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Using Human Cancer Cell Lines as *In vitro* Model for Testing the Efficacy of CDBPA; a New Anticancer Drug

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Abstract

The aim of the present study was to test the efficacy of cis-coordinated complexes of platinum (II) with the polymer of benzene-poly-carboxylic acids derived from lignin (CDBPA) (laboratory code BP-C1), an innovative anticancer compound, on the growth of several solid human cancer cell lines: bladder cancer, chondrosarcoma, colonic cancer, head and neck cancer, hepatic cancer, ovary cancer, pancreatic cancer and prostatic cancer. Furthermore, the effect of CDBPA on non-Hodgkin lymphoma cell lines was also tested. The effect of CDBPA on cell viability was detected by XTT assay and toxicity was detected by measuring the leakage of Lactate dehydrogenase from the cells to the media. The present study has demonstrated that CDBPA is not toxic and able to reduce cell viability substantially and significantly in various human cancer cell lines. When comparison of viability in percentage of the controls at the maximum given dose of CDBPA for each type of cancer cell line, it was found that the largest impact on the viability was on sarcoma, and then decreases via breast, prostatic, head and neck-, pancreatic, colonic cancer and finally ovarian cancer. In addition, the effect of CDBPA on non-Hodgkin lymphoma cell lines was similar to that found in sarcoma cells. We conclude that the effect of CDBPA on cell viability is different and may be dependent on genotype of the cancer cell type. This may indicate different mechanisms of action in the different cancer types. The results obtained from the *in vitro* studies are important for designing future *in vivo* studies using animal models and to predict the clinical outcome in human cancer.

Keywords: Human cancer cell lines; CDBPA; BP-C1; Cell viability; LDH; Apoptosis

Abbreviations: CDBPA (BP-C1): Cis-coordinated Complexes of Platinum (II) with Polymer of Benzene-poly-carboxylic Acids Derived from Lignin; LDH: Lactate Dehydrogenase; HCC: Hepatic Cancer; PI: Propidium Iodide; AUC: Area Under the Viability Curves; V: Viability

Introduction

The use of tissue culture methods in oncology has been performed for the last many decades, where Beebe and Ewing tried to grow lymphosarcoma in dogs [1]. An increasing variety of *in vitro* tests are used in cancer research to detect the optimal therapy and to study cancer cell functions [2]. *In vitro* experiments indicate that these *in vitro* techniques are often the leading techniques initially when a potential anticancer drug is going to be tested for its ability to kill cancer cells [2-5]. Improved cell culture techniques and application of molecular biology and genetic techniques will be more and more useful in the efficacy studies of anti-cancer drugs research as recommended by the European Community (Europe against cancer) [6]. However, we agree with Weaseling and Draws, that *in vitro* tests can`t abolish *in vivo* studies in testing any cancer agents nowadays [7]. But we feel that *in vitro* testing can reduce the number of painful animal experiments to be reduced substantially. This together with a methodology approach created to reduce the number of animals for research is an important issue [8]. We have used *in vitro* studies to determine the potential of the new innovative anti-cancer substance CDBPA (BP-C1) cis-coordinated complexes of platinum (II) with polymer of benzene poly-carboxylic acids derived from lignin, on the viability of human breast cancer cells. [2] This study was important for the following Phase I and II studies in patients with advanced breast cancer stage IV [9,10]. The aim of the present study was to test the efficacy of CDBPA to induce cell death of the following solid cancer cell lines: bladder cancer, colonic cancer, chondrosarcoma, head and neck cancer, hepatic cancer (HCC), ovarian cancer, pancreas cancer, and prostate cancer. Furthermore, the efficacy of CDBPA was also investigated in the two hematological cancer cell lines, non-Hodgkin lymphomas such as BL-2 and Ramos cell lines.

Material and Methods

Chemical and biological reagents

All cell culture media, Fetal Calf Serum, antibiotics and reagents were purchased from biological industries Beit Haemik, Israel) CDBPA (Figure 1) was obtained from Meabco A/S, Copenhagen, Denmark.

