MedChemComm

CONCISE ARTICLE

View Article Online
View Journal



Cite this: DOI: 10.1039/c4md00257a

# Design, synthesis and biological evaluation of novel 1,2,4-triazolo and 1,2,4-triazino[4,3-*a*]quinoxalines as potential anticancer and antimicrobial agents†

Doaa A. E. Issa,*[ab] Nargues S. Habib[a] and Abeer E. Abdel Wahab[c]

In an effort to find new leads as anticancer or antimicrobial agents, the present work deals with the synthesis of some novel 1-substituted 1,2,4-triazolo[4,3-*a*]quinoxalines **7**, **9a,b**, and **14–19** and 1,2,4-triazino[4,3-*a*] quinoxalines **10a–c** as well as 2-[5-amino-3-(4-chlorophenyl)pyrazol-1-yl]-3-benzylquinoxaline **13**. These were synthesized using the key intermediate 3-benzyl-2-hydrazinoquinoxaline **6** with various reagents. Ten compounds, namely **7**, **9a**, **10b**, **11**, and **13–18** were chosen by the National Cancer Institute of Bethesda (NCI) for evaluation of their anticancer activity. The results indicated that **9a** was the most active and was further evaluated for *in vitro* five dose assay against 60 human cell lines. It was proven to possess the highest broad spectrum anticancer activity. It showed particular effectiveness towards leukemia SR, non-small cell lung cancer HOP-92, NCI-H460, colon cancer HCT-116, HCT-15, CNS cancer U251, melanoma LOX IMVI, renal cancer A498, prostate cancer PC-3, and breast cancer MDA-MB-468 cell lines ($GI_{50}$ = 3.91, 3.45, 3.49, 3.21, 1.96, 5.18, 3.69, 1.80, 5.19, and 5.55 μM, respectively). All new compounds were screened for their antimicrobial activity and were very active against *P. aeruginosa*. Compounds **10a** and **16** were twice as active as ampicillin against *P. aeruginosa*. Five compounds, **9a**, **b**, **10b**, **13**, and **14** were equipotent to ampicillin against *P. aeruginosa*. In addition, compound **16** showed a broad spectrum antimicrobial activity. Furthermore, compound **9a** showed dual activity as an anticancer and antimicrobial agent.

Received 16th June 2014
Accepted 26th September 2014

DOI: 10.1039/c4md00257a

www.rsc.org/medchemcomm



## 1. Introduction

The quinoxaline pharmacophore, being isosteric to purine antimetabolites, developed an appealing platform for the discovery of active chemotherapeutic agents. Several anticancer drugs containing a quinoxaline ring have been reported along with their pharmacological data, activity against solid tumours and clinical trials studies.[1] The antineoplastic antibiotic quinoxaline, Triostatin A, (Fig. 1) showed considerable interest. It is characterized by cross-linked octapeptide rings bearing two quinoxalines and is stabilized at its centre by a cysteine pair (disulfhydryl covalent bonds). The two quinoxaline rings represent the planar aromatic ring structure which is a major requirement for intercalation.[2] Besides the two antineoplastic quinoxalines topoisomerase II poisons, **XK469** ((±)2-[4-(7-chloro-2-quinoxalinyloxy)phenoxyl]propanoic acid) and **CQS** (5-chloro quinoxalin-2-yl)-(4-aminobenzene sulfonamide) (Fig. 1) showed difference in DNA site specificity of topoisomerase II poisoning. This may be caused by differences in their geometry, side chains or electronic structure.[3] In addition, XK469 induced apoptosis of human ovarian cancer cell line PA1.[4] Several efforts have been made to search for new quinoxaline anticancer agents; thus a series of 2-alkylcarbonyl and 2-benzoyl-3-trifluromethylquinoxaline-1,4-di-*N*-oxide derivatives was found to inhibit the growth of Leukemia cell lines.[5]

Recently, a series of 5,7-diamino-3-phenyl-2-benzylamino and 5,7-diamino-3-phenyl-2-benzyloxy, substituted quinoxalines of the general formula (**A**) (Fig. 1) has been synthesized. Among them two compounds showed promising anticancer activity.[6] 4-Substituted anilino-1,2,4-triazolo[4,3-*a*]quinoxalines of the general formula (**B**) (Fig. 1) exhibited prominent cytotoxicity against mock-infected M.T-4 cells.[7] Whereas, 3-benzoyl-2-piperazinylquinoxalines of the general formula (**C**) (Fig. 1) showed anticancer activity against melanoma, renal and colon cancer.[8] Quinoxalines are currently recognized to display good affinity to the ATP-binding site of the c-kit tyrosine protein. Their activity as antitumor agents may be due to their ability to inhibit protein tyrosine kinases.[9]

