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RESEARCH ARTICLE

INFLUENCE OF TWO PROMISING PRODUCTS (SILIBININ AND TRANS-RETINOIC ACID) ON IMMUNOPHENOTYPIC CHARACTERIZATION OF LEUKEMIC CELLS PROPAGATED EX-VIVO

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ABSTRACT

Background: Leukemias are a group of diseases characterized by increased numbers of abnormal white cells in the blood and bone marrow. Two promising products such as silibinin and trans-retinoic acid have found to have anti-cancer effects.
Aims and Objectives: This work aims to study the effect of silibinin, and trans-retinoic acid on leukemia cells (LC) propagated ex-vivo in comparison with normal cells.
Materials and Methods: This study was done on 75 subjects that divided into three groups: 25 AML patients group, 25 CML patients group and 25 healthy controls group enrolled in a prospective study. Blood samples were collected and LC was isolated from whole blood samples. Haemocytometer cell count and viability studies, immunophenotypic characterization levels were detected by flowcytometry, and -IFN-γ level was performed by ELISA technique.
Results: Silibinin and TRA treatment show significant differences in the level of cell density, γ-IFN, and CD4% among AML and CML patients compared to normal healthy control persons , on the contrary CD8 did show insignificant difference among the previous mentioned groups due to the previous mentioned treatment.
Conclusion: We may suggest that combination therapy of silibinin and TRA could be useful treatment of CML and AML patients.

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INTRODUCTION

Leukemia is a disease of the blood or bone marrow, which is characterized by increased numbers of abnormal white blood cells. The abnormality of leukemic cells lies in their inhibited differentiation and increased proliferation rate, and it is classified clinically on the basis of the character of the disease into acute or chronic, or classified according to the type of cells involved into myeloid, lymphoid or monocytic (Pujo-Menjouet, 2004; and Hoffbrand, 2006). Silibinin is a natural polyphenolic flavonoid derived from the fruits and seeds of milk thistle that has received special attention of many chemists and clinicans since it has been shown to have cancer preventive and anticarcinogenic effect, as well as, hepatoprotective effect (Saller, 2008).

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Silibinin has demonstrated anti-cancer effects against various types of malignancies (Katiyar et al., 1997; Yang, 2003; Tyagi, 2004; and Singh, 2007). These features of silibinin have made it used as an effective agent for chemoprevention and chemotherapy (Flora, 1998; Luper, 1998; and Ting, 2016). Retinoids regulate growth and differentiation of normal and malignant cells. In the haematopiotic system, TRA has been shown to inhibit growth, induce differentiation of myelomonocytic progenitor cells, and to enhance self-renewal of more immature multipotent stem cells. So, some authors hypothesized that TRA is used to treat acute promyelocytic leukemia (Huang, 1988; and Castaigne, 1990). Generation of both humoral and cellular immune responses depends on the activation of $CD4^+$ T helper (T_H) cells. A distinct population of 5% to 10% of the total $CD4^+$ T cells has a constitutive expression of CD25 (the alpha chain of the IL-2 receptor), which is only expressed by other $CD4^+$T cells after TCR activation (Ribas, 2003). Previous studies have shown that

interferons (IFNs) are pleiotropic cytokines secreted by several cell types as part of the innate immune response. They share antiviral, immunomodulatory, and anti-proliferative effects (Stark, 1998 and Pestka, 2004). So, the aim of the current work is to study the possible effect of Silibin in and TRA individually or in combinations on the immunophenotypic profile for CD4+, CD8+, and CD25+ levels, and the differentiation of AML, and CML cells propagated ex-vivo.

MATERIALS AND METHODS

Study population

Seventy five individuals were involved in the present study from the Medical Oncology Department of the National Cancer Institute (NCI) - Cairo University. The study protocol and informed consent were approved by the NCI's Ethics Committee. They were classified into the following 3 groups; Group A including 25 Normal control leukocytes (NCL) group that subdivided into; A1: NCL cultured without treatment, A2: NCL treated with 1μM trans retinoic acid (TRA), A3: NCL treated with 10μg /ml silibinin, and A4: NCL treated with 1μM trans retinoic acid +10 μg/ml silibinin. Group B including 25 AML group that subdivided into; B1: AML without treatment used as a control culture, B2: AML treated with1μM Trans Retinoic acid, B3: AML treated with10μg/ml silibinin, and B4: AML treated with10μg /ml silibinin+1μM Trans Retinoic acid. Group C including 25 CML group that subdivided into; C1: CML without any treatment used as a control culture, C2: CML treated with 1μM trans retinoic acid, C3: CML treated with 10μg /ml silibinin, and C4: CML culture treated with 10 μg /ml silibinin+1 μM trans retinoic acid. All cultures of normal control leukocytes, AML, and CML were cultured for 2 days. Retinoic acid (Sigma Chemical Company, St. Louis, MQ) was added from a 1mM stock of RA dissolved in DMSO stored protected from light at -20^{0}C and used at a final concentration of 1 μM (Ortega, 2005). On the other hand, silibin in (Sigma Chemical Company, St. Louis, MQ)was also dissolved in DMSO to make a stock solution of 40 mg/ml. protected from light at -20^{0}C and used at a final concentration of 10 μg /ml (Kang, 2001).

