unites.uqam.ca/biologie_moleculaire/images/chromosome.gif) .ca/biologie_moleculaire/images/chromosome.gif)

indispensable for normal cell physiology [2]. The apparent tight regulation of DNA methylation is believed to rely on the well-coordinated activity of several enzymes. These include the recently identified DNMT3A and 3B, acting as *de novo* DNA methyltransferases, as well as the ubiquitously expressed DNMT1, maintaining previously established methylation patterns due to its strong preference for hemi-methylated DNA [3,4]. Genomic demethylation also occurs during development, although the existence of a corresponding enzyme is controversial at this point. The methyltransferases, histone deacetylases, methyl-CpG binding proteins and transcription repressors cooperate in a multi-unit complex regulating proper establishment and maintenance of the transcriptionally repressed chromatin [5].

Cancer, the Wild West of epigenetics

This orchestrated mechanism goes awry in cancer. Neoplastic cells are characterized by overall genomic demethylation with concomitant elevated levels of DNA methyltransferases and regional CpG island hypermethylation [2,6]. Each of these phenomena may contribute to carcinogenesis. Genome-wide demethylation was shown to significantly increase the frequency of chromosomal aberrations [7]. The proposed mechanisms involve altered chromatin packaging, possibly facilitated by additional growth-stimulating factors or inappropriate cell division. Hypomethylation can also be linked with overexpression of an oncogene [8], or lead to loss of imprinting in uniparentally expressed loci as the insulin-like growth factor II (*IGF2*) gene in Wilms' tumor and in sporadic colorectal cancers [9]. Demethylation is also deemed responsible for disinhibition of transposons and viral sequences in cancer cells, which may lead both to insertional mutagenesis and to promoter activation of these elements with deleterious effect on expression of nearby genes [10]. Elevated and inappropriately targeted DNMTs may result in higher mutation rates in critical loci in the genome as a consequence of spontaneous deamination of methylcytosine. The corresponding CG to TG or CA point mutations are among the most common in human disorders, with CpG mutation hotspots in the *p53* gene being the best documented example [11]. An even more important consequence of the DNMTs deregulation, the transcriptional silencing of tumor suppressor genes by promoter hypermethylation, has been observed in many malignancies including breast, colon and prostate cancers, leukemias, retinoblastoma and other familial cancers. This epigenetic inactivation is a functional alternative to sequence mutations and can deliver equally severe "hits" in Knudson's model of carcinogenesis. The list of CpG islands specifically affected by aberrant methylation in cancer is constantly growing. It includes promoter regions of genes responsible for DNA repair, angiogenesis, metastasis, drug resistance/detoxification, apoptosis, cell cycle and differentiation regulation, signal transduction, and transcription factors. Some of these aberrations precede tumor formation and are found in adjacent normal tissues, probably as a consequence of carcinogen exposure and age-related hypermethylation documented for several loci. These epigenetic events could predispose to neoplastic growth initiation by conferring a selective advantage to such cells or by destabilizing their genomes due to deficient DNA repair [12]. Promoter inactivation of tumor suppressor genes also occurs later in multistep carcinogenesis, thus contributing to malignant transformation and drug resistance.

The emerging concept of cancer epigenetics as a key aspect of tumor biology has attracted considerable research effort worldwide. Recently proposed to be termed "methylomics" [6], the field was brought to a new technical level by the advent of bisulfite modification-based techniques of methylation analysis. For the first time a detailed and quantitative study of each CpG in an entire promoter area became feasible, with a further possibility to focus on cloned individual alleles. On a more global level, methods of scanning the genome for abnormally methylated sequences have been applied successfully and are constantly improving [13,14]. Important insights on the interrelation of the genetic and epigenetic factors in cancer were gained following identification of the *hMLH1* methylation-related defects of DNA mismatch repair and the discovery of *DNMT3B* mutations underlying the extreme chromosomal abnormalities in lymphocytes of patients with ICF syndrome (**i**mmunodeficiency, **c**entromeric instability, and **f**acial anomalies) [3,15]. Mouse models and comparison of familial and sporadic cancers also proved to be most valuable [4,12].