During our ongoing research program on quinoxaline derivatives,[10–13] we were able to synthesize new lead structures. In particular: 1-(3-methoxyphenyl)-4-phenyl-1,2,4-triazolo[4,3-*a*] quinoxaline (**D**), 1-(substituted methyl)-4-phenyl-1,2,4-triazolo

[a]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Alexandria, 21521, Alexandria, Egypt*

[b]*Department of Pharmaceutical Sciences, Faculty of Pharmacy, Beirut Arab University, 115020, Beirut, Lebanon. E-mail: d.issa@bau.edu.lb*

[c]*Genetic Engineering and Biotechnology Research Institute (GEBRI), City for Scientific Research and Technology Application, Borg El-Arab, 21934, Alexandria, Egypt*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4md00257a

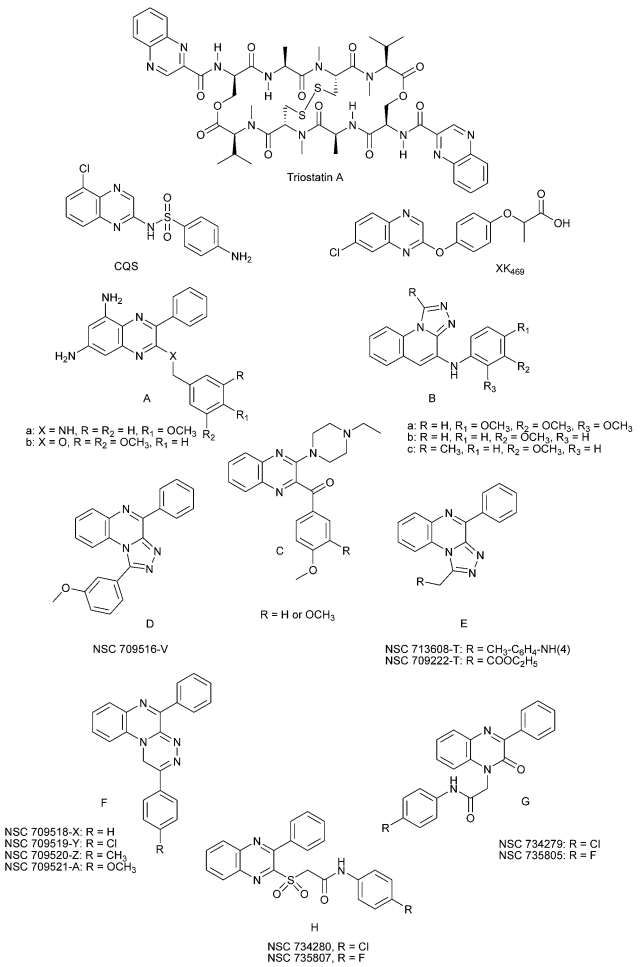



Fig. 1 Some quinoxaline derivatives having anticancer activity.

[4,3-*a*]quinoxalines (**E**), 2-(4-substituted phenyl)-5-phenyl-1*H*-1,2,4-triazino[4,3-*a*]quinoxalines (**F**),[12] 1-(*N*-arylcarbamoylmethyl)-3-phenylquinoxaline-2(1*H*)-ones (**G**),[13] and 2-(*N*-arylcarbamoylmethylsulfonyl)-3-phenylquinoxaline (**H**) (Fig. 1).[14] The antimicrobial activity of the triazolo and triazinoquinoxalines is well documented.[10,11,13–15]