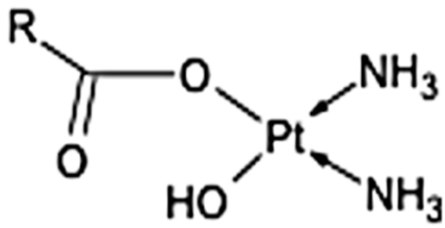

Figure 1: Simplified structural formula of CDBPA. R represent radical (polymer of benzene polycarboxylic acids).

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Cells and cell culture

All human cancer cell lines used in the present study: bladder (T24P), breast (MCF-7, T47D), chondrosarcoma (SW-1353), colon (HCT-116), head and neck (Fadu), liver (HepG2), ovary (SK-OV3), pancreas (PL-45, and APAF2) and prostate cancer cells (PC-3) were purchased from American Tissue Culture Collection (ATCC, Bethesda, MD) just as, non-Hodgkin lymphoma cell lines (Ramos and BL-2 cells). Human normal fibroblast cells were prepared at the Department of Molecular Genetics, Carmel Medical Center, Haifa, Israel. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in DMEM medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM of L-glutamine and 10% fetal calf serum (Biological Industries, Beit Haemek, Israel). To the growing media of MCF7 cells, 0.25 U/ml of insulin was added.

Cell viability

Cells were seeded in 96-well plates (2×10^3 cells/well). The following day, cells were treated with CDBPA in a spectrum of concentrations ranging from 10 to 250 µg/ml for 72 hours and cell viability was detected using Cell Proliferation Assay, XTT (Biological Industries, Beit Haemek, Israel) as previously described [11]. The XTT (sodium 3'-1-(phenylaminocarbonyl)-3,4-tetrazolium-bis (4-methoxy-6-nitro) benzene sulfonic acid) cell proliferation assay is an effective method to measure cell growth and drug sensitivity in tumor cell lines. XTT is a colorless or slightly yellow compound that when reduced becomes brightly orange. Briefly, XTT is cleaved by the mitochondrial dehydrogenase in metabolically active living cells to form an orange formazan dye. The absorbance of each sample was measured with a spectrophotometer at a wavelength of 450 nm. Each experiment was carried out in five replicates, in three different biological repeats. The results are presented as percentage of control and expressed as mean ± standard deviation of three independent experiments in which each treatment was performed in five replicates.

Detection of lactate dehydrogenase (LDH)

Cellular damage, such as necrosis, causes an elevation of the LDH concentration in the medium. The integrity of the plasma membrane following treatment with CDBPA was determined by measuring LDH activity released into the culture medium. The enzyme activity was measured using a spectrophotometric method [12].

Calculations and Statistical Analyses

Statistical model

The main variable in this study is the Viability in percent of controls for each specified cancer type and strain given CDBPA dose. The % Viability (%V) is defined as: {*[Stimulated value / control]*100}* used as the dependent variable and the CDBPA dose as the independent. A regression model has been used to express %V by a polynomial function in the CDBPA dose. The results are expressed with 95% confidence boundary [13]. In order to compare the viability for the different cancer types examined in the same dose intervals, the Area under the Viability curves (AUC) has been calculated using the Trapezium Rule [14]. %V

