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Original article

Part I: Synthesis, cancer chemopreventive activity and molecular docking study of novel quinoxaline derivatives

Shadia A. Galal [a,*], Ahmed S. Abdelsamie [a], Harukuni Tokuda [b], Nobutaka Suzuki [b], Akira Lida [c], Mahmoud M. ElHefnawi [d], Raghda A. Ramadan [d], Mona H.E. Atta [d], Hoda I. El Diwani [a]

[a] Department of Chemistry of Natural and Microbial Products, Division of Pharmaceutical and Drug Industries, National Research Centre, Dokki 12622, Cairo, Egypt
[b] Department of Complementary and Alternative Medicine, Clinical R&D, Graduate School of Medical Science, Kanazawa University,13-1 Takara-machi, Kanazawa, Japan 920-8640
[c] Faculty of Agriculture, Kinki University, 3327-20 Naka-machi, Nara, Japan 631-8505
[d] Biomedical Informatics and Chemoinformatics Group, Center of Excellence For Advanced Sciences, National Research Centre, Dokki 12622, Cairo, Egypt

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ABSTRACT

The reaction of *o*-phenylene diamine and ethyl oxamate is reinvestigated and led to 3-aminoquinoxalin-2(1*H*)-one rather than benzimidazole-2-carboxamide as was previously reported. The structure of the obtained quinoxaline has been confirmed by X-ray. The anti-tumor activity of synthesized quinoxalines **1**–**21** has been evaluated by studying their possible inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Among the studied compounds **1**–**21**, compounds **12**, **8**, **13**, **18**, **17** and **19**, respectively, demonstrated strong inhibitory effects on the EBV-EA activation without showing any cytotoxicity and their effects being stronger than that of a representative control, oleanolic acid. Furthermore, compound **12** exhibited a remarkable inhibitory effect on skin tumor promotion in an *in vivo* two-stage mouse skin carcinogenesis test using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter. The result of the present investigation indicated that compound **12** might be valuable as a potent cancer chemopreventive agent. Moreover, the molecular docking into PTK (PDB: 1t46) has been done for lead optimization of the aforementioned compounds as potential PTK inhibitors.

1. Introduction

Of the various human diseases, cancer has proven to be one of the most intractable diseases to which humans are subjected, and as yet no practical and generally effective drugs or methods of control are available. Therefore, identification of novel potent, selective, and less toxic anticancer agents remains one of the most pressing health problems [1].

Quinoxalines have been reported as candidates for the treatment of cancer and disorders associated with angiogenesis functions and several of them are currently in clinical trials. The quinoxaline ring may act as bioisostere of both pteridine and quinazoline rings present in the most representative drugs as methotrexate MTX, trimetrexate and tomude [1–10].

Quinoxalines showed to be potent inhibitors of the c-kit tyrosine kinase [11]. C-kit is a member of the family protein tyrosine kinase III. The c-kit proto-oncogene is a receptor protein-tyrosine kinase. Tyrosine kinases, such as c-kit, are proteins whose function is to transduce signals from the environment into the cell leading to complex behaviors such as proliferation, migration, survival and differentiation. Many of these actions are deregulated in cancer, which is characterized by uncontrolled proliferation, insensitivity towards death stimuli, migration of tumor cells away from the primary tumor site and in some cases also blocking of cellular differentiation leaving the cell in an immature proliferative state. Inhibition of the kinase activity, may lead to suppression of signals that support proliferation in transformed cells. The tyrosine kinase receptor c-kit is associated with several malignant human diseases [12].

Quinoxaline tyrosine kinase inhibitors, which are specific antagonists for c-kit also increase apoptosis. Quinoxalines' pattern of inhibition to c-kit is either ATP competitive or mixed-type inhibition, depending on the state of the receptor. Quinoxalines bind to the same residues that ATP binds to on the receptor [13,14].

In this work, new quinoxaline derivatives have been synthesized to study their *in vitro* promoting activity by estimating the inhibitory effect on Epstein–Barr virus antigen (EBV-EA) activation.

* Corresponding author. Tel.: +20 233371615; fax: +20 233370931.
E-mail address: sh12galal@yahoo.com (S.A. Galal).

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Epstein–Barr virus is a cancer-causing virus induced by phorbol ester tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) which stimulates cell proliferation through rapid activation of protein kinase C (PKC), followed by gradual degradation of the kinase [15].

We had for aim in this manuscript to design new quinoxaline derivatives through the following targets:

1- Introduction of different substituents in the 1, 2 or 3 positions, hydrophobic or hydrophilic groups, hydrogen bond donors or acceptors to influence and increase the affinity towards the receptor binding sites.
2- Synthesis of heterocyclic-fused quinoxalines as it is known from the literature that annelation was successful in causing selectivity and strong affinity towards the receptor.
3- Introduction of an electron rich pyrimidine ring to the quinoxaline moiety through a thio or a methyl thio linkage to increase the lipophilicity and hence the interaction with the target.

2. Result and discussion

2.1. Chemistry

Quinoxalin-2,3(1*H*,4*H*)-dione (**1**) was synthesized by the modified procedure of Obafemi and Pfeiferer [16]. Stirring a mixture of compound **1** with phosphorus oxychloride in methylene chloride at room temperature gave 3-chloroquinoxalin-2(1*H*)-one (**2**), which structure has been proven by X-ray structure. 3,4-dichloroquinoxaline (**3**) was afforded by treating compound **1** or **2** with phosphorus oxychloride in dimethylformamide [17]. The X-ray structure of compound **3** was obtained (c.f. Fig. 1). 3-Aminoquinoxalin-2(1*H*)-one (**4**) [18–20] was obtained by stirring a solution of compound **2** in ammonia and ethanol. 3-Hydrazinylquinoxalin-2(1*H*)-one (**5**) [21] can be obtained by reaction of compound **1**, **2** or **4** with hydrazine hydrate in ethanol (c.f. Scheme 1).

On the other hand, the reaction of *o*-phenylene diamine with oxamic acid ester in dimethylformamide was previously reported by Petyunin et al [22] to yield benzimidazole-2-carboxylic amides. In this

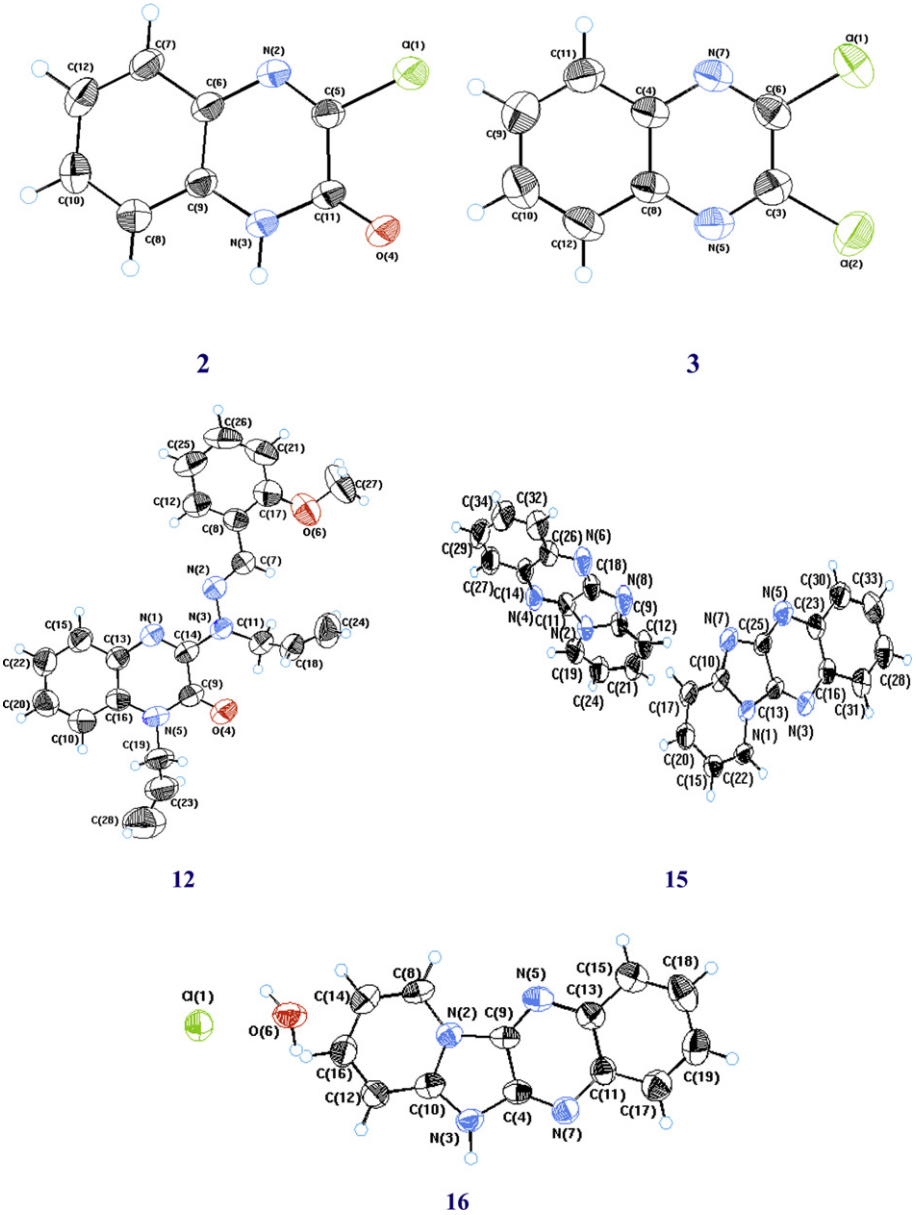
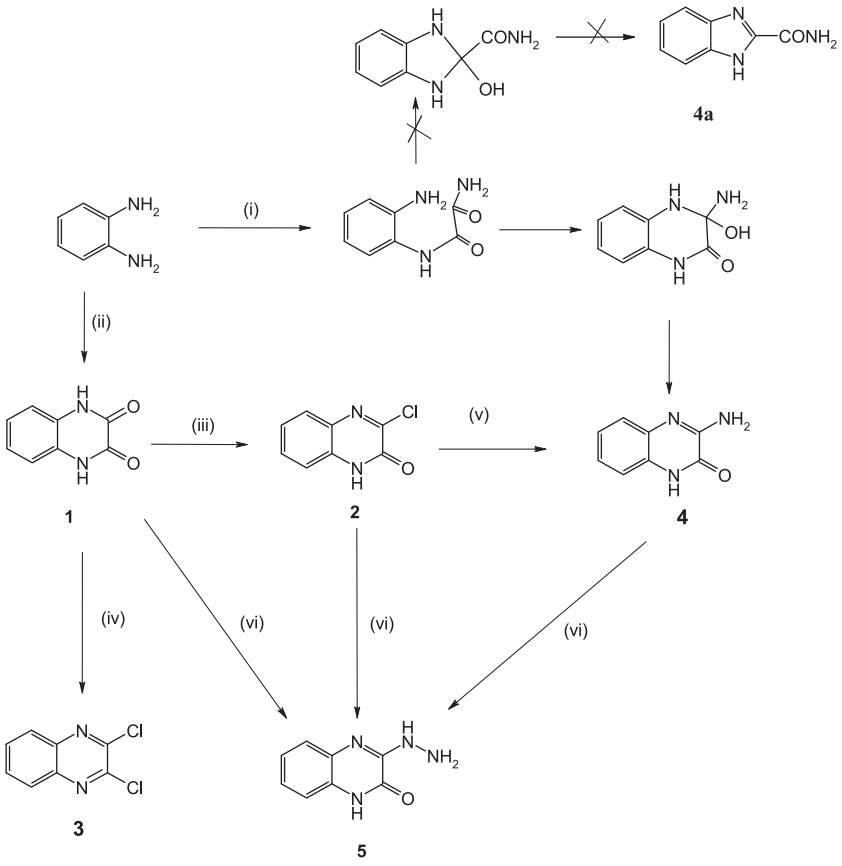


Fig. 1. X-ray structures of compounds **1**, **2**, **12**, **15** and **16**.