Apart from the accumulation of a long list of novel data, several important observations were made. The alteration of methylation patterns was found to be more important for cancer development than the absolute level of DNA methylation. In several studies the relevance of regional methylation changes was elegantly demonstrated by the inverse relationship between the genetic and epigenetic mutations in genes belonging to a common pathway. While some genes have been identified as common targets of epigenetic inactivation in many neoplasms, others were specifically affected in small subsets of tumors. The frequency of methylation alterations and the sets of silenced genes were also different in distinct cancer types. Moreover, a number of gene promoters were found to undergo methylation in a somewhat concordant rather than independent manner. For many of these genes a link between methylation and gene silencing is well established, and their role in tumorigenesis was demonstrated in mouse models, *in vitro* or in clinical samples. Therefore, the presence of specific patterns of hypermethylation in only a fraction of patients with a particular diagnosis indicates the existence of cancer subsets with different expression profiles. Thus, molecular epigenetic studies within a clinical entity may help to identify tumor subtypes with distinct molecular etiology, response to treatment, and prognosis [16–18]. With some methylation changes occurring early and specifically in oncogenesis, epigenetic markers have been proposed for monitoring malignant transformation, minimal residual disease and approaching relapse.

"Difficult problems always have simple, easy to understand incorrect solutions..."

We should beware of oversimplified conclusions in the complex field of cancer epigenetics. Although the current concepts rely on the firm body of evidence accumulated in the last two decades, the reader is advised to keep in mind several considerations for proper interpretation of individual studies. Apart from the general issues of

biased sample selection and inadequate statistical analysis, there is also some complexity inherent to the field. To begin with, hypermethylation per se does not necessarily spell gene silencing. Transcriptional inactivation usually follows only CpG island methylation that occurs 5' to the site of transcription initiation and affects positive regulators of expression (enhancers/promoters). Methylation of CpGs located downstream may have no effect or even facilitate transcription [19]. Similarly, hypermethylation of repressor sequences can cause enhanced expression of the corresponding gene. Moreover, some assays can only analyze methylation status of a single or a few cytosines in the region studied. Unless the epigenetic concordance of these sites with the rest of the CpG island is ascertained, their representative value is limited. It should also be noted that expression from many promoters was found to depend on the density of methylation and not on its topology [20]. Therefore, techniques in the analysis of entire CpG islands are superior in that respect. An ultimate mechanistic study is the one that establishes a correlation between the methylation levels and transcription activity or, most preferably, the protein product, since a fraction of genes are post-transcriptionally and/or post-translationally regulated. Use of methylation-specific primers for polymerase chain reaction amplification was shown to result in gross overestimation of the methylated template prevalence in the initial sample [21]. The cell type-dependent normal epigenetic background and the issue of origin of the detected methylated alleles should also be addressed since many samples contain mixed cell types or contaminating normal tissue. Ideally, to ascertain the prognostic significance of an epigenetic marker, treatment outcomes and survival should be correlated with response of the methylation to therapeutic modulation, as determined by multiple assessments [22]. Finally, some epigenetic changes are late and secondary in carcinogenesis. The dysfunction of methylation machinery in malignancy frequently involves alterations in apparently irrelevant loci alongside epigenetic mutations of selective value in a subset of genes [18,23].

Methylation is in our blood

A normal hematopoietic progenitor follows a tightly regulated succession of changes on its way to a mature form. The dynamic alterations of the immunophenotype reflect complex transcription pattern changes related to commitment and differentiation. A molecular aberration that modifies this expression profile may divert the developmental process to leukemogenesis. Indeed, hematologic malignancies frequently involve impairment of a wide range of factors controlling transcription, histone acetylation and chromatin structure. All the best studied genes in this group, such as the *RAR*, *AML1* and *MLL*, are affected as a result of translocations. A hallmark of leukemias, such structural and numeric chromosomal aberrations could, in turn, indicate defective chromatin structure, probably associated with abnormal regional methylation patterns. This notion and the ready availability of tumor samples have brought hematologic malignancies to the focal point of cancer epigenetics research.

Given the large number of sites for which aberrant methylation status has been reported, we selected three representative loci containing four genes that vary considerably in the specificity and importance currently attributed to their promoter hypermethylation in leukemia.

