Research Article

Design, Synthesis and Cytotoxicity Measurement of Unsymmetrical Diacylhydrazine Derivatives as Potential Anticancer Agents

Raed M. Maklad, Omar M. Aly, El-Shimaa M. N. AbdelHafez, and Dalia Abdelhamid
Department of Medicinal Chemistry,
Faculty of Pharmacy, Minia University, Egypt

Abstract
We describe the synthesis and biological evaluation of a series of tubulin polymerization potential inhibitors, analogues of combretastatin A٤ (CA-٤) which contains the bis-hydrazide linkage in replacement of the rigid cis-restricted structure of the double bond of CA-٤ I (Fig.١). The bis-hydrazides assume more than one tautomeric structure producing new tautomeric OH group(s) (Fig.٢) which exerts extra-binding affinity to colchicine binding site of tubulin protein by making strong hydrogen bonds with the active site as shown by molecular docking studies of different members of the series (table ١) as compared to common potent tubulin inhibitors like CA-٤ and its analogues (Fig.١). The bis-hydrazide nucleus do retain the rigid structure of CA-٤ by replacement of C=C by C=N resulting from tautomerism of the hydrazide functional group, molecular modeling studies show that the most stable tautomer do retain both the C=N analogous to C=C of CA-٤ and the tautomeric OH as an extra-binding group to the receptor. (Fig.٢). All the synthesized analogues show higher C-DOCKER ENERGIES than the lead CA-٤ as shown by molecular docking studies (table ١). Some of the synthesized compounds exhibited moderate cytotoxicity against a variety of cancer cell lines as shown by in-vitro cytotoxicity screening. Attachment of the ٣-$NH_٢$,٤-$OCH_٣$ group to the ring B as exemplified by compound ٧e, or ٤-OAc group as exemplified by compound ٧d conferred optimal receptor binding and optimal cytotoxicity among this series (Table ١). Nearly all of the tested compounds have moderate anti-proliferative activity against the cell lines PC-٣ (Prostate Cancer) and NCI-H٥٢٢ (Non-Small Cell Lung Cancer). Computer docking and molecular simulations of ٧e inside the colchicine binding site of tubulin enabled identification of residues most likely to interact strongly with these inhibitors **(Fig.٣)** and explain their potent anti-tubulin activity and cytotoxicity. It is hoped that results presented here will stimulate further examination of these substituted bis-hydrazides as potential anti-cancer therapeutic agents

Key words: Diacylhydrazine, tubulin inhibitors, cytotoxicity, anticancer, docking study

Introduction
Microtubules are among the most important molecular targets for anticancer agents due to their essential role in the growth and function of cells,[١] they are a key component of the cytoskeleton, and are involved in a wide range of cellular functions, including regulation of motility, cell division, organelle transport, maintenance of cell morphology, and signal transduction. The formation of microtubules is a dynamic process that involves the polymerization and depolymerization of α and β tubulin heterodimers. Tubulin inhibitors interfere with this dynamic equilibrium and thus induce cell cycle arrest, resulting in cell death.[٢] There are three previously identified binding sites in tubulin: the taxane,[٣] *vinca* alkaloid,[٤] and colchicine sites.[٥] Among the natural microtubule depolymerizing agents, combretastatin A-٤ (CA-٤) I (Fig. ١) is one of the most studied compounds. CA-٤, isolated from the bark of the South African tree Combretum Caffrum[٦], strongly inhibits polymerization of tubulin by binding to the colchicine site[٧]. CA-٤ has

been reported to exhibit potent cytotoxicity against a broad range of cancer cells including multi-drug-resistant (MDR) cell lines.[٨,٩]

Structure–activity relationship (SAR) of CA-٤ and its analogues is extensively studied. The trimethoxy substitutions on the A ring and the cis-olefinic bridge configuration have been reported as essential for potent cytotoxicity, while the B ring can tolerate modifications.[١٠] Alternatives to the olefinic bridge have been introduced; examples of heterocyclic bridges include five-membered rings such as thiophene,[١١] furan,[١١] pyrazole,[١٢] imidazole,[١٢,١٣] thiazole,[١٢a] isoxazole,[١٤] ١,٢,٣-thiadiazole,[١٥] isomeric triazoles,[١٢,١٦] and indoles. (Fig. ١)[١٧,١٨] Nonheterocyclic bridges are ethers, olefins, ketones, sulfonamides, sulfonates, amine, amide derivatives, and cyclopentanes.[١٠,١٩]

Structural modifications on the B ring suggest that the ٤-methoxy group is crucial for cytotoxicity, while the ٣-hydroxy group is optional. [٢٠,٢١,٢٢] The observed high cytotoxicity depended on the substitution pattern on the B ring, the chain lengthening from ٤-methoxy to ٤-ethoxy substituent enhanced biological activity, with the latter having the optimum length.[٢٣] The introduction of hydrogen-bonding groups in the ٣-position of B-ring may increase or retain the cytotoxicity, for example chlorine,[٢٣] fluorine,[٢٤] and the superior NH_2[٢٥] group which usually increases the cytotoxicity further than the OH of the lead compound.

In our efforts to discover novel tubulin polymerization inhibitors as potential anticancer chemotherapeutic agents, we selected the bis-hydrazide nucleus which retain the rigid structure of CA-٤ by replacement of C=C by C=N resulting from tautomerism of the hydrazide moiety, the tautomeric OH is expected to have an extra-binding affinity to the receptor. Additionally, insertion of ٣-amino group in the B ring would optimize the cytotoxicity and anticancer activity. Here we report the synthesis and in vitro cytotoxicity of a series of novel bis-hydrazide based tubulin polymerization inhibitors (Scheme ١. Compounds ٧a-e)

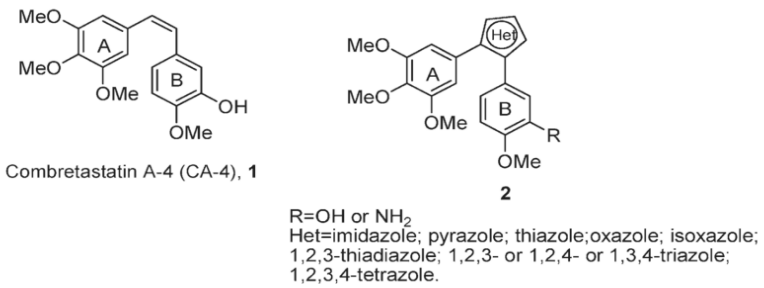


Fig.١. Common potent tubulin polymerization inhibitors

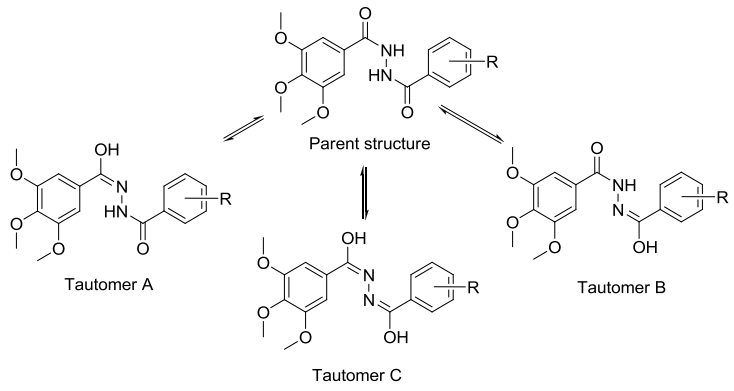


Fig.٢. Tautomerism in unsymmetrical bis-hydrazides

Results and discussion

Chemistry

Substituted benzoic acid derivatives (٣ and ٥, Scheme ١) were prepared as starting materials for their corresponding acyl chlorides used in the next step (٣ and ٥, Scheme ١). Firstly, ٤-allyloxybenzoic acid is prepared by alkylation of ٤-hydroxy-benzoic acid using allyl bromide, utilizing the high reactivity of allyl bromide toward both SN_1 and SN_2 nucleophilic substitution reaction and the good leaving group bromide ion. Here the reaction is brought to SN_2 reation conditions by in situ generation of the highly nucleophilic phenoxide ion, this was such a tedious task which require careful selection of the base used to deprotonate the phenolic OH group, the major problem was that selective deprotonation of phenolic OH group is impossible due to much higher acidity of carboxylic group, normal bases like sodium carbonate or triethyl amine are too weak to deprotonate the phenolic OH, instead they result in selective deprotonation of COOH group to its carboxylate salt which may undergo alkylation to give the ester as a major byproduct. However, when a strong base like sodium ethoxide (prepared in situ from sodium metal and ethanol) is used as a strong base, it completely deprotonates both the COOH and the phenolic OH giving the dianion which may undergo alkylation at both sites with a much higher reactivity to the most nucleophilic phenoxide ion, giving the desired ٤-allyloxybenzoic acid after acidic work up. The product is characterized by matching the melting point with the reported one, and by IR spectrometry; C=O stretching at ١٦٧٤ cm^{-1} and the C=C stretching at ١٦٠٤ cm^{-1}.

