

# DNA Binding and Antiproliferative Effects of Some Benzimidazole Retinoids

[Bazı Benzimidazol Retinoidlerin DNA'ya Bağlanması ve Antiproliferatif Etkileri]

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## ÖZET

**Amaç:** Bu çalışma üç yeni benzimidazol retinoidin HL-60 hücrelerinde çoğalmayı baskılayıcı etkisini ve DNA'ya bağlanmasını incelemeyi amaçlamaktadır.

**Metotlar:** Benzimidazol retinoidler daha önce bildirildiği biçimde sentezlendi. Hücreler % 10 fetal sığır serumu ve antibiyotik içeren besi ortamında üretildi. All-trans retinoic asit, 9-sis retinoik asit ve retinoidler farklı dozlarda hücrelere eklendi. Hücre çoğalması 4, 6-diamidino 2 phenyl indole ile boyanarak DNA ölçümü ile incelendi. Plazmid bakteride çoğaltılıp elde edildi. Retinoidler, retinoik asit, etidyum bromür ve 4, 6-diamidino 2 fenil indol, plazmidlerle 5µg DNA'ya 10<sup>-5</sup> M olacak şekilde etkileştirildi. Daha sonra agaroz jel elektroforezine uygulandı ve etidyum bromür ile görüntüldü. Kendi içinde komplementer olmayan adenin ve timinden oluşan oktametik oligonükleotitler retinoidlere uygulandıktan sonra poliakrilamid jel elektroforezinde koşuruldu ve gümüş boyama ile izlendi.

**Bulgular:** Benzimidazol retinoidler hücre çoğalmasını doza bağlı bir şekilde baskıladı. Baskılayıcı etki all-trans retinoik asit ve 9-sis retinoik asit ile oluşandan önemli bir fark göstermedi. Retinoidler pDNA'ya 4, 6-diamidino 2 fenil indol ile benzer biçimde bağlandı.

**Sonuç:** HL-60 hücreleri üzerinde önemli oranda çoğalmayı baskılayıcı etki göseren bu yeni retinoidler etkin antikanser bileşikler olabilirler ve DNA'ya bağlanma özellikleri bu hücrelerde çoğalmaya ilişkin süreçleri etkileyebileceklerine işaret ediyor olabilir. Ancak, özgün etkilerinin açığa çıkartılması gereklidir.

**Key Words:** Benzimidazol, retinoidler, HL-60 hücreleri, antiproliferatif etkiler, DNA'ya bağlanma

## ABSTRACT

**Objectives:** This study aims at analyzing the antiproliferative effects of three novel benzimidazole retinoids on HL-60 cells and their DNA binding.

**Methods:** Benzimidazole retinoids were synthesized as reported previously. Cells were grown in medium supplemented with 10 % fetal calf serum and antibiotics. All-trans retinoic acid, 9-cis retinoic acid and retinoids at different doses were added to cells. Cell proliferation is determined by measuring the amount of DNA using 4, 6-diamidino 2 phenyl indole staining. Plasmid was grown in bacteria and isolated. Retinoids, ATRA, ethidium bromide and 4, 6-diamidino 2-phenyl indole were added to the plasmids at a ratio of 10<sup>-5</sup> M to 5µg DNA. These were then electrophoresed on agarose gel and stained with ethidium bromide. Octameric oligonucleotides without self complementarity consisting of adenine and thymine were incubated with retinoids and run in polyacrylamide gel electrophoresis then visualized by silver staining

**Results:** Benzimidazole retinoids have suppressed cell growth in a dose dependent manner. Cell growth inhibition was not significantly different to those induced by all-trans retinoic acid and 9-cis retinoic acid. Retinoids were bound to plasmid DNA in a similar mode with 4, 6-diamidino 2 phenyl indole.

**Conclusion:** These novel retinoids showing significant amount of antiproliferative effects on HL-60 may be potential anticancer agents and their DNA binding potential may point out their further efficiency in interfering the growth related processes in these cells. Their specific actions however remain to be elucidated.