Reagents and conditions:

(i) Ethyl oxamate, pyridine, reflux, 8 h. (ii) oxalic acid, 4N HCl, reflux, yield 97% (iii) $POCl_3$, methylene chloride, stirring, 12h, room temp., yield 35% iv) $POCl_3$, DMF, stirring, 2h, 50 °C, yield 93% (v) Ammonia solution ethanol, stirring, yield 95% (vi) Hydrazine hydrate, ethanol, stirring, yield 60-80%.

Scheme 1. The synthesis of compounds **1**–**5**.

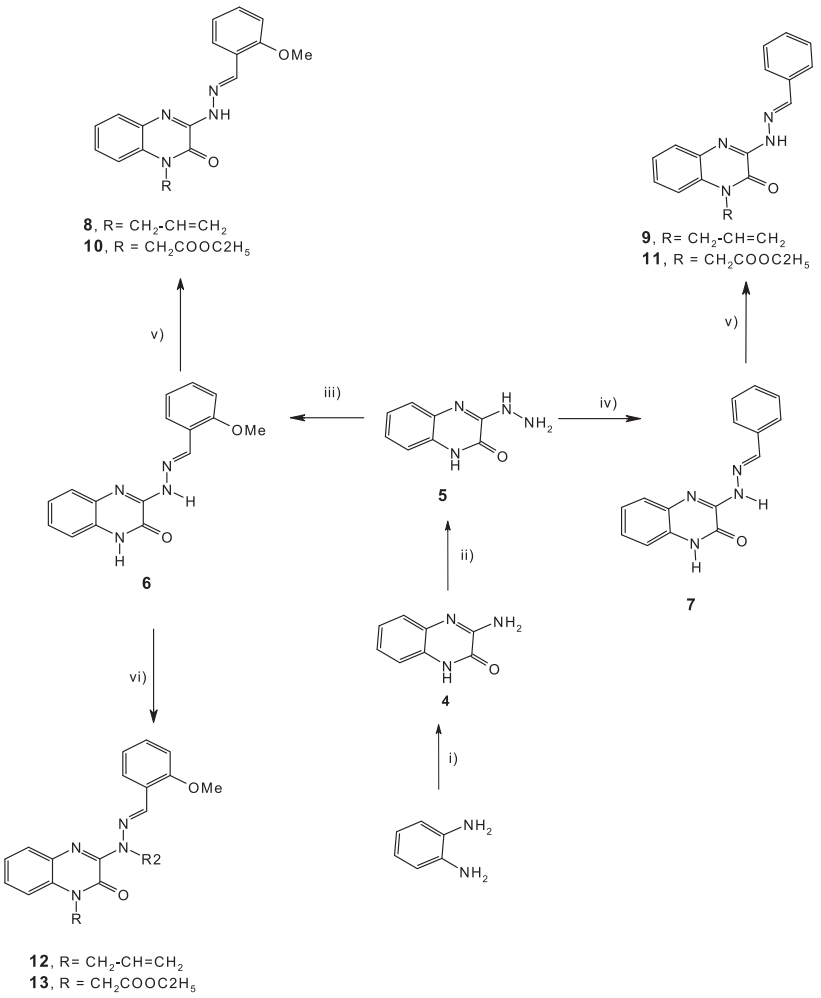
present study, the reaction of *o*-phenylene diamine and ethyl oxamate was reinvestigated in pyridine and dimethylformamide. The product obtained is believed to be 3-aminoquinoxalin-2(1*H*)-one (**4**) rather than 1*H*-benzo[*d*]imidazole-2-carboxamide (**4a**) as was previously reported [22,23]. On the other hand, compounds **4** and **4a** may have similar, spectroscopic data and their melting points were both above 300 °C but the formation of quinoxaline could be defined by its hydrazino derivative which was identical to the reported 3-hydrazinylquinoxalin-2(1*H*)-one (**5**) (c.f. Scheme 1) [21]. Furthermore, the formation of quinoxaline derivative (**4**) rather than benzimidazole derivative (**4a**) by the reaction of *o*-phenylene diamine with ethyl oxamate was confirmed by the X-ray structure of 1-Allyl-3-(1-allyl-2-(2-methoxybenzylidene)hydrazinyl)quinoxalin-2(1*H*)-one (**12**) (c.f. Fig. 1).

The reported anti-tumor activity of hydrazones [24,25] has prompted us to investigate new hydrazinyl quinoxalinones' hydrazones as 3-(2-(2-methoxybenzylidene)hydrazinyl)quinoxalin-2(1*H*)-one (**6**) and 3-(2-benzylidenehydrazinyl)quinoxalin-2(1*H*)-one (**7**). Compounds **6** and **7** were obtained by reaction of compound **5** with 2-methoxybenzaldehyde and benzaldehyde, respectively, in ethanol. Alkylation of compounds **6** and **7** with equimolar amount of allyl bromide or ethyl chloroacetate yielded compounds **8**–**11**, respectively, while using dimolar amounts of the two alkylating agents with compound **6** afforded the dialkyl derivatives **12** and **13**, respectively (c.f. Scheme 2). The structures of compounds **8**–**11**, **13** were deduced compounds displayed molecular ion peaks at appropriate *m*/*z* values. The structure of compound **12** was supported on the basis of its X-ray single crystal (Fig. 1).

Alkylation of compounds **6** and **7** using equimolar amount of allyl bromide or ethyl chloroacetate to afford compounds **8**–**11** led to significant lower frequency shift of absorption bands of ν(C=O). Also, the bands and signals due to NH of quinoxaline ring system of these compounds in both IR and ^{1}H NMR spectra disappeared with respect to that originally appearing at compounds **6** and **7**. The significant lower frequency shifts of absorption bands of ν(C=O) besides the disappearance of NH of quinoxaline ring system, indicated that alkylation took place selectively at NH of quinoxalinone rather than NH of hydrazinyl (–NHN=CH).

Attempts to form a 3-pyridyl derivative starting from the 3-chloroquinoxaline-2-amine (**14**) by chlorination of compound **4** using phosphrous oxychloride, led to the unexpected benzo[1′,2′]imidazo[4,5-*b*]quinoxaline (**15**), which structure has been proven by its X-ray single crystal (c.f. Fig. 1). The formation of compound **15** was explained to take place *via* the following mechanism (c.f. Scheme 3).

Similarly, treatment of compound **3** with pyridine afforded the unexpected quinoxalino[2,3-d]benzo[*b*]imidazolium chloride monohydrate (**16**). The structure of compound **16** has been proven by its X-ray single crystal (c.f. Fig. 1). The suggested mechanism for the formation of compound **16** is the following (c.f. Scheme 4).



Reagents and conditions:

i) Ethyl oxamate, pyridine, reflux, 8 h. ii) Hydrazine hydrate 98%, ethanol, reflux, 8h. (iii) 2-Methoxybenzaldehyde, ethanol, reflux, yield 71.3%. iv) Benzaldehyde, ethanol, reflux, yield 79.5%. v) Equimolar amounts of allyl bromide or ethyl acetate, compound **6** or **7**, potassium carbonate, DMF, stirring, room temp., vi) Compound **6**, allyl bromide or ethyl bromoacetate and potassium carbonate with molar ratio (1:2: 2), DMF, stirring, room temp., yield: 52% and 54%,respectively.

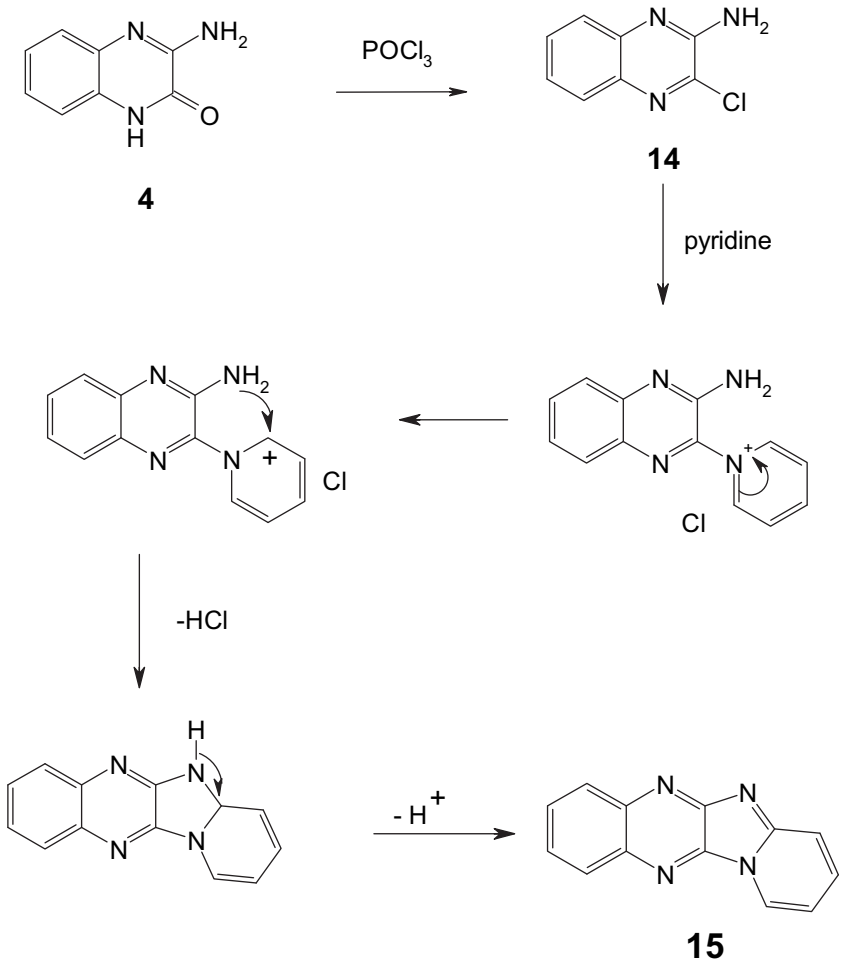
Scheme 2. Synthesis of compounds **4**–**13**.