By similar alkylation reaction under SN_2 reaction condition ٣, ٤,٥-trimethoxybenzoic acid is prepared starting from gallic acid by alkylation with the very reactive methy-lating agent dimethyl sulphate, resulting in exhaustive methylation by using large excess of the alkylating agent and of the base sodium hydroxide (١٠ equivalents) the produced ester is not easily separated and purified from the reaction mixture so it is preferably hydrolysed by excess sodium hydroxide to give the corresponding acid, which is characterized by matching the melting point with the reported one, and by IR spectrometry; C=O stretching at ١٦٨١ cm^{-1} shifted to higher frequency due to decreased hydrogen bonding.

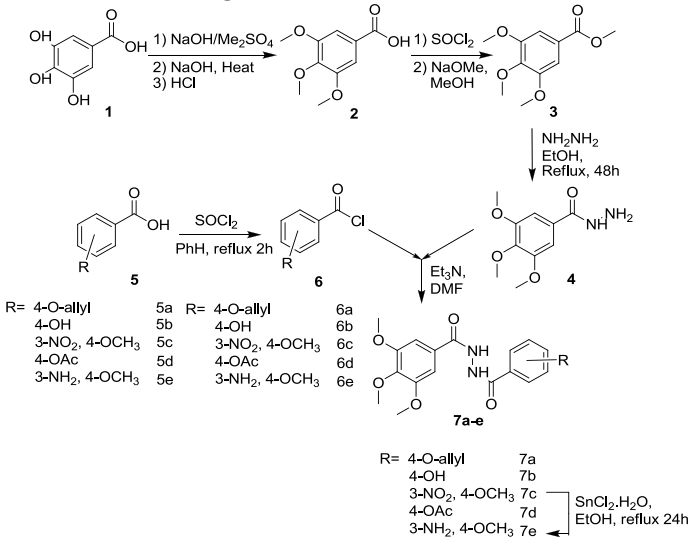
٤-methoxy-٣-nitrobenzoic acid is prepared by mild nitration of p-anisic acid using the mixed fuming nitric acid (less than two equivalents) and sulphuric acid in glacial acetic acid as solvent and selectively at the most reactive position ortho to the activating methoxy group, more vigorous nitration conditions may give the dinitro derivative or result in nonselective mono-nitration giving two isomeric nitro-acids. The desired product is characterized by matching the melting point with the reported one.

٤-acetoxybenzoic acid is prepared by direct acetylation of ٤-hydroxybenzoic acid using acetic anhydride under acid catalyst (orthophosphoric acid), the use of sulphuric acid results in partial charring or decomposition, also the acetylation with acetyl chloride in cold alkaline medium did not work. The ٤-acetoxybenzoic acid is characterized by matching the melting point with the reported one, and by IR spectrometry; C=O stretching at ١٦٧٨ cm^{-1} of the carboxylic acid dimer, phenyl ester C=O stretching at ١٧٥٤ cm^{-1} of higher frequency than normal esters.

Substituted benzoyl chlorides are prepared by treatment of the corresponding acids with excess of neat refluxing thionyl chloride, inert solvent like benzene may be used for dilution of the reaction mixture when labile functional group is present; e.g. ester as in compound ٥d which may partially undergo hydrolysis under strongly acidic conditions and allyl ether as in compound ٥a which may partially undergo hydrolysis or electrophilic addition of chlorine or thionyl chloride. Some of the crude acyl chlorides were characterized by IR spectrometry; C=O stretching at ١٧٧٠-١٧٤٥ cm^{-1} of the acyl chloride, at higher frequency due to electron-withdrawing chlorine.

Methyl ٣,٤,٥-trimethoxybenzoate ٣ was prepared by very easy method and in a quantitative yield by methanolysis of the corresponding acid chloride using in-situ-prepared sodium methoxide solution in methanol, an instantaneous precipitation of sodium chloride byproduct is a strong driving for the reaction, unlike the use of methanol only in which case hydrogen chloride gas is an interfering byproduct making the reaction slower and under equilibrium. Also the normal Fischer esterification was not favored due to lower yield, slower reaction rate and difficult work up. The produced ester is characterized by IR spectrometry; C=O stretching at ١٧١٧ cm^{-1} of the ester, at higher frequency than the starting acid due to electron-withdrawing methoxy group, also the disappearance of C=O stretching at ١٦٨٩ cm^{-1} and the very broad OH band centered at ٣١١٠ cm^{-1} of acid dimer.

٣,٤,٥-trimethoxybenzohydrazide is prepared by nucleophilic acyl substitution reaction between the corresponding ester and hydrazine (in the form of hydrate) in refluxing ethanol, the reaction is straightforward and selective to the monoacyl hydrazine due to lower nucleophilicity of the produced monohydrazide than hydrazine itself. The product is characterized by matching the melting point with the reported one, and by IR spectrometry; C=O stretching at ١٧٠٨ cm^{-1} shifted to lower frequency due to resonance and the NH_2 stretching as forked band at ٣٢٠٠-٣٤٣٥ cm^{-1}.



Scheme ١. Synthesis of unsymmetrical bis-hydrazides ٧**a-e**

Bishydrazides ٧a-e were prepared by acylation of ٣,٤,٥-trimethoxybenzohydrazide using equimolar quantity of substituted benzoyl chlorides ٦a-e in DMF solvent and using triethylamine as base, refluxing pyridine may be used as both solvent and base but the polar aprotic nature of DMF and its high solvation properties allow concentration of the reactants to be high enough for high reaction rate, thus conducting the reaction under milder conditions at room temperature. The produced bishydrazides are of the most polar compounds, so can be easily purified by several washing with boiling ethyl acetate, no cyclization of the bishydrazides is observed under the mild reaction conditions due to low reactivity of hydrazide carbonyl group due to resonance. The product is characterized by IR spectrometry; two nonequivalent C=O stretching at ١٦٨٠-١٥٥٠ cm^{-1} and the two NH stretching absorption bands at ٣٤٥٠-٣١٥٠ cm^{-1}, and by ^{1}HNMR; two offset singlets at ١٠-١١.٥ ppm of the two NH protons, singlet of ٢H at ٧.٣ ppm of the two equivalent aromatic protons of A ring, two singlets of ٣H and ٦H of the A ring

substituents ٣,٥-$(OCH_3)_2$ and ٤-OCH_3 respectively appearing at ٣.٥-٤ ppm, ^{13}CNMR; two carbonyl signals at ١٦٠-١٧٠ ppm, ٤ aromatic carbon signals of A ring at ١٠٠-١٥٥ ppm, two signals for carbons of A ring substituents ٣,٥-$(OCH_3)_2$ and ٤-OCH_3 at ٥٥-٦٠ ppm, other signals of ^{1}HNMR and ^{13}CNMR characteristic of B ring and its substituents as expected are found in the experimental part.

***In-vitro* cytotoxicity assay:**

In series ٧a-e, results showed that compound ٧e having ٣-NH_2,٤-OCH_3 groups in the B ring, showed the best anti-proliferative activity against the three of eleven tested cancer cell lines (Table ١); the best of them was the result of A٤٩٨ cell line (subtype of renal cancer) showing ٦٢.٥٤٪ growth, and also cell lines A٥٤٩/ATCC (Non-Small Cell Lung Cancer cancer) and UO-٣١ (subtype of renal cancer), indicating moderate activity of compound ٧e as a potential treatment in renal cancer and Non-Small Cell Lung Cancer. However, other tested compounds ٧a-d showed no or weaker anti-proliferative activity against the same cell lines indicating the essential role of ٣-NH_2 group in anti-proliferative activity. Unlike the whole series ٧a-e, Compound ٧d moderate anti-proliferative activity against the cell line CAKI-١ (subtype of renal cancer) (٧٣.٨٧٪ growth) indicating moderate role of ٤-acetoxy group in renal cancer. Nearly all of the tested compounds have moderate anti-proliferative activity against the cell lines PC-٣ (Prostate Cancer), the best of them are ٧b (٧٢.٦٣٪ growth), ٧e (٧٤.٥٠٪ growth) and NCI-H٥٢٢ (Non-Small Cell Lung Cancer), the best of them are ٧d (٦١.٠٩٪ growth), ٧b (٦٥.٨٥٪ growth) as shown in Table ١.