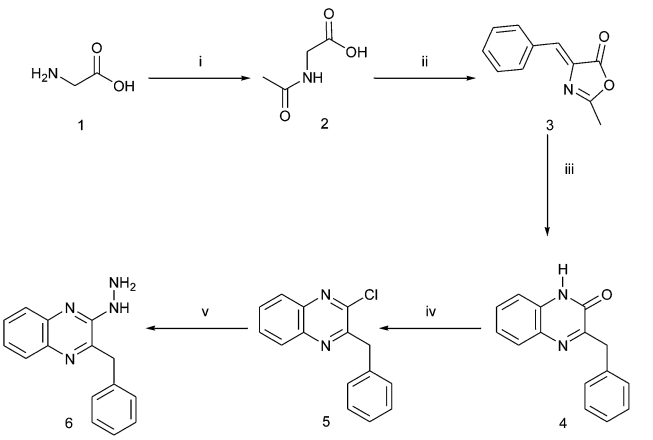
Lately the incorporation of 1,2,4-triazolo fused heterocycles in anti-proliferative and anti-microbial drug design projects revealed a very interesting scaffold to find new potential agents.[16,17] These findings prompted us to continue our investigations on quinoxalines having dual activity as anticancer and antimicrobial agents and to synthesize 1-substituted 4-benzyl-1,2,4-triazolo[4,3-*a*]quinoxalines having various pharmacophoric groups at the 1-position (**7**, **9a**, **b**, and **16–19**); also a series of 2-aryl-5-benzyl-1*H*-1,2,4-triazino[4,3-*a*]quinoxalines **10a–c** were synthesized besides 2-[5-amino-3-(4-chlorophenyl) pyrazol-1-yl]-3-benzylquinoxaline **13** and 4-benzyl-1,2,4-triazolo [4,3-*a*]quinoxaline-1(2*H*)-one **15.**

## 2. Results and discussion

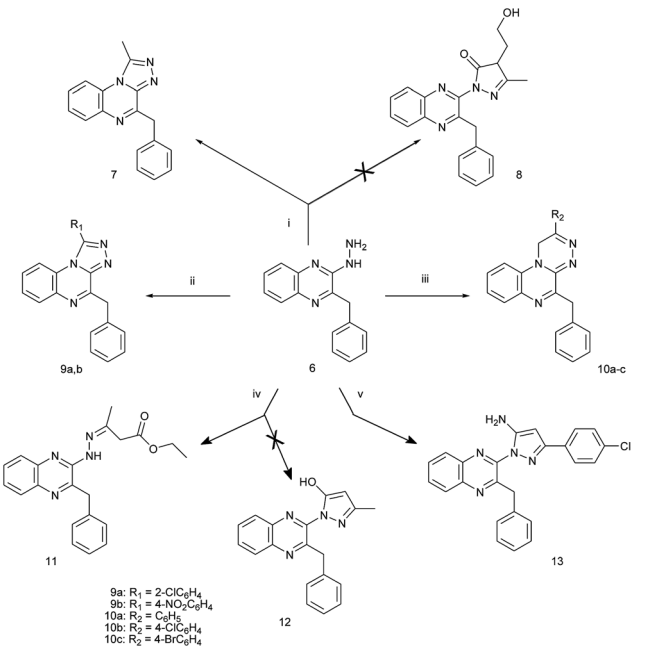
### 2.1. Chemistry

Target 1,2,4-triazolo[4,3-*a*]quinoxalines **7**, **9a**, **b**, **14–19** and 1,2,4-triazino[4,3-*a*]quinoxalines **10a–c** as well as 2-[5-amino-3-(4-chlorophenyl)pyrazol-1-yl]-3-benzylquinoxaline **13** were synthesized by the reactions depicted in Schemes 1–3.

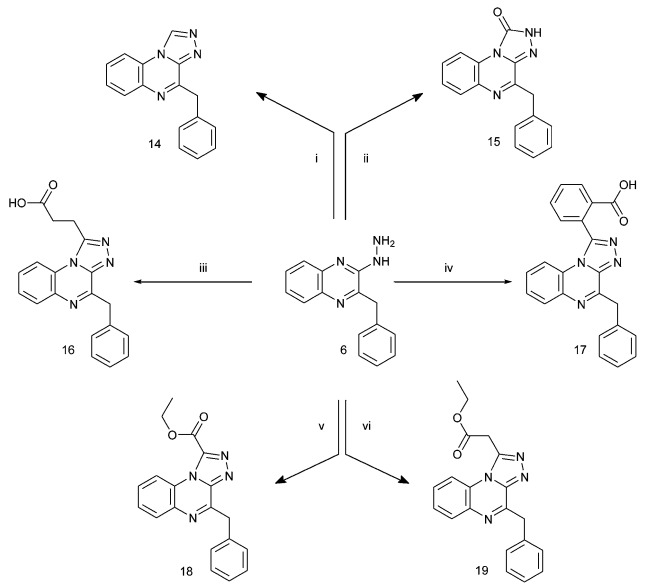
Preparation of the hydrazino key intermediate **6** was accomplished according to the sequence of reactions of Scheme 1. The azlactone of α-acetamidocinnamic acid **3** has been prepared according to the literature.[18,19] Its condensation with *o*-phenylenediamine achieved ring closure to quinoxalinone **4**




