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An Innovative Epigenetic Merge in Treatment of AML Patients Correlates with Better "Molecular and Clinical" Outcomes

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ABSTRACT

PURPOSE: Epigenetic gene silencing due to promoter hypermethylation & histone acetylation influence various molecular pathways in leukemogenesity of Acute Myeloid Leukemia (AML). Combined use of DNA methyltransferases & histone deacetylases inhibitors proved to reverse the methylomic phenotype of myeloid blasts & improve patient's prognosis. The study recruited 68 Adults patients 45 received novel combination of the HDACi valproic acid +/- the Dnmt1 inhibitor; epigallocatechingallate "EGCG" parallel to standard chemotherapy for 2 successive cycles. RESULTS: We recorded; 37/45 (CR), 7 (PR) &1 (RF), 60% MRD elimination. Decreased Dnmt1 & HDAC1 activities ($p<0.001$). Reversed $P15^{INK4B}$ gene methylation & expression states 58 %, ($p<0.02$) & decreased levels of VEGF, bfgf, IL-6, NF-KB, TNF, COX-2 & P65 cytokines levels in a harmonized orchestra ($p<0.01$). Survival analyses showed high significant 2 years DFS ($P<0.01$). CONCLUSION: The used epi-drug combination distinctively exerted a destructive impact on AML blasts interpreted to tumor regression, better hematological & clinical response.

Keywords: DFS: disease free survival; Dnmt1 DNA methyltransferases1; HDACi: histone deacetylases inhibitor; MRD: inimal residual disease; VEGF: vascular endothelial growth factor; NF-KB: nuclear factor KB.

INTRODUCTION

The high relapse rate in Acute Myeloid Leukemia "AML" is a significant clinical problem mainly reflects the survival of residual tumor cells after standard therapy. Simultaneous and rapid epigenetic processes such as DNA methylation and histone post-transductional modifications energize tumor cell to stop or slow its proliferation, differentiation or apoptosis. leading to chemotherapy-resistant phenotyped cells. The epigenetic mechanism of drug resistance gives no exception to abnormal methylation. A theme that is prevalent in the resistant cells and that can be overcome by combining HDAC & methyltransferases inhibitors to AML standard chemotherapy. As the most frequent alterations found in acute leukemia Promoter silencing by DNA methylation is an established mechanism to inhibit tumor suppressor genes. This inhibition reflects a state of deacetylated chromatin, a conformational change signal that is dictated by HDACs. Working in harmony to alter the transcriptome profile, DNA methylation & histone acetylation will induce pluripotent resistant phenotype that can be easily reversed targeting these two machineries.

Epigallocatechingallate "EGCG "in green tea" has been proved to inhibit Dnmt1 & reactivate methylation-silenced genes in cultured cancer cells [1, 2] and work with reduced toxicity comparing to nucleoside hypomethylating analogues [3]. As a novel histone acetyltransferase inhibitor "HATi" with global specificity for the majority of HAT enzymes, EGCG showed no activity toward epigenetic enzymes including HDAC and HMTase [4] and it needs to work in concert with a HDACi counterpart to reach the epigenetic

state of gene re-activation. Clinical implications of EGCG in treatment of cancer cells of varying origins [5, 6, 7, 8, 9, 10, 11] would in addition represent a pleiotropic impact in favor of better treatment.

Valproic acid (VPA) is a unique HDAC inhibitor that can enhance (MBD2/dMTase), induce DNA demethylation [12] target a number of non-histone proteins, induce differentiation, cell-cycle arrest [13]& apoptosis [14]. Clinical efficacy of VPA has been proved in many earlier trials, in MDS, advanced, poor risk, & refractory AML patients [15, 16]. A forceful attack to DNA methylation & histone acetylation would then be expected if these two drugs were combined with the standard AML chemotherapy. Only if they proved to work in concert and without a robust effect, a better patients prognosis would be expected.

Silencing of the tumor suppressor $\boldsymbol{p15^{INK4b}}$ gene by hypermethylation is a frequent event in various hematological malignancies especially in AML [17, 18]. It is correlated with Leukemic transformation in nearly all FAB subtypes & can be detected in bone marrow, peripheral blood cells [19]. Quantifying MRD with estrogen receptor A (ERA) and/or $\boldsymbol{p15^{INK4b}}$ methylation proved to account for a high relapse risk and reduced relapse-free survival. Reactivation of $\boldsymbol{p15^{INK4b}}$ by "EGCG" was prescribed earlier [1]. HDACIs as well, proved to induce reactivation of $\boldsymbol{p15^{INK4b}}$ through activation of its deacetylated promoter [20] and restraining the formation of a repressive complex [21].

Both EGCG and VPA proved to inhibit NF-κB activation [22, 9] which would consequently inhibit downstream proliferative & angiogenic cytokines as COX2 [23], VEGF [24]& IL-6 [9] as well as TNFα. activation [25, 26].

In the present study, we tested this hypothesis that epigenetic treatment with the combination of VPA & EGCG, parallel to the standard chemotherapy regimen (3+7) would reverse DNA methylation *in vivo*. Our data have important potential implications on the therapeutic reversal of pathological DNA methylation in acute myeloid leukemia patients.

1-PATIENTS:

This study recruited 68 unrelated newly diagnosed adults AML patients (27 females, 41 males; mean age, 36.09 years) & 23 healthy subjects for control purposes. All subjects signed a written consent [1*]. All were admitted & followed at Alexandria Faculty of Medicine, Department of Internal Medicine and Hematology between March 2006 and July 2008. Patients were classified following FAB classification to (M0, 7 patients; M1, 11; M2, 16; M3, 12; M4, 11; M5, 5; M6, 3; & M7, 3) and were categorized into three treatments arms" CHX (1^{st}), VA (2^{nd}), VG (3^{rd}); they were all treated within (3+7) protocol. The M3 subtype of AML " acute promyelocytic leukemia" treated with the drug ATRA (all-*trans*-retinoic acid) in addition to induction chemotherapy. Both 2^{nd} & 3^{rd} arms received 40 mg/kg/bwt of VPA tablets per day [2*]. Additional 25 mg/kg/day of EGCG tablets were given to 3^{rd} arm only. Epidrugs were given starting from day one of chemotherapy to day eight and for two successive THX cycles.

