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ARAŞTIRMA MAKALESİ/RESEARCH ARTICLE

SYNTHESIS AND ANTIMICROBIAL, ANTIMITOTIC ACTIVITIES AND TOXICITIES OF SOME PLATINUM COMPLEXES OF THIOSEMICARBAZIDE DERIVATIVES

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ABSTRACT

In this study, some thiosemicarbazide derivatives were synthesized by reaction of hydrazides with the appropriate arylisothiocyanate and the platinum complexes of these compounds were prepared. The structure elucidation of the compounds were performed by IR, ^{1}H-NMR spectral data and elemental analyses results. Antimicrobial activities of the ligands and complexes were examined without the reactions being interrrupted by using a bioautographic agar overlay method. Toxicities and antimycotic activities of some of these compounds were also investigated.

Key Words: Thiosemicarbazide; Pt(II) complex, Bioautographic agar overlay method, Anticancer

ÖZ

Bu çalışmada, uygun arilisotiyosiyanatlar ve hidrazidlerin reaksiyonu ile bazı tiyosemikarbazid türevleri sentezlenerek platin kompleksleri hazırlandı. Bileşiklerin yapıları IR, 1H NMR spektral verileri ve elemental analiz sonuçları ile aydınlatıldı. Ligandların ve komplekslerin antimikrobiyal aktiviteleri, reaksiyonlar kesilmeden "bioautographic agar overlay" metodu ile test edildi. Aynı zamanda bileşiklerin toksisiteleri ve antimitotik aktiviteleri araştırıldı.

Anahtar Kelimeler : Tiyosemikarbazid; Pt(II) kompleks; antimikrobiyal; antikanser

1. INTRODUCTION

Thiosemicarbazides have antibacterial, antifungal, antimycobacterial, antimalarial, anticancer activity and some of the derivatives of thiosemicarbazides are used on the chemotherapy of tuberculosis, besides, many of the biological activities of proteins and enzymes can be ascribed to the metal centers, so a lot of metal complexes have been studied extensively because of their potential use in chemotherapy, such as anticancer, antibacterial, antiviral, antiarthritic etc. (Barefoot, 2001; Demirayak et al., 1992; Sherman et al., 1987; Wong et al., 1999). Moreover, transition metal complexes of thiosemicarbazones have been found, in some instances, to have enhanced or modified activity in comparison with the uncomplexed ligand (Bindu et al.,1998; Gümüş et al., 1996; Miller et al., 1998; Offiong et al., 1996). The interesting biochemical applications of platinum group metal complexes with nitrogen and sulfur ligands led us to undertake the present systematic study of some substituted thiosemicarbazide complexes with platinum.

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In this study, N^1-aryloxyaceto-N^4-aryl-3-thiosemicarbazide derivatives were synthesized by reaction of aryloxyacetohydrazides with the appropriate arylisothiocyanate and the platinum complexes of these compounds were prepared (Atalay et al., 1998; Beraldo et al., 1998; McCaffrey et al., 1997; Mylonas et al., 1988; Nagasawa et al., 1998). The structure elucidation of the compounds were performed by using IR, ^{1}H-NMR spectral data and elemental analyses results (Bergs et al., 1997; El-Shadaw, 1991; Yalçın et al., 1993). Antimicrobial activities of the ligands and complexes were examined without the reactions being interrrupted by using a bioautographic agar overlay method (Gibbons et al., 1998; Rahalison et al., 1991) which can also be used to the search for antimicrobial activity of some of the stereochemically non-rigid metal complexes. In addition, toxic effects of these compounds were studied by MTT(3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyl tetrazolium bromide) assay, and also antimycotic effect was investigated by BrdU-proliferative assay in fibroblast like mouse embryo cells (Merante et al., 1996; Scioscia, 1997).

2. MATERIALS AND METHODS

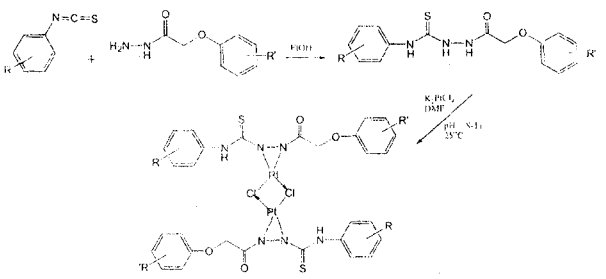
Melting points were determined by using a Gallenkamp apparatus and given uncorrected. Spectroscopic data were recorded on the following instruments : IR : Schimadzu 435 IR spectrophotometer; ^{1}H NMR: DPX 400 NMR spectrometer, Microanalyses: Leco CHNS Elemental Analyses Apparatus.