2,3-Dichloroquinoxaline (**3**) was reacted with 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile derivatives [27] to produce compounds **17**–**19** (c.f. Scheme 5). Then again, compound **3** treated with 1*H*-benzimidazole-2-thiol [28] and 2-thiomethyl-1*H*-benzimidazole [29] to yield compounds **20** and **21** (c.f. Scheme 6). The structures of compounds **17**–**21** were deduced from their elemental analyses IR, ^{1}H NMR, ^{13}C NMR data and mass spectra.

2.1.1. Inhibition of EBV-EA activation assay

A primary screening test was carried out using a short-term *in vitro* synergistic assay on EBV-EA activation [30,31]. The inhibitory effect of quinoxaline derivatives **1**–**21** on the EBV-EA activation induced by TPA and the associated viability of Raji cells was listed in Table 1. In this assay, all the tested compounds showed inhibitory effects on EBV-EA activation without cytotoxicity on Raji cells. All compounds exhibited dose dependent inhibitory activities, and the viability percentages of Raji cells treated with the test compounds (**1**–**21**) were 50, 60 or 70% at the highest concentration of 1000 mol ratio/TPA. As shown in Table 1, the inhibitory activities of compounds **12**, **8**, **13**, **18** **17** and **19** were stronger than that of oleanolic acid at the highest concentration used. Oleanolic acid is known as a representive anti-tumor promoting agent [32]. Compound **9** had comparative activity with oleanolic acid. The relative ratio of compound **12** with respect to TPA (100%) was 9.2, 41.9, 73.2 and 98.7% at the concentrations of 1000, 500, 100 and 10 mol ratio/TPA, respectively, (Table 1); meaning 91.8, 58.1, 26.8 and 0.3% inhibition of the EBV-EA activation by TPA, respectively. Compounds **8** and **13** showed 89, 57.3, 25.4 and 0%, and 88, 57.9, 25 and 0%inhibition of the EBV-EA activation by TPA, respectively, at concentrations of 1000, 500, 100 and 10 mol ratio/TPA.

As shown in Table 1, formation of the hydrazinyl derivative **5** has positive effect on the inhibitory activity on EBV-EA activation with respect to aminoquinoxalin-2(1*H*)-one (**4**). On the other hand, we have investigated the role of alkyl substitution on the hydrazinyl (N) of 3-hydrazinylquinoxalin-2(1*H*)-one (**5**) which was found to have great effect on activity.



Scheme 3. The suggested mechanism for the formation of compound **15**.

Disubstitution with alkyl groups, allyl and ethyl acetate, on both nitrogens of hydrazine and quinoxaline of compounds **12** and **8** proved to be crucial for activity especially the allyl group, as compound **12** was found to be more active than the reference. This high activity may be due to hydrophobic interaction between the alkyl groups with the hydrophobic alpha spheres of the binding site of the receptor.

The importance of the alkyl group was clear from the fact that the diaallyl compound **12** was more active than the monoallyl **8** and the diethylacetate **13** was more active than the monoethylacetate **10**.

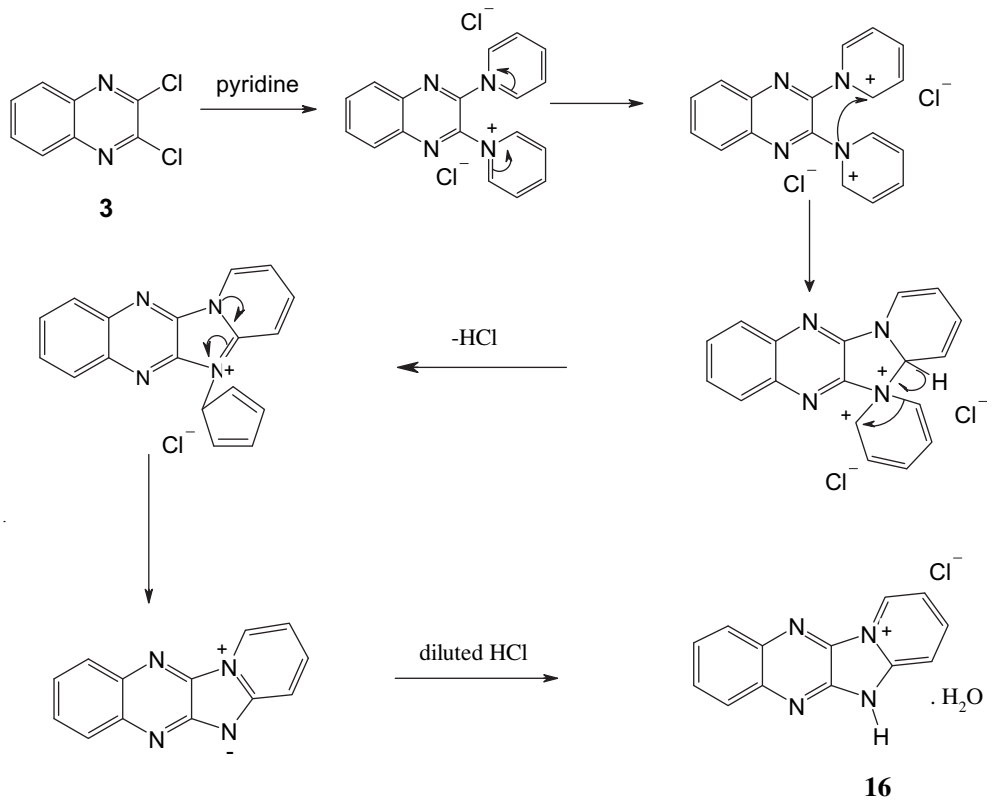
Also, the presence of methoxy group on the phenyl ring of compounds as in compounds **6**, **8** and **10** led to positive effect on the activity with respect to their analogues **7**, **9** and **11**, respectively, which may be attributed to hydrogen bonding.

Substitution with the electron rich pyrimidine ring linked to quinoxaline through sulfur seems to be interesting, as compounds **18**, **17** and **19** were found to be highly potent also. The presence of the hydroxyl group as hydrogen bonding donor was favored as compound **18** was more active than the unsubstituted phenyl compound **17** or compound **19** having the 4-fluoro substituent which is a hydrogen bond acceptor.

Annelation and formation of fused heterocyclic rings werefound to be un-favored as compounds **16** and **15** were the least active compounds.

Among the tested quinoxaline derivatives, compounds **12**, **8**, **13**, **18**, **17** and **19** exhibited the significant potency against TPA-induced activation.

Based on the results obtained *in vitro*, compound **12** was selected to examine the effect on the *in vivo* two-stage carcinogenesis test focusing on mouse skin papillomas induced by DMBA as an initiator and TPA as a promoter (c.f. Table 2) [33–35]. During the *in vivo* assay, the body-weight gains of the mice were not influenced by the treatment with the test compound and no toxic effects, such as lesional damages and inflammation (edema, erosion and ulcer) were observed on the areas of mouse skin topically treated with the test compound. Figs. 2 and 3 demonstrate the results of the papilloma formation in the skin of mice treated with compound **12**. The papilloma-bearing mice in the positive control group treated with DMBA (390 nmol) and TPA (1.7 nmol, twice/week) appeared as early as week 6, and the percentages of the papilloma-bearers increased rapidly to reach 100% after week 10. On the other hand, the treatment with compound **12** (85 nmol) along with DMBA/TPA inhibited the formation of papillomas until week 11 and the first papilloma appeared as late as at week 8. The percentage of papilloma-bearers in the mice of this group was 86.6% over the period of week 20. As shown in Fig. 3, in the positive control group with DMBA/TPA, the number of papillomas formed per mouse increased rapidly after week 5 to reach 8.0 papillomas/mouse at week 20, whereas the mice treated with compound **12** bore only 3.7 papillomas even at week 20. These results suggested that the inhibitory effect of compound **12** on two-stage carcinogenesis test was of potent activity *in vitro* and *in vivo*. Thus, compound **12** can be considered an appropriate lead compound to develop more potent agents with anti-tumor promoting activity for clinical use.



Scheme 4. The suggested mechanism for the formation of compound **16**.

2.2. Molecular modeling: docking study

The tyrosine kinase receptor c-kit is associated with several malignant human diseases the inhibition of the kinase activity become a target for therapeutic intervention. Quinoxaline tyrosine kinase inhibitors are specific antagonists for c-kit therefore, the synthesized qunioxaline derivatives **1**–**21** are investigated for the binding affinity of these into c-kit receptor (PDB code: 1t46) for the purpose of lead optimization and to find out the interaction between compounds **1**–**21** and the c-kit receptor.

Molecular modeling calculations and local docking were done by using MOE (molecular modeling environment) to evaluate the binding free energies of these inhibitors into the target c-kit kinase receptor.

2.2.1. Validation of the docking performance and accuracy

To validate the docking accuracy of the program used, docking of the native co-crystallized STI-571 ligand (Imatinib or Gleevec) was done into its binding site of c-kit receptor.

The docked ligand was exactly superimposed on the native co-crystallized one with RMSD being 0.40 Á and binding free energies of (−20.04 kcal/mol). The hydrogen bonds between the docked ligand and the amino acids were the same as those between the native ligand and the amino acids.

2.2.2. The binding affinities of the synthesized compounds *1*–*21* into c-kit kinase receptor

Molecular docking study was done to find out interactions between ligand and receptor and to compare affinities of the synthesized compounds to the target c-kit receptor. For the docking calculations, the protein structure (PDB code: 1t46) was first separated from the inhibitor molecule and refined using molecular minimization with added hydrogen.

Docking calculations were carried out using standard default variables for the MOE program. The binding affinity was evaluated by the binding free energies (S-score, kcal/mol), hydrogen bonds, and RMSD values. All synthesized compounds were docked into same groove of the binding site of the native co-crystallize STI-ligand.