**Anahtar Kelimeler:** Benzimidazole, retinoids, HL-60 cells, DNA binding, antiproliferative effects, DNA binding.

## Introduction

It is well known that all-trans-retinoic acid (ATRA) and related natural retinoids are biological regulators of differentiation, proliferation, apoptosis and synthetic analogs which have been used for many years as monotherapy and/or in combination for treatment of acute premyelocytic leukemia and the treatment of dermatological diseases [1, 2]. (ATRA), 13-cis retinoic acid (9-cis RA) induces several cancer cell lines including HL-60 to differentiate into granulocytes, which subsequently die by apoptosis [3–5]. Both natural and synthetic retinoids have been shown to inhibit the growth of different pre-neoplastic and neoplastic cell types and to suppress the induction of growth-related properties [6]. Therefore, retinoids have recently received considerable attention as agents that may have utility for both cancer prevention and treatment due to their regulatory properties in cell differentiation/proliferation [7,8] and inducing apoptosis [3–5]. However, widespread use of these retinoids has been limited due to the observation of numerous undesirable side effects, due in part to their high hydrophobicity, such as hypervitaminosis A, which includes mucocutaneous irritation, cornification disorder, alopecia, teratogenicity and metabolic disorders [9]. Thus, several retinoids have been designed in order to improve the clinical efficacy of retinoids. We have developed conformationally constrained retinoids consisting of tetrahydro-tetramethyl-naphthalene moiety which is integrated with benzimidazole ring system. Tetrahydro-tetramethyl-naphthalene moiety is extensively studied among retinoidal compounds and also exists within the structure of one of the potent retinoid agonist [10,11].

We have previously described the synthesis and antioxidant activity of a tetrahydro-tetramethyl-naphthalene benzimidazole compound which showed a potential antioxidant activity on hepatic cytochrome P450 (CYP) dependent ethoxyresorufin O-deethylase (EROD) and pentoxyresorufin O-deethylase (PROD) enzyme activities [12]. We have extended this observation to further synthesis of derivatives of the above mentioned compound of which synthesis and antioxidant activities are reported elsewhere [13, 14]. Here, we report their antiproliferative effects with comparison to that of (ATRA) and (9-cis RA) on HL-60 cells and their binding to plasmid DNA (pDNA) and synthetic AT nucleotides.

## Materials and Methods

### Retinoids

The compounds were synthesized using  $\text{NaHSO}_3$  addition product of 5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalene-carboxaldehyde as starting material, and were prepared as described [14, 15]. The structures of retinoids are shown in Figure 1. (ATRA) and (9-cis-RA) (Sigma Chem. Co) and the retinoids were dissolved in 70% ethanol, stored as 100 mM stock solutions and kept in dark at  $-20^\circ\text{C}$ .

### Cell Culture

The human myelocytic leukemia cell line HL-60 were grown in RPMI-1640 medium (Gibco Grand Island, New York, USA) supplemented with 10 % heat-inactivated fetal calf serum (Gibco), 2mM glutamine, penicillin (100 U/ml) and streptomycin (100  $\mu\text{g}/\text{ml}$ ) by incubating in 5 %  $\text{CO}_2$  humidified atmosphere at  $37^\circ\text{C}$ . The total HL-60 cells dispensed in tissue culture wells at a density of 500000 cells/well (6 well trays, Costar, Cambridge, MA, USA).

### Drug Treatment

(ATRA), (9-cis-RA) and retinoids indicated as BITN, BITNm and BITNe each at three different doses;  $10^{-4}$  M,  $10^{-5}$  M,  $10^{-6}$  M were added to HL-60 cells in culture. Retinoids were dissolved in ethanol; they were further diluted in the culture medium. Therefore ethanol never exceeded 0.1 % and this percentage did not effect cell growth.

### Cytotoxicity assay

Cell viability was determined by trypan blue dye exclusion assay. To evaluate the cell growth ATRA, 9-cis RA and the retinoid treatment,  $10^{-5}$  of each compound was added per 500 000 HL-60 cells and incubated for 48 hours. Cells were then collected and the amount of DNA was indicated by 4, 6-diamidino 2 phenyl indole (DAPI) staining and measuring the fluorescence at 360 nm and 500 nm excitation and emission respectfully [15].

To evaluate the dose dependent cytotoxicity, HL-60 cells were dispensed in 6 well culture plates at a density of 500.000 cells/ml. Retinoids were applied at doses of  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$ . At 24, 48 and 96 hours of treatment growth is expressed as a percentage of control cells.

