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**DNA DAMAGE AND APOPTOSIS INDUCTION IN L1210
CELLS BY *CIS*-DIAMMINEDICHLOROPLATINUM(II)
AND ITS NEW AMINOFLAVONE ANALOGUE**

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Abstract: This work compares the biological properties of *cis*-diammine-dichloroplatinum (cisplatin) and its new analogue *cis*-[Pt(AF)₂Cl₂] (AF stands for 3-aminoflavone), which contains two aminoflavone substituents as non-leaving ligands. Both compounds were tested for their antiproliferative activity against cultured L1210 cells, and their DNA interstrand crosslinking activity in cells and in a cell-free system. Cisplatin was found to be an approximately 6 times more cytotoxic drug than its new analogue. Platinum complexes reacted with purified calf thymus DNA in a cell-free system producing DNA interstrand crosslinks. The kinetics of crosslink formation was very similar for both compounds but the maximal level of crosslinks was 20% higher for cisplatin. In cells, however, crosslinks were produced by cisplatin, whereas this type of DNA lesion was almost undetected in cells treated with the aminoflavone analogue as assayed by DNA alkaline elution. At higher drug concentrations, strong degradation of DNA was observed in L1210 cells treated with *cis*-[Pt(AF)₂Cl₂] but not in the cells incubated with cisplatin. This DNA degradation seems to reflect very efficient apoptosis induction by *cis*-[Pt(AF)₂Cl₂] as the electrophoretic patterns of DNA from cells incubated with this drug showed a ladder typical for apoptotic cells.

Key Words: Cisplatin Analogue, DNA Damage, Apoptosis

INTRODUCTION

The antineoplastic agent *cis*-diamminedichloroplatinum (cisplatin) has been shown to be active against several human malignant diseases, such as: lung, bladder, neck and head, ovarian and especially testicular cancers [1]. Despite the clinical success of cisplatin, its effectiveness is limited due to some nephrotoxic side effects, probably the main problem with this drug [2]. The mechanism of cisplatin cytotoxic action is not fully understood but it is rather generally accepted that the ultimate cellular target for this drug is DNA [3]. The reaction of cisplatin with DNA leads to the binding of Pt to guanine(s) at N7 sites giving several types of DNA adducts: monoadducts, interstrand crosslinks, intrastrand crosslinks and DNA-protein crosslinks. As a result of the formation of DNA intrastrand crosslinks a specific distortion of DNA occurs, which seems to be linked with the cytotoxicity and antitumor activity of cisplatin [3]. Although the mechanism of cisplatin-derived nephrotoxicity is not clear, there are numerous data suggesting that the inactivation of some kidney enzymes, which occurs by reaction of cisplatin or its active species with the sulphhydryl groups of proteins, may be responsible for this side effect [4]. Thus, obtaining coordination complexes of a reduced rate of reaction with sulfur compounds may be the rationale behind the design of new platinum drugs. One of the possible ways to synthesize platinum compounds with such properties is to introduce bulky, planar amine ligands instead of aliphatic amines into the Pt complex [4].

In this work a new cisplatin analogue *cis*-[Pt(AF)₂Cl₂], which contains two bulky aminoflavone substituents in the place of ammonia groups (Fig. 1), was tested for its ability to produce DNA damage in a cell-free system as well as in L1210 cells. Cisplatin was used as a reference compound in all the experiments.

MATERIALS AND METHODS

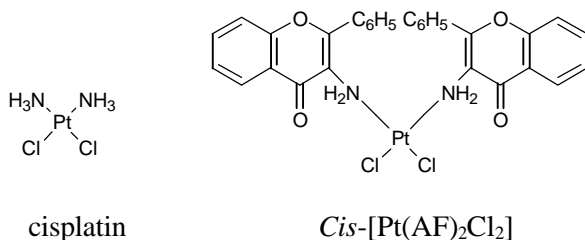


Fig. 1. The structure of the platinum coordination complexes

Cisplatin was synthesized according to Dhara [5]. *Cis*-[Pt(AF)₂Cl₂] was synthesized as described earlier [6]. A water solution of K₂PtCl₄ (0.415 g, 1mM) was added to the solution of 3-aminoflavone (0.474 g, 2 mM) in ethanol

(80 ml). The reaction mixture was stirred for 6 h at room temperature. The precipitate of *cis* - [Pt(AF)₂Cl₂] was filtered, washed with ethanol and dried in a vacuum. The purity was checked by IR, ¹H NMR and ¹⁹⁵Pt NMR spectroscopy. The drugs were dissolved in dimethylsulfoxide (ICN, USA) immediately before use.