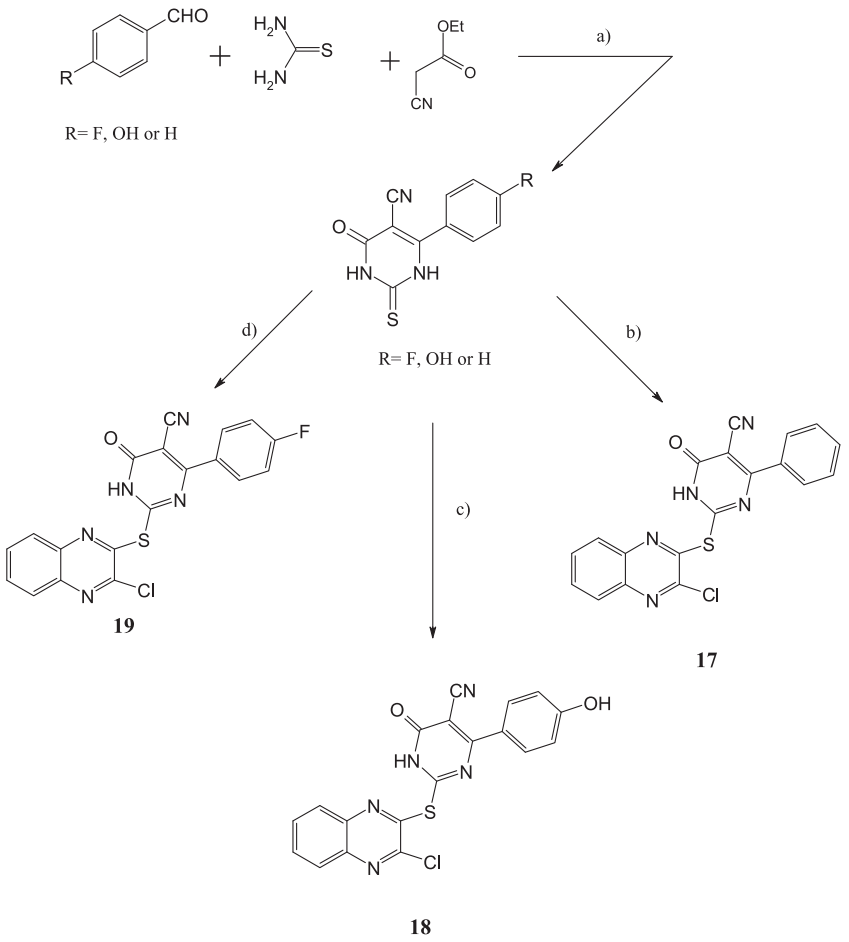
The compounds which gave the best docking scores based on the binding free energy and H-bonds with its distance between the amino acids in the receptor and RMSD from the native ligand were compounds **17**, **18**, **12**, **8**, **10** and **19**, respectively. H-bonds between all synthesized compounds and the amino acids in c-kit were the same of those between the native co-crystallized ligand **STI** with the amino acids of the receptor; this was represented in Fig. 4–7.

The ligand interaction of compound **17** with c-kit receptor clarified the significance of 4-hydroxypyrimidine-5-carbonitrile moiety in compounds **17**–**19** (c.f. Fig. 5).

The ligand interaction of compounds **12** and **8** with c-kit receptor explained the importance of both methoxy and allyl functional groups for activity (c.f. Figs. 6 and 7).

3. Conclusion

The reaction of *o*-phenylene diamine and ethyl oxamate in pyridine or dimethylformamide led to 3-aminoquinoxalin-2(1*H*)-one (**4**) rather than 1*H*-benzo[*d*]imidazole-2-carboxamide (**4a**) as was previously reported. Alkylation of compounds **6** and **7** using equimolar amount of allyl bromide or ethyl chloroacetate took place selectively at NH of quinoxalinone rather than NH of hydrazinyl (−NHN=CH). Reflux of 3-chloroquinoxaline-2-amine (**14**) in pyridine led to the unexpected benzo[1′,2′]imidazo[4,5-*b*]quinoxaline (**15**). Reaction of 2,3-dichloroquinoxaline **3** with pyridine afforded also the unexpected quinoxalino[2,3-d]benzo[*b*]imidazolium chloride monohydrate (**16**). The presence of methoxy group on the phenyl ring of compounds **6**, **8** and **10** led to positive effect on the inhibitory effects on EBV-EA activation with respect to their analogues **7**, **9** and **11**, respectively. The optimum activity observed in compound **12** with the presence of two allyl function groups. Compounds **12**, **8**, **13**, **17** and **18**, respectively,



Reagents and conditions:

(a) EtOH, K_2CO_3, 6h, reflux, yield 88% (b) 4-oxo-6-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile, compound **3**, K_2CO_3, DMF, stirring, 12h., yield 63.4%. (c) 6-(4-hydroxyphenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile, compound **3**, K_2CO_3, DMF , stirring, 10h., yield: 52%. (d) 6-(4-fluorophenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile, compound **3**, K_2CO_3, DMF, stirring, 10h., yield 49.3%.

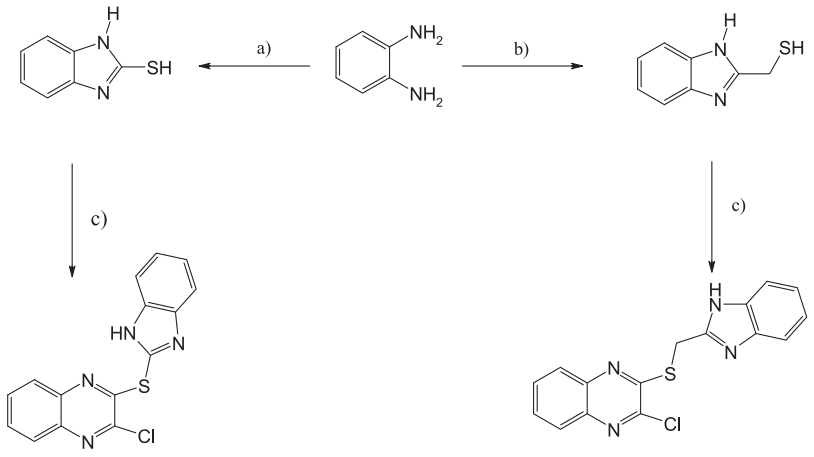
Scheme 5. The synthesis of compounds **17**–**19**.

demonstrated strong inhibitory effects on the EBV-EA activation without showing any cytotoxicity, their effects being stronger than that of a representative control, oleanolic acid. Compound **12** on *in vivo* two-stage carcinogenesis test was of significant activity. Thus, Compound **12** can be considered to become an appropriate lead compound to develop more potent agents with anti-tumor promoting activity for clinical use. The Molecular Docking investigation of the synthesized derivatives was carried out for lead optimization and they docked into c-kit protein-tyrosine kinase. The correlation between the binding affinities of the synthesized compounds **1**–**21** into c-kit kinase receptor predicted by MOE and the inhibition ratio of the EBV-EA activation by TPA with respect to positive control was good.

4. Experimental

4.1. Physical measurements

Microanalyses, spectral data and X-ray structures of the compounds were performed in the Central service and X-ray Laboratories, National research centre, Cairo, Egypt. The IR spectra (4000-400 cm^{-1}) were recorded using KBr pellets in a Jasco FT/IR 300E Fourier transform infrared spectrophotometer on a Perkin Elmer FT-IR 1650 (spectrophotometer). The ^1H and ^{13}C NMR spectra were recorded using Joel EX-270 MHz and 500 MHz NMR spectrophotometers. Chemical shifts are reported in parts per million (ppm) from the tetramethylsilane resonance in the indicated solvent. Coupling constants are reported in Hertz (Hz), spectral splitting partners are designed as follow: singlet (s); doublet (d); triplet (t); multiplet (m). Column chromatography was performed on Merck silica gel 60 (200–400 mesh).The mass spectra were carried out using Finnigan mat SSQ 7000 (Thermo. Inst. Sys. Inc., USA) spectroscopy at 70 ev. Crystal and molecular structures prepared by maXus Computer Program for the Solution and Refinement of Crystal Structures. All diagrams and calculations were performed using maXus (Bruker Nonius, Delft & MacScience, Japan). Extinction correction: none. Atomic scattering factors from Waasmaier & Kirfel, 1995. Data collection: KappaCCD. Cell refinement: HKL Scalepack and Data reduction: Denzo Program(s) used to solve structure: SIR92 and Scalepak Program(s) used to refine structure: maXus, Molecular graphics: ORTEP, Software used to prepare material for publication: maXus [36–39]. Crystal data, fractional atomic coordinates and equivalent isotropic thermal parameters, anisotropic displacement



20 **21**

Reagents and conditions:

(a) EtOH, KOH, CS_2, 4h, reflux, yield: 89%, (b) mercaptoacetic acid, 4N HCl, 6h, reflux, yield: 83%. (c) Compound **3**, K_2CO_3, DMF, stirring, 12h, yield 52% and 57% for compound **20** and **21**, respectively

Scheme 6. The synthesis of compounds **20** and **21**.

parameters and geometric parameters of compounds **2**, **3**, **12**, **15** and **16** are given in supplementary data.

*4.1.1. Preparation of 3-chloroquinoxalin-2(1H)-one (**2**)*

A mixture of quinoxalin-2,3(1*H*,4*H*)-dione (**1**) [16] (40 mmol) and phosphorus oxychloride (60 mmol) in methylene chloride (50 mL) was stirred at room temperature for 12 h. The excess solvent was evaporated under reduced pressure. The residue was dissolved in ice/water and neutralized with ammonia solution 30%. The formed precipitate was filtered off and purified on column chromatography by using petroleum ether (60–80): ethyl acetate (7:3) as an eluent. Crystal structure of compound **3** obtained.

Table 1
The relative ratio[a] of EBV-EA activation with respect to positive control (100%) in the presence of compounds **1**–**21** and oleanolic acid.