Cell Culture Techniques

Fresh AML and CML cells (at least 70% of leukemic cells present) were obtained from patients did not receive any kind of therapy. The heparinized blood samples were diluted with RPMI-1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and then overlaid on 6 ml of Ficoll-Hypaque (Phanacia Fine Chemicals, UPP, Sweden) and centrifuged at 500xg for 30 min. The mononuclear cells were washed twice and suspended in RPMI -1640 medium supplemented with 10% fetal bovine serum, plated in culture dishes at 6x10^6 cells/ ml, and incubated at 37^{0}C in a humidified atmosphere of 5% CO2. Cells were harvested after 2 days for analysis. A sample from the supernatant was stored at -80^{0}C (Yamada, 1998). Cell density was determined by daily counts using a haemocytometer and cell viability was determined by trypan blue exclusion (Schokowski, 2004).

Detection of Interferon Gamma (IFN-γ)

IFN-γ was detected in all samples using IFN-γ kit (CYTELISA, USA), and detection of its level was done by ELISA procedure (Ralph, 2002).

Detection of CD4%, CD8%, and CD25% levels by flowcytometry technique

CD4%, CD8%, and CD25% immunophenotypic characterization levels were detected by flowcytometry. Monoclonal antibodies (from Dako Ltd.,Cambridge, UK) were used that bind to specific cell surface antigens (CD4, CD8, and CD25), and 2 flouresenic dyes are applied to label. Flouresenic isothiocyanate (FITC) for CD4 & CD25 and Phytoerytherin (PE) for CD8. Calculations of the concentration of those cell surface markers were done according to the previous study (Papa, 2002).

Statistical Analysis

Data were statistically described in terms of range, mean, standard error (±S.E.), and frequencies (number of cases) when appropriate. Comparison of variables between the study groups was done using analysis of variance (ANOVA) test. A probability value (P-value) less than 0.05 was considered statistically significant. Statistical calculations were done using statistical computer program: SPSS 17 (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics of the studied group

Age and sex of fifty patients and twenty five healthy control individuals were summarized in Table1. There were no significant difference in sex and age of patients with AML and CML compared to their controls (p>0.05).

Effect of Silibinin and Trans-Retinoic acid (TRA) on leukemic cells and normal mononuclear leukocytes in relation to

Cell Density (CD): There were significant differences in (CD) count of AML patients when compared Control Untreated cultured cells with RA treated cultured cells alone or in combination with Silibinin treated cultured cells (P= 0.043, P=0.012; respectively). Whereas, a high significant difference (P=0.004) was found to be among cultured cells from CML patients when compared Control Untreated cultured cells with Silibinin plus RA treated cultured cells. There were no significant differences (P> 0.05) in (CD) count of controls when compared Control Untreated cultured cells with Silibinin treated cultured cells, RA treated cultured cells, and Silibinin plus RA treated cultured cells, as shown in Table 2.

IFN-γ level: There were high significant differences in case of AML patients when comparing Control Untreated cultured cells with Silibinin treated cultured cells, RA treated cultured cells, and Silibinin plus RA treated cultured cells (P= 0.002, P=0.043, P=0.000; respectively). Also, there were high significant differences in case of CML patients when comparing Control Untreated cultured cells with Silibinin treated cultured cells, and Silibinin plus RA treated cultured cells (P=0.000, P=0.004; respectively). Results added that there were high significant differences (P=0.000) in case of Controls when comparing Control Untreated cultured cells with Silibinin treated cultured cells, and Silibinin plus RA treated cultured cells. So, it was deduced that both separated and combinated therapy with Silibinin (and/or) RA has a potent therapeutical effects on AML patients.