Table (١): Docking study results versus % growth of cancer cells treated with ١.٠٠E-٥ molar solution of test compound as compared to standard CA-٤, ND; not determined.

Cpd. name	C-Docker Energy	C-Docker Interaction Energy	% Growth of cancer cells treated with ١.٠٠E-٥ Molar solution of test compound										
			A٥٤٩ / ATCC	NCI-H٥٢٢	MOLT-٤	HCT-١١٦	BT-٥٤٩	MDA-MB-٤٦٨	CAKI-	TK-١٠	A٤٩٨	UO-٣١	PC-٣
CA-٤	-١٧.٢٢	-٤٣.٣٨	<١٠٪	<١٠٪	<١٠٪	<١٠٪	<١٠٪	<١٠٪	<١٠٪	<١٠٪	<١٠٪	<١٠٪	<١٠٪
٧a	-٢٥.٣٤٠٥	-٥٣.٢٢١٧	٧٩.٠٤	٧٨.٩٦	٨٢.٥٩	٦٩.١٣	٧٠.٥٤	٩٨.٢٣	٩١.٨٦	٩٥.٨٦	٨٤.٢٥	٨٢.٣٤	٧٨.٨١
٧b	-٣٠.٥١٣٣	-٤٩.٠٧٧٥	٨٥.٩١	٦٥.٨٥	٨٧.٤٥	٩٩.٩٨	٩٦.٠٠	٩٦.٧٤	٨٩.٧٢	٩٠.٨١	١٠٤.٠	٨٥.١٢	٧٢.٦٣
٧c	Lipinski Failure; hydrogen bond acceptors above ١٠		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
٧d	-٣٧.٥٢٤٩	-٥٥.٢٨٤٤	٨٥.٨٨	٦١.٠٩	٨٢.٥٩	٩٠.٠٢	٩٩.١٣	١٠٤.٢٤	٧٣.٨٧	٧٧.٢٧	٩٧.٤٥	٨١.٣٧	٧٧.٨١
٧e	-٣١.٠٣٠٨	-٥٠.٨٤٣٥	٧١.٠٩	٨٠.٤٥	٩١.٧٣	٨٦.١٥	٩٩.٩١	١١٦.٠٩	٨٧.٨٧	٩٦.٦٢	٦٢.٥٤	٧٨.٠٢	٧٤.٥٠

Molecular Modeling:

The most interesting is that the bis-hydrazide analogues give much better binding to the Colchicine-Binding Site than most of the know potent tubulin inhibitors including the superior one CA-٤, as shown by the results of molecular modeling studies. In case of compound **٧e**, we can observe the following drug receptor interactions;

- Hydrogen bond between the oxygen of the hydrazide group with Hydrogen of the OH group of SER١٧٨ of the alpha subunit of tubulin
- Hydrogen bond between the tautomeric hydrogen of the hydrazide group with carbonyl oxygen of LYS٣٥٢ of the beta subunit of tubulin
- Hydrogen bond between the tauto-meric hydrogen of the hydrazide group with carbonyl oxygen of THR٣٥٣ of the beta subunit of tubulin
- Hydrogen bond between the hydrogen atom of meta amino group (analogous to aminocombretastatin A-٤) with the carbonyl oxygens of the aminoacids VAL٢٣٨ and ALA٣١٦ of beta subunit of tubulin
- Hydrogen bond between the nitrogen atom of meta amino group (analogous to aminocombretastatin A-٤) with the mercapto group of the aminoacids CYS٢٤١ of beta subunit of tubulin
- Hydrogen bond between the oxygen atom of para methoxy group of Ring B with the mercapto group of the aminoacids CYS٢٤١ of beta subunit of tubulin.

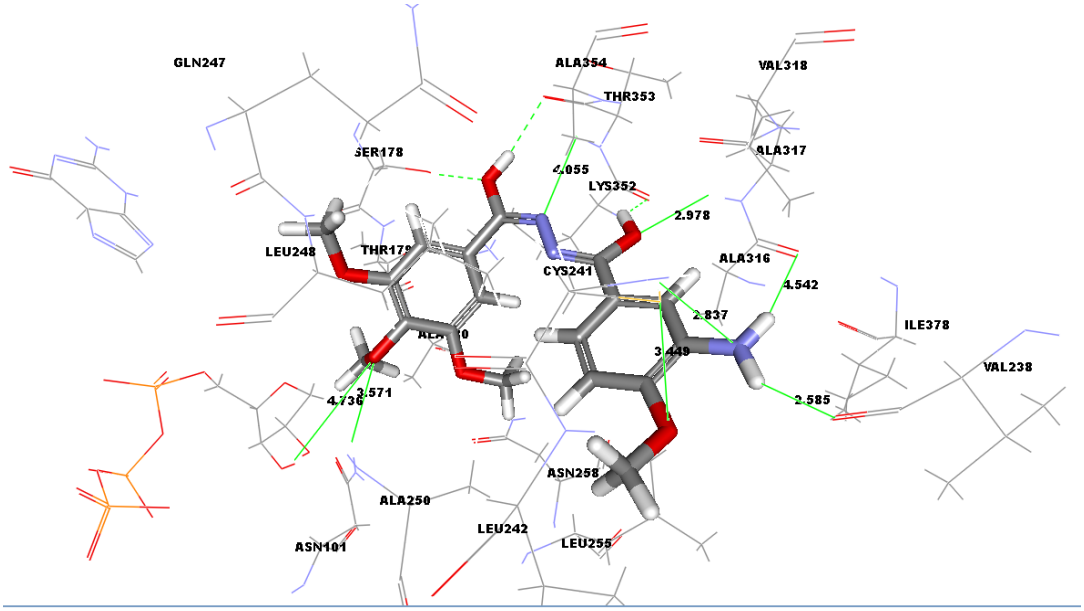


Fig. (٣a) Tautomer C of compound **٧e** inside colchicine-binding site of tubulin with close aminoacid residues

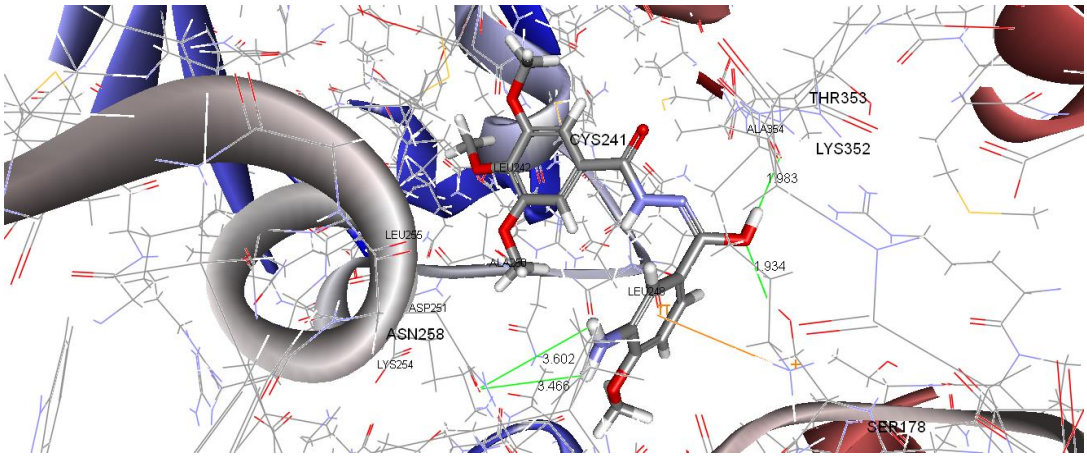


Fig. (٣b) Tautomer B of compound **٧e** inside colchicine-binding site of tubulin

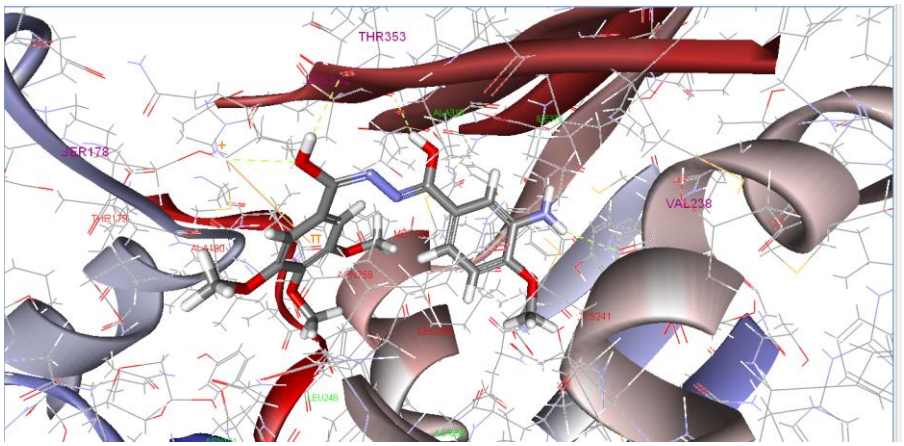


Fig. (٣c) Tautomer C of compound **٧e** inside colchicine-binding site of tubulin

Fig. (٣): Docking of different tautomers of the most potent analogue **٧e** inside colchicine-binding site of tubulingreen lines in green indicate H-bonds and orange lines in orange indicate π interactions.