Compound #	% To control (% viability) 1000 mol ratio/TPA[b,c]	500 mol ratio/TPA[b]	100 mol ratio/TPA[b]	10 mol ratio/TPA[b]
1	14.2(60)	46.8	78.5	100
2	16.7(50)	48.5	80.2	100
3	18.9(50)	51.7	81.5	100
4	15.6(60)	47.2	79.1	100
5	14.9(60)	46.5	78.1	100
6	17.2(60)	47.0	82.1	100
7	18.5(60)	48.1	83.2	100
8	11.0(60)	42.7	74.6	100
9	13.0(60)	43.0	75.2	100
10	13.0(60)	43.0	75.2	100
11	13.9(60)	44.3	76.8	100
12	9.2 (60)	41.9	73.2	98.7
13	12.0(60)	42.1	75.0	100
15	18.1(60)	50.3	83.2	100
16	20.2(60)	51.2	84.6	100
17	12.4(60)	54.3	87.0	100
18	12.1(60)	42.6	77.6	100
19	12.5(60)	55.8	88.0	100
20	13.4(60)	51.6	78.6	100
21	13.7(60)	47.2	84.1	100
Oleanolic acid	12.7 (70)	3 0.0	80.0	100

[a] Values represent percentages relative to the positive control value (100%).
[b] TPA concentration was 20 ng/mL (32 pmol/mL).
[c] Values in parentheses are the viability percentages of Raji cells.

*4.1.2. Preparation of 2,3-dichloroquinoxaline (**3**) [17]*

A mixture of quinoxalin-2,3(1*H*,4*H*)-dione (**1**) [16] or compound **2** (40 mmol) and phosphours oxychloride (100 mmol) in dimethyl foramide (20 mL) was stirred at 50 °C for 4 h. The reaction mixture was added portion wise to ice/water and neutralized with ammonia solution 30%. The formed precipitate was filtered off and purified on column chromatography by using petroleum ether (60–80): ethyl acetate (9:1) as an eluent.

*4.1.3. Preparation of 3-aminoquinoxalin-2(1H)-one (**4**)*

4.1.3.1. Method A. A mixture of *o*-phenylene diamine (40 mmol) and ethyl oxamate (60 mmol) in pyridine (25 mL) was stirred under reflux for 8 h. The reaction mixture was poured into water and the precipitate formed was filtered off, washed and crystallized from ethanol as

Table 2
Inhibitory effects of compound **12** on two-stage mouse skin carcinogenesis.

Weeks of treatment	Positive control		TPA + (85 nmol) of Compound **12**	
	DMBA (390 nmol) + TPA (1.7 nmol)			
	Papillomas (%)	Papillomas/ mouse	Papillomas (%)	Papillomas/ mouse
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	6.6	0.4	0	0
7	20.0	0.9	0	0
8	40.0	1.8	13.3	0.2
9	73.3	2.4	13.3	0.6
10	86.6	3.5	20.0	1.1
11	100	3.9	26.6	1.4
12	100	4.3	33.3	1.7
13	100	5.2	46.6	1.9
14	100	6.0	60.0	2.3
15	100	6.6	60.0	2.5
16	100	6.8	66.6	2.7
17	100	7.1	73.3	2.9
18	100	7.5	80.0	3.2
19	100	7.8	86.6	3.4
20	100	8.0	86.6	3.7

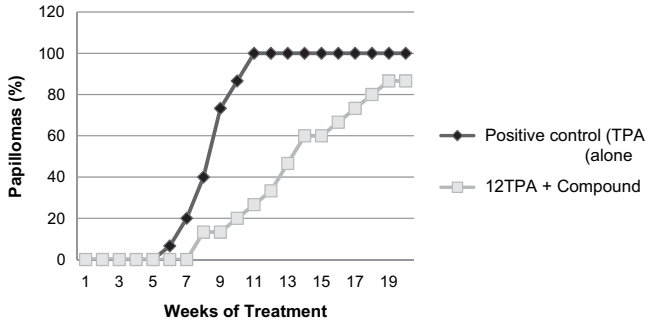


Fig. 2. Inhibition of TPA-induced tumor promotion by multiple application of compound **12**. All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA, given twice weekly starting 1 week after initiation. Statistically different from the positive control ($P < 0.01$, using Student's t-test).

a buff powder. (The preparation of compound **3** was also performed in dimethylformamide (instead of pyridine) under reflux for 48 h).

4.1.3.2. Method B. A mixture of compound **2** (40 mmol) in ammonia solution (50 mL) in ethanol (100 mL) was stirred at 0–5 °C for 8 h. The mixture was evaporated under reduced pressure. The residue was chromatographed on a silica gel column $R_f = 0.36$ (petroleum ether/ethyl acetate/methanol, 1: 1: 0.1). Yield: 95%, m.p. > 300 °C (lit., m.p. > 350 °C [18–20]). ^{1}H NMR (500 MHz, DMSO-d_6): 7.25(m, 4H); 11.21(br., 2H, NH_2, D_2O exchangeable), 13.19(br., 1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 115.85, 123.95, 125.8, 129.1, 131.6, 142.7, 155.3, 157.5. IR (cm^{-1}): 3492.45(NH quinoxaline), 3288, 3208 (NH_2), 3050, 2968 (CH, aromatic), 1682 (C=O), 1613 (C=N), 1529 (C=C). MS: [*m/z* (rel. abundance)]: 161(M^+, 100%). Anal. Calcd for $C_8H_7N_3O$ (FW: 161.16): C, 59.62; H, 4.38; N, 26.07. Found: C, 59.58; H, 4.41; N, 26.19.

*4.1.4. Preparation of 3-hydrazinylquinoxalin-2(1H)-one (**5**)*

A mixture of compound **1**, **2** or **4** (7 mmol), ethanol (50 mL) and hydrazine monohydrate 98% (10 mL) was stirred at room temp. for 3 h, 0.5 h, or 5 h, respectively, according to the starting agent. The reaction mixture was evaporated under reduced pressure. The solid residue was washed with ethanol and recrystallized from dimethylformamide as a yellowish white powder. $R_f = 0.39$ (petroleum ether/ethyl acetate/methanol, 1: 1: 0.25). Yield: 60–80%, m.p. > 300 °C (lit. > 360 °C [21]). ^{1}H NMR (500 MHz, DMSO-d_6): 4.52 (br., 2H, NH_2, D_2O exchangeable), 7.11(m, 1H), 7.27(m, 1H); 7.34 (m, 1H), 7.6(m, 1H); 8.75(br., NH, D_2O exchangeable), 12.17(br., NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 115.91, 123.89, 125.98, 129.11, 131.66, 142.65, 153.5, 159.23. IR (cm^{-1}): 3404 (NH quinoxaline), 3332–3200 ($NHNH_2$), 3050, 2968 (CH, aromatic),

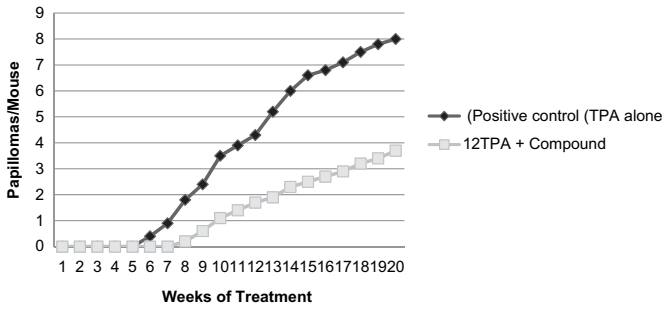


Fig. 3. Inhibition of TPA-induced tumor promotion by multiple application of compound **12**. All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA, given twice weekly starting 1 week after initiation. Statistically different from the positive control ($P < 0.01$, using Student's t-test).

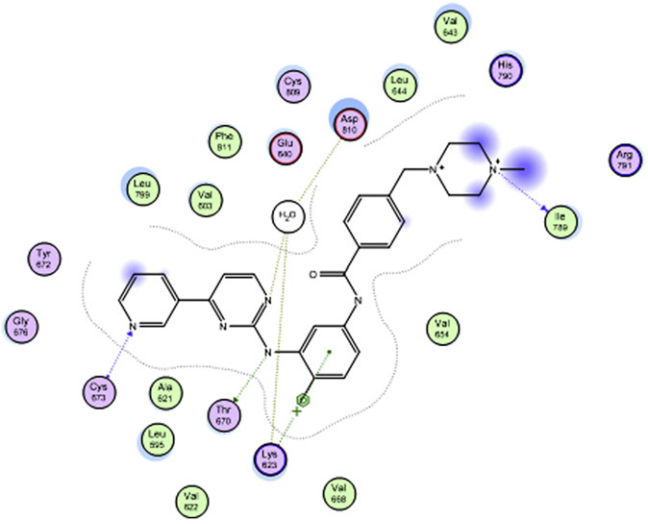


Fig. 4. The ligand interaction and the binding mode of the native ligand **STI** with C-kit, it exhibited 3 H-bonds with CYS 673, THR 670 and ILE 789.

1680 (C=O), 1615 (C=N), 1580, 1500 (C=C). MS: [*m/z* (rel.abundance)]: 176(M^+, 55%). Anal. Calcd for $C_8H_8N_4O$ (FW: 176.07): C, 54.54; H, 4.58; N, 31.80. Found: C, 54.51; H, 4.65; N, 31.71.

*4.1.5. Preparation of compounds **6–8***

A mixture of compound **5** (50 mmol) in absolute ethanol (150 mL) and 2-methoxybenzaldehyde or benzaldehyde (50 mmol) was refluxed for 7 h. After reaction completed, the solvent was evaporated and the solid residue washed with water. The solid was filtered off and recrystallized from ethanol.

*4.1.5.1. 3-(2-(2-Methoxybenzylidene)hydrazinyl)quinoxalin-2(1H)-one (**6**).* $R_f = 0.65$ (petroleum ether/ethyl acetate, 1: 3), yield: 71.3%, m.p. 157–159 °C. ^{1}H NMR (270 MHz, DMSO-d_6): 3.68(s, 3H, OCH_3), 7.15(m, 5H); 7.45(m, 2H); 8.0 (m, 1H); 8.8(s, 1H); 11.24(br., NH, D_2O exchangeable), 12.4(br., NH, D_2O exchangeable). ^{13}C NMR (270 MHz, DMSO-d_6): 56.1, 112.2, 115.0, 117.1, 120.7, 123.0, 124.65, 125.49, 128.76, 131.13, 132.96, 142.46, 146.29, 148.5, 150.89, 157.6. IR (cm^{-1}): 3401(NH quinoxaline), 3315 (NH), 3050, 3010 (CH, aromatic), 2846 (CH, aliphatic), 1678 (C=O), 1618 (C=N), 1579(C=C). MS: [*m/z* (rel. abundance)]: 294(M^+, 5%), 176(m*, 100). Anal. Calcd for $C_{16}H_{14}N_4O_2$ (FW: 294.11): C, 65.30; H, 4.79; N, 19.04. Found: C, 65.38; H, 4.75; N, 19.15.

