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EVALUATION OF ANTIPROLIFERATIVE POTENCY AND INDUCED BIOCHEMICAL PARAMETERS OF NOVEL PYRIDINE DERIVATIVES AGAINST LEUKEMIA, LUNG, BREAST AND COLON CANCER CELL LINES

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ABSTRACT

On the basis of monitoring the inhibition of the growth of human cancer cells, a series of novel Pyridine derivatives reacted with different phosphorus nucleophiles to afford novel phosphanylidene compounds that thought to possess a broader spectrum of antitumor activity and fewer toxic side effects than traditional anticancer drugs were synthesized. Nine of the newly synthesized compounds (**5a**, **5b, 6, 12a, 12b, 12d, 15b, 16, 21)** were subjected to a screening system for investigation of their antiproliferative potency against human promyelocytic leukemia (HL-60) in comparison to the traditional anticancer drugs: cisplatin (CIS) and doxorubicin (DOX). Results showed that the highest *in vitro* cytotoxic activity against HL-60 cell line (IC_{50} lower than 12 µg/ml) revealed compounds: **5a, 6, 5b**. Those compounds were chosen for testing their activity towards lung (A549), breast (T-47D) and colon (LoVo) cancer cell lines. Compound 6 showed the highest potency against all tested cancer cells. Moreover, the biochemical effects of the selected Pyridine derivatives on some enzymes such as aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP), in addition to albumin, globulins, creatinine, total lipids, cholesterol,

triglycerides and bilirubin in serum of mice were studied in comparison to Cisplatin and Doxorubicin.

On the other hand, results of the biochemical investigations indicated that Cisplatin and Doxorubicin caused significant changes in the level of all parameters tested while treatment with the selected compounds showed slight, moderate or no significant changes. In this study, we have identified phosphanylidene derivatives as a novel class of compounds possessing anti-proliferative activity. The lead compounds **5a and 6** were the most potent in the biological assay employed (e.g.: similar or improved growth inhibition potential as compared to the reference anticancer drugs cisplatin and doxorubicin).These experimental findings may provide support for the use of these novel compounds as anticancer agents.

KEYWORDS: Phosphanylidene derivatives, Cytotoxic activity, leukemia (HL-60), lung cancer (A549), breast adenocarcinoma (T-47D), human colon cancer cells (LoVo).

INTRODUCTION

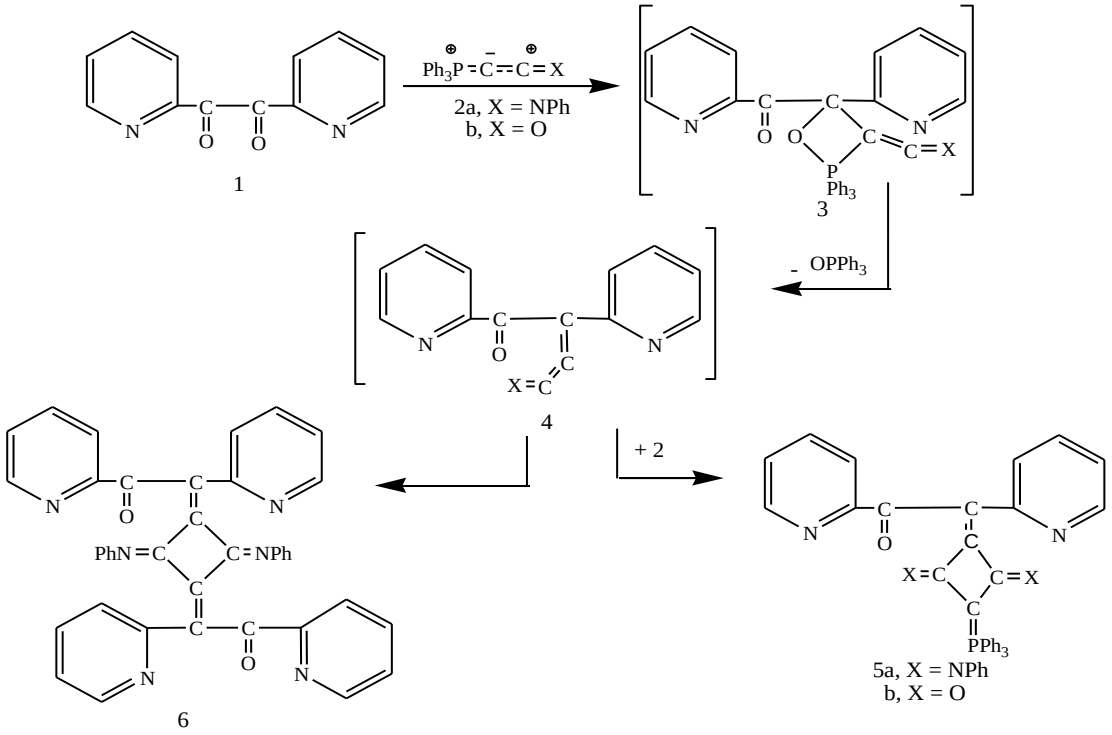
Pyridine derivatives have attracted intense interest in recent years due to their broad spectrum of biological and pharmacological activities as they are widely used in medicine as anticancer drugs.[1] antihypertension medications.[2] antibacterial.[3] and antiviral.[4] On the other hand, Phosphorus ylides were used in a wide variety of reactions of interest to synthetic chemists, especially in the synthesis of naturally occurring compounds with biological and pharmacological activity.[5-11]