Scheme 1 Synthesis of key intermediate **6**. Reagents and conditions: (i) $(CH_3CO)_2O{\cdot}H_2O$, rt, 2 h, 91%; (ii) $C_6H_5CHO$, $(CH_3CO)_2O$, $CH_3CO_2Na$, 100 °C, 2 h, 86%; (iii) *o*-$C_6H_4(NH_2)_2$, $CH_3CH_2OH$, reflux, 5 h, 79%; (iv) $POCl_3$, 100 °C, 2 h, 75%; (v) $NH_2NH_2{\cdot}H_2O$, $CH_3CH_2OH$, reflux, 2 h, 92%.




Scheme 2 Synthesis of the target compounds **7–13**. Reagents and conditions: (i) $(CH_3CO)_2O$ or 2-acetylbuterolactone, dry xylene, reflux, 2–3 h, 55–77%; (ii) 2-Cl or 4-$NO_2C_6H_4COOH$, $POCl_3$, 100 °C, 2 h, 92–95%; (iii) 4-$RC_6H_4COCH_2Br$, dry dioxane, reflux, 1 h, 64–73%; (iv) $CH_3COCH_2COOC_2H_5$, 160–170 °C, 1 h, 69%; (v) 4-$ClC_6H_4COCH_2CN$, $C_2H_5OH$, $CH_3COOH$, reflux, 2 h, 97%.




Scheme 3 Synthesis of the target compounds **14–19**. Reagents and conditions: (i) HCOOH, reflux, 3 h, 57%; (ii) $ClCOOC_2H_5$, $C_2H_5OH$, reflux, 3 h, 69%; (iii) succinic anhydride, gl AcOH, reflux, 5 h, 57%; (iv) phthalic anhydride, gl AcOH, reflux, 5 h, 59%; (v) $(COOC_2H_5)_2$, dry xylene, reflux, 3 h, 68%; (vi) $CH_2(COOC_2H_5)_2$, 160–170 °C, 1 h, 87%.

in good yield, the product was identical to that previously described by Moffitt and Schultz obtained from the hydrolysis and decarboxylation of 3-(α-carboxamido)benzyl-2(1*H*)-quinoxalinone.[20] Earlier, 3-benzyl-1*H*-quinoxalin-2-one **4** has been differently prepared from *o*-phenylenediamine and phenylpyruvic acid.[8] Treatment of **4** with phosphorus oxychloride gave the chloroquinoxaline derivative **5**. Replacement of the chlorine atom of **5** with the hydrazine moiety resulted in the key intermediate **6**.

In the present work we studied the reaction of **6** with a variety of carbonyl compounds. The synthetic routes adopted to obtain the newly synthesized compounds are depicted in Schemes 2 and 3. Treatment of **6** with 2-acetylbutyrolactone failed to afford the corresponding 2-(pyrazol-1-yl)-3-benzylquinoxaline derivative **8** as reported for the preparation of related compounds.[21] Instead, 4-benzyl-1-methyl-1,2,4-triazolo[4,3-*a*]quinoxaline **7** was obtained. The structure of **7** was confirmed by IR and $^1$H-NMR spectra and chemically through its parallel synthesis from **6** and acetic anhydride.[15] Cyclization of **6** using aromatic acids and phosphorus oxychloride yielded 1-aryl-4-benzyl-1,2,4-triazolo[4,3-*a*]quinoxaline **9a,b** where **9a** is reported to be differently prepared.[22] The 1,2,4-triazino[4,3-*a*]quinoxalines **10a–c** were obtained through the reaction of **6** with phenacyl bromides. Fusion of a mixture of **6** and ethyl acetoacetate failed to afford 2-[5-hydroxy-3-methylpyrazol-1-yl]-3-benzylquinoxaline **12** as previously reported for analogous compounds.[11] It yielded ethyl 3-[(3-benzylquinoxalin-2-yl)hydrazono]butyrate **11**. Reacting **6** with 4-chlorophenyl-ω-cyanoacetophenone yielded 2-[5-amino-3-(4-chlorophenyl)pyrazol-1-yl]-3-benzylquinoxaline **13**.