*4.1.5.2. 3-(2-Benzylidenehydrazinyl)quinoxalin-2(1H)-one (**7**).* $R_f = 0.165$ (petroleum ether/ethyl acetate, 1: 3), yield: 79.5%, m.p. 240–242 °C. ^{1}H NMR (270 MHz, DMSO-d_6): 7.05(m, 2H); 7.50 (m, 5H); 7.98 (m, 2H); 8.65(s, 1H); 11.23(s, 1H, NH, D_2O exchangeable), 12.4(s, 1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 112.1, 116.2, 121.6, 123.6, 124.9, 125.49, 128.56, 131.2, 133.5, 142.54, 146.43, 150.9, 155.3, 157.5. IR (cm^{-1}): 3376 (NH quinoxaline), 3334 (NH), 3050 (CH, aromatic), 1689 (C=O), 1613 (C=N), 1565(C=N and C=C). MS: [*m/z* (rel. abundance)]: 264(M^+, 38%), 176(m*, 100). Anal. Calcd for $C_{15}H_{12}N_4O$ (FW: 264.10): C, 68.17; H, 4.58; N, 21.20. Found: C, 68.19; H, 4.61; N, 21.16.

*4.1.6. Preparation of compounds **8–11***

General procedure: A mixture of compound **5** or **6** (4 mmol), K_2CO_3 (4.2 mmol) and allyl bromide or ethyl bromoacetate (4.2 mmol) in 10 ml of DMF was stirred for 10–12 h. The inorganic salt was filtered off and the solution is evaporated under the reduced pressure. The precipitate was washed with water and crystallized from acetone.

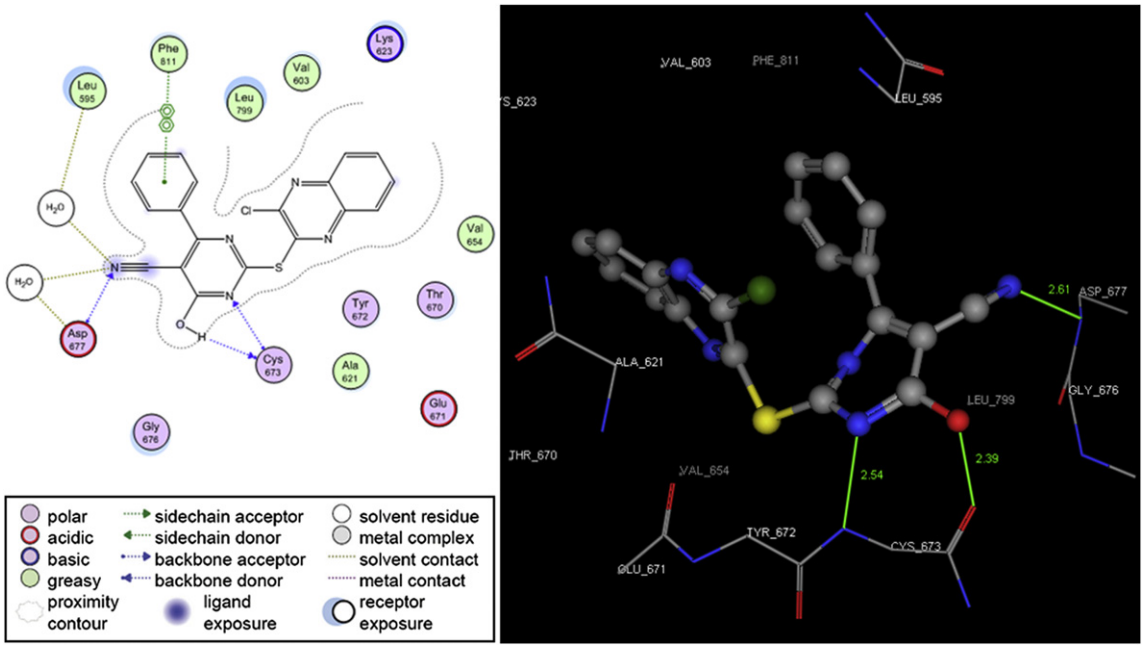


Fig. 5. Ligand interaction and the binding mode of compound **17** with c-kit receptor, it exhibited 3 H-bonds with the amino acids in C-kit two of them with CYS 673 and one with ASP 677, the hydrogen bonds formed colored in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

*4.1.6.1. 1-Allyl-3-(2-(2-methoxybenzylidene)hydrazinyl)quinoxalin-2(1H)-one (**8**).* R_f = 0.24 (petroleum ether/ethyl acetate, 1: 3), yield: 48.5%, m.p. 174–176 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 3.7 (s, 3H, OCH_3), 4.9(d, 2H, J = 16 Hz, CH_2), 5.16(d, 2H, J = 10 Hz, CH_2=), 5.94(m, 1H, CH=), 7.25(m, 5H); 7.55(m, 2H); 7.9 (m, 1H); 8.6(s, 1H); 11.06(s,1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 52.5, 56.21, 111.8, 115.6, 116.7, 120.3, 122.5, 123.4, 125.2, 125.8, 126.1, 128.33, 131.21, 132.76, 142.55, 146.9, 149.8, 152.89, 153.2. IR (cm^{-1}): 3260 (NH, hydrazide), 3029 (CH, aromatic), 2931 ($CH=CH_2$), 1650 (C=O), 1611 (C=N), 1568(C=C). MS: [*m*/*z*(rel. abundance)]: 334(M^+, 8%), 176(m*, 100). Anal. Calcd for $C_{19}H_{18}N_4O_2$ (FW: 334.14): C, 68.25; H, 5.43; N, 16.76. Found: C, 68.31; H, 5.49; N, 16.69.

*4.1.6.2. 1-Allyl-3-(2-benzylidenehydrazinyl)quinoxalin-2(1H)-one (**9**).* R_f = 0.65 (petroleum ether/ethyl acetate, 1: 3), yield: 76.5%, m.p. 160–162 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 5.09(d, 2H, J = 16 Hz, CH_2), 5.17(d, 2H, J = 10 Hz, CH_2=), 5.95(m, 1H, CH=), 7.25 (m, 2H); 7.42(m, 4H);7.69(m, 1H); 7.7 (m, 2H); 8.5(s, 1H); 11.26(s,1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 52.5, 112.5,

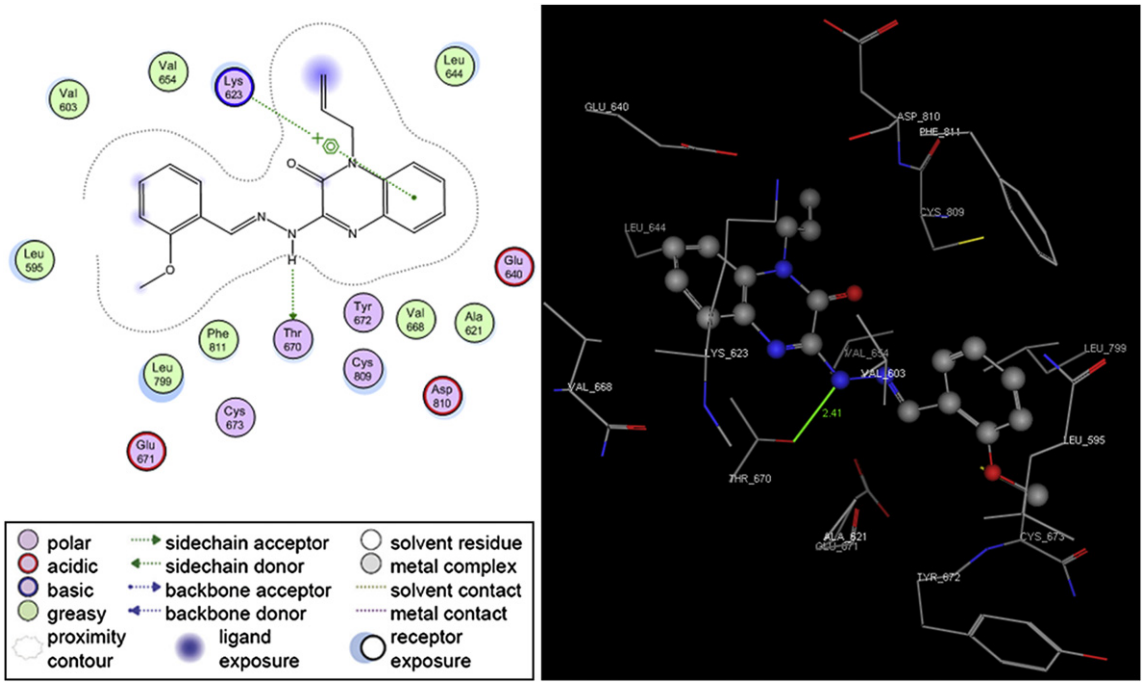


Fig. 6. Ligand interaction and the binding mode of compound **8** with c-kit receptor, it exhibited 1 H-bond with THR 670, the hydrogen bonds formed colored in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

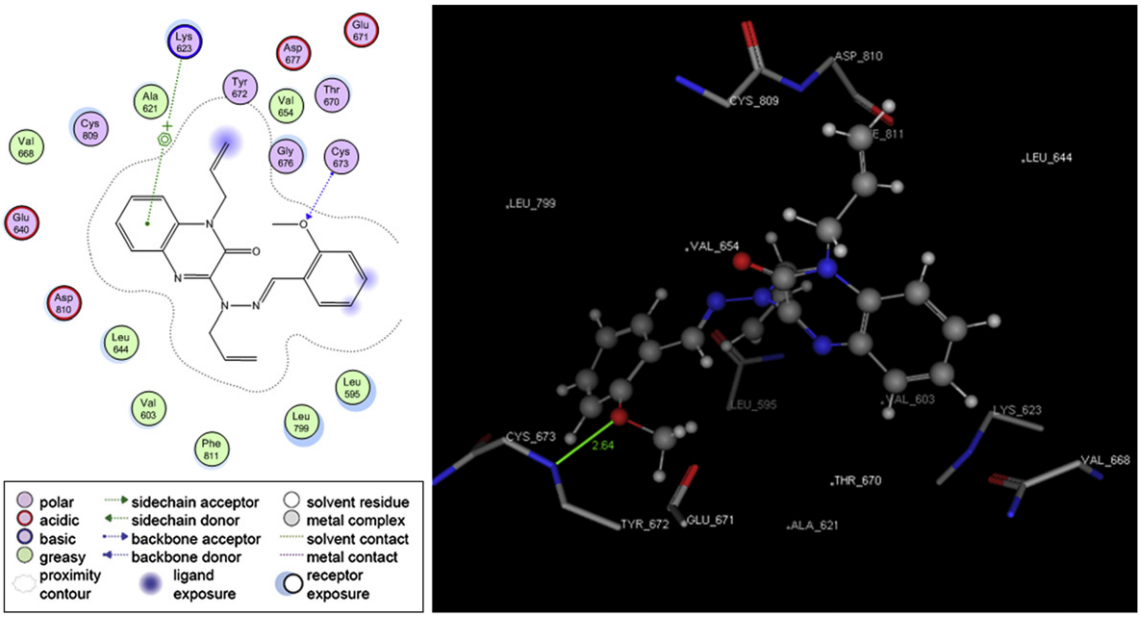


Fig. 7. Ligand interaction and the binding mode of compound **12** with c-kit receptor, it exhibited 1 H-bond with them with CYS 673.