Based on these findings the present work aimed to investigate the in vitro cytotoxic activity of newly synthesized phosphanylidene derivatives.[12] against promyelocytic leukemia (HL-60), lung carcinoma (A549), breast adenocarcinoma (T-47D) and human colon cancer cells (LoVo) cancer cell lines in comparison to the traditional anticancer drugs Cisplatin and Doxorubicin. Moreover, the biochemical effects of some selected phosphanylidene derivatives on some enzymes such as aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP), in addition to albumin, globulins, creatinine, total lipids, cholesterol, triglycerides and bilirubin in serum of mice were studied in comparison to Cisplatin and Doxorubicin.

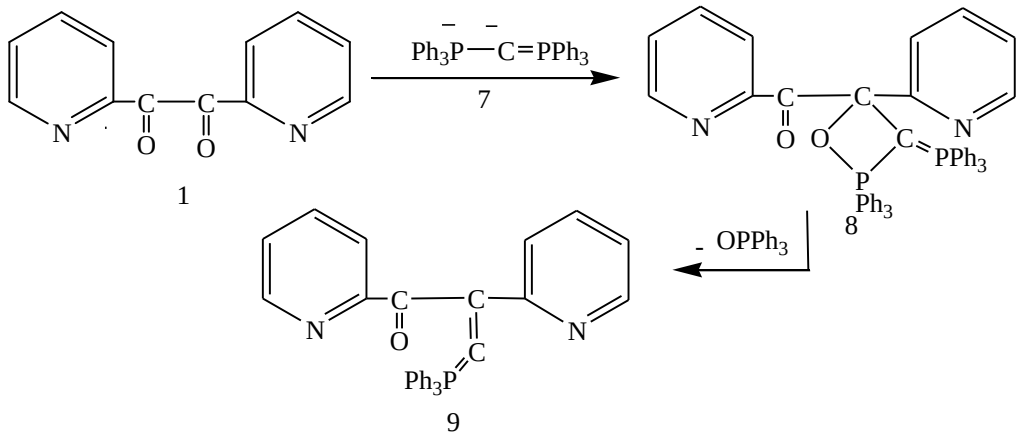
MATERIALS and METHODS

1- Synthesis of phosphanylidene derivatives

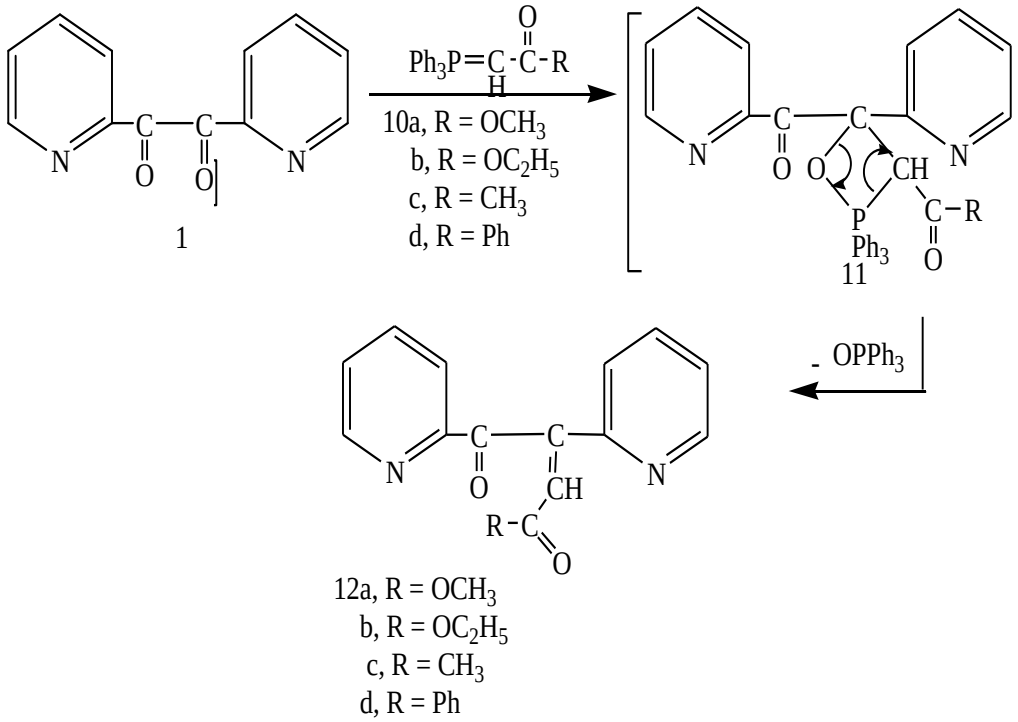
The rationales for the synthesis and characterization of the selected phosphanylidene derivatives (compounds **5a**, **5b, 6, 12a, 12b, 12d, 15b, 16, 21**), by elemental analysis, infrared, electronic spectra, room temperature magnetic measurements and powder X-ray diffraction were previously published by Soliman *etal*.[12]