Cells and cytotoxicity assay

Mouse leukemia L1210 cells were cultured in RPMI 1630 medium (Sigma, USA) supplemented with 10% foetal calf serum (Gibco, USA), gentamycin (50 µg/ml) and 0.02 M HEPES buffer (Gibco, USA). Cytotoxic effects were assayed by measuring the inhibitory effects on L1210 cell proliferation. In this assay, cells were seeded in 2 ml aliquots in 6 ml tissue culture tubes (Corning, USA) at a concentration of 5x10³ cells/ml and exposed to drugs for 72 h at 37°C. After this time the cell number relative to the control was determined by the colorimetric tetrazolium dye method [7].

DNA interstrand crosslinking

DNA crosslinking in a cell-free system was determined by the thermal denaturation method [8]. Calf thymus DNA (Worthington, England) at a concentration of 500 µg/ml in 0.05 M Tris/HCl pH 7 was incubated with platinum compounds for 24 h at 37°C. Aliquots of DNA solutions were taken at different times of incubation and DNA was precipitated with ice-cold ethanol, then dissolved in a 0.05 M Tris/HCl pH 7 buffer. Samples of DNA were heated in boiling water for 10 min in sealed glass ampoules and then cooled in an ice-water bath. The fraction of crosslinked DNA was estimated by measuring the A₂₆₀ of DNA solutions before denaturation and after renaturation [8].

The presence of DNA interstrand crosslinks in cells was assayed by means of alkaline elution [9]. L1210 cultures at a density of 2x10⁵ cells/ml were incubated with 0.02 µCi/ml of [¹⁴C]thymidine (Chemapol, Czech Republic) for 24 h and then treated with drugs for 2 h at 37°C. The suspensions were centrifuged, the pellets were washed with cold PBS and alkaline elution was performed essentially as described [9], except that the pumping rate was 0.1 ml/min and the tetrapropylammonium hydroxide in the elution buffer was replaced by tetraethylammonium (Sigma, USA). Lysates from cells treated with platinum coordination complexes were deproteinized by 30-min digestion at room temperature with 0.5 mg/ml of proteinase K (Sigma, USA) dissolved in 0.01 M EDTA pH 10. In the interstrand crosslink assay, the control and drug-treated cells were irradiated on ice with a dose of 3 Gy of γ radiation.

DNA degradation

To measure the content of fragmented DNA in the cell population, cells were labelled as described in the above paragraph and treated with drugs for 3 h at 37°C in a growth medium. Then cells were lysed on polycarbonate filters

(Millipore, USA, pore diameter = 0.8 μm) with 5 ml of 2% sodium dodecyl sulphate dissolved in 0.01 M EDTA pH 10). DNA degradation was expressed as the percentage of DNA which passed through the filter.

In order to check whether DNA degradation may be a result of apoptosis, gel electrophoresis of DNA extracted from cells was performed according to Gong *et al.* [10]. In these experiments L1210 cells were treated with platinum compounds, collected by centrifugation and fixed in 70% ethanol. The cells were then centrifuged at 1500 rpm for 5 min to remove the ethanol. The cell pellets were resuspended in 0.5 ml of pH 8 buffer (45 mM Trisphosphate-borate, 1 mM EDTA, 0.25% Nonidet) and digested by DNase-free RNase A (Sigma, USA, 1 mg/ml) for 30 min at 37 $^{\circ}\text{C}$ and later by proteinase K (1 mg/ml, 30 min at 37 $^{\circ}\text{C}$). After digestion, 0.1 ml of loading buffer (0.25% bromophenol blue, 30% glycerol) was added and 70 μl of DNA solutions were applied on the 1.8% agarose gel containing 0.5 $\mu\text{g/ml}$ of ethidium bromide. Electrophoresis was performed at 1.2 V/cm for 17 h. The DNA in the gels was visualized under ultraviolet light and photographed using Ilford FP4 negative film (England).

RESULTS

Cytotoxic activity

Both compounds were tested for their antiproliferative activity against cultured L1210 cells. Cisplatin was found to be more cytotoxic than its new analogue with bulky aminoflavone substituents. In terms of IC_{50} (the drug concentration inhibiting 50% of the cell growth after 72 h exposure of L1210 cells to the drug) cisplatin was about 6 times more active (Fig. 2). The IC_{50} values for cisplatin and *cis*-[(AF) $_2$ PtCl $_2$] were 0.65 μM and 3.5 μM respectively.