115.1, 117.2, 120.9, 121.5, 123.8, 124.2, 126.7, 127.83, 130.61, 132.7, 135.43, 143.43, 145.8, 148.6, 152.22, 153.8. IR (cm^{-1}): 3259 (NH), 3029 (CH, aromatic), 1643 (C=O), 1604 (C=N), 1568(C=C). 1568 (C=C). MS: [*m*/*z* (rel. abundance)]: 304(M^+, 40%), 176(m*, 100). Anal. Calcd for $C_{18}H_{16}N_4O$ (FW: 304.13): C, 71.04; H, 5.30; N, 18.41. Found: C, 71.10; H, 5.28; N, 18.52.

*4.1.6.3. Ethyl 2-(3-(2-(2-methoxybenzylidene)hydrazinyl)-2-oxoquinoxalin-1(2H)-yl)acetate (**10**).* R_f = 0.65 (petroleum ether/ethyl acetate, 1: 3), yield: 76.5%, m.p. 196–198 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 1.18(t, 3H, CH_3), 3.76(s, 3H, OCH_3), 4.16(q, 2H, CH_2), 5.1(s, 2H, CH_2), 6.67(m, 3H), 7.25(m, 2H), 7.32(m, 1H), 7.53(m, 2H); 8.41(s, 1H); 10.98(s, 1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 14.58, 48.95, 52.5, 55.9, 111.8, 115.6, 116.7, 120.3, 120.5, 123.4, 123.9, 126.1, 128.33, 131.21, 132.76, 142.55, 146.9, 152.89, 153.2, 169.2. IR (cm^{-1}): 3271(NH, hydrazide), 3070 (CH, aromatic), 2837(CH, aliphatic), 1750 (C=O), 1653 (C=O), 1604 (C=N), 1569(C=C). MS: [*m*/*z*(rel. abundance)]: 380(M^+, 6%), 176(m*, 100). Anal. Calcd for $C_{20}H_{20}N_4O_4$ (FW: 380.15): C, 63.15; H, 5.30; N, 14.73. Found: C, 63.22; H, 5.41; N, 14.79.

*4.1.6.4. Ethyl 2-(3-(2-benzylidenehydrazinyl)-2-oxoquinoxalin-1(2H)-yl)acetate (**11**).* R_f = 0.54 (petroleum ether/ethyl acetate, 1: 3), yield: 88%, m.p. 210–212 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 1.19(t, 3H, CH_3), 4.15(q, 2H, CH_2), 5.12(s, 2H, CH_2), 7.26(m, 1H); 7.42(m, 6H); 7.69 (m, 2H); 8.57(s, 1H); 11.31(s, 1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 14.4, 50.5, 59.8, 111.8, 115.6, 116.7, 120.3, 120.5, 123.4, 123.9, 126.1, 128.33, 131.21, 132.76, 142.55, 146.9, 152.77, 153.9, 168.35. IR (cm^{-1}): 3281(NH, hydrazide), 3061 (CH, aromatic), 2826 (CH, aliphatic), 1749 (C=O), 1643(C=O), 1602 (C=N), 1571(C=C). MS: [*m*/*z*(rel. abundance)]: 350(M^+, 14%), 176(m*, 100). Anal. Calcd for $C_{19}H_{18}N_4O_3$ (FW: 350.14): C, 65.13; H, 5.18; N, 15.99. Found: C, 65.22; H, 5.21; N, 15.85.

*4.1.7. Preparation of compounds **12** and **13***

General procedure: A mixture of compound **5** (4 mmol), K_2CO_3 (8.5 mmol) and allyl bromide or ethyl bromoacetate (8.5 mmol) in 20 mL of DMF was stirred for 10 h. After reaction completed, the inorganic salt was filtered off and the solution is evaporated under the reduced pressure. The precipitate was washed with water and crystallized from acetone.

*4.1.7.1. 1-Allyl-3-(1-allyl-2-(2-methoxybenzylidene)-hydrazinyl)quinoxalin-2(1H)-one (**12**).* R_f = 0.51 (petroleum ether/ethyl acetate, 1: 3), yield 52%, m.p. 230–232 °C.

*4.1.7.2. Ethyl 2-(3-(1-(2-ethoxy-2-oxoethyl)-2-(2-methoxybenzylidene) hydrazinyl)-2-oxoquinoxalin-1(2H)-yl) acetate (**13**).* R_f = 0.52 (petroleum ether/ethyl acetate, 1: 3), yield 54%, m.p 218–221 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 1.17(t, 3H, CH_3), 1.20(t, 3H, CH_3), 3.72 (s, 3H, OCH_3), 3.86(s, 3H, OCH_3), 4.16(q, 2H, CH_2), 4.28(q, 2H, CH_2), 4.9(s, 2H, CH_2), 5.1(s, 2H, CH_2), 6.66(m, 3H), 7.23(m, 2H); 7.51(m, 3H); 8.49(s, 1H). ^{13}C NMR (500 MHz, DMSO-d_6):15.2, 50.2 50.7, 59.9, 111.8, 114.6, 116.7, 120.3, 121.5, 123.4, 124.0, 126.1, 128.33, 131.21, 132.76, 142.55, 146.9, 151.89, 153.2, 167.9, 169.2. IR (cm^{-1}): 3047(CH, aromatic), 2826(CH, aliphatic), 1750 (C=O), 1648 (C=O), 1604 (C=N), 1565(C=C). MS: [*m*/*z*(rel. abundance)]: 466(M^+, 5%), 176(m*, 100). Anal. Calcd for $C_{24}H_{26}N_4O_6$ (FW: 466.19): C, 61.79; H, 5.62; N, 12.01. Found: C, 61.83; H, 5.57; N, 12.13.

*4.1.8. Preparation of 3-chloroquinoxalin-2-amine (**14**) [26]*

A solution of compound **4** (40 mmol) and phosphours oxychloride (60 mmol) in methylene chloride (50 mL) was stirred at room temperature for 12 h. The excess solvent was evaporated under reduced pressure. The residue was dissolved in ice/water and neutralized with ammonia solution 30%. The formed precipitate was filtered off and purified on a silica gel column (eluent: methanol–dichloromethane: 5/95) to give compound **13**. m.p.138–140 °C (lit. 139 °C).

*4.1.9. Preparation of compounds **15** and **16***

A solution of compound **14** or **3** (10 mmol) in pyridine (10 mL) was refluxed for 6 h. After cooling, it was poured on ice/water (200 mL) and acidified with diluted HCl (pH = 6) the product extracted by ethyl acetate (100 × 3). Solution of ethyl acetate dried over sodium acetate anhydrous (50 g) for 2 h, and then the solid is filtered off. A solution of ethyl acetate is evaporated under reduced pressure and crystals are collected.

*4.1.9.1. Benzo[1′,2′]imidazo[4,5-b]quinoxaline (**15**).* R_f = 0.45 (petroleum ether/ethyl acetate, 1: 3), yield: 88%, m.p. 256–258 °C.

*4.1.9.2. Quinoxalino[2,3-d]benzo[b]imidazolium chloride monohydrate (**16**).* $R_f = 0.18$ (petroleum ether/ethyl acetate, 1: 3), yield: 72%, m.p. > 300 °C.

*4.1.10. Preparation of compounds **17**–**21***

A mixture of 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile derivatives [27], 1*H*-benzimidazole-2-thiol [28] or 2-thiomethyl-1*H*-benzimidazole [29] (4 mmol), K_2CO_3 (4.2 mmol) and compound **3** (4.2 mmol) in 10 mL of DMF was stirred for 10–12 h. The inorganic salt was filtered off and the solution is evaporated under the reduced pressure. The solid residue was washed with water and crystallized from acetone.

*4.1.10.1. 2-(3-Chloroquinoxalin-2-ylthio)-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile (**17**).* $R_f = 0.42$ (petroleum ether/ethyl acetate, 1: 3), yield: 63.4%, m.p. 269–271 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 7.49(m, 3H), 7.51(m, 2H), 7.82 (m, 2H), 8.12(m, 2H), 11.86 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 95.3, 115.7, 127.9, 128.5, 129.2, 131.5, 134.7, 135.5, 136.9, 141.3, 143.5, 145.8, 153.1, 161.4, 164.2, 170.2 IR (cm^{-1}): 3344 (NH), 3053(CH aromatic), 2222 (CN), 1603 (C=N), 1576 (C=N, C=C pyrimidine). MS: [*m*/*z*(rel. abundance)]: 390 ($M^+ - 1$, 24%), 196(m*, 100). Anal. Calcd for $C_{19}H_{10}ClN_5OS$ (FW: 391.03): C, 58.24; H, 2.57; Cl, 9.05; N, 17.87; S, 8.18. Found: C, 58.31; H, 2.61; Cl, 9.16; N, 17.82; S, 8.21.

*4.1.10.2. 2-(3-Chloroquinoxalin-2-ylthio)-4-(4-hydroxyphenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**18**).* $R_f = 0.51$ (petroleum ether/ethyl acetate, 1:3), yield: 52%, m.p. > 300 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 6.88(m, 2H), 7.32(m, 2H), 7.68 (m, 2H), 7.97 (m, 2H), 10.54(s, 1H, OH, D_2O exchangeable), 11.33 (br., 1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 92.3, 115.4, 127.1, 128.3, 129.1, 129.6, 130.4, 140.2, 142.5, 146.7, 145.8, 153.1, 158.4, 163.9, 165.3, 170.3. IR (cm^{-1}): 3452 (OH), 32836 (NH), 3037(CH aromatic), 2210 (CN), 1661.7 (C=O), 1610 (C=N), 1579 (C=N, C=C pyrimidine). MS: [*m*/*z*(rel. abundance)]: 407(M^+, 8%), 245(m*, 100). Anal. Calcd for $C_{19}H_{10}ClN_5O_2S$ (FW: 407.02): C, 55.96; H, 2.47; Cl, 8.69; N, 17.17; S, 7.86. Found: C, 55.82; H, 2.39; Cl, 8.72; N, 17.21; S, 7.87.