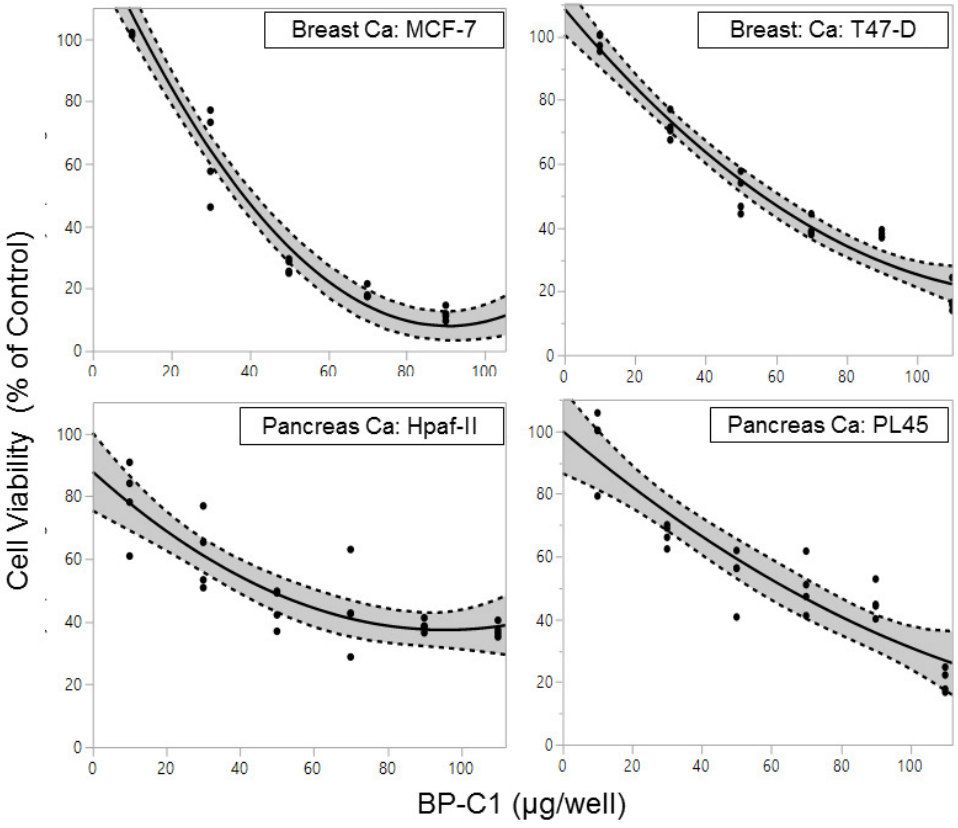

Figure 2: Development of Viability in percentage of the controls for Breast cancer MCF-7 & T470 and Pancreatic cancer Hpaf-II & PL45. The dots shows the observed values of the cancer strains. The full line illustrates the estimated development as a function in CDBPA (BP-C1) dose µg/Well and the surrounding dotted lines the 95% confidence boundary. The results are presented as percentage of untreated control cells of three independent experiments in which each treatment was performed in five replicates.

at given maximal CDBPA dose and AUC are expressed by mean values with SD in bracket and 95% confidence intervals [15].

Results

The Viability in percentage of the controls for the breast cancer strains MCF-7 and T47D was significantly expressed by a third- and a second-degree polynomial function in CDBPA dose (Figure 2). The dose explained 96 and 95% of the variations. The Viability for MCF-7 and T47D representing a mean percent reduction during the dose increase of 47.4 and 59.7%, respectively. The pancreatic cancer strains Hpaf-II and PL45 (Figure 2), as well as the hepatic HEPG-2, and the prostatic PC3 (Figure 3) cells were significantly expressed by second-degree polynomial functions in CDBPA dose. The CDBPA dose explained 85, 74, 90 and 87%, respectively, of the variations. This represents a mean percent reduction during the increase in dose of 44.5, 49.2, 47.3 and 46.6%, respectively (Table 1).

The Viability for the colon cancer strain HCT-116 and the Head & Neck strain FaDu were significantly expressed by a fifth-degree polynomial function (Figure 3). The CDBPA dose explained 98 and 95% of the variation and represents a mean percent reduction during the dose increase of 66.3 and 79.4%, respectively.

In accordance with the percent Viability obtained at the highest used CDBPA dose, the largest reduction was detected for the Breast cancer strain MCF-7, the prostatic PC3 and the head & neck FaDu

Viability (% of Control)	R2	Mean
Breast		
A-T47D	0.95	52.7
B-MCF-7	**0.96**	40.3
Pancreatic		
PL45	0.85	55.5
Hpaf-11	0.74	50.8
Colon		
HCT-116	0.9S	33.7
Head & Neck		
FaDu	0.95	20.6
Prostatic		
PC3	0.87	53.4
non-Hodgkin lymphoma		
BL -2	**9.76**	3.9
RAMOS	94.6	26.6
Sarcoma		
SW -1353	98.3	52.7

Table 1: Viability in % of control. The mean % viability over the given dose interval and R^2 expressing the percent of the variability in the efficacy explained by the CDBPA (BP-C1) dose.