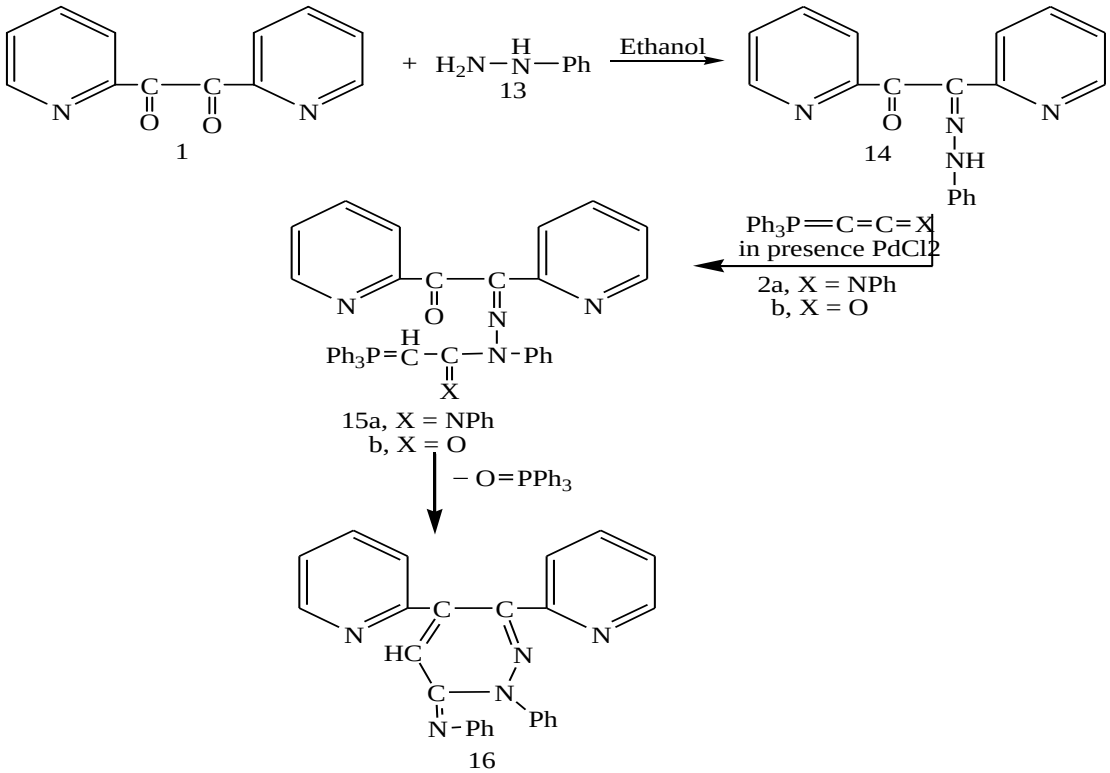
Scheme 1: Synthesis of Compounds 5a,b and 6 Cited from Soliman et al.[12]