*4.1.10.3. 2-(3-Chloroquinoxalin-2-ylthio)-4-(4-fluorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**19**).* R_f = 0.48 (petroleum ether/ethyl acetate, 1: 3), yield: 49.3%, m.p. 277–279 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 6.99(m, 2H,); 7.46 (m, 2H); 7.78 (m, 2H); 8.09 (m, 2H); 11.33 (br., 1H, NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 93.3, 114.2, 115.9, 127.1, 128.4, 129.0, 129.7, 130.6, 132.4, 140.2, 143.5, 146.7, 145.8, 151.6, 158.4, 161.8, 163.3, 165.7, 170.2. IR (cm^{-1}): 32836 (NH pyrimidine); 2208. (CN); 1661 (C=O); 1610. (C=N); 1549 (C=N, C=C). MS: [*m*/*z*(rel. abundance)]: 409(M^+, 11.6%), 247(m*, 100). Anal. Calcd for $C_{19}H_9ClFN_5OS$ (FW: 409.02): C, 55.68; H, 2.21; Cl, 8.65; F, 4.64; N, 17.09; S, 7.82. Found: C, 55.75; H, 2.31; Cl, 8.55; N, 17.17; S, 7.89.

*4.1.10.4. 2-(1H-Benzo[d]imidazol-2-ylthio)-3-chloroquinoxaline (**20**).* R_f = 0.28 (petroleum ether/ethyl acetate, 1: 3), yield: 52%, m.p. > 300 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 7.19(m, 2H), 7.55(m, 2H); 7.61(m, 2H); 7.81(m, 2H); 12.91(br., NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 115.3, 123.7, 128.5, 129.7, 130.1, 130.8, 133.0, 138.2, 140.4, 142.1, 143.4, 147.7, 152.1. IR (cm^{-1}): 3421 (NH); 3041(CH, aromatic), 1622 (C=N), 1565(C=C). MS: [*m*/*z*(rel. abundance)]: 312 (M^+, 22%), 150(m*, 100). Anal. Calcd for $C_{15}H_9ClN_4S$ (FW: 312.02): C, 57.60; H, 2.90; Cl, 11.33; N, 17.91; S, 10.25. Found: C, 57.53; H, 2.85; Cl, 11.28; N, 17.86; S, 10.29.

*4.1.10.5. 2-((1H-Benzo[d]imidazol-2-yl)methylthio)-3-chloroquinoxaline (**21**).* $R_f = 0.61$ (petroleum ether/ethyl acetate, 1: 3), yield: 57%, m.p. > 300 °C. ^{1}H NMR (500 MHz, DMSO-d_6): 4.72 (s, 2H, CH_2), 7.23 (m, 2H), 7.60(m, 2H); 7.65(m, 2H); 7.83(m, 2H); 12.33(br., NH, D_2O exchangeable). ^{13}C NMR (500 MHz, DMSO-d_6): 35.9, 115.3, 123.7, 128.2, 129.6, 131.3, 138.2, 141.4, 142.1, 143.4, 148.7, 152.1. IR (cm^{-1}): 3426 (NH); 3043(CH, aromatic), 2839 (CH, aliphatic), 1625 (C=N), 1571(C=C). MS: [*m*/*z*(rel. abundance)]: 326 (M^+, 19%), 132 (m*, 100). Anal. Calcd for $C_{16}H_{11}ClN_4S$ (FW: 326.04): C, 58.80; H, 3.39; Cl, 10.85; N, 17.14; S, 9.81. Found: C, 58.89; H, 3.42; Cl, 10.87; N, 17.15; S, 9.90

4.2. Chemopreventive activity

4.2.1. Cells

EBV genome-carrying lymphoblastoid cells (Raji cells derived Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 under the conditions described previously [30]. Spontaneous activation of EBV-EA in our subline of Raji cells was less than 0.1%.

4.2.2. Animals

Specific pathogen-free (SPF) female ICR and female SENCAR mice (6 weeks old, respectively) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and maintained under SPF conditions in Animal Center of Kyoto Prefectural University of Medicine. The mice were housed five per polycarbonate cage in a temperature controlled room at 24 ± 2 °C and given food, Oriental MF (Oriental Yeast Co., Tokyo, Japan), and water or aqueous sample solution adlibitum during the experiments. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kanazawa University of Medicine.

4.2.3. Inhibition of EBV-EA activation assay

Inhibition of EBV-EA activation was assayed using Raji cells (Virus nonproducer type), an EBV genome-carrying human lymphoblastoid cell, which were cultivated in 10% fetal bovine serum. (FBS) RPMI-1640 medium. The indicator cells (Raji, 1 μ 10^6/mL) were incubated at 37 °C for 48 h in 1 mL of medium containing n-butyric acid (4 mM as trigger), TPA (32 pM = 20 ng in 2 μL of DMSO as inducer), and various amounts of the test compounds dissolved in 5 μL of DMSO (ca. 0.7% DMSO). Smears were made from the cell suspension. The EBV-EA inducing cells were stained with high titer EBV-EA positive serum from NPC patients and detected by an indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. Triplicate assays were performed for each data point. as a relative ratio to the positive control experiment (100%), which was carried out with n-butyric acid (4 mM) plus TPA (32 pM). In the experiments, the EBV-EA induction was normally around 35%, and this value was taken as the positive control (100%). n-Butyric acid (4 mM) alone induced 0.1% EA-positive cells. The viability of treated Raji cells was assayed by the trypan blue staining method. The cell viability of the TPA positive control was greater than 80%. Therefore, only the compounds that induced less than 80% (% of control) of the EBV-activated cells (those with a cell viability of more than 60%) were considered able to inhibit the activation caused by promoter substances. Student's t-test was used for all statistical analyses [30,31].

4.2.4. Two-stage mouse skin carcinogenesis model induced by DMBA/TPA

Animals (6 weeks old SPF female ICR mice for 1, 6 weeks old SPF female SENCAR mice for 6 and 7) were divided into five experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with DMBA (100 mg, 390 nmol) in acetone (0.1 mL) as an initiation treatment. For group Ia (positive control group of the ICR mice) and group Ib (positive control group of the SENCAR mice), one week after the initiation,

papilloma formation was promoted twice a week by the application of TPA (1 mg, 1.7 nmol) in acetone (0.1 mL) on the skin. For groups II, III, IV and V, test sample compound **12** (85 nmol each)] in acetone (0.1 mL) were topically applied for 1 h before the each promotion treatment. The incidence of papilloma-bearers and numbers of papillomas per mouse were observed weekly for 20 weeks: the percentage of mice bearing papillomas and the average number of papillomas per mouse were recorded. A pathologist checked the type of tumor in these experiments by histological examination. Statistical significance was determined using Student's t-test [33,34].

4.2.5. Two-stage mouse skin carcinogenesis test induced by peroxynitrite and TPA

Animals (6 weeks old SPF female SENCAR mice) were divided into five experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with acetone (0.1 mL) and after 10 s, peroxynitrite (33.1 μg, 390 nmol in 0.1 mL of 1 mM NaOH) as an initiation treatment. For group I (positive control group), one week after the initiation, papilloma formation was promoted by the twice weekly application of TPA (1 μg, 1.7 nmol) in acetone (0.1 mL) on the skin (no papilloma formation was seen with topical application of the acetone solvent alone). For groups II, III, IV and V, test sample of compound **12** (0.0025% in drinking water) were orally administered (average 7.5 mL per mouse per day) for two weeks before the promotion treatment (from one week before initiation to one week after initiation). Subsequently, each group was promoted by the twice a week application with TPA (1 μg, 1.7 nmol) in acetone (0.1 mL). The incidence of papilloma-bearers and numbers of papillomas per mouse were detected weekly for 20 weeks: the percentage of mice bearing papillomas and the average number of papillomas per mouse were recorded. Student's t-test was used for statistical analyses of the numbers of papillomas per mouse. The animal weights were not statistically different between any of the groups in all in vivo assays [35].

4.3. Molecular docking study

The docking studies were carried out using Molecular Operating Environment (MOE) 2008.10 (Moe source: Chemical Computing Group Inc., Quebec, Canada, 2008). First, a Gaussian Contact surface around the binding site was drawn. The surface surrounds the van der Waals surface of a molecule (filling in solvent inaccessible gaps). Then docking studies were carried out to evaluate the binding free energy of the inhibitors within the macromolecules. The Dock scoring in MOE software was done using London dG scoring function and has been enhanced by using two different refinement methods, the Force-field and Grid-Min pose have been updated to ensure that refined poses satisfy the specified conformations. We allowed rotatable bonds; the best 10 poses were retained and analyzed for the binding poses best score. The database browser was used in MOE to compare the docking poses to the ligand in the co-crystallized structure and to get RMSD of the docking pose compared to the co-crystal ligand position. The affinity of the compounds is represented with the hydrogen bonds with the target receptor and root mean square deviation from the co-crystallized ligand are given in supplementary data.

4.3.1. Preparation of ligands and target protein-tyrosine kinase

The compounds involved in this study as ligands are 21 compounds which were studied for their binding affinity into PTK. The Molecule Builder tool in MOE was used to construct a three-dimensional model of the structures. Energy minimization was done through Force-field MMFF94x Optimization using gradient of 0.0001 for determining low energy conformations with the most favorable (lowest energy) geometry. The crystal structures of c-kit receptor protein-tyrosine kinase in complex with STI-571 (Imatinib or Gleevec) were obtained from the Protein Data Bank (PDB) http://www.rcsb.org/pdb/Welcome.do (PDB code: 1t46). Hydrogen atoms and partial charges were added to the protein with the protonation 3D application in MOE. This application is performed to assign ionization states and position hydrogen atoms in the macromolecular structure. As most of protein structures obtained from the Protein Data Bank contain little or no hydrogen coordinate data due to limited resolution. Yet, the hydrogen bond network and ionization states can have a dramatic effect on simulations results.

4.3.2. Molecular modeling and analysis of the docked results

The binding free energy was used to rank the binding affinity of the synthesized compounds to PTK protein. Also, Hydrogen bonds between the ligand and amino acids in PTK were used in the ranking of the compounds. Evaluation of the hydrogen bonds was done by measuring the hydrogen bond length which doesn't exceed 3 Å. RMSD of the docking pose compared to the co-crystal ligand position was used in the ranking. The mode of interaction of the native ligand (STI-571) within the crystal structure of c-kit receptor protein-tyrosine kinase was used as a standard docked model as well as for RMSD calculation.

Appendix. Supporting information

Supporting information associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2010.11.022.

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