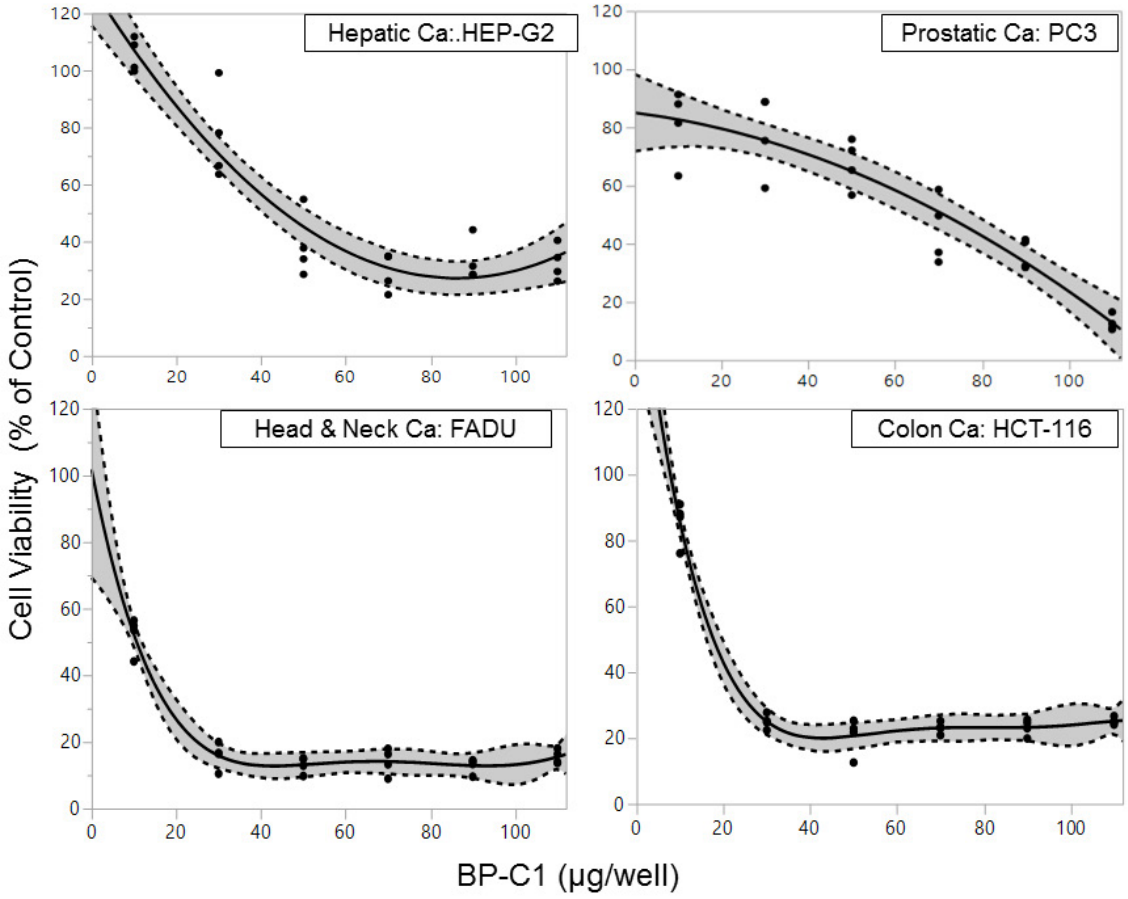

Figure 3: Development of Viability in percentage of the controls for a Hepatic cancer HEP-G2, Colon cancer HCT-116, Head & Neck cancer FADU and Prostatic cancer PC3. The dots shows the observed values of the cancer strains. The full line illustrates the estimated development as a function in CDBPA (BP-C1) dose µg/Well and the surrounding dotted lines the 95% confidence boundary. The results are presented as percentage of untreated control cells of three independent experiments in which each treatment was performed in five replicates.