2.1. Synthesis of the ligand N^1-aryloxyaceto-N^4-aryl-3-thiosemicarbazide derivatives (I-IV)

A mixture of suitable aryloxyacetohydrazide (5 mmol) and arylisothiocyanate (5 mmol) in ethanol (100 ml) was refluxed for 2 h. The precipitate was filtered and crystallised from methanol-water mixture (yield ~80%) (Table 1).

2.2. Preparation of the Pt complexes (Ia-IVa)

N^1-aryloxyaceto-N^4-aryl-3-thiosemicarbazide derivatives (0.5 mmol) and K_2PtCl_4 (0.5 mmol) in DMF (5 ml) were stirred at 25°C for 8h. The solution was filtered and then kept in the refrigerator for 24 h. The complexes were precipitated after the addition of sodium acetate as a buffering agent and filtered. After washing several times with ethanol and diethyl ether, the final products were dried in vacuo (yield ~50%) (Table 1). Reactions were showed in the Scheme 1.



Scheme 1.

I : IR(KBr)n_{max}(cm^{-1}): 3275-3050 (N-H), 1671 (C=0), 1240 (C=S), 838 (1,4-disübs), 741,681 (monosübs.). ^{1}H-NMR s(ppm): 2.23 (3H, s), 4.52 (2H, bs), 6.8-7.32 (9H, m), 9.5 (2H, s), 10.25, (1H, s). El. An. Calc. (% C-H-N): 60.93-5.43-13.32; Found(%C-H-N) : 60.71-5.20-13.40.

II : IR(KBr)n_{max}(cm^{-1}): 1647 (C=0), 1482 (C=N). 1160 (C-O), 845 (1,4-disübs.). 771,722 (monosübs.), 330 (M-N), 292 (M-Cl), ^{1}H-NMR s(ppm): 2.44 (3H, s), 3.52 (2H, br.s), 7.98-8.52 (9H, m). El. An. Found(%C-H-N) : 36.71-2.30-8.41.

III : IR(Kbr)n_{max}(cm^{-1}): 3375-3000 (N-H), 1664 (C=0), 1245 (C-S), 829 (1,4-disübs.). 744 (1,2-disübs.). ^{1}H-NMR s(ppm): 4.71 (2h, bs). 6.59-7.45 (8H, m), 9.71 (2H, s), 10.16, (1H, s). El. An. Calc.(%C-H-N): 53.65-4.20-12.51; Found(%C-H-N) : 53.31-4.46-12.66.

IV : IR(KBr)n_{max}(cm^{-1}): 1607 (C=0), 1483 (C=N), 813 (1,4-disübs.), 751 (1,2-disübs.), 339 (M-N), 293 (M-S). ^{1}H-NMR s(ppm): 5.01 (2H, s), 6.05-8.64 (8H, m). El. An. Found(%C-H-N) : 34.55-2.60-7.41.

V : IR(KBr)n_{max}(cm^{-1}): 1664 (C=0), 1485 (C=N), 854 (1,4-disübs.), 747 (1,2-disübs.), 336 (M-N), 296 (M-S). ^{1}H-NMR s(ppm): 2.49 (3H, s), 3.24 (2H, br.s), 8.06-8.60 (8H, m). El. An. Found(%C-H-N) : 23.71-1.87-5.41.

VI : IR(KBr)n_{max}(cm^{-1}): 1604 (C=0), 1483 (C=N), 750,687 (monosübs.), 745 (1,2-disübs.), 321 (M-N), 285 (M-S). ^{1}H-NMR s(ppm): 5.02 (2H, br.s), 6.70-7.90 (9H, m). El. An. Found(%C-H-N) : 23.71-1.87-5.41.

VII : IR(KBr)n_{max}(cm^{-1}): 3305-3110 (N-H), 1660 (C-0), 1245 (C=S), 838 (1,4-disübs.), 740 (1,2-disübs.). 1H-NMR s(ppm): 2.30 (3H, s), 4.78 (2H, bs), 6.98-7.46 (8H, m), 9.63 (2H, s), 10.18, (^{1}H. s). El. Cn. Calc.(%C-H-N) : 54.93-4.61-12.01: Found(%C-H-N) : 54.91-4.45-11.78.

VIII : IR(KBr)n_{max}(cm^{-1}): 3380-3160 (N-H), 1669 (C=0), 1293 (C=S), 800,695 (monosübs.), 748 (1,2-disübs.), ^{1}H-NMR s(ppm): 4.73 (2H, bs), 6.93-7.41 (9H, m), 9.62 (2H, s), 10.15, (1H, s). El. An. Calc.(%C-H-N) : 54.93-4.61-12.01; Found(%C-H-N) : 54.91-4.45-11.78.

Table 1. Some Characteristics of The Compounds.