### DNA binding assays

PGEM plasmid was grown in E coli and isolated by fast alkaline lysis [16]. Retinoids, (ATRA), ethidium bromide (EtBr) and (DAPI) were added to the plasmids at a ratio of  $10^{-5}$  M to 5 $\mu\text{g}$  DNA. Adducts were incubated for 1 hr at  $37^\circ\text{C}$  in the dark. These were then electrophoresed in 0.08 % agarose in 1xTBE for 2 hours at 70 mV. Gels were stained with (EtBr) for 20 minutes and visualized after destaining O/N. Octameric oligonucleotides without self complementarity consisting solely of adenine and thymine were designed and synthesized at 0.186  $\mu\text{g}/\mu\text{l}$  concentration. Complementary AT octamers were incubated at  $20^\circ\text{C}$  to form duplexes. Retinoids were incubated with AT octamers and AT octameric duplexes. Adducts were then run in 20 % polyacrylamide gel electrophoresis (PAGE) in 1X TBE and bands were visualized by silver staining [17].

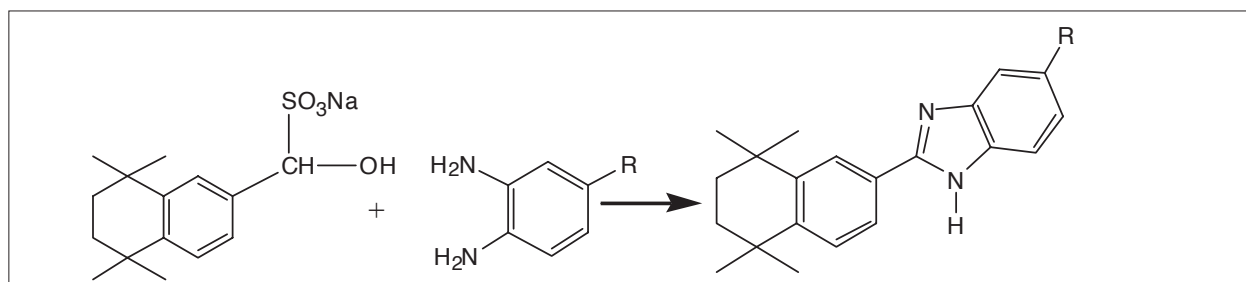


Figure 1. Structures of the retinoidal compounds.

## Results

Antiproliferative effects of retinoids: Retinoidal compounds exhibited strong cytotoxicity on HL-60 cells; however the magnitude of inhibition was not significantly higher than that of (ATRA). Among the three retinoids BITNm's cytotoxic effect was found to be significantly more than the other two (Figure 2). The cytotoxic effects of retinoids were found to be dose dependent as shown in Figure 3.

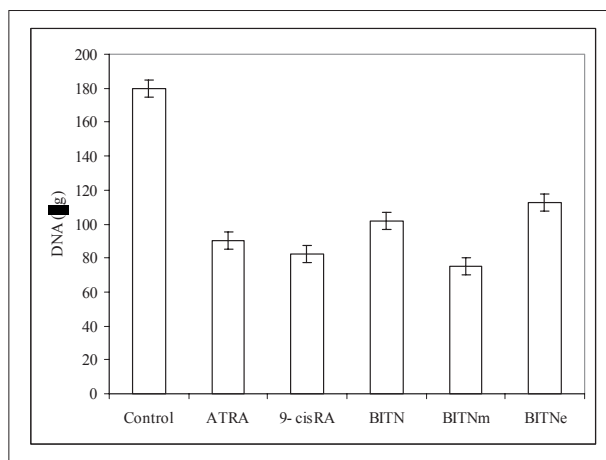
### DNA binding:

**Plasmid DNA:** Agarose electrophoresis of pGEM plasmids reveal linear and supercoiled forms of bacterial DNA (pDNA). (EtBr), (DAPI) and ATRA and the retinoids are incubated with (pDNA) and analyzed at 0.8 % agarose gel. Figure 4 shows the electrophoretic profile of (pDNA) adducts. Treatment of (pDNA) with (EtBr) and (ATRA) lead to quenching of distinct bands while (DAPI) had a rather weak effect on (pDNA) profile in electrophoresis similar to those of retinoids.

**Oligonucleotides:** Retinoids were incubated with AT octamers an AT duplex and adducts were run in 20% polyacrylamide gel and stained with silver. The gel retardation of retinoid bound nucleotides is shown in Figure 5. AT octamers and duplexes treated with retinoids were both retarded in gel as compared to that of untreated ones.