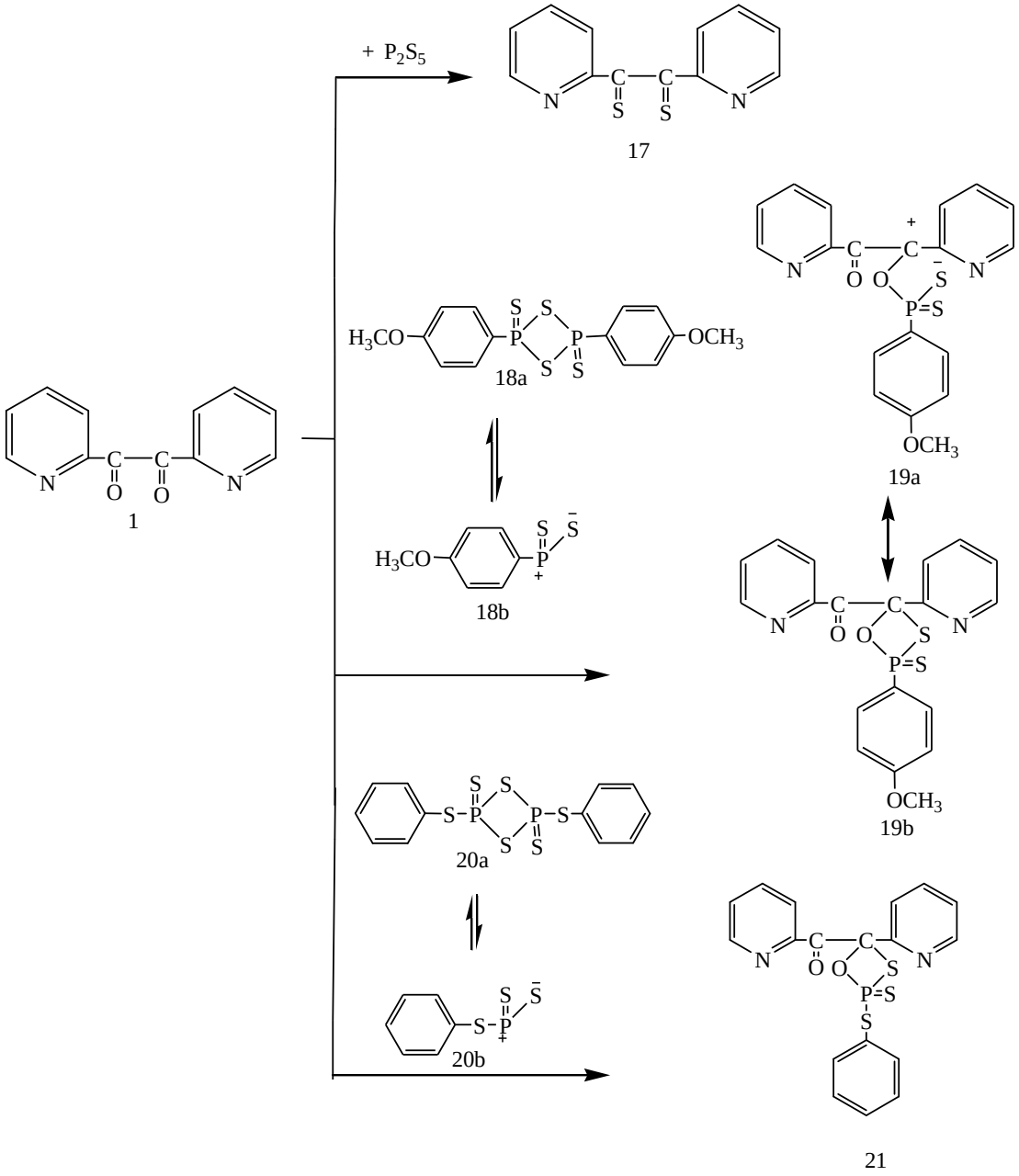
Scheme 2: Synthesis of Compounds 8 and 9 Cited from Soliman et al.[12]



Scheme 3: Synthesis of Compounds 12a-d Cited from Soliman et al.[12]



Scheme 4: Synthesis of Compounds 14-16 Cited from Soliman et al [12]



Scheme 5: Synthesis of Compounds 17, 19 and 21 Cited from Soliman et al [12]

2- Antiproliferative Activity

Nine of the newly synthesized compounds (**5a**, **5b, 6, 12a, 12b, 12d, 15b, 16, 21)** were selected to test their antiproliferative potency towards the human promyelocytic leukemia (HL-60) and the most active compounds (**5a, 5b, 6)** were tested on lung cancer (A549), breast adenocarcinoma (T-47D) and human colon cancer cells (LoVo) in comparison with the known anticancer drugs: cisplatin (CIS) and doxorubicin (DOX) using MTT and SRB assays.

Structure formula of the selected phosphanylidene derivatives (compounds **5a**, **5b, 6, 12a, 12b, 12d, 15b, 16, 21**), selected from Schemes 1 - 5, are as follows**:**

5a:*2,4-Bis(phenylimino)-1-(2-pyridyl)-3-(triphenyl-λ5-phosphanylidene)cyclobutyl]-(2-pyridyl)methanone.*

5b:*2-(pyridine-2-carbonyl)-2-(2-pyridyl)-4-(triphenyl-λ^5-phosphanylidene)cyclobutane-1,3-dione* .