Comp	R	R'	Mp. (°C)	Yield(%)	Formula	Mol. Mass
I	H	CH_3	171	82	$C_{16}H_{17}N_3O_2S$	315.384
II	H	CH_3	>300	44	$C_{16}H_{15}N_3O_2SPt$	508.458
III	Cl	CH_3	158	73	$C_{16}H_{16}N_3O_2SCl$	349.827
IV	Cl	CH_3	>300	48	$C_{16}H_{14}N_3O_2SClPt$	542.901
V	Cl	Cl	169	85	$C_{15}H_{13}N_3O_2SCl_2$	370.244
VI	Cl	Cl	>300	50	$C_{15}H_{14}N_3O_2SCl_2Pt$	563.318
VII	Cl	H	140	76	$C_{15}H_{14}N_3O_2SCl$	335.797
VIII	Cl	H	>300	43	$C_{15}H_{14}N_3O_2SClPt$	528.871

2.3. Antimicrobial activity assay

TLC bioautographic overlay assay of Gibbons and Gray was used to determine the antimicrobial activity of ligands and the platinum complexes of thiosemicarbazide derivatives. *Staphylacoccus aureus* ATCC 6538P, *Escherichia coli* ATCC 25922, *Pseudomonas aureuginosa* ATCC 27853, *Enterobacter feacalis* ATCC 29212 were obtained from Ege University, Faculty of Science and *Candida albicans* was kindly obtained from Osmangazi University Medical Faculty.

A base of nutrient agar was poured into a dish and allowed to solidify. The compounds were run on a TLC plate with petroleum ether:ethyl acetate (1:1 and 2:1) as a developing solvent. An inoculum of the cultures at a titer of 10^5cfu/ml in Müeller Hintan Broth (Merck) was prepared and nutrient agar was added at 7.5 g/lt to thicken the medium. TLC plate was placed on the nutrient agar base and then the medium containing test organism was poured over the plate and incubated at 37°C for 24 hour. A solution of tetrazolium salt (2,3,5-triphenyltetrazolium) at %1 concentration was sprayed onto the face of the medium. Zones of inhibition appeared as clear zones against a purple background. The assay was carried out in dublicate.

2.4. Cell Culture

F2408 (fibroblast like rat embriyo) and 5RP7 (H-ras oncogene active fibroblast cells) were maintained in Dulbecco Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% (v/v) foetal calf serum (FCS) (Gibco), penicillin/streptomycin at 100 units/ml and glutaminase as adherent monolayers. Cells were incubated at 37°C under 5% CO_2/95% air in a humidified atmosphere.

2.5. MTT Dye Reduction Assay

This assay is based on the conversion of a yellow, water soluble monotetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MTT] to an insoluble purple formazan when reduced. The mitochondrial dehydrogenases are involved in MTT reduction via electron transport from NAD or NADP diaphorases and only living, not dead, cells are able to reduce MTT. Cells must be in an exponential phase of growth when MTT is added and MTT dye reduction should be linear with respect to cell number. It was therefore crucial that the optimum cell number and length of assay were determined for each cell line used.

Monolayer F2408 fibroblast cells in exponential growth phase were harvested and resuspended in fresh medium to give a density of $1x10^4$ /ml and incubated with various dilutions of compound I, II, III, IV, V and VI for 24, 48 and 72 hrs at 37°C. Aliquots (200µl) of the cell suspensions were placed into each of 88 wells of a 96 well microtitre plate. The initial row of 8 wells was filled with 200 µl of medium alone to serve as a blank. Plates were then incubated in a 8% CO_2 atmosphere at 37°C. The number of living cells was measured each day. At the end of the exposure time 20µl of MTT dye solution (5 µg/ml in sterile PBSA) were added to each well and the plates were incubated for a further 2 h. Under these conditions, MTT was reduced by living cells into an insoluble blue formazan product. The medium containing MTT solution was then gently removed from the wells by aspiration leaving the reduced tetrazolium salt present as blue crystals in the wells.The tetrazulium salt was then solubilised by addition of 200 µl of DMSO to each well, followed by agitation using a plate shaker for 10 min. Absorbance at 540 nm was determined by use of a Dynatech., MR5000 (Dynatech Lab, USA) plate reader with a reference beam of 690 nm. Values obtained for the medium blanks automatically were subtracted. Each drug concentration was repeated 4 times per experiment. The results of repeat wells within the same experiment were averaged and the SD within each experiment was always <10%.

2.6. Analysis of DNA Synthesis (Antimithotic Activity)

Cell proliferation assay was performed in 96-well plates (Falcon, Beckton Dickinson) and the BrdU colorimetric kit (Boehringer Mannheim) was used to determine the DNA synthesis by the method as given by the manufacturer. F2408 and 5RP7 cells were cultured as detailed above. The cells were detached with 0.25% trypsin/EDTA and $1x10^3$ cells/ml were transferred into each well containing 10% FCS and 1% FCS plus 1µl DEX.