Compounds **14** and **15** were obtained by the reaction of **6** with formic acid and ethyl chloroformate, respectively. Compound **14** was previously reported to be synthesized from 2-benzyl-3-(2-methylenehydrazinyl) quinoxaline by its pyrolysis in dimethylformamide or its acylation with acetic anhydride in pyridine.[22] Compound **15** was reported to be synthesized through the reaction of 2-chIoro-3-benzylquinoxaline with semicarbazide hydrochloride.[15] Compounds **16** and **17** were obtained by cyclization of **6** using succinic or phthalic anhydride, respectively, in glacial acetic acid. Furthermore reacting **6** with diethyl oxalate or diethyl malonate in boiling dry xylene gave compounds **18** and **19** respectively as described for the synthesis of analogous compounds.[13]

## 2.2. Biological evaluation

**2.2.1. Preliminary *in vitro* anticancer screening.** Out of the newly synthesized compounds, ten candidates, namely: **7**, **9a**, **10b**, **11**, and **13–18** were selected by the National Cancer Institute (NCI) Bethesda-Maryland, USA, to be evaluated for their *in vitro* antitumor activity through the *in vitro* disease-oriented human cells screening panel assay. An effective one-dose assay has been added to the NCI-60 cell screen in order to increase compound throughput and reduce the data-turnaround time to suppliers while maintaining efficient identification of active compounds.[23,24] All compounds submitted to the NCI-60 cell screen are now tested initially at a single high dose (10 μM) in the full NCI-60 cell panel including leukemia, non-small cell lung, colon, CNS melanoma, ovarian, renal, prostate, and breast cancer cell lines. Only compounds which satisfy pre-determined threshold inhibition criteria would proceed to the five-dose screen. The threshold inhibition criteria for proceeding to the five-dose screen were designed to efficiently capture compounds with anti-proliferative activity, and are based on careful analysis of historical Development Therapeutic Program (DTP) screening data. Data are reported as a mean graph of the percent growth of treated cells, and presented as percentage growth inhibition (GI%) caused by the test compounds (Table 1). Moreover, three response parameters ($GI_{50}$, TGI, and $LC_{50}$) were calculated for each cell line for compound **9a** (Table 2). The $GI_{50}$ value corresponds to the compound concentration causing a 50% decrease in net cell growth. The TGI value is the compound concentration resulting in total growth inhibition and the $LC_{50}$ value is the compound concentration causing a net 50% loss of initial cells at the end of the incubation period (48 h). Subpanel and fullpanel mean-graph midpoint values (MG-MID) for certain agents are the average of individual real and default $GI_{50}$, TGI, or $LC_{50}$ values of all cell lines in subpanel and fullpanel, respectively.[25]

As revealed from Table 1 showing the percentage growth inhibition (GI%) caused by the test compounds, compound **9a**, having the 2-chlorophenyl moiety at the 1-position of the triazoloquinoxaline, was the most active compound. It showed a broad spectrum anticancer activity against most cell lines, namely Leukemia HL-60(TB), K-562, MOLT-4, RPMI-8226 and SR cell lines with a growth inhibition of 46.36%, 78.62%, 60.41%, 63.81% and 84.89%, respectively. Compound **9a** was

Table 1 *In vitro* percentage growth inhibition (GI%) caused by the test compounds against some selected tumor cell lines at the single dose assay[a]