Valproic acid dose used herein was preceded with pilot studies. Doses 10,20,40 mg/kg.bwt were given for two cycles with chemotherapy. LDH & IL-6 were tested as prognostic indicators. 40 mg/kg.bwt dose was chosen. As drug level is considered to be a factor favoring toxicity or G_1 arrest VPA dose was just within therapeutic levels for epilepsy and thus appears clinically acceptable.

Sequential bone marrow aspirates were obtained when possible. Blood samples were withdrawn before & after each treatment cycles "for the first two cycle". All samples were handled, and coded. Laboratory tests were done according to the designed protocol and all data were recorded "each in the relevant report. Patients were followed up for 18 after end of their consolidation therapy " each patient received 2 induction & 3 to 4 consolidation cycles, median of 6 months"

cases with three time points of follow-up were selected for analysis [3*]

METHODS

3.1. Preparation of cell pellets: Blood was collected from patient on EDTA and samples were centrifuged at 4°C & 2000 r.p.m. for 5 min. plasma was then withdrawn, RBCs were lysed and white blood cell were isolated by centrifugation and preserved in PBS at (-80°C) until use.

3.2. Preparation of Nuclear Extract & Quantifying Protein Concentration: Following the method described [27]. Absence of cytosolic LDH was a purity-indicator. And, proteins were quantified in 10 µl aliquots of nuclear extracts [28]: by using a Bradford assay using the Biorad Dye Reagent Concentrate (BIO-RAD Laborites. Cat. # 500-000l). BSA as standard. Spectrophotometeric measures were done for standard and samples against blank at O.D 595 on (Ultraspec® 1000 Pharmacia Biotech.U.K). ALL chemicals were purchased from Sigma Aldrich.

3.3. RT-PCR for $p15^{INK4b}$ gene: Total RNA was isolated from peripheral blood and from white blood cells pellets using TRIzol® LS reagent (Invitrogen, Paisley, UK. Cat. No. 10296010) as described [29]. Reverse transcription-PCR was done by using the Sigma Enhanced Avian HSRT-PCR kit Proceedure was done in One-Step RT-PCR reaction. 0.5 µL from each $p15^{INK4b}$ primers were purchased from Sigma (sense & anti-sense 100 pmoles/µl) assayed as described (30) & amplification was performed using PXE 0.2 thermal-cycler (Thermo Electron Co.)

3.4. MS-PCR for $p15^{INK4b}$ gene: 5µg of genomic DNA samples were treated with the chemical bisulfite to convert unmethylated cytosines into uracils. Samples were purified using the "EZ Bisulfite DNA Clean-up Kit™" from ZYMO Research (Cat.D5025) & amplified as described [31]. Primers used for p15 unmethylated reaction were: p15 UM were: Sense 5'-TGT GAT GTG TTT GTA TTT TGT GGT T-3' &Antisense 5'-CCA TAC AAT AAC CAA ACA ACC AA-3'. And p15 M: sense 5'-GCG TTCGTATTTTGC GGT T-3' &Antisense 5'-CGT ACA ATA ACC GAA CGA CCG A-3' .GAPDH, 5'-CGGAGTCAACGGATT TGGTCGTAT-3' & 5'-AGCCTTCTCCATGGTG G TGAAGAC-3'. PCR conditions were as follows: 95°C for 3 min, then 40 cycles at 95°C for 40 s, 60°C for 40 s and 72°C for 40 s, and a final extension of 3 min at 72°C. Then 5-6% PAGE was used for detection as described (30).

3.5. Dnmt1 Activity Assay*:* This assay is based on methods pre-described [32, 33] using a sensitive, UV-based, enzyme coupled assay. DNA methyltransferase activity was determined in 10 µL of nuclear extracts. the nuclear extracts were incubated for 1.5 or 2 hrs at 37°C with the Substrate 0.66 µM of poly(dI-dC)·poly(dI-dC) and 10 µM of *S*-adenosyl-L-methyl-methionine in a total volume of 10 µl of a pH 7.4 buffer, containing 20 mM Tris-HCl, 25% glycerol (v/v), 10 mM EDTA, 0.2 mM PMSF, 0.02% DMSO, and 2 mM MgCl2 as a buffer additive]. Reaction incubated for 2 hrs The reaction was initiated by the addition of nuclear extracts and stopped by mixing with 300 µl of a solution containing 1% SDS, 0.25 mg/ml carrier salmon testes DNA and 1 mg/ml proteinase K & chilling in ice. 6.5 µl of solution (a) containing ADA (15U/1mg; Worthington Biochem.) & S.adenosyl-L-homosycteine nucleosidase (1U) were buffered in 173.5 µl solution (b) [50% glycrol (v/v) , 0.01 mM KH2PO4 PH 6.0]. The mixture is pre warmed to R.T., and 180 µl of that mix is added per well, in a final volume of 200 µl per well. We immediately made wells to zero point and began to measure absorbance at 510 nm. For the background control, aliquots of 10 µl acceptor substrate were added to aliquot 10µl SAM MTase Assay Buffer into each background control well. The background control was subtracted from samples and then a curve of protein content against absorbance was plotted representing rate of the enzyme activity. Except of ADA all other chemicals were purchased from Sigma.

3.6. HDAC Activity Assay: This assay was performed using Biomol HDAC Colorimetric Activity/Inhibition Assay Drug Discovery Kit (Biomol® International LP. Cat. No. AK-501) & according to the manufacturer' instruction.

The HDAC reaction was initiated by addition of diluted *Color de Lys* ™ substrate and stopped after appropriate time by addition of diluted *Color de Lys* ™ developer (containing Trichostatin A). Absorbance was read at 405 nm. A curve of protein content against absorbance was plotted representing rate of the enzyme activity. HDAC activity was signified as a function of O.D. 405.

3.7. Determination of IL-6 in patients' sera: This determination was set using BioLegend's ELISA MAX™ Set Standard Human IL-6 kit (Cat. 430502) & according to the manufacturer' instruction. Tween-20 was purchased from Sigma (Cat. # P-7949) TMB Substrate & Assay Diluent were purchased from BioLegend's Cat. No. 421101, & 421201 respectively. The sensitivity of the kit was 1 pg/ml of IL-6. Absorbance was read at 450 nm with (BioTek Elx-800, Germany micro-plate reader), and concentration of IL-6 in sera was determined in pg/ml using standard curve.