All compounds were dissolved in DMSO. To investigate the effects of these compounds on DNA synthesis, the cells were incubated with 10 or 25 µg/ml of compound in DMSO for various periods of time. These doses were chosen according to MTT assay as described above. After each day, the cells were labeled with 10 µl BrdU solution at 37°C for 2 hrs and then fixed with the addition of fixdenat solution for 30 min at room temperature. After removing the fixdenat solution, cells were treated with 100 µl of anti-BrdU-working solution for 90 min at room temperature. Then the cells were washed three times with PBSA and incubated with substrate solution until the color is sufficient for photometric detection that was predetermined. The absorbance of the samples was measured in an ELISA reader (Organom, Technica) at 492 nm.

3. RESULTS AND DISCUSSION

3.1. Synthesis

The structure of the compounds obtained were elucidated by spectral data and elemental analyses. The IR spectrum of ligands showed three bands between 3375-3000 cm^{-1} due to n(N-H), 1675-1600 cm^{-1} due to ν(C=O) 1220-1240 cm^{-1} due to ν(C=S) which disappeared in the spectrum of platinum complexes. No bands exist in the 2600-2400 cm^{-1} region which are due to S-H vibrations in the IR spectrum of the ligands. New bands in the low frequency regions at 340-310 cm^{-1} and 310-290 cm^{-1} assignable to ν(M-N), ν(M-S) were observed.

In the ^{1}H-NMR spectra, the peaks of ethylene protons were observed about 3.4 and 4.4 ppm. Aromatic protons were observed about 6.70-7.60 ppm as multiplets. The peaks of N-H protons of the ligands were obtained between 9.5-10.25 ppm which disappeared in the spectrum of complexes.

3.2. Antimicrobial activities

The antimicrobial activities of the compounds tested is shown in Table 2. As it is seen from Table 2, platin complexes showed more inhibition of microorganism than ligands.

3.3. Toxicity

Cytotoxicity of the compounds I and II; III, IV, V and VI used in cell proliferation assay was determined with a tetrazolium (MTT) assay as described in material and methods. MTT is commonly employed as an indicator of cell number and viability, since it is converted to a coloured formazan derivative via mitocondrial dehydrogenase activity only by viable cells (Pagliacci et al., 1993). F2408 fibroblast cell line was incubated with various concentrations of Compound I (μg) for 3 days. 10 mg and 25 mg compound I and II, the concentrations used in other experiment showed between 10-20% toxicity after 24 hrs. Interestingly

Table 2. Antibacterial Activity of The Compounds.

Comp.	S. Aureus		Ecoli		Ps. Aeroginosa		E. Feacalis		C. Albicans	
I	+	+	-	-	+	+	+	+	-	-
II	-	-	+	+	-	-	-	-	-	-
III	-	-	+	+	+	+	-	-	-	-
IV	-	-	-	-	-	-	-	-	-	-
V	-	-	+	+	+	+	+	+	-	-
VI	-	-	-	-	+	+	-	-	-	-
VII	+	+	+	+	+	+	+	+	-	-
VIII	-	-	-	-	+	+	+	+	-	-
Fluconazole	-	-	-	-	-	-	-	-	-	-
Chloramphenicole	-	-	-	-	-	-	-	-	-	-

(-) Inhibition of growth (+) Non-inhibition of growth

even higher concentration of compound II showed a little toxicity on F2408 fibroblast cell line (Figure 1. panel A and B) MTT assay also failed to show toxic effects of compound III at 10 μg/ml and 25 μg/ml over the 24 hr incubation period. 10 μg/ml Compound IV showed 20% toxicity after 24 hrs, however the toxicity was increased up to 80% for 2 and 3 days (Figure 1. panel C and D).

Compound V and VI showed similar toxicity to compound I and II. 10 μg/ml and 25 μg/ml of compound V, VI, the concentrations used in cell proliferation assay, showed no toxicity after 24 hrs. (Figure 1. panel E and F).

3.4. Antimitotic activity

Normal fibroblast cells (F2408) and H-ras transfected fibroblast cells (5RP7) were used to determine the effect of compounds I, II, III, IV, V and VI on cell proliferation.

BrdU labelling fibroblast cells were incubated with anti-BrdU antibody and washed several times with PBSA. After the last washed step, the cells were incubated with substrat solution for 10 min. And the colour change, which is related to DNA synthesis, in the 96-cell plates were measured at 492 nm by spectrophotometer. The results, in Figure 2, panel A and B, show that cell proliferation of normal fibroblast cells (F2408) was not changed during all three days, in the presence of either compound I (S120) or compound II (S120Pt) when compared to control cells. In contrast the compound II appeared to block the cell proliferation of 5RP7 cells during the incubation time. This meant that at a concentration of 25 μg/ml of compound II, the cell proliferation capacity of these cells was reduced by about 75%. Interestingly, compound I was a little effect of equal doses in the cell proliferation capacity. Both compounds (I and II) of 10 μg was no effect on cells proliferation of 5RP7 cancer cell line. 5RP7 Fibroblast cancer cells also showed a dose dependent decrease in proliferation using compound II (approximately 30% at 10 mg and 75% at 20 μg).