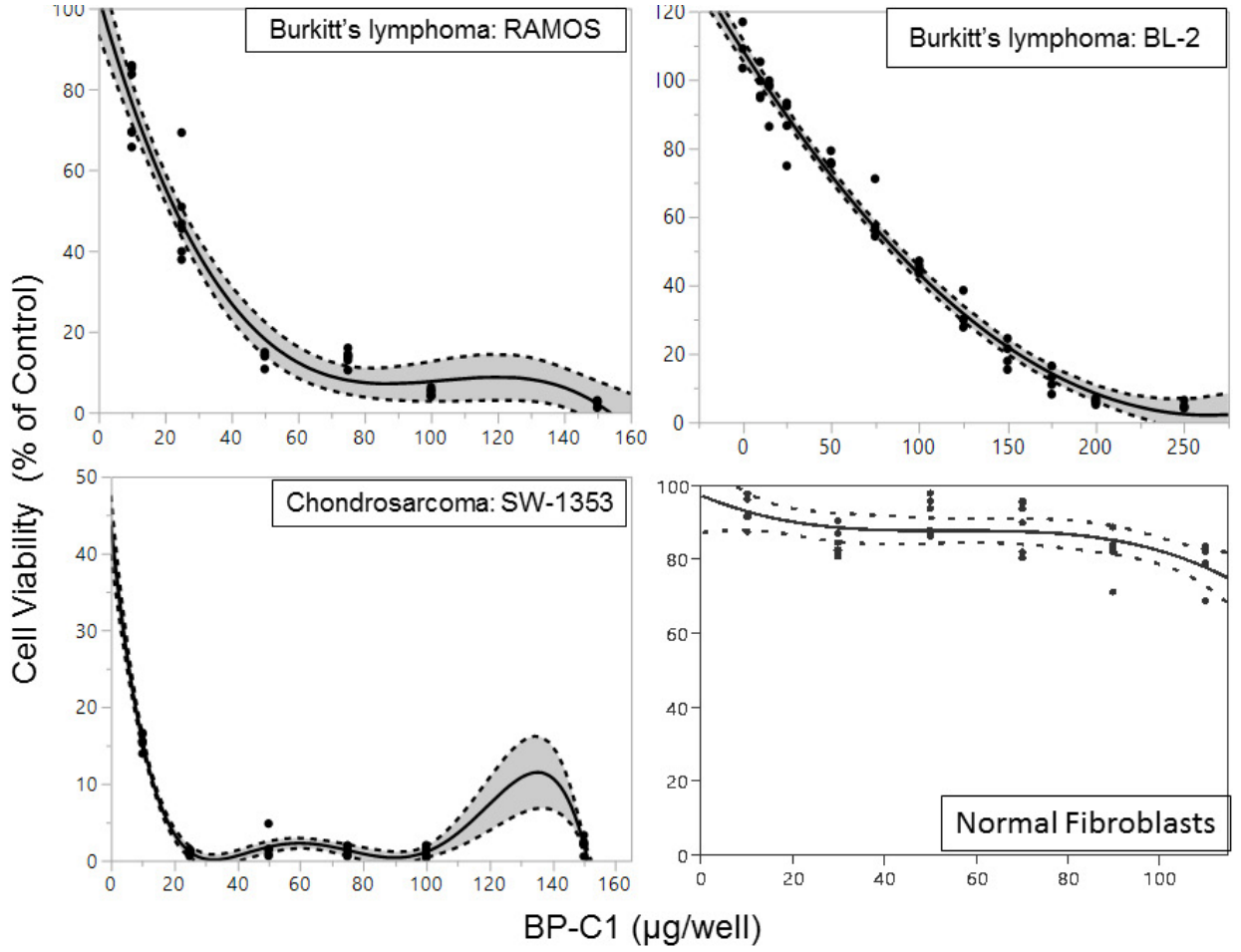

Figure 4: Development of Viability in percentage of the controls for (a) and Burkitt's lymphoma BL-2 and RAMOS, chondrosarcoma SW-1353 and normal fibroblast cells. The dots show the observed values of the cancer strains. The full line illustrates the estimated development as a function in CDBPA (BP-C1) dose µg/Well and the surrounding dotted lines the 95% confidence boundary. The results are presented as percentage of untreated control cells of three independent experiments in which each treatment was performed in five replicates.