| Cpd | NSC no. | Panel | Subpanel tumor cell lines (% growth inhibitory activity) |
|---|---|---|---|
| **7** | 761 669 | Non-small cell lung cancer | HOP-92 (17.65); NCI-H226 (15.11) |
| | | CNS cancer | SNB-75 (21.94) |
| | | Ovarian cancer | NCI/ADR-RES (12.55) |
| **9a** | 761 676 | Leukemia | HL-60(TB) (46.36); K-562 (78.62); MOLT-4 (60.41); RPMI-8226 (63.81); SR (84.89) |
| | | Non-small cell lung cancer | EKVX (48.06); HOP-92 (50.11); NCI-H460 (84.44) |
| | | Colon cancer | HCT-116 (65.87); HCT-15 (55.37); KM12 (51.07) |
| | | CNS cancer | SF-268 (47.55); U251 (57.23) |
| | | Melanoma | LOX IMVI (60.62) |
| | | Renal cancer | A498 (61.07) |
| | | Prostate cancer | PC-3 (60.87) |
| **10b** | 761 674 | Leukemia | HL-60(TB) (74.81); K-562 (69.87); RPMI-8226 (50.59); SR (74.57) |
| | | Non-small cell lung cancer | NCI-H522 (61.57) |
| | | Colon cancer | HCT-116 (46.86) |
| | | Renal cancer | CAKI-1 (44.63) |
| **11** | 761 673 | Renal cancer | A498 (17.75); UO-31 (15.85) |
| **13** | 761 675 | Non-small cell lung cancer | A549/ATCC (21.96); EKVX (21.36); NCI-H226 (15.73); NCI-H23 (18.49) |
| | | Colon cancer | HCT-116 (27.18); HCT-15 (25.56); HT29 (30.32) |
| | | CNS cancer | SNB-75 (15.58) |
| | | Renal cancer | CAKI-1 (19.3); RXF 393 (21.29) |
| | | Breast cancer | MDA-MB-231/ATCC (24.88) |
| **14** | 761 678 | Non-small cell lung cancer | NCI-H226 (15.47) |
| | | CNS cancer | SNB-75 (23.49) |
| | | Renal cancer | A498 (38.71); UO-31 (22.68) |
| | | Prostate cancer | PC-3 (14.24) |
| **15** | 761 677 | Renal cancer | A498 (28.58); UO-31 (35.5) |
| **16** | 761 671 | Non-small cell lung cancer | HOP-92 (18.87) |
| | | CNS cancer | SNB-75 (17.71) |
| | | Renal cancer | UO-31 (12.22) |
| **17** | 761 670 | Non-small cell lung cancer | NCI-H522 (14.55) |
| | | CNS cancer | SF-268 (14.1); SNB-75 (24.41) |
| | | Ovarian cancer | OVCAR-3 (20.22) |
| | | Renal cancer | UO-31 (18.01) |
| | | Prostate cancer | PC-3 (43.92) |
| **18** | 761 672 | CNS cancer | SNB-75 (10.34) |
| | | Renal cancer | UO-31 (15.06) |

[a] The data obtained from NCI *in vitro* disease-oriented human tumor cell screen at 10 μM concentration.

Table 2 Growth inhibitory action ($GI_{50}$) of some selected *in vitro* tumor cell lines (μM)[a] for compound **9a** (NCS 761676)

| Panel | Subpanel cell lines (cytotoxicity $GI_{50}$ μM) |
|---|---|
| Leukemia | CCRF-CEM (7.11); HL-60(TB) (32.5); K-562 (5.55); MOLT-4 (9.04); RPMI-8226 (5.64); SR (3.91) |
| Non-small cell lung cancer | A549/ATCC (8.77); EKVX (5.96); HOP-62 (12.4); HOP-92 (3.45); NCI-H226 (9.96); NCI-H322M (53.9); NCI-H460 (3.49); NCI-H522 (53.9) |
| Colon cancer | COLO 205 (8.77); HCC-2998 (42.8); HCT-116 (3.21); HCT-15 (1.96); HT29 (9.15); KM12 (9.48); SW-620 (43.4) |
| CNS cancer | SF-295 (12.3); SF-539 (24.2); SNB-19 (27.8); SNB-75 (6.12); U251 (5.18) |
| Melanoma | LOX IMVI (3.69); MALME-3M (23.8); M14 (36.7); SK-MEL-2 (55.8); SK-MEL-5 (5.90); UACC-257 (34.3); UACC-62 (7.96) |
| Ovarian cancer | IGROV1 (27.7); OVCAR-3 (21.7); OVCAR-4 (8.01); OVCAR-8 (7.21); NCI/ADR-RES (7.04); SK-OV-3 (23.9) |
| Renal cancer | 786-0 (10.4); A498 (1.80); ACHN (34.1); CAKI-1 (9.71); RXF 393 (8.45); TK-10 (37.4); UO-31 (25.0) |
| Prostate cancer | PC-3 (5.19); DU-145 (44.2) |
| Breast cancer | MCF7 (22.2); MDA-MB-231/ATCC (6.24); HS 578T (9.66); BT-549 (6.72); T-47D (6.82); MDA-MB-468 (5.55) |

[a] Data obtained from NCI *in vitro* disease-oriented human cell screen.

remarkably active against non-small cell lung cancer EKVX, HOP-92 and NCI-H460 cell lines with a growth inhibition of 48.06%, 50.11% and 84.44%, respectively. The colon cancer HCT-116, HCT-15 and KM12 cell lines showed a growth inhibition of 65.87%, 55.37%, and 51.07%, respectively. In addition CNS cancer SF-268 and U251 cell lines were reasonably inhibited by compound **9a** with a growth inhibition of 47.55% and 57.23%, respectively. Also compound **9a** manifested appreciable activity against melanoma LOX IMVI, renal cancer A498 and prostate cancer PC-3 cell lines with a growth inhibition of 60.62%, 61.07% and 60.87%, respectively.