These results strongly suggest that cell proliferation of 5RP7 fibroblast cancer cells was inhibited by the presence of S120Pt.

The effects of compound III and IV on normal and cancer cell line were investigated using the BrdU labeling agents. Figure 3 illustrates the effects of these two compounds on F2408 (panel A) and 5RP7 cancer cells (panel B). Both compound III (25 μg) and compound IV (5 μg) had little or no effect upon the proliferation of F2408 cells but the higher concentration of compound IV had a significant inhibitory effect on the cell prolif-

eration of 5RP7 cancer cells on day 3. According to results obtained from MTT, two different concentration of compound IV also used in these experiment. Because of the high toxicity of these compounds experiments carried out for only 24 hrs. Results also showed in figure 3. The effects of compound IV (either 10 or 25 μg) on 5RP7 cell proliferation were of a substantially greater magnitude. Thus lower concentration of compound IV was showed inhibitory effect which rely on longer incubation time. In apparent contrast the effects of high concentration of compound IV appeared as a early effect.

In contrast to effects on cell proliferation obtained from other compounds, the proliferation of 5RP7 cells was increased by 30% in the presence of both compounds V and VI (Figure 4). This meant that at a concentration of 25 mg compound V and VI may be mitogenic activity on these cell line. Interestingly, there was no effect of equal doses in the proliferation capacity of F2408 cell line (Figure 4 panel A)

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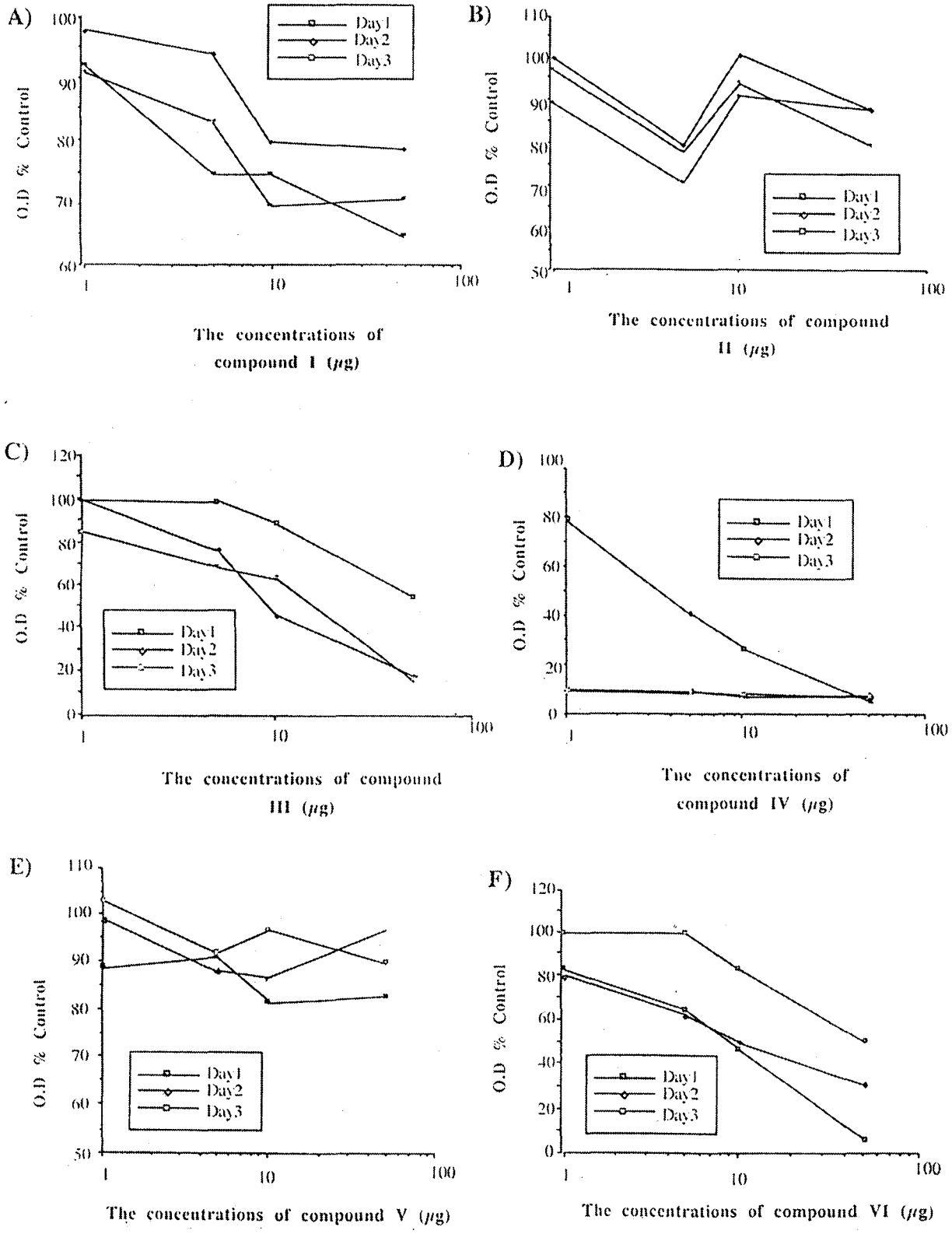