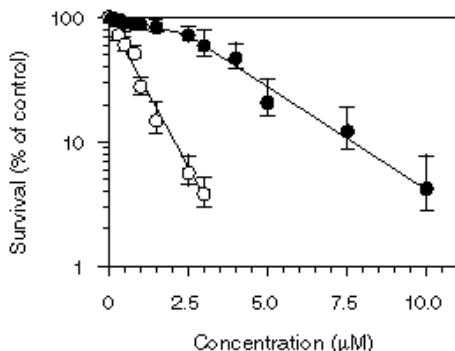


Fig. 2. The inhibitory effects of cisplatin and *cis*-[(AF) $_2$ PtCl $_2$] on the proliferation of cultured L1210 cells. Cells were exposed to platinum complexes for 72 h in a growth medium. Relative cell number was estimated by a colorimetric assay as described in Materials and Methods. Empty symbols cisplatin, filled symbols *cis*-[(AF) $_2$ PtCl $_2$]. Points represent data from three independent experiments \pm S.D.

DNA interstrand crosslink formation in a cell-free system

Both platinum coordination complexes react with DNA producing DNA interstrand crosslinks. Cisplatin and *cis*-[Pt(AF)₂Cl₂] exhibited a similar rate of crosslink formation and reached a plateau at 6 hours of incubation. However, cisplatin was more active and produced about a 20% higher level of crosslinks (Fig. 2).

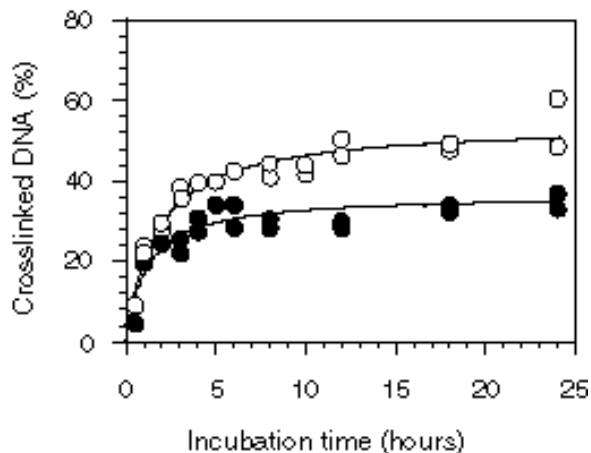


Fig. 3. The kinetics of calf thymus DNA crosslinking by platinum complexes. Calf thymus DNA (500 $\mu\text{g/ml}$) in 0.05 M Tris/HCl pH 7 was incubated with 100 μM platinum at 37°C. DNA was precipitated with ice-cold ethanol and dissolved in a 0.05 M Tris/HCl buffer. The fraction of crosslinked DNA was estimated by the thermal denaturation method. Empty symbols cisplatin, filled symbols *cis*-[(AF)₂PtCl₂]. Points represent data from two independent experiments.

DNA interstrand crosslinking in L1210 cells

The presence of interstrand crosslinks in L1210 cells treated with platinum coordination complexes was assayed by means of alkaline elution. As crosslink formation in the cell by cisplatin is a rather slow process, cells were exposed to the drugs for three hours and then incubated in a drug-free medium up to 12 hours. 2.5 μM cisplatin induced a high frequency of crosslinks which reached a maximal level at 6 h of post-drug incubation. Crosslinks induced by 2.5 μM *cis*-[(AF)₂PtCl₂] could be detected only at the beginning of post-drug incubation. After three hours of incubation in a drug-free medium these lesions could not be detected (Fig. 4). When *cis*-[(AF)₂PtCl₂] was used at concentrations higher than 2.5 μM a strong degradation of DNA occurred, which masked the possible presence of DNA crosslinks.

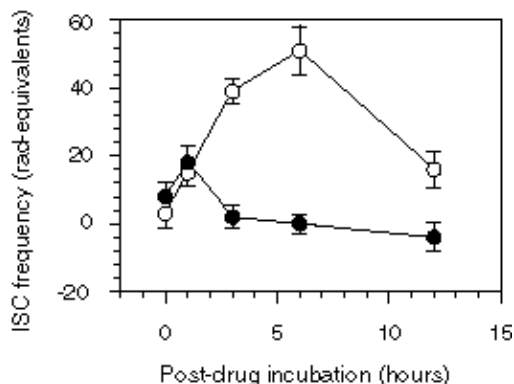


Fig. 4. The kinetics of DNA interstrand crosslink formation and removal in L1210 cells treated with platinum coordination complexes. Cells were incubated with drugs for 3 hours at 37 °C and then post-incubated in a drug-free medium up to 12 h. Crosslink frequencies were estimated by DNA alkaline elution. Empty symbols cisplatin, filled symbols *cis*-[(AF)₂PtCl₂]. Data are the means of two independent experiments ± range of results.