Table 1. Demographic features of the studied group

Groups Parameters	Healthy Controls (n=25)	AML Patients (n= 25)	CML Patients (n=25)	P-value
Sex (M/F)	15/10	16/9	15/10	> 0.05**
Age (Mean±SE*)	48.20± 0.93	49.92± 1.05	51.88±1.59	> 0.05**
Range	(38 – 56)	(42 – 58)	(32 – 65)	

(SE); Standard error, (> 0.05)**; Non-significant difference.*

Table 2. Effect of Silibinin and RA on cell density of the studied groups

Treatment Groups	a) Control Untreated cultured cells	b) Silbinin treated cultured cells	c) RA Treated cultured cells	d) Silibinin +RA treated cultured cells	P-value
AML Mean + SE	1.46+ 0.40	0.71+ 0.22	0.54+ 0.15	0.79+ 0.25	> 0.05 (a,b) 0.043* (a,c) 0.012* (a,d)
CML Mean + SE	4.04+ 0.78	2.70+ 0.67	2.41+ 0.54	1.50+ 0.29	> 0.05 (a,b) > 0.05 (a,c) 0.004* (a,d)
Control Mean + SE	0. 64+ 0.03	0.67+ 0.03	0.68+ 0.06	0.62+ 0.03	> 0.05 (a,b) > 0.05 (a,c) >0.05 (a,d)

- Cell density of cultured cells of AML, CML, and Controls ×10^6

- (S.E.); Standard error, (*); represents significant difference (P<0.05), (a,b), Relation between Control Untreated cultured cells and Silibinin treated cultured cells, (a,c), Relation between Control Untreated cultured cells and RA Treated cultured cells, (a,d), Relation between Control Untreated cultured cells and Silibinin plus RA treated cultured cells.

Table 3. Effect of Silibinin and RA on IFN- γ levels of the studied groups

Treatment Groups	a) Control Untreated cultured cells	b) Silbinin Treated cultured cells	c) RA treated cultured cells	d) Silibinin +RA treated cultured cells	P- Value
AML Mean + SE	85.89± 10.13	142.56± 25.34	145.65± 15.36	153.2± 12.69	0.002* (a,b) 0.043* (a,c) 0.000* (a,d)
CML Mean + SE	205.31±13.52	193.98± 14.01	267.62± 9.08	520.86± 6.48	0.000* (a,b) > 0.05 (a,c) 0.004* (a,d)
Control Mean + SE	226.10± 6.41	189.72± 17.33	304.47± 2.58	273.06± 3.57	0.000* (a,b) > 0.05 (a,c) 0.000* (a,d)

(S.E.); Standard error, (*); represents a significant difference (P<0.05), (a,b), Relation between Control Untreated cultured cells and Silibinin treated cultured cells, (a,c), Relation between Control Untreated cultured cells and RA Treated cultured cells, (a,d), Relation between Control Untreated cultured cells and Silibinin plus RA treated cultured cells.

Table 4. Effect of silibinin and RA on the immunophenotyping characterization of leukemic cells (LCs) and MNLs isolated from patients with AML, CML and normal controls

Groups Treatments	Control healthy persons (n=25) Mean ± SE			AML patients (n=25) Mean ± SE			CML Patients (n=25) Mean ± SE		
	CD4 %	CD8 %	CD25 %	CD4 %	CD8 %	CD25 %	CD4 %	CD8 %	CD25 %
a)Cultured cells alone (Control)	54.73± 0.04	24.53± 0.94	6.62± 0.10	41.3± 0.3	40.30± 1.55	7.52± 0.97	44.66± 0.95	22.29± 0.16	1.81± 0.00
b)Cultured cells treated sili(10µg/mL)	43.28± 0.59	22.57± 1.91	3.02± 0.39	24.08± 0.37	41.22± 2.67	7.76± 0.86	31.22± 0.08	2.36± 0.17	0.04± 0.00
c)Cultured cells treated with TRA (1µM)	53.06± 0.32	24.19± 0.73	5.32± 0.14	43.29± 0.54	42.29± 0.69	4.42± 0.37	47.81± 0.77	25.40± 1.60	0.62± 0.06
d)Cultured cells treated with sili (10µg/mL) + TRA (1µM)	42.64± 1.53	23.49± 1.74	1.80± 0.27	30.38± 1.02	42.82± 0.82	4.66± 0.21	39.97± 0.39	2.47± 0.25	0.94± 0.01
P. value	0.000*(a,b) 0.000*(a,c) 0.000*(a,d)	0.05* (a,b) 0.05* (a,c) 0.05* (a,d)	0.000* (a,b) 0.000* (a,c) 0.000* (a,d)	0.000* (a,b) 0.003* (a,c) 0.000* (a,d)	0.05* (a,b) 0.05* (a,c) 0.05* (a,d)	0.05* (a,b) 0.004* (a,c) 0.006* (a,d)	0.000* (a,b) 0.014* (a,c) 0.000* (a,d)	0.000*(a,b) 0.000* (a,c) 0.000*(a,d)	0.000* (a,b) 0.000* (a,c) 0.000* (a,d)