## Discussion

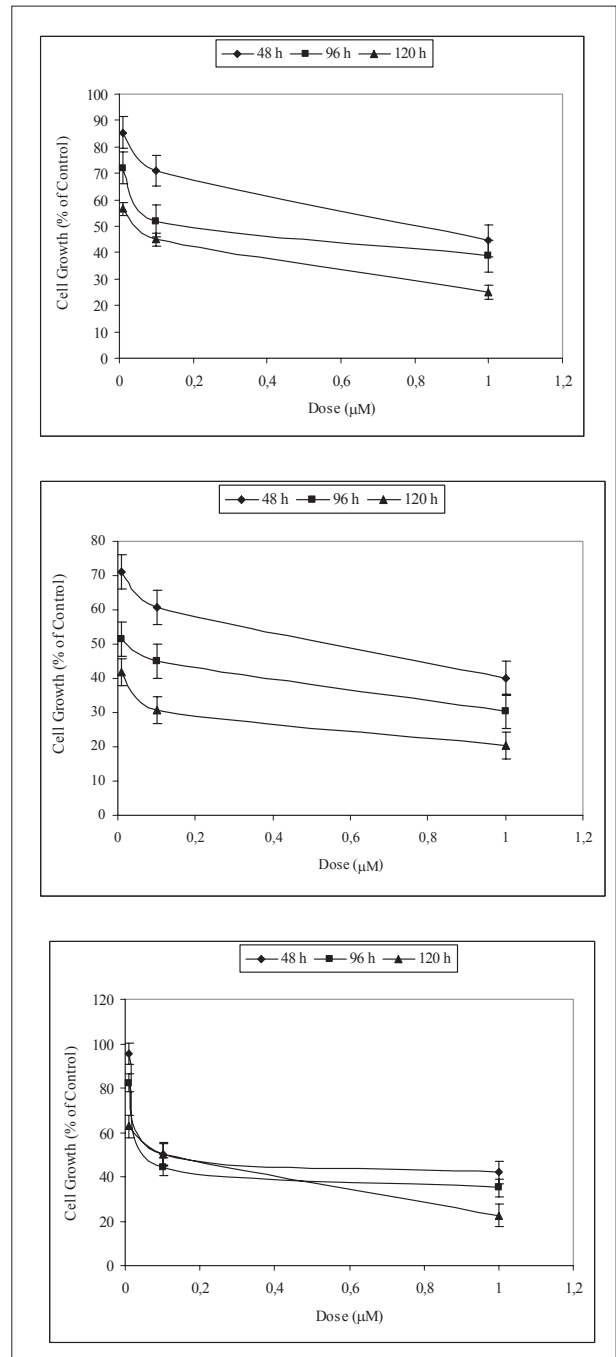
Benzimidazole derivatives are a group of molecules with diverse biological activities ranging from antitumor to antiviral effects [19–21]. We have investigated the antitumor effects and DNA binding of three compounds having benzimidazole rings. Benzimidazole retinoids BITN, BITNm and BITNe have exerted strong antiproliferative effects on HL-60 cells. They suppressed cell growth by 56.5 %, 41.8 % and 62.7 % of control respectively. Cell growth inhibition was not significantly different to those induced by ATRA and 9-cis-RA. The difference between the antiproliferative effect of BITNm was significantly higher than the effects of BITN and BITNe. This may be due to the greater affinity towards retinoid receptors or other intracellular mediators of cell death due to the ter-



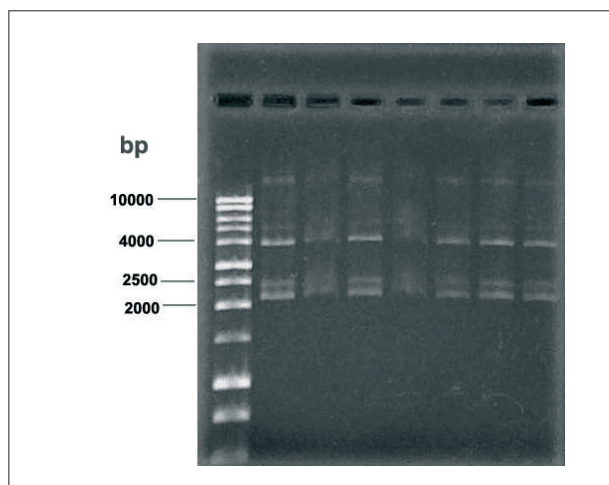
**Figure 2.** Effect of ATRA, 9-cis RA and retinoidal compounds BITN, BITN-m and BITN-e on the proliferation of HL-60 cells after 48 hours. Proliferation is expressed as the amount of DNA as determined by DAPI staining. The concentration of each chemical is  $10^{-5}$  M. Bars represent the mean and S.D. of six different experiments.

minimal methyl group in the molecule. Further analysis of their receptor selectivities may explain this difference. The antiproliferative effects of retinoids are found to be dose dependent. BITN and BITNm demonstrates a highly dose dependent effect during the 5 day period, while BITNe becomes more dose dependent at higher doses (1  $\mu$ M).