6:*2-[3-[2-oxo-1,2-bis(2-pyridyl)ethylidene]-2,4-bis(phenylimino)cyclobutylidene]-1,2-bis(2-pyridyl)ethanone.*

12a: *Methyl 4-oxo-3,4-bis(2-pyridyl)but-2-enoate* .

12b: *Ethyl 4-oxo-3,4-bis(2-pyridyl)but-2-enoate* .

12d: *4-phenyl-1,2-bis(2-pyridyl)but-2-ene-1,4-dione* .

15b:*N-[2-oxo-1,2-bis(2-pyridyl)ethylidene]amino]-N-phenyl-2-(triphenyl-λ^5-phosphanylidene)acetamide.*

16: *N,2-diphenyl-5,6-bis(2-pyridyl)pyridazin-3-imine* **.**

17: *1,2- Bis(2-pyridyl))ethane-1,2-dithione* **.**

21:*[2-phenylsulfanyl-4-(2-pyridyl)-2-thioxo-1,3,2λ^5-oxathiaphosphetan-4-yl]-(2 pyridyl)methanone* **.**

Tested compounds

Series 1: **5a**, **5b, 6, 12a, 12b, 12d, 15b, 16, 21**

Series 2: **5a**, **5b, 6**

Compounds from series 1 were tested on the human promyelocytic leukemia (HL-60) and compounds from series 2 were tested on lung cancer (A549), breast adenocarcinoma (T-47D) and human colon cancer cells (LoVo). Reference compounds were cisplatin and doxorubicin, whereas control of the dissolvent was DMSO, tested in the same concentration as compound probes. Test solutions of the 23 compounds tested (1 mg/ml) were prepared by dissolving the substances in 100 µl of DMSO completed with 900 µl of tissue culture medium. Afterwards, the tested compounds were diluted in culture medium to reach the final concentrations of 10, 1, 0.1, 0.01 and 0.001 µg/ml.

Cell lines

Established *in vitro*, human cell line: HL-60 (human promyelocytic leukemia), A549 (lung cancer), T-47D (breast adenocarcinoma) and LoVo (human colon cancer cells) were used.

These lines were maintained at the Institute of Immunology and Experimental Therapy (IIET), Wroclaw, Poland.

HL-60 cell line was cultured in RPMI 1640 medium (Gibco, Scotland, UK) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 1.0 mM sodium pyruvate, 10% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). LoVo cells were cultured in RPMI 1640+Opti-MEM (1:1) (both from IITD, Wroclaw, Poland), supplemented with 2 mM L-glutamine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 5% fetal bovine serum (Thermo-Fisher Scientific Oy, Vataa, Finland) and with 1.0 mM of sodium pyruvate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). A549 cells were maintained in RPMI-1640 GlutaMAX (Gibco, Scotland, UK) medium containing 1.0 mM sodium pyruvate, 4.5 g/L glucose (both from Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and supplemented with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). T47D cells were cultured in RPMI 1640+Opti-MEM (1:1) (both from Gibco, Scotland, UK), supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, 10% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The culture of T47D cells was supplemented with 0.8 mg/L of insulin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All culture media were supplemented with 100 units/ml penicillin, and 100 µg/ml streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland). All cell lines were grown at 37°C with 5% CO_2 humidified atmosphere.

Anti-proliferative assay in vitro

Twenty four hours prior to the addition of the tested compounds, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of 1×10^4 or 0.5×10^4 cells per well. The assay was performed after 72 h of exposure to varying concentrations of the tested agents. The *in vitro* cytotoxic effect of all agents was examined using the MTT (HL-60) or SRB (A549, T-47D, LoVo) assay. The results were calculated as an IC_{50} (inhibitory concentration 50) – the dose of tested agent which inhibits proliferation of 50% of the cancer cell population. Each compound in each concentration was tested in triplicate in a single experiment, which was repeated at least 3 times.

MTT assay[13]

This technique was applied for the cytotoxicity screening against leukemia cells growing in suspension culture. An assay was performed after 72-hours exposure to varying

concentrations (from 0.001 to 10 µg/ml) of the tested agents. For the last 3-4 hours of incubation 20 µl of MTT solution were added to each well (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; stock solution: 5 mg/ml, Sigma-Aldrich, Germany). The mitochondria of viable cells reduce the pale yellow MTT to a navy blue formazan. When incubation time was completed, 80 µl of the lysing mixture were added to each well (lysing mixture: 225 ml dimethylformamide, POCh, Gliwice, Poland, 67.5 g sodium dodecyl sulphate, Sigma-Aldrich, Germany, and 275 ml of distilled water). After 24 h, when formazan crystals had been dissolved, the optical densities of the samples were read on Synergy H4 photometer (BioTek Instruments, USA) at 570 nm wavelength. Each compound in given concentration was tested in triplicates in each experiment, which was repeated at least 3 times.