3.8. Determination of TNF-α in patients' sera: This quantifying was done using Human TNF-α ELISA Development Kit (Peprotech, cat # 900-K25). BSA, & ABTS Liquid Substrate Solution were purchased from Sigma Cat # A-7030, & A3219); Where Dulbecco's PBS [10x] was obtained from (Gibco BRL Cat. # 14200-075). Absorbance was read at 405 nm with wavelength correction set at 650 nm. The sensitivity of the kit was within the range of 32–2000pg/ml.

3.10. Determination of VEGF& bFGF: These assays were set as described [34] using Human EG-VEGF ELISA development Kit (Peprotech, Cat. # 900 K-433) for the quantitative measurement of natural h EG-VEGF in a sandwich ELISA format. The sensitivity of the kit was within the range of 16–1000pg/ml. Absorbance was read using the former BioTek ELISA plate reader BioTek at 405 nm with λ correction at 650 nm. bFGF assay was performed using quantitative sandwich enzyme immunoassay technique. The Human FGF basic EIA Kit from ALPCO Diagnostics Cat. No. 45-FGFHU-E01 was used for the assay. Absorbance was read at 450 nm within against blank. FGF-b concentrations for unknown samples and controls were extracted from the standard curve & values obtained were multiplied by 2 to correct for the 1:2 dilution.

3.11. Estimation of NF-κB in Nuclear Extract: Procedure was performed as described [35] for the estimation of the activated NF-κB in nuclear extract. The probe 2 pmole of 22 bp of the sequence [5'-AGTT GAG GGG ACT TTC CCA GGC-3'] was manufactured by Linilab., Cairo, Egypt. And the following chemicals were purchased: Streptavidin-coated plate (Sigma Cat # M5432 Sigma Screen), Rabbit Anti-NF-κB antibody (BioLegend Cat # 622601), Peroxidase-Conjugated goat anti-rabbit IgG (Cayman Chem. Cat # 10003401), & TMB substrate (BioLegend B104903). The developed yellow color was read using BioTek ELISA plate reader at 450 nm with a reference wavelength (λ) 655 nm. The

Intensity of the colored product was directly proportional to the concentration of the activated NF-κB in the original sample.

3.12. Western blotting for COX2 and p65: COX2 and p65 immunoblots were performed on prepared nuclear extract. Following method pre-described [36]. Protein were quantified in nuclear extracts and assayed in 20µl protein lysates. Antibodies for COX2, P65, & lamin B were purchased from (Santa Cruz Biotechnology Inc. Cat. # sc-1745, 1:300, sc-109, 1:300, &sc-6217,1:300 respectively). DTT, Bromophenol blue.& TEMED from (Sigma Cat # D 9760, 114391 & T9281 respectively),HRP-conjugated secondary Ab from abcam (1:1000; ab 6721. Abcam U.S.A.). Ab labeling was visualized by Chemiluminescence Reagent Plus (PerkinElmer Life Science's Cat. # NEL103), and membrane was exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y., USA).

RESULTS AND DISSCUSSION

Valproic acid (VPA) & green tea extract have been used as well-tolerated drugs with various therapeutic effects [5-14]. In this study we combined these two epidrugs as an epigenetic "parallel treatment" protocol with the standard

AML chemotherapy (3+7). Peripheral blood samples were collected & tested for effect on the DNA methyltransferase1 "Dnmt1" activity. Methylation activity assay was performed as described previously (Section 3.5) to determine the relative levels of activity following our treatment. Lanes (1) in Fig. (1a) shows result obtained from control patients' samples. Lanes (2, 4 & 6) represents means levels of enzyme activity before treatment in all groups. While lanes (3, 5 & 7) represents activity after 2 treatment cycles. Results showed an approximately one third reduction in activity (34%) in lane (7) represents VG group (3rd arm of treatment, received VPA + EGCG in addition to chemotherapy) comparing to a minimal & imperceptible decrease by 7% in lane (5) represents VA group; (2nd arm of treatment, received VPA in addition to chemotherapy) & a noticeable increase in 1st arm category who received chemotherapy alone by 16%; lane (3).

Study recorded *"for the first time to our knowledge"* a slight decrease in Dnmt1 activity in category of 2nd treatment arm due to VPA treatment which implies that Dnmt1 gene is possibly regulated by histone acetylation mechanism bearing results recorded earlier (37). Yet this effect was absent in samples from patients treated with doses <40 mg/kg/BWT "pilot study". A possible mechanism discussed earlier of docking of EGCG in the catalytic pocket of the enzyme *in vitro* (1) may stood for inhibition of the enzyme *in vivo* as in 3rd arm (VG group) as well.

The effect of inhibitors represented in Figure (1b) shows The Eadie-Hofstee plots for the inhibition of "Dnmt1"& determining the Michaelis Constant Km and the Limiting Velocity Vmax after 2 cycles of THX. 3rd arm gave the greatest inhibition effect on the enzyme reducing Vmax to 0.42 pmol /min/mg instead of 0.93 pmol/min/mg in 1st arm.

Questioning synergism between DNA methylation and histone modifications, effect on HDAC activity was measured. Figure (2) shows a marked decrease in activity in both 2nd & 3rd arms of treatment (≈ 60 %) with P <0.0001, suggesting that a mechanism of epigenetic gene control alterations by EGCG might be exerted corresponding to almost equal rise in activity in 1st arm.

To determine if there was a re-activation of silenced genes in Leukemic Blasts, methylation analysis of $p15^{INK4b}$ gene was performed. Methylation analyses for p15 gene in the recruited subjects were done at two points before & after end of treatment (A & B), Fig. (3a) represents MS-PCR for p15gene. Results showed: A decrease in methylation level of $P15^{INK4b}$ in 3rd arm (lanes 6, 7, 8) more that that recorded in 2nd arm (lanes 3, 4, 5) while no difference at all was recorded in 1st arm - group received CHX alone-(lanes 1, 2). Hypomethylation of the $P15^{INK4b}$ gene was accordant to retain the gene expression in 2nd & 3rd arms as seen in fig. (3b). Figures (3c) & (3d) shows the rest MS-PCR results for 10 & 9 cases of both 2nd & 3rd arms respectively (P<0.02). Table (1) shows number of cases recorded before treatment with increased *P15* methylation & reduced *P15* expression in treated groups and the change in these two parameters post treatment. The pattern of Dnmt1 activity inhibition underlined the change in $P15^{INK4b}$ methylation status in each case "results not shown".