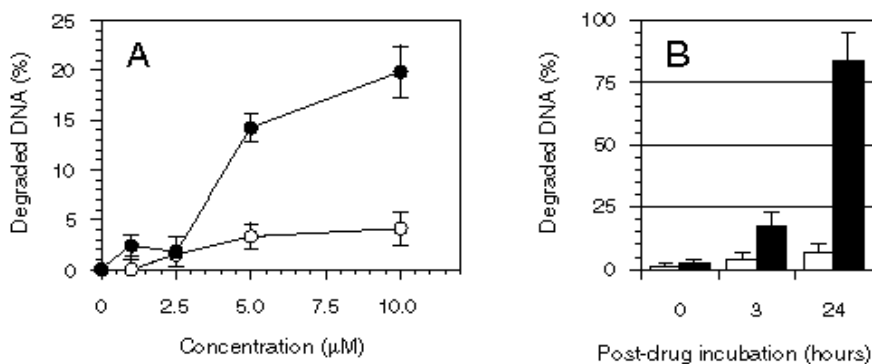


Fig. 5. Degradation of DNA in L1210 cells incubated with platinum coordination complexes. Cells with ¹⁴C labelled DNA were incubated with drugs for 3 h at 37 °C and then post-incubated in a drug-free medium up to 24 h. Cells were lysed on polycarbonate filters as described in the Materials and Methods section. The fraction of high molecular DNA adsorbed on the filters and the fraction of degraded DNA which passed through the filter in the lysis solution were estimated according to the procedure of Kohn [7]. Panel A – dependence of the amount of degraded DNA on platinum complex concentration. Panel B – increase of DNA degradation during post-drug incubation. Cells were treated with 10 µM drugs and then post-incubated in a fresh medium. Empty symbols and bars cisplatin, filled symbols and bars *cis*-[(AF)₂PtCl₂]. Data are the means of two independent experiments ± range of results.

DNA degradation

A significant difference in the DNA breakage potency of platinum complexes was found. At concentrations exceeding 2.5 μM , *cis*-[(AF)₂PtCl₂] produced very extensive DNA fragmentation in L1210 cells whereas cisplatin was barely active in this process (Fig. 5A). This fragmentation was not detected immediately after drug treatment (data not shown) but during post-drug incubation (Fig. 5B). DNA breakage produced by *cis*-[(AF)₂PtCl₂] was detected at pH 10, when the DNA helix exists in its double stranded form. This result shows that DNA degradation caused by *cis*-[(AF)₂PtCl₂] resulted from the formation of double strand breaks.

Degraded DNA may reflect the presence of necrotic or apoptotic cells in the cell population. In cells undergoing apoptosis, a fraction of the nuclear DNA is cleaved, giving short fragments of size equivalent to DNA in mono- or oligonucleosomes. Therefore, DNA extracted from apoptotic cells and subjected to agarose gel electrophoresis gives the characteristic ladder pattern of DNA fragments. To distinguish whether DNA degradation caused by *cis*-[(AF)₂PtCl₂] corresponds to apoptosis or necrosis, DNA from L1210 cells was analysed by agarose gel electrophoresis. As seen in Fig. 6 (lines 6, 8, and 10) characteristic DNA laddering was observed in the case of DNA from the cells treated with *cis*-[(AF)₂PtCl₂]. No such effects were observed in DNA from cells treated with cisplatin under the same conditions (data not shown).

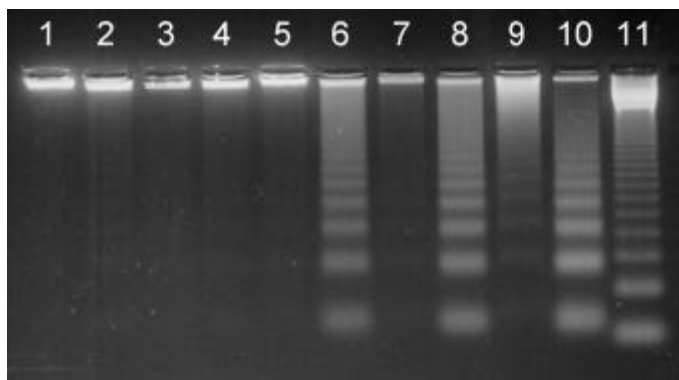


Fig. 6. Apoptotic DNA fragmentation caused by *cis*-[Pt(AF)₂Cl₂] in L1210 cells. The image shows the results of electrophoresis of DNA from cells treated with the drug.