SRB assay[14]

This technique was applied for the cytotoxicity screening against cells growing in adherent culture. The cytotoxicity assay was performed after 72-hour exposure of the cultured cells to varying concentrations (from 0.01 to 10 µg/ml) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% TCA (trichloroacetic acid, Aldrich-Chemie, Germany) on the top of the culture medium in each well. The plates were incubated at 4°C for 1h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.1% sulforhodamine B (SRB, Sigma, Germany) dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 minutes. Unbound dye was removed by rinsing (4x) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base (Sigma, Germany) for determination of optical density (at 540 nm) on Synergy H4 photometer (BioTek Instruments, USA).

3- Biochemical Analysis

Animals

Male albino mice weighing 18-20 g were used in the present study. Mice were divided into three main groups as follows:

1- Group (1): Untreated or control group (5 mice).
2- Group (2): divided into two subgroups (5 mice for each subgroup) and treated with CIS or DOX as reference anticancer drugs.

3- Group (3): divided into eight subgroups (5 mice for each subgroup) and treated with the selected pyridine derivatives (compounds **5a**, **5b, 6, 12a, 12b, 12d, 15b, 16, 21)**.

Treatment

Group (1): each mouse was given a single intraperitoneal injection of 0.1ml DMSO.

Group (2): each mouse was given a single intraperitoneal injection of 0.1ml containing 12 mg/kg body weight CIS or DOX dissolved in sterile water.

Group (3): each mouse was given a single intraperitoneal injection of 0.1ml containing 12 mg/kg body weight of the selected pyridine derivatives (compounds **5a**, **5b, 6, 12a, 12b, 12d, 15b, 16, 21** respectively) dissolved in DMSO.

Blood was collected after 7 days from all mice groups.

The biochemical effects of the selected pyridine derivatives on some liver enzymes such as aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP), were done using blood auto analyzer (Olympus AV 400, Japan).[15] Moreover, albumin,[16] globulins.[17] creatinine,[18] total lipids,[19] cholesterol,[20] triglycerides,[21] and bilirubin.[22] in serum of mice were evaluated in comparison to cisplatin (CIS) and doxorubicin (DOX).

Statistical analysis of the results was performed using Chi-square values (SPSS computer program).

RESULTS

The results of the studies on *in vitro* cytotoxic activity are summarized in Tables 1 and 2**.**

Table 1. Antiproliferative activity of synthesized compounds against HL-60 (human promyelocytic leukemia) cell line.

COMPOUNDS	HL-60
	IC_{50} ((µg/ml)
5a	1,22 ± 0,87
6	2,84 ± 0,39
5b	11,63 ± 6,63
12a	26,01 ± 8,72
12b	41,35 ± 5,51
12d	24,45 ± 7,60
15b	---
16	48,95 ± 26,65
21	39,37 ± 2,58
CISPLATIN	0,27 ± 0,03
DOXORUBICIN	0,06 ± 0,06

Results illustrated in table 1 showed that the highest *in vitro* cytotoxic activity against HL-60 cell line (IC_{50} lower than12 ug/ml) was shown by compounds: **5a, 6, 5b** respectively. These compounds were chosen for the next study against A549 (lung cancer), T-47D (breast adenocarcinoma) and LoVo (human colon cancer cells).

Table 2. Antiproliferative activity of selected compounds against A549, T-47D and LoVo cancer cell lines.

COMPOUNDS	A549	T-47D	LoVo
	IC50 ((µg/ml)	IC50 ((µg/ml)	IC50 ((µg/ml)
5a	5,23 ± 0,32	0,45 ± 0,05	20,28 ± 3,05
6	4,53 ± 0,65	0,54 ± 0,08	6,87 ± 4,56
5b	33,96 ± 3,85	13,09 ± 3,12	36,91 ± 3,99
CISPLATIN	2,36 ± 0,53	2,27 ± 0,25	0,88 ± 0,18
DOXORUBICIN	0,20 ± 0,06	0,09 ± 0,03	0,09 ± 0,06

Data on tables 1 and 2 indicated that compounds 6 and 5a showed the highest potency against all tested cancer cells.

Effect of selected new derivatives on the biochemical parameters in mice.