Table 1: Methylation analysis data for all treated groups. X_1 & Y1 :no of patients with reduced /absent P15 mRNA expression & P 15 methylation respectively at diagnosis. X_2 & Y2: no of patient with increased mRNA expression & P 15 methylation respectively at end of treatment. (R%): percent increase in P15 mRNA expression. (M%): percent decrease in P 15 methylation. : CHX: chemotherapy; VA: group treated with chemotherapy + valproic acid; VG: group treated with chemotherapy + valproic + green tea.

Methylation analysis data for all treated groups

	X1	X_2 (R%) Post THX	Y1	Y2 (M%) Post THX
CHX	18 / 23	No change	12 / 18	No change
VA	18 / 23	10 (55%) , 4 (25%)	16 / 18	5 (13 %), 1 (25%) 2 (52%)
VG	16 / 22	11 (80%) , 5 (33%)	15 / 16	7 (80%), 3 (40%) 3 (60%), 2 (33%)

To determine whether treatment down regulated proliferation of leukemia cells we measured IL-6 level. Results indicated that despite the different pattern

of IL-6 in each FAB subtype the overall result was the down-regulation of IL-6. The change in IL-6 level was regarded as separate category per subtype. IL-6 mean values variation in all treated patients represented in fig. (4a) shows tendency toward applying the proposed regimen with $p \leq 0.006$ in 2nd arm & $p \leq 0.003$ in 3rd arm. This decrease in IL-6 came accordant to patient prognosis and lower levels were associated with lower percentage of blasts in the peripheral blood & with decrease in the methyltransferase activity" not shown". Results supported evidence of IL-6 cytokine-mediated alteration of methyltransferase gene expression (38, 39, 40).

TNFα production was proved to accompany myeloid differentiation (41) and vascularity in the bone marrow as well as increased levels of various angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) (42). Fig. (4b) shows collective results of mean values of TNFα, VEGF & bFGF in (pg/ml) after each treatment cycle. Upon detecting TNF-α level represented the highest decrement percent reaching 57 % after 2nd cycles in 3rd arm that received VPA+ green tea extract with chemotherapy. While in VA group the percent was 44 %, & only 29% in CHX. The output of Angiogenesis- related cytokines in 2nd & 3rd arm was reduced by 60 % & 69 % respectively in case of VEGF, comparing to 37 % in CHX group. While bFGF reduced by 48 % & 39 % respectively comparing to 32 % in CHX group. The marked level of VEGF decrease rather than bFGF supposed to be due the relationship between VEGF secretion and VPA (43) which was lesser in case of EGCG integration.

Investigating the proliferation suppressing potential of the applied regimen, we tested samples for NF-κB inhibition. Figure (5a): represent results of inhibition of NF-κB in recruited subjects, showing at $p = 0.01$, there was no significant difference in group received the standard chemotherapy. In 2nd & 3rd arms, we recorded 44 %& 53 % decrease in NF-κB level, respectively. Suppression of NF-κB limits the proliferation of cancer cells & has potential therapeutic consequences. Down regulation of NF-κB, would then increase the cellular susceptibility to TNF-induced apoptosis. Western Blot analysis for the change of COX2 & P65 protein levels showed reduction of both COX-2 & P65 in 2nd arm in 18/23 & 12/23 cases respectively ($p<0.001$), while in 3rd arm reduction was recorded in 21/22 & 17/22 cases respectively. There was no significant change in 1st arm. Figure (5b) represents results of 5 cases in 2nd & 3rd arms before (A) & at the end of treatment (B). Reduction of COX-2 may be due to bilateral inhibition that might be triggered by both HDACi blocking to TNF-α activation (44) and green tea catechins suppressive effect on NF-κB (45) Reduction of the P65 in 2nd arm, would further be associated with sequential IκB kinase suppression & suppression of NF-κB trans-activation, consequently COX2 inhibition forcing cells to commend apoptosis. An effect that possibly increased in 3rd arm upon combining green tea. (46), Clinical data showed that this impact on methylation level had a desirable outcome in patients prognosis, the majority in epigenetically treated groups show rehearse of complete remission with one refractory case in 2nd arm and no deaths, third arm actually showed no RF cases (Table 2). Hematological data[4*] as represented in fig. (6) "upper left " shows a notable decrease in minimal residual disease (MRD) in these groups (MRD≤ 1%, p=0.004; 0.0036) in 2nd & 3rd arms respectively with complete elimination of MRD (in 17 / 37 CR patients). Samples with reduced/ negative reversal of p15 methylation mirrored clinical data for patients with PR:RF "the majority were in 1st arm of treatment.

According to MRD status during/after therapy, two groups of patients. In 7/45 PR cases in both epigenetically treated arms who had MRD levels of less than 10% in relation to all time points tested after therapy, 6 experienced relapse before 15 months of follow up in 2nd arm & 1 in 3rd arm. Other who experienced relapse came after 15 month, with median remission duration of

17& 24 months in 2nd & 3rd arms respectively.

Table 2: Patient prognostic indices/ FAB Subtype in all treated groups. Abbreviations: CHX: chemotherapy; VA: group treated with chemotherapy + valproic acid; VG: group treated with chemotherapy + valproic + green tea, (m0-m7): FAB subtypes of AML, CR: complete remission; PR, partial remission; RA, refractory anemia;

Groups	CR	PR	RF	Failed
CHX	1(M1), 3(M2), 2(M3), 2(M4),	2(M2), 2(M3), 3(M4),	2(M1), 2(M2), 1(M5),1(M6)	1(M0), 1(M7),
VA	2(M0), 2(M1), 5(M2), 6(M3), 2(M4), 1(M5)	2(M0),1 (M1), 1(M4)	1(M7)	
VG	2(M0), 3(M1), 4(M2), 3(M3), 4 (M4), 2(M5), 1(M7)	1(M5), 2(M6)		

Survival analyses (fig. 6) provided evidences on rehearse of remission end-point & decreased risk of relapse. Progress-Free Survival (PFS) curve "upper right" shows that third arm of treatment "VG group" had the fastest & highest ratio of complete remission between groups. Disease-Free Survival (DFS) curve "lower right" showed that the fastest relapse was in CHX group, "that relapse delay came consistent with both drawbacks in $p15^{INK4b}$ methylation and MRD".