Cancer	Strain	Mean (SD)	95% Confidence Interval	Maximum dose	Efficacy range
Normal Fibroblast Cells		79.3 (5.0)	13.4- S).1	110 Well	
Breast	B-MCF-7	17.6 (4.6)	10.4 - 24.9	110 pg Well	7(4)
	B-MCF-7	10.9 (1.5)	8.5 -13.3	110 lig Well	4(1)
Pancreatic	Hpaf-11	37.3 (2.3)	33.6 - 40.9	110 lig Well	11(8)
	PL45	20.3 (3.8)	14.3- 26.3	110 pg Well	-5
Hepatic	HEPG-2	32.6 (6.2)	22.7 - 42.5	110 pg Well	10(7)
Color	HCT-116	25.1 (1.1)	23.3- 26.8	110 pg Well	9(6)
Head& Neck	FaDu	15.5 (2.1)	12.1 - 1S.S	110 pg Well	6(3)
Prostatic	PC3	12.6 (2.7)	8.3 - 17.0	110 pg Well	5(2)
Chondrosarcoma	SW -1353	10.7 (6.6)	7.2- 14,2	250 pg Well	3
Non-Hodgkin lymphoma	BL -2	2.1 (0.9)	1.2 - 3.1	150 pg Well	1
	RAMOS	2.4 (0.6)	1.7 - 3.1	150 tig Well	2
Ovary	SKOV-3	70.3 (6.7)	62.0- 78.6	75 ng Wells*	
Bladder	T24P	12.6(1.3)	11.4-14.1	75 µg Well*	

* Only one single dose of 75 µg/Well CDBPA (BP-C1) is given

Table 2: Comparison of Viability in percentage of the controls at the maximum given dose of CDBPA (BP-C1) for each type of cancer strain. The results expressed by mean values and 95% confidence intervals. Except for Ovary and Bladder cancer which is only tested for a single dose, the efficacies of CDBPA (BP-C1) are ranged between the strains. The ranges given in brackets represent the results when excluding Chondrosarcoma and non Hodgkin lymphoma.

Cancer	Strain	Mean (SD)	95% Confidence Interval	BP -C1 efficacy range
Breast	A-T47D	5158 (275)	4720 -5598	**7**
	B-MCF-7	3629 (338)	3091 -4166	3
Pancreatic	Hpaf-II	4935 (305)	4499 -5421	4
	PL45	5495 (514)	4677-6313	8
Hepatic	HEPG-2	4940 (862)	3568 -6311	5
Colon	HCT-116	2940 (V)	2633 -3248	2
Head & Neck	FaDu	1792 (43)	1724-1861	1
Prostatic	PC3	5471 (378)	4870 -6072	6

Table 3: Comparison of CDBPA (BP-C1) effect expressed by the Area under the curves (AUC) of each Cancer strain.

where the effect on normal fibroblast cells is minor (Table 2). The AUC of the Viability curve take into account the development including all the CDBPA doses used. The rank based on AUC shows best effect on the head & neck cancer strain FaDu, the colon strain HCT-116 and the breast strain B-MCF-7 (Table 3).

The Viability for the chondrosarcoma strain SW-1353 was significantly expressed by second-degree polynomial functions in CDBPA dose explaining 98.3% of the variations (Figure 4) and represents a reduction during the increase in dose of 47.3% (Table 1).

The leukemia strains BL-2 and RAMOS was significantly expressed by a fifth- and a third-degree polynomial function in CDBPA dose (Figure 4), respectively. The dose explained 97.6 and 94.6% of the variations. The Viability of BL-2 and RAMOS representing a mean percent reduction during the dose increase of 97.9 and 97.6%, respectively. Interestingly the effect of CDBPA on viability of normal fibroblast cells is minor (Figure 4).

The Ovary Cancer strain SKOV-3 and the Bladder strain T24P were stimulated by 75 µg/Well CDBPA causing a mean percentage Viability of 70.3 and 12.8 %, representing a reduction of 29.7 and 87.2%, respectively (Table 2).

Discussion

This study has demonstrated that the novel new anticancer product, CDBPA, is able to reduce cell viability substantially in various human cancer cell lines besides breast cancer cell lines as previously shown [2]. CDBPA is capable of reducing cell viability substantially in the following solid cancer cell lines; Bladder, colon cancer, chondrosarcoma, osteosarcoma, head and neck squamous cell carcinoma, hepatocellular carcinoma, ovarian cancer, pancreatic cancer and prostate cancer.