Data showed in table 3 presents the liver enzymatic activities (ALT, AST and ALP) in serum of control and treated groups of mice. The results showed that the values recorded for AST, ALT and ALP activities were significantly higher ($P < 0.001$) with CIS and DOX treated groups of mice than the control. On the other hand, treatment with the new derivatives (compounds **5a**, **5b, 6, 12a, 12b, 12d, 15b, 16, 21**), caused inverse effects, where some values recorded for AST and ALT were non significant (n.s.) or slightly higher ($P < 0.01$) in comparison to control. Data listed in table 4 demonstrates the comparison between the levels of total lipids, cholesterol, triglycerides and bilirubin in serum of treated mice and the control group. It can be deduced from the present data that CIS and DOX caused a significant increase in the level of these parameters while treatment with the selected new derivatives, showed moderate or no significant changes. Table 5 represents a comparison between the levels of albumin, globulins and creatinine in serum of control and treated groups of mice. It is clear from the results in the table that there was a slight increase in the level of albumin and creatinine and globulins in the CIS and DOX treated groups while there were moderate or non significant changes in pretreated groups with the selected new derivatives.

Table 3: Biochemical effects of CIS, Dox. and Pyridine derivatives on serum ALT, AST and ALP in mice.

Biochemical Parameters / Mice Groups	Alanine amino transferase Mean ± SD ALT (IU/ml)	Aspartate amino transferase Mean ± SD AST (IU/ml)	Alkaline phosphatase Mean ± SD ALP (k.k./dl)
Control	43.5 ± 2.03	108.32 ± 4.19	18.70 ± 1.10
Cisplatin P<	65.7±19.09 0.001	137.43 ± 8.92 0.001	29.48 ± 6.03 0.001
Doxorubcin P<	59.26 ± 12.03 0.001	147.22 ± 16.34 0.001	30.317 ± 5.14 0.001
5a P<	33.42 ± 7.05 n.s.	123.9 ± 11.4 0.01	18.83 ± 6.29 n.s.
5b P<	46.09±6.13 n.s.	110.54±12.7 0.01	19.94±4.35 n.s.
6 P<	39.56±6.7 n.s.	107.81 ± 4.25 n.s.	21.94 ± 3.4 0.01
12a P<	61.6±11.8 0.001	136.72±22.09 0.001	32.53±8.24 0.001
12b P<	43.73±4.7 n.s.	112.81±9.88 n.s.	21.59±3.42 n.s.
12d P<	73.09±14.2 0.001	140.09±31.01 0.001	43.7 ± 8.36 0.001
15b P<	39.56±6.7 n.s.	111.2 ± 11.05 n.s.	19.94±4.35 n.s.
16 P<	58.36±9.7 0.001	131.8±26.43 0.001	39.82±8.5 0.001
21 P<	68.34 ± 11.9 0.001	146.4 ± 28.1 0.001	44.26±7.01 0.001

Data are expressed as Mean + S.D.

P< 0.01: significant, P< 0.001: highly significant, n.s. : non significant

Table 4: Biochemical effects of CIS, Dox. and Pyridine derivatives on serum total lipids, cholesterol, triglycerides and bilirubin in mice.

Biochemical Parameter / Mice Groups	Total Lipids mg/dl	Cholestrol mg/dl	Triglycerides mg/dl	Bilirubin mg/dl
Control	323.41 ± 27.1	94.32 ± 13.5	108.7 ± 16.8	0.63± 0.04
Cisplatin P<	375.2±31.4	107.9±11.7	129.5±19.4	0.84± 0.10
Doxorubcin P<	366.7 ± 6.10	109.3 ± 14.2	137.8 ± 17.10	0.81± 0.19
5a P<	329.34 ± 19.7 n.s.	116.4 ± 8.3 0.01	98.4 ± 10.6 n.s.	0.65 ± 0.9 n.s.
5b P<	331.63± 17.5 n.s.	95.3 ± 9.4 n.s.	119.7 ± 18.8 0.01	0.68± 0.11 n.s.
6 P<	317.4 ± 30.7 n.s.	96.4± 10.5 n.s.	118.6± 19.70 0.01	0.51± 0.08 0.01
12a P<	367.52± 31.7	119.6± 23.8 0.001	127.8± 20.4 0.001	0.91± 0.1 0.001
12b P<	326.32± 19.3	96.5± 19.4 n.s.	114.6± 10.7 n.s.	0.66± 0.08 n.s.
12d P<	328.3 ± 13.7 n.s.	110.9± 31.2 0.01	152.6± 34.5 0.001	0.77± 0.2 0.01
15b P<	331.63± 17.5	96.4± 10.5 n.s.	118.6± 19.70 0.01	0.68± 0.11 n.s.
16 P<	368.71± 32.2	111.5± 16.9 0.01	92.3± 9.60 n.s.	0.91± 0.4 0.001
21 P<	382.09 ± 23.6 0.001	123.3 ± 26.09 0.001	112.3 ± 10.6 n.s.	0.79± 0.1 0.01

Data are expressed as Mean + S.D.