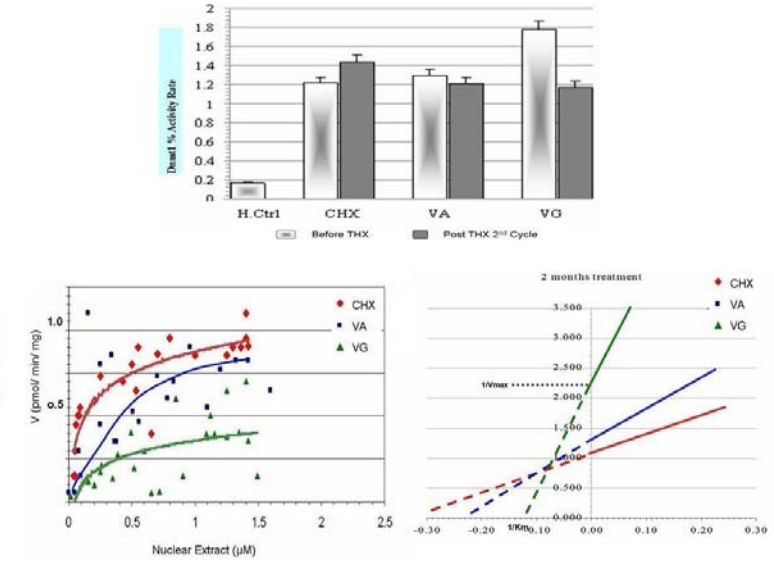

Fig. (1): Inhibition of 5-cytosine DNA methyltransferase "Dnmt1" activity. **(a)** Inhibition of Dnmt1 activity rate in recruited subjects, represented as mean values before & at the end of treatment. Abbreviations: H.Ctrl: healthy control; CHX: chemotherapy; VA: group treated with chemotherapy + valproic acid; VG: group treated with chemotherapy + valproic + green tea.. The assay was done as prescribed in methods. Results are mean of 2 determinations, ($P \leq 0.01$). **(b)** Eadie-Hofstee plots for the inhibition of "Dnmt1". Each data point is the mean of duplicate determinations. At 95% confidence interval, in **CHX** group *Km* = 0.82 + 0.006 µM & *Vmax* = 0.93 pmol/min/mg. In **VA** group *Km* = 0.43 + 0.008 µM & *Vmax* = 0.78 pmol/min/mg. In **VG** group *Km* =0.33 + 0.008 µM & *Vmax* = 0.42 pmol/min/mg. Data were analyzed by Two-way ANOVA (V. 8.0 SR2, 2008).

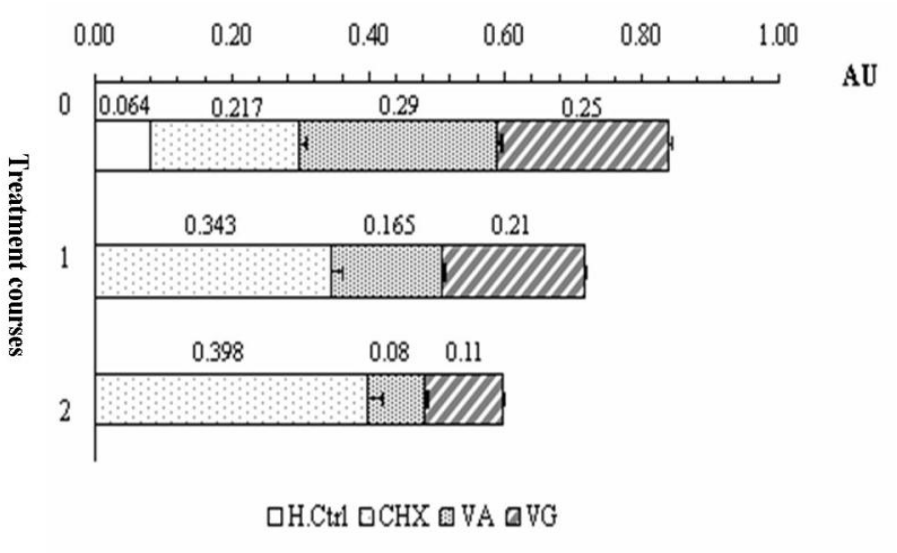

Fig. (2): Inhibition of HDAC activity in recruited subjects. Abbreviations: (0,1, &2) : Time courses of treatment; H.Ctrl: healthy control; CHX: chemotherapy; VA: group treated with chemotherapy + valproic acid; VG: group treated with chemotherapy + valproic + green tea; significance between 0, 2 cycles ($p \leq 0.0001$) in VA & VG groups. Each data point represents the mean SD of 3 determinations. mean values cumulative bars shows HDAC activity is more reduced when EGCG in green tea extract was added.