Figure 1. Toxicity of The Compounds Were Determined By MTT Assay For F2408 Fibroblast Cell Line. Predetermined Cell Numbers Were Incubsted With Various Concentrations of Compounds During The Experiment at 37°C. After Each Day, 20 μl of 5μg/ml MTT Was Added To Each Well and Incubated For A Further 2 hr. Medium Was Discarded and 200 μl of DMSO Was Used to Dissolve The Dye. Density of Dye Was Measured By Plate Reader (at 570nm). Results Are The Mean of Quadruplicate Wells (standard deviation less than 10%).

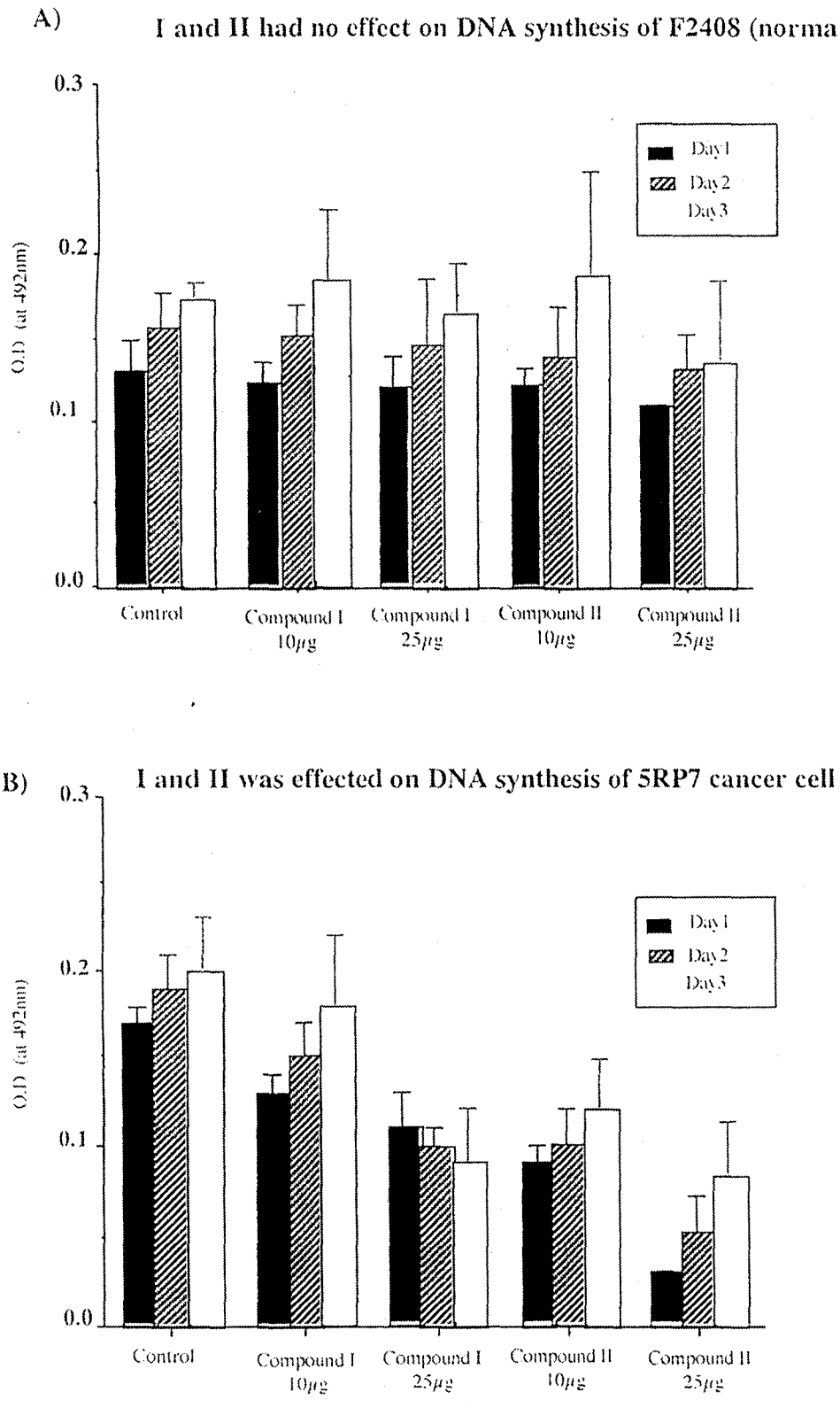

Figure 2. The Effect of Compound I and II On Cell Proliferation Was Determined By BrdU-labeling Fluoresans Technique For Both F2408 (panel A) and 5RP7 (panel B) Fibroblast