Conclusion

We have synthesized and biologically evaluated a series of novel tubulin polymerization inhibitors that contain a core bis-hydrazide ring to which retain the cis configuration required for bioactivity via tautomerism with the more stable hydroxyimino tautomer (Fig. ٢, Fig. ٣). Attachment of the ٣-$NH_٢$,٤-$OCH_٣$ group to the ring B as exemplified by compound ٧e, or ٤-OAc group as exemplified by compound ٧d conferred optimal receptor binding and optimal cytotoxicity among this series (Table ١). Nearly all of the tested compounds have moderate anti-proliferative activity against the cell lines PC-٣ (Prostate Cancer) and NCI-H٥٢٢ (Non-Small Cell Lung Cancer). Computer docking and molecular simulations of ٧e inside the colchicine binding site of tubulin enabled identification of residues most likely to interact strongly with these inhibitors (Fig.٣) and explain their potent anti-tubulin activity and cytotoxicity.

Experimental

All melting points were determined on an electro thermal melting point apparatus (Stuart Scientific, Model SMP١, U.K). Pre-coated silica gel plates (TLC aluminum sheets, silica gel ٦٠ F٢٥٤, thickness ٠.٢ mm, Merck, Germany) were used to follow up reactions, test the purity of the products and to identify the most possible byproducts by comparison with references. Visualization of the spots was effected by

UV-lamp at λ = ٢٥٤ nm or by staining solution of p-anisaldehyde/sulphuric acid. IR spectra were recorded on Nicolet IS٥ FT-IR spectrometer at Minia University. NMR spectra were carried out using Bruker® Advance III (٤٠٠ MHz) spectrophotometer (Bruker AG, Switzerland) at faculty of Pharmacy Bani-Suief University Egypt. NMR spectra were carried out using Brucker Advance ٤٠٠ MHz NMR spectrometer, using TMS as internal reference, Chemical shifts (δ) values are given in parts per million (ppm) relative to TMS, $CDCl_٣$ (٧.٢٩ for proton and ٧٦.٢٦ for carbon) or DMSO-d٦ (٢.٥٠ for proton and ٣٩.٥٠ for carbon) and coupling constants (J) in Hertz. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet ; dd, doublet of doublet; m, multiplet, brs; broad singlet. Elemental analysis was performed on Vario El Elementar CHN Elemental analyzer, organic microanalysis section, Cairo University, Egypt.

Synthesis of substituted benzoic acids (٥a-e)

٤-(allyloxy)benzoic acid (٥a)

In ٢٥٠-ml round-bottom flask is placed ١٥٠ ml of methanol, to which is added cautiously and very slowly ٣.٣٣ g. (١٤٤ mmole, ٢ equiv. ٤-hyroxybenzoic acid) very finely divided pieces of sodium metal, ice- bath is used to control the exothermic reaction, slow stirring, left till all sodium metal dissolved and evolution of hydrogen gas subsided. To the above solution is added very slowly with vigorous stirring and in a dropwise manner a solution of ٤-hyroxybenzoic acid ١٠ g. (٧٢.٤ mmole) dissolved in ٥٠ ml methanol, the rate of addition must be too slow to prevent sudden precipitation of sodium bromide which may cause clogging of the magnetic stirrer bar. After complete addition, allyl bromide ٩.٦٣ g. (٧٩.٦٤ mmole, ١.١ equiv.) is added portionwise, and then reaction is heated at reflux for ٧ h. The progress of the reaction is monitored by TLC, using petroleum ether/ethyl acetate system as eluent, the product alkylated acid appears as dark spot at ٢٥٤nm having higher Rf than the starting hydroxyacid indicating lower polarity of the product. After complete disappearance of the TLC spot of the start material, the reaction is cooled down, methanol evaporated using the rotavap, ٣٥ ml of distilled water is added while stirring to dissolve sodium bromide by product, and then the aqueous alkaline reaction mixture is washed with DCM ٢x٣٥ ml, organic layer discarded, and during stirring of the aqueous one is added cautiously ٦M hydrochloric acid (١.١ equivalent the theoretical amount) , the heavy precipitate formed is filtered under suction, pressed through filter paper, left under suction for ٣ h for air drying, dried in the oven below ١٠٠ºC. Yield: (٩.٢٤ g, ٧١.٦ of theoretical), IR; C=O stretching at ١٦٧٤ $cm^{-١}$ and the C=C stretching at ١٦٠٤ $cm^{-١}$.

٤-acetoxybenzoic acid (٥d)

In ١٠٠ ml round bottom flask is placed ٥.٣٩ g (٣٩.٠١ mmol) of ٤-hydroxybenzoic acid and ٢٥ g (excess) of acetic anhydride, the reaction mixture is kept at ٧٥ C by means of glycerin bath while stirring till nearly all the ٤-hydroxybenzoic acid has dissolved, then ٢٠ drops of ٨٥٪ orthophosphoric acid added dropwise while stirring and the temperature of glycerin bath is raised to ٨٥-٩٠ºC for ٨h. Reaction progress is followed up by treatment of a few drops of the reaction mixture with distilled water then extraction with ethyl acetate and TLC of the extract, using petroleum ether/ethyl acetate system as eluent, the produced O-acetylated derivative acid appears as dark spot at ٢٥٤ nm having higher R_f than the starting hydroxyacid indicating lower polarity of the product.

After disappearance of the spot of the lower R_f of the starting material, the reaction is cooled down to ٥٠C, and then distilled water (١٠٠ ml) is added portionwise very cautiously under well-ventilated fume hood to avoid acetic acid vapors, the precipitated solid is kept heated for ١h at that temperature for decomposition of excess acetic anhydride, then the reaction mixture is cooled down by ice bath then filtered under suction and the precipitate is pressed through filter paper, air dried and then dried below ١٠٠°C in the oven to give greenish white powder, TLC single spot. M.p ١٩٠-١٩٢°C (reported ١٩٠-١٩٤ºC), IR ١٧٥٤ C=O ester, ١٦٧٨ C=O acid dimer.

If needed, further degree of purification is carried out by recrystallization from the lowest possible amount of water to give needle crystals.