Whereas, Silibinin separated alone or combinated therapy (Silibinin plus RA) have potent therapeutical effects on CML patients, as shown in Table 3.

$CD4^+$, $CD8^+$, and $CD25^+$ immunophenotypic profile: A significant decrease of CD4+ level was found in AML, and CML patients treated with silibinin alone or in combination (p=0.000), while it is significantly increased in AML, and CML patients (p=0.003, p=0.014, respectively) treated with TRA. On the other hand, a non- significant increase of CD8+ level in AML patients was found when treated with silibinin, TRA alone or in combination (p>0.05).whereas, a significant increase in CD8+ level of CML patients treated with silibinin, TRA alone or in combination. For T-reg (CD25+) of AML, CML, and controls, there was a significant decrease in CD25+ level of AML patients treated with TRA alone or in combination (p<0.005), while, it increased non-significantly in AML patients treated with silibinin (p>0.05). Moreover, a

significant decrease of CD25+ level was found in CML patients and control group treated with silibinin, TRA alone or in combination (P=0.000), as shown in Table 4.

DISCUSSION

Leukemia like other cancers, results from somatic mutations in DNA which activate proto-oncogenes or deactivate tumor suppressor genes and disrupt the regulation of signal transduction cell cycle and programming cell death, differentiation or division. These mutations may occur spontaneously or as a result of exposure to radiation or carcinogenic substances and are likely to be influenced by genetic factors (Miw, 1994). Recently, a number of natural products isolated from herbs have been found to inhibit proliferation, induce apoptosis, suppress angiogenesis, retard metastasis and enhance chemotherapy, exhibiting anti-cancer potential both in vitro and in vivo. The current study hypothesized that there were significant differences ($p<0.05$) when comparing Control Untreated cultured cells with TRA treated cultured cells, and Silibinin plus TRA treated cultured cells among the AML patients. This result was supported by previous studies demonstrated that combination of the dietary plant silibinin at non-cytotoxic concentration produced a synergistic antiproliferative effect and a massive apoptotic cell death in HL-60 and KG-1a human AML cells (Pesakhov, 2010). On the other hand, Biological agents such as: Trans-retinoic acid are a step forward in supportive care to reduce the hematological toxicity of high-dose chemotherapy and to manage the frequent infectious complications (Agarwal, 2008). Experience accumulated over the last 10 years of has clearly shown that the combination of trans retinoic acid and chemotherapy gives better survival in newly diagnosed Acute promyelocytic leukemia than chemotherapy alone because of fewer relapses and a higher complete remission rate experienced by these patients (Laurent, 2001). So, RA separated therapy or combined therapy with Silibinin plus RA is available treatment in case of AML patients.