Lines: **1** - control; **2** - control (20 h post-incubation); **3** - control + DMSO (4 h treatment); **4** - control + DMSO (4 h treatment + 20 h post-incubation); **5** - 5 μM *cis*-[Pt(AF)₂Cl₂] (4 h treatment); **6** - 5 μM *cis*-[Pt(AF)₂Cl₂] (4 h treatment + 20 h post-incubation); **7** - 10 μM *cis*-[Pt(AF)₂Cl₂] (4 h treatment); **8** - 10 μM *cis*-[Pt(AF)₂Cl₂] (4 h treatment + 20 h post-incubation); **9** - 50 μM *cis*-[Pt(AF)₂Cl₂] (4 h treatment); **10** - 50 μM *cis*-[Pt(AF)₂Cl₂] (4 h treatment + 20 h post-incubation); **11** - marker (DNA ladder 123 bp. DMSO – dimethylsulfoxide).

DISCUSSION

The replacement of ammonia groups in the cisplatin molecule by bulky aromatic amines may influence the rate of the reaction of the platinum complex with DNA [4]. This study focused on the DNA damaging properties of *cis*-[Pt(AF)₂Cl₂], a novel analogue of cisplatin with two aminoflavone rings used as non-leaving ligands (Fig. 1). The binding stoichiometry of *cis*-[Pt(AF)₂Cl₂] to DNA was not estimated in this work but it seems that the presence of aminoflavone rings reduces the yield of the reaction of platinum coordination complexes. This is suggested by the fact that the level of DNA interstrand crosslinks induced in a cell-free system by *cis*-[Pt(AF)₂Cl₂] was lower in the case of cisplatin (Fig. 3). This observation fits with the cytotoxicity of *cis*-[Pt(AF)₂Cl₂] being lower than that of cisplatin (Fig. 2).

A striking difference between cisplatin and its analogue was observed at the cellular level of their action. It is well known that cisplatin induces DNA intrastrand crosslinks and interstrand crosslinks, which were postulated as cytotoxic lesions [3, 11]. Both tested compounds were active in DNA interstrand crosslink formation in L1210 cells; however, they exhibited very different crosslink formation and removal kinetics (Fig. 4). Crosslinks produced by *cis*-[Pt(AF)₂Cl₂] disappeared 3 hours after drug removal, whereas crosslinks produced by cisplatin could be detected after 12 hours of post-drug incubation. This result does not necessarily mean that DNA interstrand crosslinks caused by *cis*-[Pt(AF)₂Cl₂] are repaired quickly. This is because *cis*-[Pt(AF)₂Cl₂] produced a strong degradation of DNA during post-drug incubation (Fig. 5). As the presence of DNA breaks is known to interfere with the interstrand crosslinks assay, giving an artificially low level of crosslinks [9], it seems possible that crosslinks produced by *cis*-[Pt(AF)₂Cl₂] are still present in the cellular DNA, but are undetectable due to the high DNA break frequency.

DNA breakage by *cis*-[Pt(AF)₂Cl₂] appears to be its major cellular lesion. The mechanism by which *cis*-[Pt(AF)₂Cl₂] produces DNA breaks is not clear. For some compounds, DNA breaks may be the primary effect of their action, as in the case of the generators of reactive oxygen species [12]. There are also DNA interacting agents forming DNA adducts which induce secondary DNA breaks as a result of repair processes, spontaneous chemical reactions [12, 13], and even necrosis or apoptosis. DNA breaks caused by free radicals or breaks occurring in necrotic cells can easily be distinguished from those appearing in cells which undergo apoptosis. Whereas DNA breaks are distributed randomly in necrotic cells and in cells subjected to agents which generate free radicals, breaks in apoptotic cells are localized in the internucleosome sections [12, 13]. Therefore, DNA extracted from necrotic cells or cells subjected to free radicals action gives a smear without distinct bands. In contrast, DNA from apoptotic cells gives a characteristic ladder consisting of many DNA bands of size corresponding to mono- and oligonucleosomes [8, 14]. This type of laddering

was seen on the agarose gel where DNA from cells treated with *cis*-[Pt(AF)₂Cl₂] was analysed (Fig. 6). Thus, this data suggest that DNA breaks produced by the novel analogue of cisplatin are the secondary lesion presumably reflecting the apoptosis process induced by this compound. As Sorenson *et al.* [17] demonstrated, cisplatin induces apoptosis in L1210 cells. This process was found to be rather slow, with DNA breakage only becoming visible two days after drug treatment [17]. As post-drug incubation was limited to 24 hours in our experiments, DNA breaks were not detected in the cells treated with cisplatin.