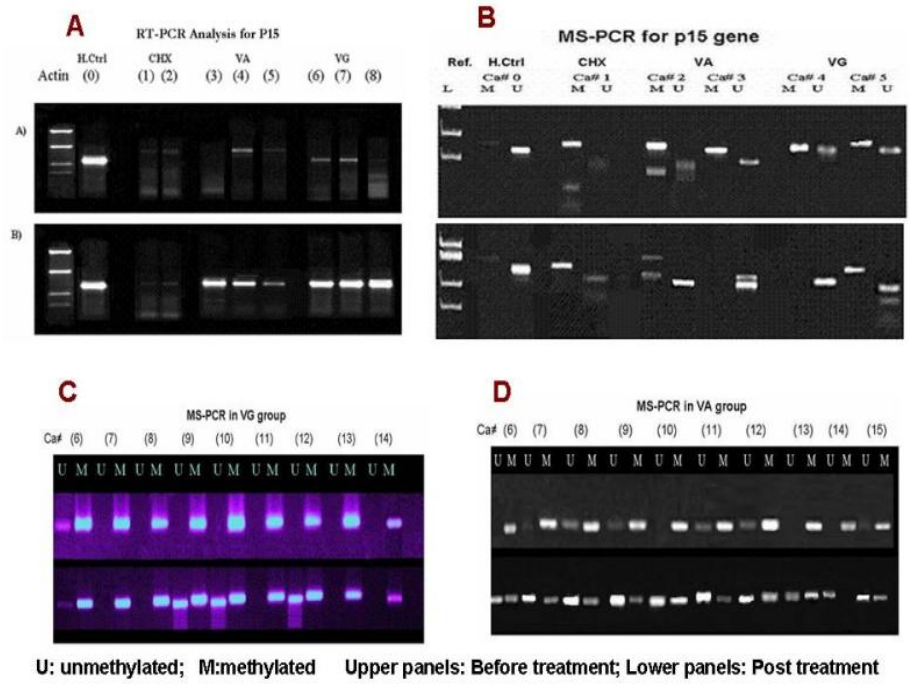

Fig. (3): Alterations of methylation status and mRNA expression levels of p15INK4b gene in AML patients. For all divisions "upper panels" represent before treatment & "bottom panels" represent end of treatment. **(a)** Shows the mRNA expression levels in 9 cases of recruited subjects. 4µg of m.RNA was used in a reverse transcription reaction as per protocol with β-actin as internal control. Products were electrophoresed on 3% agarose gel, stained with EtBr. (0- 8) cases number. **(b) (c) & (d):** Shows methylation change in AML patients after epigenetic treatment. 5 µl of chemically modified genomic DNA was used in a methylation specific PCR reaction as per protocol using GADP as control & electrophoresed using PAGE. H.Ctrl: healthy control; CHX: chemotherapy; VA: group treated with chemotherapy + valproic acid; VG: group treated with chemotherapy + valproic + green tea. methylated (M), unmethylated bands (U). [Ca#] case number; (0) H.ctrl; (1) CHX gp; (2,3) VA gp; (4,5) VG gp. No change was recorded in H.Ctrl (ca # 0).

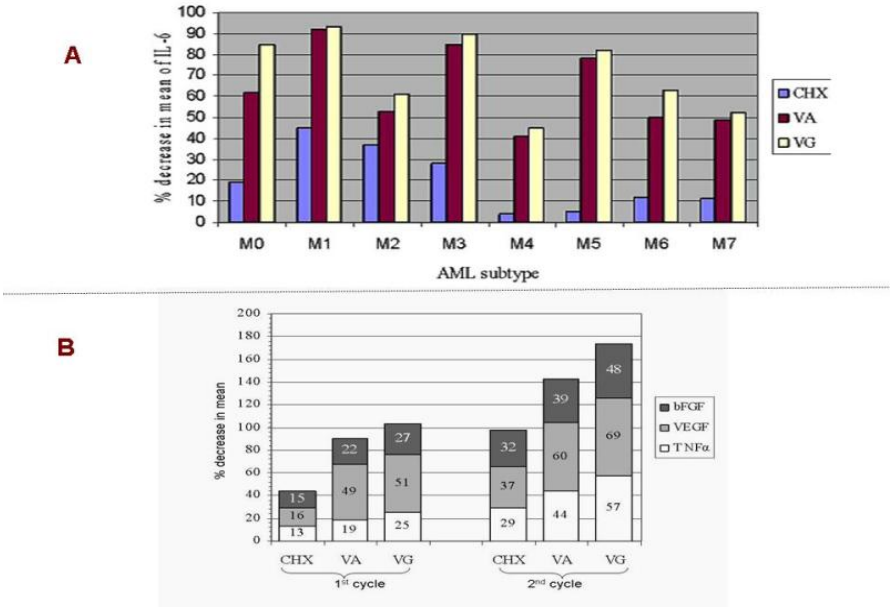

Fig. 4: Detection of cytokines inhibition in epigenetically–treated AML patients. IL-6, TNFα, VEGF & bFGF activities were measured as described in the text. Activity of each was taken as 100%. **(a):** IL-6 mean values variation in all treated patients. Mean values ranged between (439.0 - 764.0) in M0; (97.5 - 694.6) in M1; (3296.0 - 697.0) in M2; (69.0 – 477.0) in M3; (33.2 - 53.0) in M4; (2050.0 – 3116.0) in M5; (61.0 – 2504.0) in M6; (42.6 – 63.0) in M7. Decrease in mean within the same FAB subtype in each treatment group was in the order CHX<VA<VG ($p \leq 0.006$ in 2nd arm & $p \leq 0.003$ in 3rd arm). **(b):** Collective results of mean values of TNFα, VEGF & bFGF (pg/ml) after each treatment cycle in all treated patients. Each data point represents the mean SD of 3 determinations, ($P \leq 0.01$). Abbreviations: (M0-M7): FAB subtype; H.Ctrl: healthy control; CHX: chemotherapy; VA: group treated with chemotherapy + valproic acid; VG: group treated with chemotherapy + valproic + green tea. Results are expressed as means ± SEM. Each data point represents the mean SD of 3 determinations.

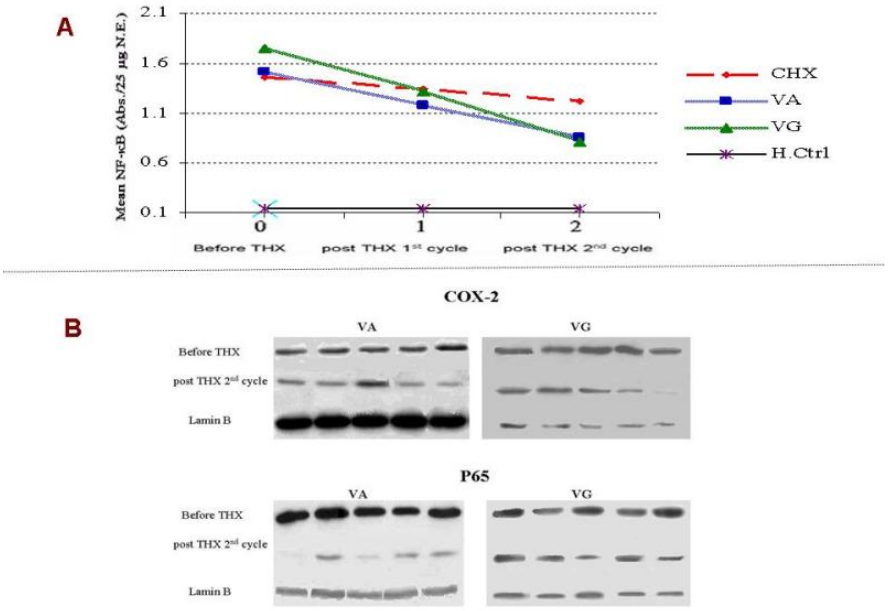

Fig. 5: **(a):** Mean values of inhibition of NF-κB in all recruited subjects before (0) & after (1,2) treatment cycles. H.Ctrl: healthy control; CHX: chemotherapy; VA: group treated with chemotherapy + valproic acid; VG: group treated with chemotherapy + valproic + green tea. **(b):** Western Blot analysis for the change of COX2 & P65 protein levels represents results of 5 cases in 2nd & 3rd arms before (A) & at the end of treatment (B). Protein were quantified in nuclear extracts and assayed in 20μl protein lysates as mentioned in methods and compared to Lamin B ($P \leq 0.008$).