One of the first promoter CpG islands demonstrated to be hypermethylated in cancer, the calcitonin (*CALC1*) gene is located on the short arm of chromosome 11, which contains several growth regulation-related genes [24]. Therefore, despite the apparent lack of its causal role in carcinogenesis, hypermethylation of this promoter is an important indicator of the epigenetic status of the adjacent potential tumor suppressor genes in solid and hematologic neoplasms. An early event in lymphoid leukemogenesis, *CALC1* 5' methylation is associated with T cell lineage and with enhanced risk of relapse in both pediatric and adult patients. The latter finding could be attributed to the 100% correlation of calcitonin methylation with down-regulation of the adjacent cyclin-dependent kinase inhibitor (CDKI) *p57KIP2* gene that controls the G1/S transition [25]. The calcitonin gene is also increasingly methylated during progression of the chronic myeloid leukemia to blastic crisis [26] and might indicate likely leukemic transformation in myelodysplastic syndrome. A very common finding already at diagnosis of acute myeloid leukemia and acute lymphoblastic leukemia, methylation of *CALC1* does not correlate with any clinical or hematologic parameter [23]. To summarize, in hematologic disorders this modification appears to be a marker of malignant clones, and therefore its elevated levels may reflect increased tumor mass and hence be associated with a less favorable prognosis. Calcitonin and *p15* belong to the subset of genes commonly methylated in AML, with frequent evidence of apparent bi-allelic methylation or loss of heterozygosity [18].

The *p15*(INK4B) and *p16*(INK4A) tumor suppressor genes are also often abnormally hypermethylated in hematopoietic neoplasms, but their involvement in oncogenesis is more direct and significant. Despite the common location at 9p21, these negative regulators of cell cycle display remarkably independent modes of inactivation that vary significantly between distinct cancer types. While intragenic mutations of *p15* and *p16* are rare in acute leukemias, homozygous deletions inactivate one or both of these genes in a significant proportion of adult and childhood ALL, especially of T cell lineage. Specific or concomitant promoter methylation in these loci was demonstrated in myeloid, lymphoid and biphenotypic forms of acute leukemia. Overall, 70–80% of AML cases and the majority of ALL patients are characterized by various degrees of *p15* methylation positivity, with much lower levels of *p16* methylation of uncertain significance [18]. However, the adult T cell leukemias were reported to contain methylated p16 promoters in 75% of cases, with no evidence of *p15* involvement [27]. The methylation of *p15* and *p16* is associated with loss of transcription, and for *p15* promoter in acute leukemia the inactivation was shown to occur when more than 40% of CpG sites in the island are methylated [20]. A potential mechanism of the epigenetic inactivation is suggested in the recent report of higher levels of DNMT1 and

AML = acute myeloid leukemia
ALL = acute lymphoblastic leukemia

3B in AML patients with methylated *p15* gene [28]. While the prognostic value of *p15/p16* methylation in the different forms of acute leukemia is uncertain, the common *p15* methylation was proposed as a frequently available molecular marker for clinical monitoring.

In contrast to the wide range of solid tumors and hematologic malignancies demonstrating promoter methylation of the aforementioned loci, epigenetic inactivation of the *ABL (ABL1, c-ABL)* gene appears to be exclusively associated with leukemias positive for the reciprocal translocation t(9;22)(q34;q11). This cytogenetic marker, the Philadelphia chromosome (Ph'), is found in more than 95% of CML cases and in 25% of adult ALL patients. At the molecular level, the translocation produces the fusion *BCR-ABL* oncogene. A grave prognostic factor in ALL, this fusion tyrosine kinase is solely responsible for initiation of the chronic phase of CML and also plays a major role in its subsequent progression via the accelerated phase to the fatal blastic crisis. The ABL protein is ubiquitously expressed in normal human cells and plays a role in stress response and cell differentiation, division and adhesion [29]. The gene has two active alternative promoters, 1a and 1b. The entire proximal (1a) transcriptional unit of *ABL* is incorporated into the Ph' chromosome in 90% of all CML cases. Nevertheless, our group found complete epigenetic inactivation of the translocated *ABL* promoter in cell lines of blast crisis CML [30]. We subsequently reported a correlation between the disease stage and methylation of the 1a region in 99 CML patients and samples from 12 control subjects [31]. Absent to low levels of methylation were observed in the chronic phase (74% and 26% of samples, respectively), with intermediate levels found in the accelerated phase samples and extensive methylation in as much as 93% of blast crisis samples. In contrast, no *ABL* methylation was detected in Ph-negative controls. Within the chronic-phase patient subset, the 1a methylation was significantly associated with disease of longer duration. A gradual increase in *ABL* promoter methylation with time elapsed since diagnosis was demonstrated in sequential samples from the same individuals. Finally, treatment with interferon gamma, but not the less effective hydroxyurea therapy, prevented or reversed hypermethylation of this locus in CML patients. Thus, we found promoter methylation of *ABL* to increase with time following diagnosis and during disease progression to blastic crisis CML [31].