Cell Lines. Predetermined Cells Numbers Were Incubated With Either 10μg/ml and 25 μg/ml of Both Compounds For Period of Time At 37°C. Experiment Was Carried Out As Described In Section of Materials and Methods. Density of Dye Was Measured By A Plate Reader (ratio between OD of plates at 492 nm). Results Are The Mean of Triplicate Wells.

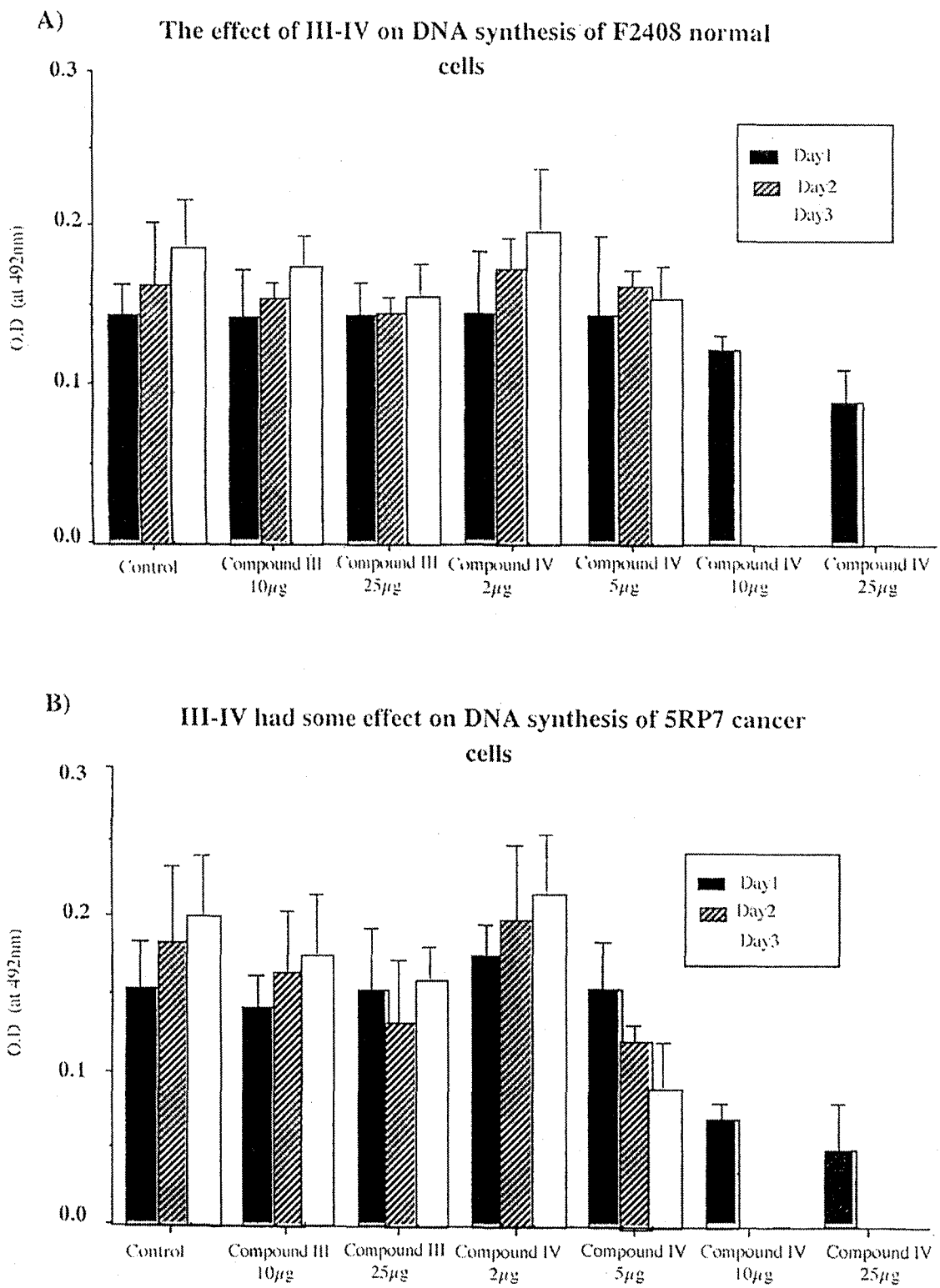

Figure 3. The Effect of Compound III and IV On Cell Proliferation Was Determined For Both F2408 (panel A) and 5RP7 (panel B) Fibroblast Cell Lines. Cells Were Incubated With Either 10μg/ml and 25 μg/ml of Compound III and 2μg/ml, 5μg/ml, 10μg/ml and 25 μg/ml of Compound IV For Period of Time at 37°C. Results Are The Mean of Triplicate Wells.