٤-methoxy-٣-nitrobenzoic acid (٥c)

In ٢٠ ml round bottom flask is placed ٠.٣٨٤٤g (٢.٥٢٦mmol) of ٤-methoxybenzoic acid and ٢ ml of glacial acetic acid, the reaction mixture is heated by means of glycerin bath kept at ١٠٥C while stirring till nearly all the ٤-methoxybenzoic acid has dissolved, then the reaction mixture is cooled down by cold water bath until just before the precipitation of the dissolved acid. The reaction mixture is kept at this temperature, and while vigorous stirring is added dropwise a previously ice-cooled mixture of red fuming nitric acid ٩٧٪, d=١.٤٧ g/ml (٠.١٨ ml, ٤ mmol) and concentrated sulphuric acid ٩٨٪ (٠.٢٥ ml). After complete addition of the nitrating mixture the glycerin bath is adjusted to ٨٥C for ٢h. Reaction progress is followed up by dilution of a few drops of the reaction mixture with methanol then TLC of the resulting solution, using petroleum ether/ethyl acetate system as eluent, the produced nitro derivative appears as yellow-colored spot at ٢٥٤nm and visible light having lower Rf than the starting acid indicating higher polarity of the nitro-containing derivative. After disappearance of the spot of the starting material, the reaction is cooled down to ٥٠°C, and then poured into ١٠٠ ml beaker containing vigorously-stirred ice distilled water (٢٥ ml), the precipitated solid is filtered under suction and the precipitate is pressed through filter paper, air dried as long as possible, drying in the oven is not recommended for nitro compounds to prevent darkening of their color, TLC single spot. Yield ٠.٣٦ g (٧٢.٣٪) , m.p. ١٩٠°C (reported ١٩٢-١٩٤ °C), If needed, further degree of purification is carried out by recrystallization from the lowest possible amount of aqueous ethanol ٨٥٪ to give white shiny crystals m.p °C

٣,٤,٥-trimethoxybenzoic acid (٢)

To a cold solution of ٨٠ g. (٢ moles) of sodium hydroxide in ٥٠٠ cc. of water in a ١-l. flask is added ٥٠ g. (٠.٢٦٦ mole) of gallic acid. The flask is immediately tightly stoppered, and the mixture shaken occasionally until all the acid has dissolved; ٨٩ g. (٦٧ ml) of dimethyl sulfate (٠.٧١ mole) is then added and the flask is shaken for twenty minutes, being cooled by means of cold water in order that the temperature does not rise above ٣٠–٣٥°. Occasionally the stopper is raised to release any pressure. A second portion of ٨٩ g. of dimethyl sulfate is then added and shaking continued for ten minutes longer. During this second addition the temperature may rise to ٤٠ ٤٥°. The flask is then fitted with a reflux condenser and the contents boiled for two hours. In order to saponify the small amount of ester which is produced, a solution of ٢٠ g. of sodium hydroxide in ٣٠ cc. of water is then added and boiling continued for two additional hours. The reaction mixture is then cooled and acidified with dilute hydrochloric acid; the precipitated trimethylgallic acid is filtered with suction and washed well with cold water. The product, which melts at ١٥٧–١٦٠°, is sufficiently pure for many purposes. It weighs ٥٠–٥٢ g. (٨٩–٩٢ per cent of the theoretical amount). It may be purified by recrystallization from ٢ l. of boiling water with the use of decolorizing carbon, the filtration being carried out in a steam-jacketed funnel. In this way ٤١–٤٣ g of colorless needles melting at ١٦٧° is obtained. IR; C=O stretching at ١٦٨١ cm^{-1} shifted to higher frequency due to decreased hydrogen bonding.

General procedure for synthesis of substituted benzoyl chlorides (٦a-e)

A mixture of the aromatic acid (٢.٥mmol), thionyl chloride (٧.٥mmol, ٠.٩g) and benzene (٧ml) was heated under reflux for ٣h, benzene and the excess of thionyl chloride was removed by repeated distillation under reduced pressure, with repeated addition of fresh benzene before each distillation till the disappearance of the suffocating odor of thionyl chloride. Reaction progress is followed up by instantaneous methanolysis of a few drops of the reaction mixture containing the acid chloride then TLC of the resulting ester, using petroleum ether/ethyl acetate system as eluent, the produced ester appears as dark spot at ٢٥٤nm having higher Rf than the starting acid, complete disappearance of

the spot of the acid is a sign for the end of the reaction. The crude acid chloride is weighed by difference and used directly in the next step without any further purification. Otherwise, further purification may be performed by recrystallization from hexane. Yield ٧٠-٩٥٪ for all derivatives

Methyl ٣,٤,٥-trimethoxybenzoate (٣)

In ٥٠٠ ml round bottom flask is placed ٣٠٠ ml of absolute methanol, to which is carefully added ٦.٥٥ g (٠.٢٨٤٨ mol) of finely divided sodium metal, ice bath is used to control the exothermic reaction. After all the sodium metal dissolved giving sodium methoxide solution, the methanolic solution of the acid chloride (……) is added very carefully and dropwise while vigorous stirring. The reaction mixture is left stirring for ٢ h, and a precipitate of sodium chloride is observed. After the end of conversion of acid chloride to ester as indicted by TLC, the reaction mixture is filtered under suction, washed with fresh methanol (٣x١٠٠ ml) and the combined filtrate and methanol washings are evaporated under reduced pressure till a super-saturated solution of the ester is obtained, then gradual cooling gives shiny crystalline ester as first crop (٣٨.٦ g, ٧٢.٤٪ of theoretical yield) . A second crystallization of the filtrate is done similarly to give a total **yield** of (٥١.٩ g, ٩٧.٤% of theoretical)

R_f ٠.٥٥ (EtOAc-Pet. ether, ١:١) m.p.: ٨٥°C (lit ٨٢–٨٣°C). **IR**: ١٧١٧. ^{1}**H-NMR**: ١.٦٠ (s, ٣H), ٣.٩٢ (s, ٩H), ٧.٣٣ (s, ٢H).

٣,٤,٥-trimethoxybenzohydrazide (٤)

In ٢٥٠ ml round bottom flask is added methyl ٣,٤,٥-trimethoxybenzoate (٠.٢٦ mol, ٥٨.٩٤ g) and absolute ethanol (٢٠٠ ml), stirred till all the ester dissolves, then excess hydrazine hydrate ١٠٠٪ (٠.٨٥mol, ٤٢.٥g, ٣.٢٦ equiv.) is added portionwise, after complete addition, reaction mixture is heated by glycerin bath adjusted at ١٨٠C to obtain gentle reflux for ٤٢h, reaction progress is followed up by TLC, using petroleum ether/ethyl acetate system as eluent, the produced hydrazide appears as dark spot at ٢٥٤nm having lower Rf than the starting ester, complete disappearance of the spot of the ester is a sign for the end of the reaction. The solvent and the excess of hydrazine is evaporated under high vacuum using water aspirator pump till no more distillate comes out, the remaining traces of hydrazine are washed out with water, washings decanted, then the crude product is allowed to crystallize from a super-saturated ethanolic solution at ٧٠C, only the first crop of crystalline hydrazide is collected by vacuum filteration, air drying then drying in the oven at ١٣٠C for ٣h, TLC single spot.

Yield ٤٩.١٩ g (٨٣.٤%) R_f ٠.٥٥ (MeOH-EtOAc-Pet. ether, ١.٥:٤:٤.٥) m.p.: ١٥٨–١٦٠ °C (lit ١٦٨° C). IR: ٣٢٠٠-٣٤٣٥, ١٧٠٨. ^{1}H-NMR: ٣.٨٠ (s, ٣H), ٣.٩٠ (s, ٦H), ٧.١٨ (s, ٢H), ٩.٥٥ (brs, NH)

General procedure for synthesis of unsymmetrical bis-hydrazides (٧a-e)

In ٥٠ ml stoppered conical flask is added ٣,٤,٥-trimethoxybenzohydrazide (٢ mmol, ٠.٤٥٢٥ g) and DMF (١٥ml), stirred till all the hydrazide dissolves, then triethylamine (٢.٢ mmol, ٠.٢٣ g) is added, to this mixture is added in a dropwise manner and very slowly a solution of the appropriate acid chloride (٢.١mmol) in DMF (١٠ml), reaction mixture is stoppered and left stirring for ٧٢h. reaction progress is followed up by TLC, using petroleum ether / ethyl acetate system as eluent, the produced hydrazide appears as dark spot at ٢٥٤nm having intermediate R_f value between the starting acid chloride (high Rf) and the starting ٣,٤,٥-trimethoxy-benzohydrazide (low R_f) due to higher polarity of the bis-hydrazide than the acid chloride and lower ability of H-bonding than the monohydrazide, complete disappearance of the spot of the monohydrazide start material is a sign for the end of the reaction. The reaction mixture is then poured into a ٢٥٠ml beaker containing ١٥٠ ml of ice water while vigorous stirring for ١٥ minutes, then left in the freezer at ٥-١٠C for ٧٢h, the precipitate filtered under suction, washed several times with water, pressed through filter paper, air-dried under suction for ٣h, then dried in the oven below ١١٠C for several hours, and finally recrystallized from hot saturated acetonitrile solution or washed with boiling ethyl acetate then filtered and dried. The produced powder may be further recry-stallized to give final bis-hydrazide, TLC single spot.