Our subsequent findings in clinical samples and single hematopoietic progenitor-derived colonies demonstrated extensive hypermethylation along the promoter region (with more than 90% of all CpGs affected) lacking any distinct regional pattern [32]. The experiments also showed apparently allele-specific methylation of the translocated *ABL* promoters in the vast majority of colonies from blast crisis, but not chronic-phase CML. We also demonstrated that *ABL* methylation in CML does not reflect a generalized process and may be unique among DNA repair/genotoxic stress response genes. While low to absent levels of *ABL* promoter methylation are present in chronic-phase samples, by the time of blastic transformation extensively methylated alleles are found in all samples and are twice as prevalent as in the initial stage. Hence, specific hypermethylation of the Ph'-associated *ABL* allele is a necessary (but probably not sufficient) event for CML clonal evolution [32]. Despite its association with disease progression, the prognostic significance of this epigenetic aberration in CML awaits thorough evaluation [22].

The next step of the *ABL* promoter methylation study was suggested by the occurrence of Ph' chromosome in about 30% of acute lymphocytic leukemia cases in adults. Approximately half of these patients present with the P210 form of BCR-ABL similar to the one typical of CML; the remaining 50% express the P190 type of the oncoprotein, which is more potent as demonstrated by animal models and *in vitro* experiments. Determination of the *ABL* 1a promoter methylation status in ALL revealed that epigenetic alteration of this locus is associated mainly with the P210 type of BCR-ABL and not the P190 form [33]. In addition, the extent and relative abundance of *ABL* promoter methylation were estimated in several Ph'-positive ALL samples and compared to the methylation patterns in chronic, accelerated and blastic crisis phases of CML. While the topology and gene specificity of the *ABL* methylation were similar in ALL samples and in all phases of CML, its allelic prevalence in the acute P210-positive leukemias was twice as high as in chronic-phase CML. The results lead us to a hypothesis that correlates the different types of leukemias with the levels of *ABL* promoter methylation. In this model the P210 form of BCR-ABL may need this epigenetic modification to cause a fully malignant phenotype [33]. The mechanisms of translocated *ABL* hypermethylation may be related to an abnormal chromatin structure in the vicinity of the fusion point, as suggested by aberrant methylation patterns in several regions of the *BCR* counterpart. *BCR-ABL* oncogene and *ABL* tumor suppressor gene appear to exert opposing and antagonistic actions on the cell-cycle regulation, apoptosis, and genomic instability [29,34]. Epigenetic silencing of the 1a *ABL* promoter may alter the BCR-ABL/ABL expression ratio, which is a useful parameter for risk assessment in CML. Accordingly, the prevalence of the methylated *ABL* clone may reflect the stage of a Ph-positive disorder and facilitate monitoring the response to therapy and minimal residual disease.

Reversing the methylation process

In normal cells, CpG island methylation is essentially irreversible, and the aforementioned demethylating effect of interferon gamma on the 1a *ABL* promoter in CML might be attributed to mere shrinkage of the methylated clone. However, a growing number of substances display hypomethylating activity as their principal mode of action. Initially employed for epigenetic research, these agents are currently studied for their possible application to anticancer therapy. The rationale for this approach resides in the fact that the inactivated gene sequence remains intact following promoter methylation. Once re-expressed, it yields a fully functional wild-type protein. Hence, it is plausible to revert an epigenetic inactivation of a tumor suppressor gene in leukemic blasts. Moreover, since most CpG islands are normally unmethylated, induction of moderate hypomethylation may cause only minimal side effects related to abnormal disinhibition of numerous loci.

CML = chronic myelocytic leukemia

Nevertheless, the optimal degree of methyltransferase inactivation remains to be established, as indicated by p53-dependent apoptosis and epigenetic deregulation of up to 10% of loci in primary mouse embryonic fibroblasts with conditionally inactivated DNMT1 [4]. The most potent hypomethylating agents, 5-azacytidine and 5-aza-2′-deoxyazacytidine (decitabine), are cytidine analogues exerting their effects by blocking DNA and protein synthesis as well as by irreversible inhibition of DNA methyltransferases. Depending on the dosage and protocol used, the drugs can elicit a cytotoxic response, induce differentiation, or inhibit growth of tumor cells. The results of clinical trials in patients with leukemias and myelodysplastic syndromes are encouraging, with strong indication for combination therapy that includes one of the hypomethylating agents [35]. Other potential synergistic treatments include chromatin remodeling with histone deacetylase inhibitors and promoter transcription activation with specific agents (e.g., ATRA in acute promyelocytic leukemia) [36,37]. Screening for epigenetically inactivated tumor suppressor genes would assess an individual patient's eligibility for such a therapy.