Our results suggested that there was a highly significant difference among CML patients on comparing the untreated cultured cells of the controls with Silibinin plus RA treated cultured cells. This result is going well with the previous studies that suggested the potential role of Silibinin in Myeloid Leukemia treatment. Treatment of HL-60 cells with silibinin inhibits cellular proliferation and induces differentiation predominantly into monocytes (Kang, 2001). Previous studies have shown that TRA can exert dose-dependent inhibitory and granulo-monocytic differentiating effects on different leukemic cell lines and on CML progenitor cells (Nilsson, 1984). It can also act synergistically with IFN to induce both growth inhibition and differentiation (Sagayadan, 1991 and Zheng, 1996). In addition it can induce apoptosis both in normal hematopoietic progenitors and in leukemic ones (Guglielmo, 1995). So, we may suggest that combination therapy with Silibinin and RA is the best treatment in case of CML patients in the level of cell density. Whereas, RA separated therapy or combined therapy with Silibinin plus RA is available treatment in case of AML patients in the level of cell density. Our results showed also that there were high significant differences among both AML and CML on comparing untreated cultured cells of the controls with combined or separated Silibinin and RA treated cultured cells in the level of IFN-γ. These results were supported by previous studies demonstrated that Silibinin and RA treatments can induce immune system by increase IFN-γ release. Combination of the chemotherapeutic agent all-trans retinoic acid (ATRA) and the immunotherapeutic agent IFN-γ could concurrently induce differentiation, apoptotic death, and immune components in two different human cell lines. We propose that combination of a TRA and IFN-γ may become an effective chemoimmunotherapy for the treatment of human cancer (Haque, 2006). This previous results suggested that IFN-γ is an important biomolecule for positive regulation of the MHC presentation machinery. The treatment of tumor cells with IFN- γ induces apoptosis and the extent of cell death is enhanced by pretreatment with a TRA. Authors also showed that a combination of ATRA and IFN- γ expressed higher levels of HLA class II and HLA-DM molecules. When treated with IFN- γ or combination of a TRA and IFN-γ, tumor cells also expressed an important enzyme, gamma interferon-inducible lysosomal thiolreductase (GILT), which might be useful for enhancing the presentation of self-antigens via the HLA class II pathway (Haque, 2006). Our result is in contradiction with a previous study indicating that Silibinin inhibit gamma interferon, IL-2, IL-4, and augment synthesis of IL-10 (Wang, 2005).Regarding the effect of Silibinin and RA on immunophenotypic characterization levels of AML, and CML patients, the current results showed that Silibinin treated cultured cells, RA treated cultured cells, and Silibinin and RA treated cultured cells have significant difference in CD4 and CD8 levels of CML patients only as compared to untreated cultured cells of the controls. Whereas, Silibinin treated cultured cells, RA treated cultured cells, and Silibinin and RA treated cultured cells have significant difference in CD4 levels of AML patients only as compared to untreated cultured cells of the controls.

All previous observations illustrated the potential role of Silibinin and Retinoic Acid in cure and clearance of Myeloid Leukemia including: AML and CML. The previous therapeutic strategy leads to immune system activation and CD4/CD8 cells induction. Previous authors have also hypothesized this strategy. Schurmans et al. (2001), suggested that CD4 T cells play a crucial role in the control of tumor growth in both animal models and cancer patients. They believed that CD4 T cells provide help required for the induction of CD8 T cell responses with the ability to lyse tumor cells in a MHC, Fas ligand and perforin dependent manner. T helper (Th) 1 cells can also directly eradicate tumor cells without any significant involvement of CD8 T cells. Lymphocytes contribute to the tumor microenvironment through immunity and inflammation. CD8+ CTLs can directly kill target cells by releasing granules including membrane lytic materials such as perforin and granzymes in acquired immune response, thereby playing a central role in antitumor immunity.The induction of effective anti-tumor immune response requires the activation of both helper CD4+ and CD8+ T-cells (Mautner *et al*, 2005). Chemo immunotherapy could simultaneously reduce the burden of tumor growth and overcome immune tolerance so as to eliminate the tumor altogether. Thus, it was suggested that, in the level of CD4% and CD8%, separated or combined therapies with Silibinin (and/or) RA have therapeutical effects on CML patients. Whereas, in the level of CD4%, separated or combined therapies with Silibinin (and/or) RA have therapeutically effects on AML patients.The present study suggested that RA treated cultured cells, and combination therapy with Silibinin and RA have significant differences in CD25% level of AML patients as compared to untreated cultured cells of the controls. Moreover, there were high significant difference in case of CML patients when comparing

control untreated cultured cells with Silbinin treated cultured cells, RA Treated cultured cells, and Silibinin and RA treated cultured cells. Previous authors suggested that a distinct population of 5% to 10% of the total CD4+ T cells has a constitutive expression of CD25 (the alpha chain of the IL-2 receptor), which is only expressed by other CD4+T cells after TCR activation(Ribas, et al.,2003). The depletion of these cells leads to the development of autoimmune diseases such as colitis or encephalitis and the potentiation of antitumor responses (Shevach, 2001). So, it was suggested that, in the level of CD25%, combination and separate therapy with Silibinin and RA has a therapeutical effect on CML patients. On the other hand, the current results also demonstrated that there were therapeutical effects of separated RA and combined Silbinin plus RA on AML patients. In conclusion,we can conclude that combination therapy with Silibinin and RA is the best treatment in case of CML and AML patients in the level of cell density, γ-IFN, CD4%, and CD25%. Moreover, separated or combined Silibinin and RA treated cultured cells in the level of CD8% cannot be used as a therapy for AML

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