N'-(٤-(allyloxy)benzoyl)-٣,٤,٥-trimethoxybenzohydrazide ٧a.
Yellowish white powder; (٠.٧٠٧ g, ٩١.٤% yield); mp = ١٠٠-١٠٣°C; IR (neat) υmax = two broad bands ٣٤٢٦,٣١٩٥ cm^{-1} (NHNH), ١٦٦٩,١٦٥٤ cm^{-1} (two C=O hydrazide); ^{1}**H NMR** (٤٠٠ MHz, DMSO-d_6) δ ١٠.٤٠ (br.s, ١H, NH), ١٠.٥١ (br.s, ١H, NH), ٨.٠٧ (d, J = ٨.٩ Hz, ٢H, ArH), ٧.٣٤ (s, ٢H, ArH), ٧.٠٥ (d, J = ٨.٩ Hz, ٢H, ArH), ٦.٠٧ (ddt, J = ١٧.٢ trans coupling, ١٠.٥ cis coupling, ٥.٣ Hz vicinal coupling, ١H olefinic), ٥.٤٥ (dd, J = ١٧.٣ trans coupling, ١.٥ Hz SP_2 germinal coupling, ١H olefinic), ٥.٣٤ (dd, J = ١٠.٥ cis coupling, ١.٣ Hz SP_2 germinal coupling, ١H olefinic), ٤.٦٣ (d, J = ٥.٣ Hz vicinal coupling, ٢H, allylic CH_2), ٣.٩٧ (s, ٦H, ٣,٥-$(OCH_3)_2$), ٣.٩٣ (s, ٣H, ٤-OCH_3). ^{13}**C NMR** (١٠١ MHz, DMSO-d_6) δ ١٦٨.٧٢, ١٦٤.٢٩, ١٦٣.٧٥, ١٥٤.٠٩, ١٥٣.٧٢, ١٥٢.٤٥, ١٥١.٥١, ١٢٦.٢٠, ١٢١.٧٥, ١١٨.٩٨, ١١٦.٦٠, ١١٢.٦٣, ٦١.٠٥, ٥٦.٤٦, ٥٦.١٤. **Anal. Calcd.** For $C_{20}H_{22}N_2O_6$ (٣٨٦.١٥): C, ٦٢.١٧; H, ٥.٧٤; N, ٧.٢٥; O, ٢٤.٨٤ **Found**: C, ٦٢.٣٩; H, ٥.٩٥; N, ٧.١٢.

N'-(٤-hydroxybenzoyl)-٣,٤,٥-trimethoxybenzohydrazide ٧b.
White powder; (٠.٤٥٣ g, ٦٩% yield); mp = ١٥٢-١٥٤°C; IR (neat) υmax = broad band centered at ٣٢١٨cm^{-1} (NH,NH,OH), ١٦٠٠,١٥٦٣ cm^{-1} (two C=O hydrazide); ^{1}**H NMR** (٤٠٠ MHz, DMSO-d_6) δ ١٠.٥٦, ١٠.٤٢, ١٠.٢٩ (٣ broad singlets, ٣H, NH,NH, OH), ٧.٩٢ (d, J = ٨.٧ Hz, ٢H, ArH), ٧.٣٤ (s, ٢H, ArH), ٧.٠٥ (d, J = ٨.٧ Hz, ٢H, ArH), ٣.٩١ (s, ٦H, ٣,٥-$(OCH_3)_2$), ٣.٨٥ (s, ٣H, ٤-OCH_3). ^{13}**C NMR** (١٠١ MHz, DMSO-d_6) δ ١٦٧.٤٠, ١٦٦.١٩, ١٦٣.٠١, ١٦٢.٩٧, ١٥٤.٠٦, ١٣١.١٤, ١٣٠.٣٦, ١٢٤.٢٣, ١٢٢.٩٩, ١١٤.٦٩, ٥٦.٣٧, ٥٦.٣٦. **Anal. Calcd**. For $C_{17}H_{18}N_2O_6$ (٣٤٦.١٢): C, ٥٨.٩٦; H, ٥.٢٤; N, ٨.٠٩; O, ٢٧.٧٢. **Found:** C, ٥٩.٠٢; H, ٥.٢٧; N, ٨.١٥.

٣,٤,٥-trimethoxy-N'-(٤-methoxy-٣-nitrobenzoyl)benzohydrazide ٧c.
Greenish white powder; (٠.٦١٩ g, ٧٦.٣% yield); mp = ١١٨-١٢٠°C; IR (neat) υmax = two broad bands at ٣٢٢٣,٣٠٠٣cm^{-1} (NHNH), ١٦٧٤,١٦١٧ cm^{-1} (two C=O hydrazide), ١٤٨٩ cm^{-1} (NO_2); ^{1}H NMR (٤٠٠ MHz, DMSO-d_6) δ ١٠.٧٥ (s, ١H, NH), ١٠.٥٩ (s, ١H, NH), ٨.٥١ (d, J = ١.٩ Hz meta coupling, ١H, ArH), ٨.٢٩ (dd, J = ٨.٨ Hz ortho coupling, ١.٩ Hz meta coupling, ١H, ArH), ٧.٥٨ (d, J = ٨.٩ Hz ortho coupling, ١H, ArH), ٧.٣٢ (s, ٢H, ArH), ٤.٠٦ (s, ٣H, ٤-OCH_3 ring B), ٣.٨٩ (s, ٦H, ٣,٥-$(OCH_3)_2$ ring A), ٣.٧٧ (s, ٣H, ٤-OCH_3 ring A). ^{13}**C** NMR (١٠١ MHz, DMSO-d_6) δ ١٦٦.٢٠, ١٦٤.٦٢, ١٥٥.٥٨, ١٥٣.٧٠, ١٤١.٤٧, ١٣٩.٧٤, ١٣٤.٥٧, ١٢٨.٤١, ١٢٥.٤٧, ١٢٥. ٣٦, ١١٥.٥٨, ١٠٦.٠٠, ٦١.١١, ٥٨.١٧, ٥٧.٠٢. Anal. Calcd. For $C_{18}H_{19}N_3O_8$ (٤٠٥.١٢): C, ٥٣.٣٣; H, ٤.٧٢; N, ١٠.٣٧; O, ٣١.٥٨. Found: C, ٥٣.٣٥; H, ٤.٧٩; N, ١٠.٤٣.

٤-(٢-(٣,٤,٥-trimethoxybenzoyl)hydrazinecarbonyl)phenyl acetate ٧d.
White powder; (٠.٥٢ g, ٦٧% yield); mp = ١٣٨-١٤٠°C; ^{1}H NMR (٤٠٠ MHz, DMSO-d_6) δ ١٠.٥١ (s, ١H, NH), ١٠.٤٢ (s, ١H, NH), ٨.٠١ (d, J = ٨.٢ Hz, ٢H, ArH), ٧.٣٤ (s, ٢H, ArH), ٧.٣٣ (d, J = ٨.٢ Hz, ٢H, ArH), ٣.٩٦ (s, ٦H, ٣,٥-$(OCH_3)_2$), ٣.٩٠ (s, ٣H, ٤-OCH_3), ٢.٣٥ (s, ٣H). ^{13}C NMR (١٠١ MHz, DMSO-d_6) δ ١٦٩.٩٦, ١٦٦.٣٠, ١٦٦.١٩, ١٦٣.٠١, ١٦٢.٩٣, ١٥٤.٠٦, ١٣١.١٤, ١٣٠.٣٤, ١٢٥.٦٢, ١٢٢.٩٩, ١١٤.٦٩, ٥٦.٣٧, ٥٦.٣٦, ٢١.٨٥. Anal. Calcd. For $C_{19}H_{20}N_2O_7$ (٣٨٨.١٣): C, ٥٨.٧٦; H, ٥.١٩; N, ٧.٢١; O, ٢٨.٨٤. Found: C, ٥٨.٨٣; H, ٥.٢٧; N, ٧.٢٩

N'-(٣-amino-٤-methoxybenzoyl)-٣,٤,٥-trimethoxybenzohydrazide ٧e.
Dark yellow powder; (٠.٣٩٨ g, ٥٣% yield); mp = ١٥٤-١٥٦°C; IR (neat) υmax = ٣١٥٠-٣٣١٨ cm^{-1} broad forked band (NH^2), two broad bands (two NH), ١٦٦٧,١٦٥١ cm^{-1} (two C=O hydrazide); ^{1}**H NMR** (٤٠٠ MHz, DMSO-d_6) δ ١٠.٣٧ (br.s, ١H, NH), ١٠.٣٠ (br.s, ١H, NH), ٧.٤٥ (dd, J = ٨.٤ ortho coupling, ١.٧ Hz meta coupling, ١H, ArH), ٧.٤٠ (d, J = ١.٨ Hz meta coupling, ١H, ArH), ٧.٣٠ (s, ٢H, ArH), ٧.٠٦ (d, J = ٨.٥ Hz ortho coupling, ١H, ArH), ٣.٨٨٣ (s, ٦H, ٣,٥-$(OCH_3)_2$), ٣.٨٧٨ (s, ٣H, ٤-OCH_3), ٣.٧٧ (s, ٣H, ٤-OCH_3), ٣.٧٧ (br.s, ٢H, NH_2). **Anal. Calcd**. For $C_{18}H_{21}N_3O_6$ (٣٧٥.١٤): C, ٥٧.٥٩; H, ٥.٦٤; N, ١١.١٩; O, ٢٥.٥٧. **Found**: C, ٥٧.٦٥; H, ٥.٦٩; N, ١١.١٥.