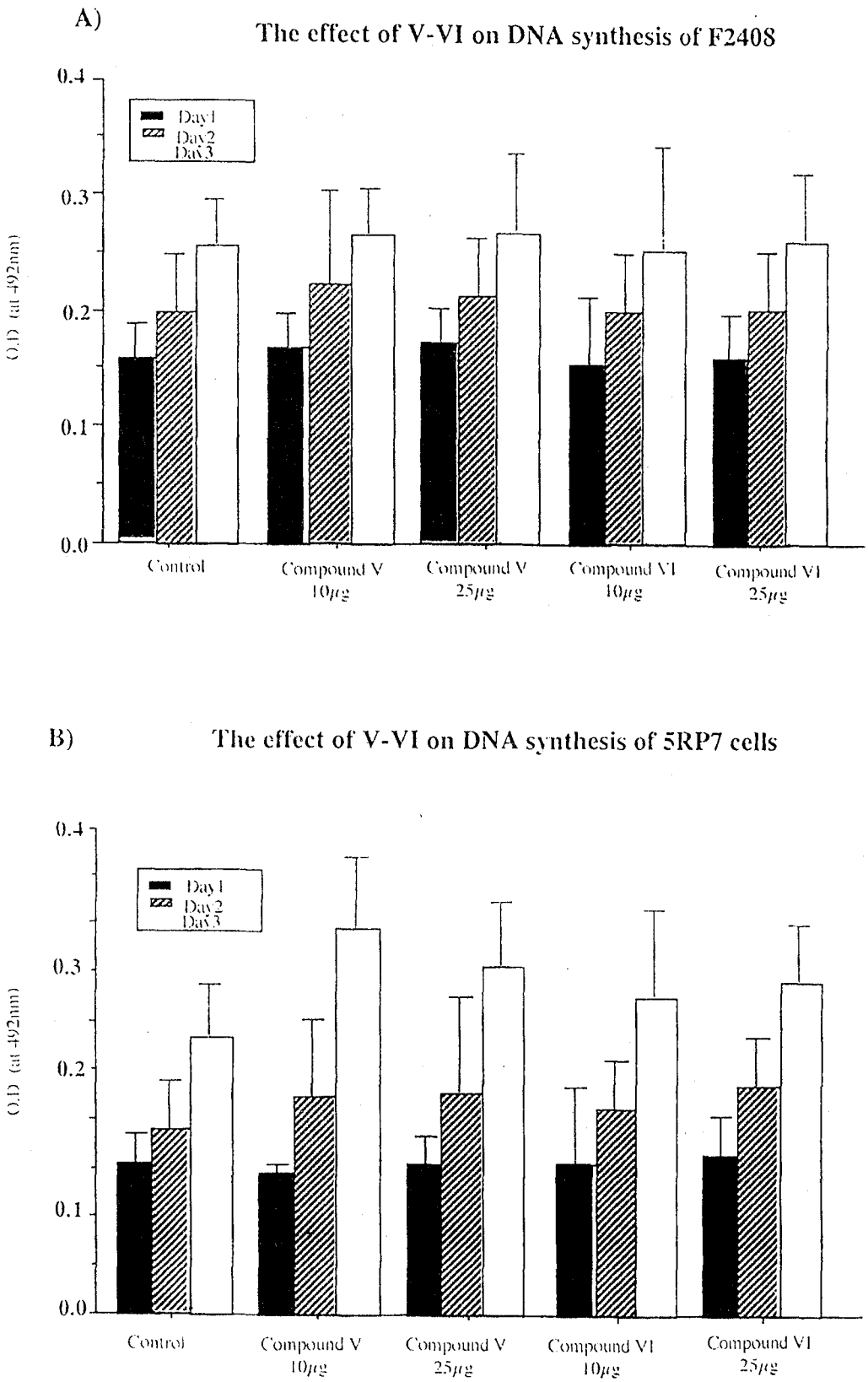

Figure 4. The Effect of Compound V and VI On Cell Proliferation Was Determined For Both F2408 (panel A) and 5RP7 (panel B) Fibroblast Cell Lines. Cells Were Incubated With Either 10μg/ml of 25 μg/ml of Both Compounds For Period of Time at 37°C. Density of Dye Was Measured By A Plate Reader (ratio between OD of plates at 492 nm). Results Are The Mean of Triplicate Wells.