Replacement of 2-chlorophenyl with 2-carboxyphenyl at the 1-position of the triazoloquinoxaline decreased the activity where compound **17** showed only activity against CNS cancer SNB-75, ovarian cancer OVCAR-3 and prostate cancer PC-3 cell lines with a growth inhibition of 24.41%, 20.22% and 43.92%, respectively. The unsubstituted triazoloquinoxaline **14** displayed moderate activity against CNS cancer SNB-75, renal cancer A498 and UO-31 cell lines with a growth inhibition of 23.49%, 38.71% and 22.68% respectively. Compound **15** having a carbonyl group at the 1-position of the triazoloquinoxaline was fairly active against renal cancer A498 and UO-31 cell lines with 28.58% and 35.5% growth inhibition, respectively. The 1-methyltriazoloquinoxaline **7** showed weak activity against the CNS cancer SNB-75 cell line with a growth inhibition of 21.94%. Conversely the triazinoquinoxaline **10b** having 4-chloro substitution demonstrated a significant growth inhibition of 74.81%, 69.87%, 50.59%, 74.57%, 61.57%, 46.86% and 44.63% against leukemia HL-60(TB), K-562, RPMI-8226, SR, non-small cell lung cancer NCI-H522, colon cancer HCT-116 and renal cancer CAKI-1 cell lines, respectively.

Furthermore the pyrazolylquinoxaline **13** showed some activity against non-small cell lung cancer, colon cancer, renal cancer and breast cancer specifically non-small cell lung cancer A549/ATCC, EKVX, colon cancer HCT-116, HCT-15, HT29, renal cancer RXF 393 and breast cancer MDA-MB-231/ATCC cell lines with a growth inhibition of 21.96%, 21.36%, 27.18%, 25.56%, 30.32%, 21.29% and 24.88%, respectively.

Only compound **9a** fulfilled the requirements of selection for five-dose assay. Further interpretation of the five-dose screening data for compound **9a** (Table 2) revealed that it was the most active lead in this study with a broad spectrum anticancer activity against most of the tested subpanel tumor cell lines with particular effectiveness against leukemia CCRF-CEM K-562,