Conclusions

DNA methylation is intricately involved in a wide diversity of cellular processes. Abnormal methylation patterns play a variety of roles in different stages of oncogenesis in many cancer types. The most important of these mechanisms is inactivation of tumor suppressor genes by means of promoter hypermethylation. Apart from its importance for cancer biology research, this epigenetic pathway of oncogenesis can provide valuable markers for malignant progression, residual disease and response to treatment. The reversibility of inactivation by methylation suggests that in future it will be feasible to reactivate tumor suppressor genes by therapeutic hypomethylating agents.

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Research Project

Cytotoxic activity of phenotiazines and related neuroleptics: Evidence for a potent apoptotic effect on brain-derived tumors

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Background: Neuroleptics are drugs used for the treatment of schizophrenia and other affective and behavioral disorders. Epidemiologic studies show a low occurrence of cancer among schizophrenic patients, and recent studies revealed an antiproliferative activity for some neuroleptics. Ben Shahar et al. found that haloperidol and phenotiazines induced a marked increase in iron transport across the blood-brain barrier in rats and mice, resulting in increased oxidative stress in the basal ganglia; and we and other investigators showed that the neuroleptics haloperidol, perphenazine, and other phenothiazines induced a marked dose-dependent neurotoxic effect in primary brain culture, in neuroblastoma and in glioma cell-lines.

Objectives: To evaluate the cytotoxic effect of different neuroleptics in neuroblastoma and glioma cell-lines, to determine the mechanism of action of active agents, and to select agents with potential antitumor effect in brain tissue.

Methods: We assessed the cytotoxic effect (neutral red and alamar blue methods) of neuroleptics belonging to different pharmacologic classes: perphenazine, haloperidol, clozapine, sulpiride, and risperidone (10-100 μM), on human neuroblastomas (SK-N-SH) and (SHSY-5Y), and rat glioblastoma (C6) cell lines. Mechanism of cell death was determined using flow cytometry of neuroblastoma cells (SK-N-SH) stained with propidium iodide, and software was used for data acquisition and cell cycle analysis (Becton and Dickenson).

Results: Perphenazine induced a potent cytotoxic activity in all the lines (IC50 10–15 μM), clozapine and haloperidol had a moderate effect (IC50 60–80 μM), and sulpiride and risperidone were not toxic. Co-administration of perphenazine and dopamine (0.125 mM) in the neuroblastoma culture potentiated the perphenazine-induced toxicity (IC50 5 μM) or did not alter the effect in the glioma cell line. We further screened the effect of other drugs belonging to the phenotiazines class: fluphenazine, chlorpromazine and thioridazine, and found that all had a marked cytotoxic and antiproliferative activity assessed by the thymidine incorporation method (IC50 range 10–20 μM). Flow cytometry of neuroblastoma cells treated with perphenazine 20–40 μM showed a marked increase in fragmented DNA: 80.0–90% (typical apoptotic) as compared to 5.3% in control cells. Analysis of the cell cycle of neuroblastoma cells treated with lower concentrations of perphenazine (2.5–20 μM) (relevant to serum levels) revealed a dose-dependent increase in the G1, and a decrease in the S phase, suggesting a cell cycle-arresting activity and inhibition of DNA synthesis.

Conclusions: Different neuroleptics possess cytotoxic activity in brain-derived tumors (neuroblastomas and gliomas). The toxic effect of the drugs is independent of their anti-DA effect which mediates the antipsychotic activity. The effect of phenothiazines is associated with DNA fragmentation and arrest of cell cycle. Finally, we suggest that some cytotoxic neuroleptics could play a role in the therapy of brain-derived tumors.

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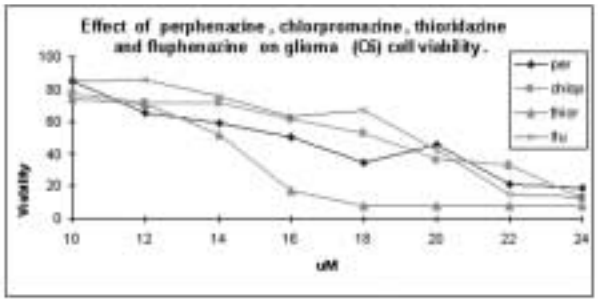

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