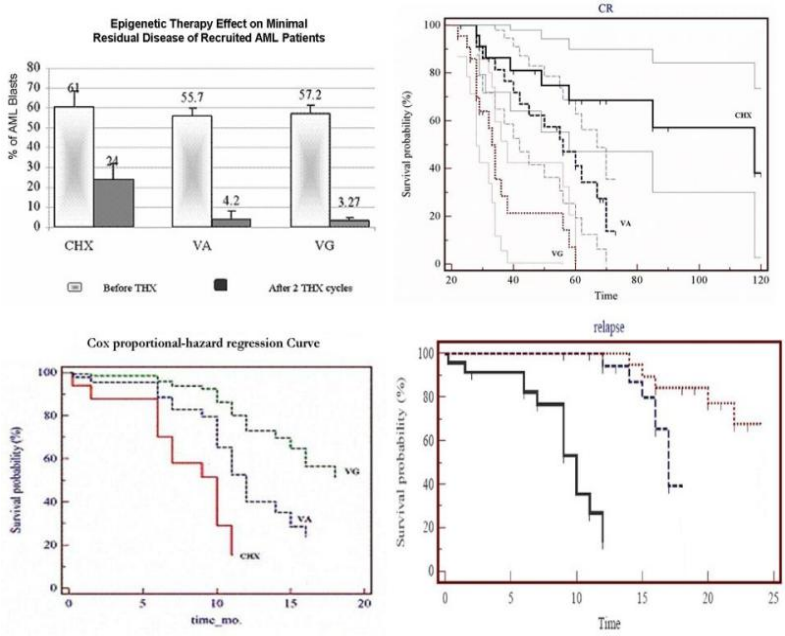

Fig. (6): Clinical & survival Data: "***upper left"*** represents Epigenetic Therapy Effect on Minimal Residual Disease "MRD" of Recruited AML Patients. MRD in all recruited subjects before (0) & after 2 treatment cycles are expressed as percents of means ± SEM. Each data point represents the mean SD of 3 determinations. (MRD≤ 1%, p=0.004; 0.0036) in 2nd & 3rd arms respectively. In 18/23 CR cases in 2nd arm, 6 showed 0 blasts in bone marrow after 1st cycle; situation didn't change after 2nd cycle. One more case progressed to "0 blast point" after 2nd cycle. In 19/22 cases in 3rd arm, 11 showed 0 blasts in bone marrow after 2nd cycle. 8 cases came after 2nd cycle. In 7/45 PR cases in both epigenetically treated arms, number of blasts ≤ 6 % . ***"upper right":*** Kaplan Meier's Progress-Free Survival (PFS) curve shows median times to reach complete remission (45, 65, 110 days) in 1st, 2nd, & 3rd arms of treatment respectively. Third arm of treatment "VG group" showed the fastest & highest ratio of complete remission between groups. ***"Lower right":*** Kaplan Meier's Disease-Free Survival (DFS) curve shows 1st arm of treatment with significantly decreased relapse-free survival (mean, 285 days; P = 0.06) & high levels of p15 methylation, while in 2nd & 3rd arms, relapse delayed with (mean, 510 days, P = 0.005)& (mean, 720 days; P =0.002) respectively. With 95% confidence, risk of relapse is approximately 1.8 & 2.5 times in CHX group than the risk in VA& VG groups. The majority of patients with high levels of $p15^{INK4B}$ methylation in remission have relapsed, whereas patients with low levels of methylation have not, the majority was in 2nd & 3rd arm groups (DFS =24 months chi-square value of 15.35 and p<0.001). ***"Lower left":*** Cox-regression Curve for covariates [LDH, NF-κB, VEGF, TNFα levels and MRD] showed a "*within group expo"*. Cumulative hazard factor equals 0.3446 upon increase of the studied variables. All variables used were found to significantly contribute to the prediction of time, and were all included in the model. A HR <1 (Exp(b) = 0.3446 with increasing 95% CI of Exp(b) = 0.1608 to 0.7384 means that our treated VA & VG groups of interest when compared to the reference CHX group are less likely have a shorter time to end event. Abbreviations: CHX: chemotherapy; VA: group treated with chemotherapy + valproic acid; VG: group treated with chemotherapy + valproic + green tea.

The majority of patients with high levels of p15INK4B methylation in 1st arm of treatment in remission (12 /18, 67%) experienced shorter time to relapse (mean 285 days) with statistical significance (P = 0.06), whereas patients with PR in 2nd arm "all had a reversed levels of methylation" had relapsed later (mean, 510 days, P = 0.005). A notable significant difference in relapse-free survival was observed in 3rd arm (mean, 720 days; P =0.002). Both arms when compared to CHX group are less likely have a shorter time to end event which is relapse. Cox-regression Curve for covariates [LDH, NF-κB, VEGF, TNFα levels and MRD] showed (P = 0.00641; HR <1 (Exp(b) = 0.3446 with increasing 95% CI of Exp(b) = 0.1608 to 0.7384) With 95% confidence, risk of relapse is approximately 1.8, 2.5 times in CHX group than the risk in VA& VG groups respectively. For the considered regression coefficients: p< .01, with increasing 95% CI of Exp (b) values were in the positive direction.