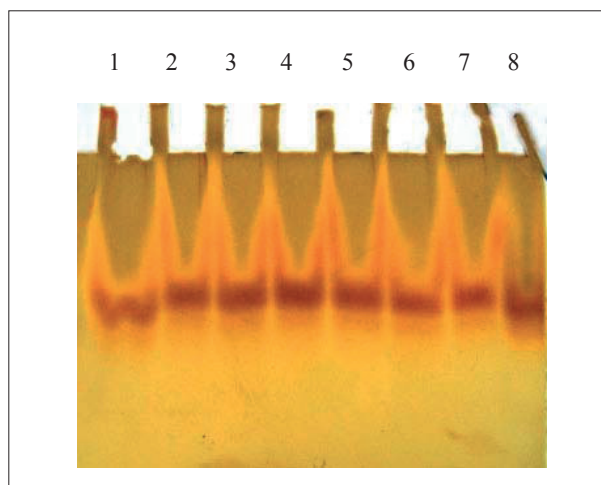
A great majority of cancer drugs and antimicrobial drugs exert their actions through DNA binding. This is believed to be the result of inhibition of gene expression or change in availability of certain parts of genome for regulatory protein recognition



**Figure 3.** The dose dependent antiproliferative effect of BITN (a), BITNm (b) and BITNe (c) on HL-60 cells. The growth inhibition is determined by measuring the amount of DNA and presented as a percentage of controls. Bars represent mean  $\pm$  SD.



**Figure 4.** DNA binding analysis. Mw marker (1), pDNA (2), EtBr (3), DAPI (4), ATRA (5) and the retinoids BITN (6), BITNm (7) and BITNe (8).



**Figure 5.** PAGE profile of retinoid bound AT nucleotides and AT duplex. AT octamer alone (1) or treated with BITN (2), BITNm (3) and BITNe (4). AT duplex treated with BITN (5), BITNm (6), BITNe (7) or alone (8). Each nucleotide is 200 ng and retinoids are 10-5 M.

[21, 22]. Major modes of DNA binding are groove binding and intercalation and intercalators may also induce strand breaks in DNA [23, 24]. Therefore, we have analyzed DNA binding of these compounds by various methods [25, 26]. All three forms of retinoids effected (pDNA) in a similar manner with (DAPI) which is a well known minor groove binder with AT sequence specificity. The DNA damage may indicate that BITN, BITNm and BITNe do not intercalate but do have affinity towards nucleotides. Thus, they may well fit into the minor groove and effect genes related to cell growth and their mode of action may be different to that of (ATRA) in a less toxic manner. Their specific actions through receptors however remain to be elucidated.