NCI anticancer screening
The methodology of the NCI anticancer screening has been described in detail elsewhere (http://www.dtp.nci.nih.gov).

Briefly, the primary anticancer assay was performed at approximately ٦٠ human tumor cell lines panel derived from nineneoplastic diseases, in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda. Tested compounds were added to the culture at a single concentration (١٠-٥ M) and the cultures were incubated for ٤٨ h. End point determinations were made with a protein binding dye, SRB. Results for each tested compound were reported as the percent of growth of the treated cells when compared to the untreated control cells. The percentage growth was evaluated spectrophotometrically versus controls not treated with test agents. The cytotoxic Experimental ١٠٨ and/or growth inhibitory effects of the most active selected compound were tested in vitro against the full panel of about ٦٠ human tumor cell lines at ١٠-fold dilutions of five concentrations ranging from ١٠-٤ to ١٠-٨ M. A ٤٨-h continuous drug exposure protocol was followed and an SRB protein assay was used to estimate cell viability or growth. Using the seven absorbance measurements [time zero (Tz), control growth in the absence of drug (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as: [(Ti - Tz)/(C Tz)] - ١٠٠ for concentrations for which Ti > Tz, and [(Ti - Tz)/Tz] - ١٠٠ for concentrations for which Ti < Tz. Three dose–response parameters were calculated for each compound. Growth inhibition of ٥٠٪ (GI٥٠) was calculated from [(Ti - Tz)/(C Tz)] - ١٠٠ = ٥٠, which is the drug concentration resulting in a ٥٠٪ lower net protein increase in the treated cells (measured by SRB staining) as compared to the net protein increase seen in the control cells. The drug concentration resulting in TGI was calculated from Ti = Tz. The LC٥٠ (concentration of drug resulting in a ٥٠٪ reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated from [(Ti Tz)/Tz] - ١٠٠ = -٥٠. Values were calculated for each of these three parameters if the level of activity is reached; however, if the effect was not reached or was exceeded, the value for that parameter was expressed as more or less than the maximum or minimum concentration tested. The log GI٥٠, log TGI, and log LC٥٠ were then determined, defined as the mean of the logs of the individual GI٥٠, TGI, and LC٥٠ values. The lowest values are obtained with the most sensitive cell lines. Compound having log GI٥٠ values -٤ and <-٤ was declared to be active.

Docking study

Docking simulation study is performed by Medicinal Chemistry Department Faculty of Pharmacy Minia University, Minia, Egypt using Acclerys Discovery Studio Client ® version ٢.٥.٠٩١٦٤. The computational software operated under "Windows ٧" installed on an Intel Pentium IV PC with a ٢.٢ GHz processor and ٨.٠٠ GB memory.

- The docking study is carried out using the software Acclerys Discovery studio ® version ٢.٥.٠٩١٦٤.
- The X-ray crystallographic structure of the target tubulin protein subunits bound to DMA-Colchicine is obtained from Protein Data Bank (PDB ID = ١SA٠); from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb)
- Prior to docking process, water molecules are deleted; the whole tubulin subunits were prepared by the protein preparation wizard using CHARM force field [١١] to generate the most stable and the least energetic conformation for tubulin protein & the ligands under investigation were also prepared in the most stable ٣D structure using the ligand preparation wizard, the preparation process was necessary for energy minimization.
- During the preparation of ligands, tautomerization of a given ligand was allowed & only the most stable tautomers were allowed to be

Docked into the receptor binding site, also ionization is allowed for both acidic and basic functional groups at physiological pH=٦.٥-٨.٥, and the Lipinski Rule is allowed.

- Binding site of DMA-Colchicine was defined by selecting the molecule (CN٢٧٠٠), then using the tool (Define Sphere from Selection), then the co-crystallized ligand, DAMA-colchicine (molecule CN٢٧٠٠) was deleted.
- The binding site sphere size was adjusted to ١٣.٩٤°A
- Docking process carried out using the protocol (Receptor Ligand interaction, C-DOCKER)
- After complete docking process, results are viewed
- Hydrogen bonds, π-cation, π-sigma and π-π interactions are monitored whenever present.
- The values of C-DOCKER ENERGY & C-DOCKER INTERATION ENERGY are recorded.
- For an energetically-favored ligand-receptor interaction, both values of C-DOCKER ENERGY & C-DOCKER INTERATION ENERGY must be negative,
- C-DOCKER INTERATION ENERGY measures the strength of ligand-receptor interaction; however, strain of ligand molecule as a result of this interaction must be also taken into consideration, so more important parameter is the C-DOCKER ENERGY.
- The higher the negative value of C-DOCKER ENERGY (i.e. - C-Docker Energy) the better unstrained drug-receptor interaction & the better the ligand.
- The close receptor residues are selected within a sphere having ٢٠°A diameter surrounding the ligand and the important amino acids are labeled.
- The results are visualized in publication quality.
- The results were saved in a DSV file; files can be visualized by Accelry Discovery Studio Client.
- The docking result of the ligands was listed in table ٢. The docking process involves a conformational search for compound which compliments a target binding site, with the aim of identifying the best matching pose along with the active site to perform docking. The stability of the docked ligand-protein complex is due to hydrogen bonding and Van der Waals interactions.

Acknowledgment

We thank the staff of the Developmental Therapeutics Program (DTP), Division of Cancer Treatment and Diagnosis, National Cancer Institute, (NIH), USA for carrying out the *in-vitro* cytotoxicity assay.

References

١. Ducki S, Mackenzie G, Lawrence NJ, Snyder JP. Quantitative structure–activity relationship (°DQSAR) study of combretastatin-like analogues as inhibitors of tubulin assembly. J. Med. Chem. ٢٠٠٥;٤٨:٤٥٧–٤٦٥.

٢. Jordan A, Hadfield JA, Lawrence NJ, McGown AT. Tubulin as a target for anticancer drugs: agents that interact with the mitotic spindle. Med. Res. Rev ١٩٩٨;١٨: ٢٥٩–٢٩٦.

٣. Andreu JM, Barasoain I. The interaction of baccatin III with the taxol binding site of microtubules determined by a homogeneous assay with fluorescent taxoid. Biochemistry ٢٠٠١; ٤٠:١١٩٧٥–١١٩٨٤.

٤. Rai SS, Wolff J. Localization of the vinblastine-binding site on beta-tubulin. J. Biol. Chem ١٩٩٦;٢٧١: ١٤٧٠٧–١٤٧١١.

٥. Ter Haar E, Rosenkranz HS, Hamel E, Day BW. Computational and molecular modeling evaluation of the structural basis for tubulin polymerization inhibition by

colchicine site agents. Bioorg. Med. Chem ١٩٩٦;٤:١٦٥٩–١٦٧١.

٦. Pettit GR, Singh SB, Hamel E, Lin CM, Alberts DS, Garcia-Kendall D. Isolation and structure of the strong cell growth and tubulin inhibitor combretastatin A-٤. Experentia. ١٩٨٩; ٤٥:٢٠٩–٢١١.

٧. Pettit GR, Singh SB, Hamel E, Lin CM, Alberts DS, Garcia-Kendall D. Isolation and structure of the strong cell growth and tubulin inhibitor combretastatin A-٤. Experentia. ١٩٨٩; ٤٥:٢٠٩–٢١١.

٨. Pettit GR, Rhodes MR, Herald DL, Hamel E, Schmidt JM, Pettit RK. Antineoplastic agents. ٤٤٥. Synthesis and evaluation of structural modifications of (Z)-and (E)-combretastatin A-٤١. J. Med. Chem ٢٠٠٥;٤٨:٤٠٨٧–٤٠٩٩.