We have previously shown that CDBPA does not release LDH from the cells indicating that CDBPA is a non-toxic drug. CDBPA mainly kills the various cells by introducing apoptosis as shown previously [2,3]. The apoptotic pathways induced by CDBPA are different between the cancer cell types. The present study has demonstrated that this is also the case on hematological cancer cells such as non-Hodgkin lymphoma. Based on these *in vitro* data obtained in breast cancer cell lines and the following clinical phase I and phase II studies we can conclude that the *in vitro* studies in human cell lines on the effect of CDBPA are an important tool to predict the clinical outcome in humans and in dogs [8-10]. CDBPA is not a cisplatin analog since its Mechanism of Action differs from that of cisplatin alone and that the benzene-polycarboxylic acid alone. However, CDBPA is thought to react with DNA to form intra - and inter-strand crosslink. Cisplatin cross links to DNA in several different ways, interfering with cell division by mitosis [16,17]. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible. Previously we have found that induced apoptosis through the extrinsic and the intrinsic pathway in human breast cancer cells through caspase activation [2]. However, it was found that CDBPA (BP-C1) induced apoptosis in lymphoma cells by caspase independent pathway [18].

When comparison of viability in percentage of the controls at the maximum given dose of CDBPA for each type of cancer cell line is performed, CDBPA has the largest impact on the viability on chondrosarcoma cells, and then decreases via breast, prostatic, head and neck-, pancreatic, colonic and finally ovarian cancer. Interestingly, CDBPA is less effective in ovarian cancer cells. This result is similar to previous study which indicated that exposure of human ovarian tumor cell lines to cisplatin led to the development of cell lines that exhibited increasing degrees of drug resistance which were closely correlated with increase of the levels of cellular glutathione [19].

However, if the efficacy of CDBPA on the various cell lines is calculated by using the area under the curve, the most effect is seen on the following row in decreasing order: Head and neck, colon, breast and liver. Overall, the most marked effect of CDBPA was found on both hematological cell lines tested, i.e. Ramos and BL2 cells. This may indicate that CDBPA acts through different molecular mechanisms dependent on the cancer cell type. Further studies are needed in order to clarify the mechanisms by which CDBPA affect cancer cell growth in each type of cancer.

Another possibility that may explain the differences of CDBPA efficacy in different cancer cells is relates to differentiating cancer-driving mutations from passenger mutations. It is generally assumed that while somatic cancer cells typically contain many genomic aberrations, only a few of them are driver mutations that provide a growth advantage for tumor cells and thus are causal with respect to cancer progression. In addition, cancer cell divisions accumulate random mutations—most of them passengers. A faulty gene in the DNA repairs mechanism or environmental factors such as UVC exposure can result in both passenger and driver mutations [20-22]. Therefore, the DNA polymorphisms were of importance for the drug response in human cancer cell lines investigated and could eventually become important for individualized drug treatment in humans. Other studies indicated that mitochondrial DNA mutations are linked to many human diseases including cancer development. The response to drug treatment was different depending on mitochondrial genotype [23]. According to these facts it is possible to assume that the genetic profile of the human cancer cell lines used in the present study is different, therefore, these cells respond differently to CDBPA.

Nonclinical as well as clinical studies are essential components of the anti-cancer drug discovery process [24]. To advance from nonclinical testing to testing of a drug in Phase II clinical studies is based on the assumption that nonclinical laboratory models are clinically predictive as previously described [24]. In our search after new and effective nontoxic anticancer drugs we have not used murine graft models to a larger extent since murine allografts models have been shown to be of limited utility in contrast to *in vitro* cell lines used under the right framework [24]. In our hands the classical nonclinical and clinical development of our compounds has followed the procedures which are practiced today. However, the present findings could contribute to a more efficient discovery of CDBPA as an anticancer drug. We are, based

on the present *in vitro* study, , to design clinical trials in head and neck cancer prostate cancer and pancreas cancer. The results of the present study may indicate that using human cancer cell lines are useful for developing an *in vitro* anticancer screening model to study the effect of various anticancer agents and various molecular pathways affected by a potential new drug. Moreover, the results obtained from the *in vitro* studies are important for designing future *in vivo* studies using animal models, if needed.

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