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## References

- [1] Hashimoto Y. (2002) Structural development of biological response modifiers based on retinoids and thalidomide. *Mini Reviews in Medicinal Chemistry*. 2(6):543-510.
- [2] Zhang CM, Duvic M. (2003) Retinoids: therapeutic applications and mechanisms. *Dermatologic Therapy*. 16 (4):322-30.
- [3] Martin SJ, Bradley JG, Cotter TG. (1990) HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. *Clin. Exp. Immunol.*, 79 (3): 448-53.
- [4] Agarwal N, Mehta K. (1997) Possible involvement of Bcl-2 pathway in retinoid x receptor alpha induced apoptosis of HL-60 cells. *Biochem. Biophys. Res. Commun.*, 13: 251-53.
- [5] Tong L, Werrbach-Perez K., Perez-Polo JR. (1997) Retinoic acid induces apoptosis in PC12 cells independent of neurotrophic factors. *J. Neurochem.*, 68: 1424-35.
- [6] Um SJ, Han HS, Kwon YJ, Park SH, Rho YS, Sin HS, Park JS. (2003) Novel retinoic acid derivative ABPN has potent inhibitory activity on cell growth and apoptosis in cancer cell. *Int. J. Cancer*, 107: 1038-46.
- [7] Lio SC, Johnson J, Chatterjee A, Ludwig JW, Millis D, Banie H, Sincar JC, Sinha A, Richards ML. (2008) Disruption of golgi processing by 2-phenyl benzimidazole analogs blocks cell proliferation and slows tumor growth. *Cancer Chemother. Pharmacol*. 61(6):1045-58.
- [8] Seaton A, Higgin C, Mann J, Baron A, Baily C, Neidle S, van den Berg H. (2003) Mechanistics and antiproliferative studies of two novel biologically active bis-benzimidazoles. *Eur. J. Cancer*. 39(17):2548-55.
- [9] Armstrong RB, Ashenfelter KO, Eckhoff C, Levin AA, Shapiro S. (1994) General And Reproductive Toxicology of Retinoids, in *The Retinoids: Biology, Chemistry and Medicine*, M.B. Sporn, A. B. Roberts, D. S. Goodman. eds., pp.545-72, Raven Press, New York
- [10] Yoshimura H, Nagai, M, Hibi S, Kikuchi K, Abe S, Hida T, Higashi S, Hishinuma I, Yamanaka T. (1995) Synthesis and characterization of highly potent and effective agonist of retinoic acid receptor. *J. Med. Chem.*, 38: 3163-73.
- [11] Eyrolles L, Kagechika H, Kawachi E, Fukasawa H, Iijima T, Matsushima Y, Hashimoto Y, Shudo K. (1994) *J. Med. Chem.*, 37:1508-17.
- [12] Ates Z., Eke BC., Suzen S., Buyukbingol E. Iscan M. (1997) Effects of a benzimidazole compound on monoxygenase activities. *II Farmaco*, 52: 703-706.
- [13] Ates-Alagoz Z, Buyukbingol E. Synthesis of some novel tetrahydronaphthalene benzimidazole derivatives (2001) *Heterocyclic Commun*. 7 (5): 455-60.
- [14] Z. Ates-Alagoz, Can-Eke B, Coban T, Iscan M, Buyukbingol E. (2004) Antioxidant properties of novel benzimidazole retinoids *Arch. Pharm.*, 337: 188-92.
- [15] Brunk C, Jones KC, James TW. (1979) Assay for nanogram quantities of DNA in cellular homogenates. *Anal. Biochem.*, 92:497-500
- [16] Birnboim HC, Doly J.(1997) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, 7 (6): 1513-23
- [17] Blum H, Beier, H. Gross HJ.(1986) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, 8(2): 93-99

- [18] Fonseca T, Gigante B, Marques MM, Gilchrist TL, De Clercq E. (2004) Antiinflammatory phospholipase-A2 inhibitors *Bio. Med. Chem.*, 12(1), 103-112.
- [19] Boufatah N, Gellis A, Maldonado J, Vanelle P. (2004) Efficient microwave-assisted synthesis of new sulfonylbenzimidazole-4,7-diones: heterocyclic quinones with potential antitumor activity. *Tetrahedron*, 60 (41), 9131-37.
- [20] Boiani M, Gonzalez M. (2005) Imidazole and Benzimidazole Derivatives as Chemotherapeutic Agents. *Mini Rev. Med. Chem.*, 5(4):409-24.
- [21] Nelson NS, Ferguson LR, Denny WA. (2004) DNA and the chromosome varied targets for chemotherapy. *Cell Chromosome*, 3(2): 1-26.
- [22] Rosenauer A, Raelson JV, Nervi C, Eydoux P, DeBlasio A and Miller WH. (1996) Alterations in expression, binding to ligand and DNA, and transcriptional activity of rearranged and wild-type retinoid receptors in retinoid-resistant acute promyelocytic leukemia cell lines. *Blood* 88(7): 2671-82.
- [23] Benod C, Subra G, Nahoum V, Mallavialle A, Guichou JF, Milhau J, Roblés S, Bourguet W, Pascussi JM, Balaguer P, Chavanieu A. (2008) N-1H-Benzimidazol-5-ylbenzenesulfonamide derivatives as potent hPXR agonists. *Bioorg. Med. Chem.* 16(7):3537-49.
- [24] Winfield LL, Smith DM, Halemama K, Leggett CS. (2008) A preliminary assessment of the structure-activity relationship of benzimidazole-based anti-proliferative agents. *Lett. Drug Des. Discov.* 5(6):369-78.
- [25] Musdal Y, Piskin, AK ( 2006) In vitro DNA binding mode analysis of some benzimidazole and thiazolidindione retinoids. *FEBS Journal suppl.1* 273: 762 .
- [26] Musdal, Y., Piskin, K., Kocum, C. (2008) Interactions of DNA by some retinoids: by scanning tunneling microscopy. *Turk J Biochem suppl.1* 33:213.