Putting forward these results, we bring to a close that targeting the DNA methyltransferase1 & histone deacetylation potential using both Valproic acid & Epigallocatechingallate " together as in the prescribed regimen" had reactivated & reversed the methylation of $\boldsymbol{p15^{INK4b}}$ gene *in vivo* of AML patients, retained TGF-ß regulatory signal. We suppose that VPA & EGCG managed together to inhibit methylation directly *via* inhibiting Dnmt1 & HDAC and indirectly by down-regulation of IL-6 (9, 22, 38). Also down-regulation of growth factors including IL-6 & TNFα which held back proliferation of AML blasts. Considering the fact that cells arrested in G_1 progress toward differentiation, and finally to apoptosis (47), the effect of TNF-α on downstream events including NF-κB cytoplasmic translocation, shared to decrease expression of target genes as COX2 "bearing in mind the direct effect of VPA & EGCG on COX-2". Reduction of COX-2 mRNA & protein shared to down-regulate the release of VEGF, drew back angiogenic potential and progression of cells from G0 to G1 phase which could free the seizing of apoptotic potential within the tumor niche, abate proliferation & angiogenic switches to the malignant epigene.

In our study, we show that the HDAC inhibitor VPA reversed p15 methylation, induced apoptosis, promoted differentiation and improved patient prognosis, all at the same dose (40 mg/kg.bwt) making a dose-dependent effect unlikely. Yet, the net effect was markedly improved when EGCG in green tea extract was introduced, a factor that reflected patients prognostic dissimilarities between the two arms.

Toxicity grading and evaluation of the applied regimen proved absence of any serious side effects which could be relate to valproic acid toxicity "results not shown", regimen already fulfilled our requirements and for this, therapy should not be further intensified.

CONCLUSION

The proposed epi-drug combination distinctively modified the malignant transcriptome of AML blasts through inhibition of DNA hypermethylation & histone acetylation and conferred positive prognostic impact on AML patients.

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FOOTNOTES

1* This study was approved by the Alexandria University G.S.R.A. Division no. (537)/4/7/20/2/1.

2* Valproic acid used as Depakene chrono 500 ® tab., purchased from Sanofi /Aventis; EGCG given as Green tea® tab. 1gm equ. to 200 mg EGCG according to the manufacturer instructions purchased from Technomad Co. An IC_{50} of ≈20 µM EGCG inhibited Dnmt1 is achievable in the oral cavity after drinking green tea and perhaps in the stomach, esophagus, and intestines where there is direct contact between EGCG and the epithelial cells. The effective concentrations of EGCG (10–50 *µ*mol/L) observed in studies with cell lines are 50 times higher than plasma and tissue levels of EGCG generally observed after ingestion of tea (1).A single, 200-mg dose of EGCG produces a plasma EGCG concentration of ~0.1 mmol/L. usually; the consumption of pharmaceutically prepared formulations of green tea polyphenols would produce plasma EGCG concentrations approaching 2 mmol/L (48). The total daily dose ranges of EGCG used to treat cancer are generally from about 10 mg to about 100,000 mg according to the U. S. Patent no.6652890 (49); administered in divided doses "parenterally or orally or topically" with a preferred total daily dose from about 0.1 mg to about 10 mg/kg/day". 3* Descriptive, inferential, and survival statistical analysis were performed using Two-Way ANOVA of Microcal Origin software [V. 8.0 SR2, 2008]. at p = 0.01 significance level. Kaplan Meier's analysis was done using [MedCalc. 7.7.4.]. Dunnett's test [KWIKSTAT WINKS SDA 6.0.] & Kruskal-Wallis test calculated at α=0.01 significance to determine specific pair wise differences "results not shown".
4* Assessment of some hematological prognostics in treated group followed IPSS score system. Toxicity grading performed according to NCI Common Toxicity Criteria (CTC) (version 2).

ABBREVIATIONS

HDACs / HDACi**:** histone deacetylases / inhibitor
HMTase: histone methyltransferase
Epigallocatechingallate: EGCG
VPA: Valproic acid
MDS: Myelodysplastic syndrome.

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ARABIC SUMMARY

علاج جديد لمرضى اللوكيميا النخاعية الحادة بإستخدام العقاقير الجينية يؤدى إلى تحسين نتائج إكلينيكية و جزيئية

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أن تثبيط الجينات بتأثير الميثيل المضافة لمحفزات الجينات المثبطة للورم وتأثيرفوق الأستلة للهيستون يقوم بالتأثير على العديد من سلاسل التفاعلات الجزيئية فى اللوكيميا النخاعية الحادة.على هذا فإن الجمع بين مثبطات كلأ من الجين الناقل للميثيل و إنزيم الهيستون دى أسيتيلاز أثبت كفاءته فى عكس المظهر الجينى للخلية و الحالة الميثيلية للخلايا الأرومية وتحسين الحالة الإكلينيكية للمرضى. وهذه الدراسة تمت على عدد ٦٨ من المرضى المصابين بسرطان الدم الحادز ٤٥ منهم تلقوا عقار الديباكين "حمض فالبرويك" كمثبط لإنزيم الهيستون دى أسيتيلاز +/- مستخلص الشاى الأخضر " إيبيجالوكاتشين جاليت " كمثبط إنزيم الناقل للميثيل "ميثيل ترانسفيرز ١" بالإضافة إلى العلاج الكيميائى ٣+ ٧. وتم تسجيل ٤٥/٣٧ حالة شفاء تام ٦٠% إنحسار عدد من هم عرضة لإرتداد المرض مرة أخرى. الحد من نشاط الإنزيم الناقل للميثيل و نشاط إنزيم الهيستون دى أسيتيلاز- ١ (٠.٠٠١)، و إعادة نسخ الحمض النووي الرايبوزي الرسول للجين (بى ١٥) مترابطاً مع إضعاف الحالة الميثيلية لمحفزالجين بنسبة ٥٨% (٠.٠٠٢).

كما تم تسجيل تناقص ملحوظ معامل النخر الورمى- ألفا، المعامل النووى- كابا- بى، إنترليوكين-٦، للتمثيل البروتينى لإنزيم الأوكسيجينات الحلقي من النوع الثانى، للـ بى _٦٥ ، بتناقص تركيز معامل النمو الأندوثيلى الوعائى وأيضاً معامل النمو الليفى القاعدى فى اوركسترا متوافق (٠.٠١)، وظهر التحسن فى آداء المرضى خلال المتابعة لعامين (٠.٠١) ، وأثبتت هذه الدراسة ان دمج العقارين معا وفقا للخطة العلاجية الموضوعة كان له تأثير إيجابى على تدمير للخلايا الأرومية و الذى أدى ألى تراجع المرض و تحسن الحالة الإكلينيكية للمرضى.