P< 0.01: significant, P< 0.001: highly significant, n.s. : non significant.

Table 5: Biochemical effects of CIS, Dox. and Pyridine derivatives on serum albumin, globulin, creatinine in mice.

Biochemical Parameters / Mice groups	Albumin mg/dl	Globulin mg/dl	A / G ratio	Creatinine mg/dl
Control	5.63 ±0.51	4.32 ± 0.9	1.3	0.69±0.03
Cisplatin P<	8.73±0.92 0.001	6.75±0.8 0.01	1.45 0.01	0.81± 0.06 0.01
Doxorubcin P<	6.37 ± 0.85 0.01	5.91 ± 0.63 0.01	1.078 0.01	0.78± 0.04 0.01
5a P<	7.2 ± 0.61 0.01	6.62 ± 0.86 0.01	1.05 0.01	0.76 ± 0.25 n.s.
5b P<	5.48 ± 0.91 n.s.	4.09± 0.63 n.s.	1.13 n.s.	0.68 ± 0.08 n.s.
6 P<	5.97± 0.34 n.s.	5.12 ± 0.9 n.s.	1.1 n.s.	0.77 ± 0.05 0.01
12a P<	6.38± 0.41 0.01	6.37± 0.81 0.01	1.002 0.001	0.78± 0.09 0.01
12b P<	5.66± 0.92 n.s.	4.87± 0.73 n.s.	1.16 n.s.	0.87± 0.3 0.01
12d P<	10.22± 1.35 0.001	8.96± 0.91 0.001	1.14 0.01	0.72± 0.21 n.s.
15b P<	5.97± 0.34 n.s.	4.09± 0.63 n.s.	1.46 n.s.	0.65± 0.09 n.s.
16 P<	6.91± 0.53 0.01	6.83± 0.90 0.01	1.01 0.001	1.53± 0.21 0.001
21 P<	11.43 ± 1.48 0.001	4.72 ± 0.91 n.s.	1.02 0.001	0.68 ± 0.08 n.s.

Data are expressed as Mean + S.D.

P< 0.01: significant, P< 0.001: highly significant, n.s. : non significant.

DISCUSSION

Cytotoxic drugs remain the main stay of cancer chemotherapy and are being administered with novel ways of therapy such as inhibitors of signals.[23] It is therefore important to discover novel cytotoxic agents with spectra of activity and toxicity that differ from those current agents.[24] It is well known that chemotherapy aims to destroy the cancer cells with various types of chemicals.[25] The substances used are supposed to target mainly the cancer cells and doses are calculated to minimize the collateral damage to surrounding tissues, which nevertheless occurs.[26] This kind of treatment increases the entropy of the organism, suppresses the immune system, and forms a toxic cell environment which may destroy surrounding healthy cells. So it is important to minimize curing doses to the least amount possible as well as trying to minimize the side effects of these drugs. For this novel derivatives of pyridine possessing a broader spectrum of antitumor activity and fewer toxic side effects than traditional anticancer drugs have been sought. The antiproliferative activities of such compounds were assessed against different cancer cell lines. Results of the present investigation indicated that the highest *in vitro* cytotoxic activity against human promyelocytic leukemia (HL-60) cell line (IC_{50} lower than12 µg/ml) was shown by compounds: **5a, 6, 5b** respectively. These compounds were chosen for further tests against human lung (A549), breast (T-47D) and colon cancer cells (LoVo) in comparison with the known anticancer drugs: cisplatin (CIS) and doxorubicin (DOX). Regarding the antiproliferative activity study, the selected compounds showed reasonable growth inhibition potency in comparison to CIS and DOX. This activity may be attributed to the presence of the phenylimino and the phosphanylidene moieties.[27]

Moreover, study of the induced biochemical parameters of the tested compounds in mice showed insignificant differences relative to the control group which indicates a moderate margin of safety for the selected compounds. Comparable to CIS and DOX, a dose augmentation of compounds 6 and 5a, searching for possible higher potency, seems, consequently, realizable without undesirable implications. Furthermore, the selected compounds have important potential advantages over CIS and DOX because of their lower toxicity and their ability to induce lower biochemical parameters. These results are similar to that obtained by other researchers worked on novel derivatives of 5-Flurouracil and found that these derivatives showed a broader spectrum of antitumor activity and fewer toxic side effects than 5- Flurouracil.[28-29]

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