It is currently unknown why *cis*-[Pt(AF)₂Cl₂] is such an effective apoptosis inducer with potency higher than that of cisplatin. As the new analogue reacts with DNA forming interstrand crosslinks and presumably also other types of adduct, it can be hypothesized that adducts with bulky aminoflavone rings are strong signals for proteins which are involved in the recognition of DNA damage and participate in triggering apoptosis. Taking into account that *cis*-[Pt(AF)₂Cl₂] has recently been found to be an active antitumor agent *in vivo* [15] and that induction of apoptosis may have clinical significance [16], the high ability of the novel analogue to induce apoptosis seems to be a very advantageous feature of this compound.

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REFERENCES

1. Loehrer, P.J. and Einhorn, L.H. Cisplatin. **Ann. Intern. Med.** 100 (1984) 704-713.
2. Wong, E. and Giandomenico, C. M. Current status of platinum-based antitumor drugs. **Chem. Rev.** 99 (1999) 2451-2466.
3. Jamieson, E.R., Lippard, S.J. Structure, recognition, and processing of cisplatin-DNA adducts. **Chem. Rev.** 99 (1999) 2467-2498.
4. Reedijk, J. Why does cisplatin reach guanine -N7 with competing S-donor ligands available in the cell? **Chem. Rev.** 99 (1999) 2499-2510.
5. Dhara, S. C. A rapid method for the synthesis of *cis*-[Pt(NH₃)₂Cl₂]. **India J. Chem.** 8 (1970) 193-194.
6. Ochocki, J., Zyner, E. Nowe związki platyny(II) z 3-aminoflawonem oraz sposoby otrzymywania nowych związków platyny(II) z 3-aminoflawonem. **Biuletyn Urzędu Patentowego Nr 17 (669)**(1999 41-42.
7. Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D. and Mitchell, J.B. Evaluation of tetrazolium-based semiautomatic colorimetric assay: assessment of chemosensitivity testing. **Cancer Res.** 47 (1987) 936-942.

8. Dall'Acqua, F., Marciani, S., Ciavatta ,L., Rodighiero, G. Formation of inter-strand cross-linking in the photoreactions between furocoumarins and DNA. **Z. Naturforsch B** 26 (1971) 561-569.
9. Kohn, K.W. Principles and practice of DNA filter elution. **Pharmacol. Ther.** 49 (1991) 55-77.
10. Gong, J., Traganos, F. and Darzynkiewicz Z. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. **Anal. Bioch.** 218 (1994) 314-319.
11. Zwelling, L.A. Cisplatin and new platinum analogs. **Cancer Chemotherapy** 8 (1986) 97-116.
12. Kohn, K.W. DNA as a target in cancer chemotherapy: measurement of macromolecular DNA damage produced in mammalian cells by anticancer agents and carcinogens. **Methods Cancer Res.** 16 (1979) 291-345.
13. Studzian, K., Kinas, R., Ciesielska, E., Szmigiero, L. Effects of alkylating metabolites of ifosfamide and its bromo analogues on DNA of HeLa cells. **Bioch. Parmacol.** 43 (1992) 937-943.
14. Arends, M.J., Morris, R.G., Wyllie, A.H. Apoptosis. The role of the endonuclease. **Am. J. Pathol.** 134 (1990) 593-608.
15. Uren, A.G., Vaux, D.L. Molecular and clinical aspects of apoptosis. **Pharmacol. Ther.** 72 (1996) 37-50.
16. Zyner, E., Graczyk J., Ochocki, J. Pt(II) and Pd(II) complexes of 3-amino-flavone: *in vitro* and *in vivo* evaluation. **Pharmazie** 54 (1999) 945-946.
17. Sorenson, C.M., Barry, M.A., Eastman, A. Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. **J. Natl. Cancer Inst.** 82 (1990) 749-745.