٩. McGown AT, Fox BW. Differential cytotoxicity of combretastatins A١ and A٤ in two daunorubicin resistant P٣٨٨ cell lines. Cancer Chemother. Pharmacol ١٩٩٠; ٢٦:٧٩–٨١.

١٠. Nam NH. Combretastatin A-٤ analogues as antimitotic antitumor agents. Curr. Med. Chem ٢٠٠٣;١٠: ١٦٩٧ ١٧٢٢.

١١. Theeramunkong, S.; Caldarelli, A.; Massarotti, A.; Aprile, S.; Caprifoglio, D.; Zaninetti, R.; Teruggi, A.; Pirali, T.; Grosa, G.; Tron, J. Med. Chem. ٢٠١٢, ٥٥, ٤٧٥–٤٨٨ G. C.; Genazzani, A.A. Regio selective Suzuki coupling of dihaloheteroaromatic compounds as a rapid strategy to synthesize potent rigid combretastatin analogues. J. Med. Chem. ٢٠١١,٥٤, ٤٩٧٧ – ٤٩٨٦.

١٢. (a) Ohsumi, K.; Hatanaka, T.; Fujita, K.; Nakagawa, R.; Fukuda, Y.; Nihai, Y.; Suga, Y.; Morinaga, Y.; Akiyama, Y.; Tsuji, T. Synthesis and antitumor activity of cis-restricted combretastatins ٥-membered heterocyclic analogues. Bioorg. Med. Chem. Lett. ١٩٨٨, ٨, ٣١٥٣ –٣١٥٨. (b) Wang, L.; Woods, K. W.; Li, Q.; Barr, K. J.; McCroskey, R. W.; Hannick, S. M.; Gherke, L.; Credo, R. B.; Hui, Y.-H.; Marsh, K.; Warner, R.; Lee, J. Y.; Zielinski-Mozng, N.; Frost, D.; Rosenberg, S. H.; Sham, H. L. Potent, orally active heterocycle-based combretastatin A-٤ analogues: synthesis, structure − activity relationship, pharmao-kinetics, and in vivo antitumor activity evaluation. J. Med. Chem. ٢٠٠٢, ٤٥, ١٦٩٧ –١٧١١.

١٣. (a) Bonezzi, K.; Taraboletti, G.; Borsotti, P.; Bellina, F.; Rossi, R.; Gavazzi, R. Vascular disrupting activity of tubulin-binding ١,٥-diaryl-١ H-imidazoles. J. Med. Chem. ٢٠٠٩, ٥٢, ٧٩٠٦ –٧٩١٠. (b) Schobert, R.; Biersack, B.; Dietrich, A.; Effenberger, K.; Knauer, S.; Mueller, T. ٤-(٣-Halo/amino-٤,٥-dimethoxyphenyl)-٥-aryloxazoles and N-methylimidazoles that are cytotoxic against combretastatin A-٤ resistant tumor cels and vascular disrupting in a cis-platin resistant germ cell tumor model. J. Med. Chem. ٢٠١٠, ٥٣, ٦٥٩٥–٦٠٠٢.

١٤. (a) Kaffy, J.; Pontikis, R.; Carrez, D.; Croisy, A.; Monneret, C.; Florent, J.-C. Isoxazole-type derivatives related to combreta-statin A-٤, synthesis and biological evaluation. Bioorg. Med. Chem. ٢٠٠٦, ١٤, ٤٠٦٧ –٤٠٧٧. (b) Lee, S.; Kim, J. N.; Lee, H. K.; Yoon, K. S.; Shin, K. D.; Kwon, B.-M.; Han, D. C. Biological evaluation of KRIBB٣ analogs as a microtubule polymerization inhibitor. Bioorg. Med. Chem.Lett.٢٠١١,٢١,٩٧٧ –٩٧٩.

١٥. Wu, M.; Li, W.; Yang, C.; Chen, D.; Ding, J.; Chen, Y.; Lin, L.; Xie, Y. Synthesis and activity of combretastatin A-٤ analogues: ١,٢,٣- thiadiazoles as potent antitumor agents. Bioorg. Med. Chem. Lett. ٢٠٠٧, ١٧, ٨٦٩ –٨٧٣.

١٦. (a) Odlo, K.; Hntzen, J.; Fournier dit Chabert, J.; Ducki, S.; Gani, O. A. B. S. M.; Sylte, I.; Skrede, M.; Florenes, V. A.; Hansen, T. V. ١,٥-Disubstituted ١,٢,٣-triazoles as cis-restricted analogues of combretastatin A-٤: synthesis,

molecular modeling and evaluation as cytotoxic agents and inhibitors of tubulin. Bioorg. Med. Chem. ٢٠٠٨, ١٦, ٤٨٢٩ –٤٨٣٨.

١٧. (b) Zhang, Q.; Peng, Y.; Wang, X. I.; Keeman, S. M.; Aurora., S.; Welsh, W. J. Highly potent triazole-based tubulin polymerization inhibitors. J. Med. Chem. ٢٠٠٧, ٥٠, ٧٤٩ –٧٥٤.

١٨. (c) Romagnoli, R.; Baraldi, P. G.; Cruz-Lopez, O.; Lopez-Cara, C.; Carrion, M. D.; Brancale, A.; Hamel, E.; Chen, L.; Bortolozzi, R.; Basso, G.; Viola, G. Synthesis and antitumor activity of ١,٥-disubstituted ١,٢,٤-triazoles as cis-restricted combretastatin analogs. J. Med. Chem. ٢٠١٠, ٥٣, ٤٢٤٨ –٤٢٥٨.

١٩. Flynn BL, Hamel E, Jung MK. One-pot synthesis of benzo-[b]furan and indole inhibitors of tubulin polymerization. J. Med. Chem ٢٠٠٢;٤٥:٢٦٧٠–٢٦٧٣.

٢٠. Liou JP, Chang YL, Kuo FM, Chang CW, Tseng HY, Wang CC, Yang YN, Chang JY, Lee SJ, Hsieh HP. Concise synthesis and structure–activity relationships of combretastatin A-٤ analogues, ١-aroylindoles and ٣ aroylindoles, as novel classes of potent antitubulin agents. J. Med. Chem ٢٠٠٤;٤٧: ٤٢٤٧–٤٢٥٧.

٢١. Maya AB, Perez-Melero C, Mateo C, Alonso D, Fernandez JL, Gajate C, Mollinedo F, Pelaez R, Caballero E, Medarde M. Further naphthylcombretastatins. An investigation on the role of the naphthalene moiety. J. Med. Chem ٢٠٠٥; ٤٨:٥٥٦–٥٦٨.

٢٢. Tron GC, Pirali T, Sorba G, Pagliai F, Busacca S, Genazzani AA. Medicinal chemistry of combretastatin A٤: present and future directions. J. Med. Chem ٢٠٠٦;٤٩: ٣٠٣٣–٣٠٤٤.

٢٣. Cushman M, Nagarathnam D, Gopal D, Chakraborti AK, Lin CM, Hamel E. Synthesis and evaluation of stilbene and dihydro-stilbene derivatives as potential anticancer agents that inhibit tubulin polymerization. J. Med. Chem ١٩٩١;٣٤:٢٥٧٩–٢٥٨٨.

٢٤. Cushman M, Nagarathnam D, Gopal D, He HM, Lin CM, Hamel E. Synthesis and evaluation of analogues of (Z)-١-(٤-methoxy-phenyl)-٢-(٣,٤,٥-trimethoxyphenyl) ethene as potential cytotoxic and antimitotic agents. J. Med. Chem ١٩٩٢; ٣٥: ٢٢٩٣–٢٣٠٦.

٢٥. Romeo Romagnoli et al. Synthesis and evaluation of ١,٥-disubstituted tetrazoles as rigid analogues of combretastatin A-٤ with potent antiproliferative and antitumor activity. J. Med. Chem. ٢٠١٢, ٥٥, ٤٧٥ –٤٨٨.

٢٦. Zhang et al. Highly potent triazole-based tubulin polymerization inhibitors. J Med Chem. ٢٠٠٧ February ٢٢; ٥٠(٤): ٧٤٩–٧٥٤.

٢٧. K. Ohsumi et al. Syntheses and antitumor activity of cis-restricted combretastatins: ٥-membered heterocyclic analogues. Bioorg. Med. Chem. Lett. ١٩٩٨ (٨) ٣١٥٣-٣١٥٨.