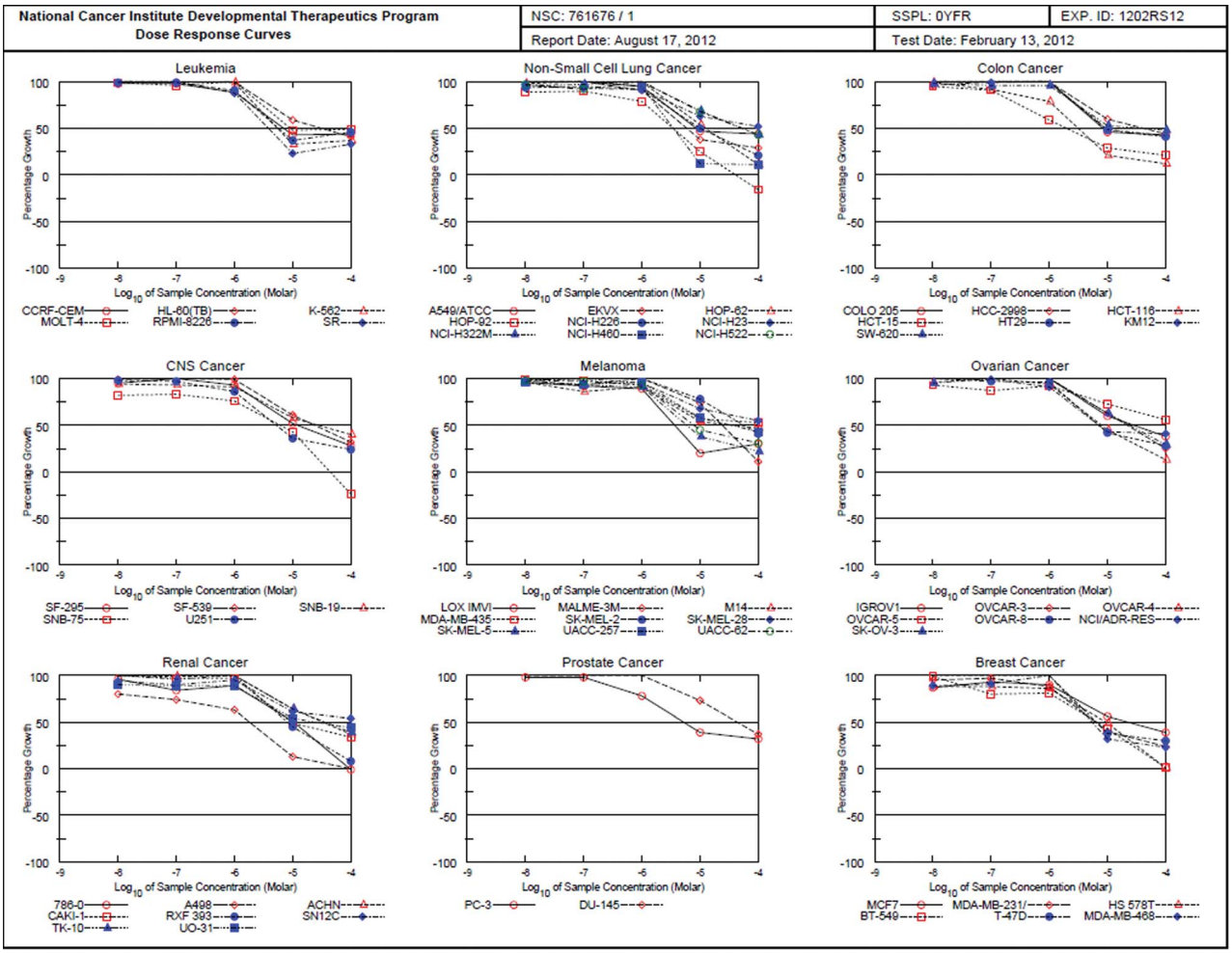



Fig. 2 Dose response curve of compound **9a** against the nine tested subpanels.

Table 3 The MG-MID ($GI_{50}$, μM) and the selectivity ratio of compound **9a** (NSC 761676)

| | Subpanel tumor cell lines[b] $GI_{50}$ MG-MID (μM) (SI)[c] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| MG-MID[a] | I | II | III | IV | V | VI | VII | VIII | IX |
| 16.80 | 10.62 (1.58) | 18.98 (0.89) | 16.97 (0.99) | 15.12 (1.11) | 24.02 (0.70) | 15.93 (1.05) | 18.12 (0.93) | 24.69 (0.68) | 9.53 (1.76) |

[a] $GI_{50}$: full panel mean-graph midpoint (μM). [b] I: leukemia; II: non-small cell lung cancer; III: colon cancer; IV: CNS cancer; V: melanoma; VI: ovarian cancer; VII: renal cancer; VIII: prostate cancer; IX: breast cancer. [c] SI: selectivity index.



MOLT-4, RPMI-8226 and SR ($GI_{50}$ = 7.11, 5.55, 9.04, 5.64 and 3.91 μM respectively). The compound also showed activity against non-small cell lung cancer A549/ATCC, EKVX, HOP-92, NCI-H226 and NCI-H460 ($GI_{50}$ = 8.77, 5.96, 3.45, 9.96 and 3.49 μM, respectively), while TGI against HOP-92 was 41.8 μM. It also illustrated significant activity against colon cancer COLO 205, HCT-116, HCT-15, HT29 and KM12 ($GI_{50}$ = 8.77, 3.21, 1.96, 9.15 and 9.48 μM, respectively). The compound displayed moderate activity against CNS Cancer SNB-75 and U251 ($GI_{50}$ = 6.12 and 5.18 μM respectively), while TGI against SNB-75 was 43.6 μM. Its $GI_{50}$ against Melanoma LOX IMVI, SK-MEL-5 and UACC-62 was equal to 3.69, 5.90 and 7.96 μM, respectively. Compound **9a** demonstrated moderate activity against ovarian cancer OVCAR-4, OVCAR-8 and NCI/ADR-RES ($GI_{50}$ = 8.01, 7.21 and 7.04 μM, respectively). The compound revealed promising activity against renal cancer A498, CAKI-1, RXF 393, prostate cancer PC-3, breast cancer MDA-MB-231/ATCC, HS 578T, BT-549, T-47D and MDA-MB-468 ($GI_{50}$ = 1.80, 9.71, 8.45, 5.19, 6.24, 9.66, 6.72, 6.82 and 5.55 μM, respectively). The dose response curve of compound **9a** is illustrated in Fig. 2.

The ratio obtained by dividing the compound fullpanel MG-MID (μM) by its individual subpanel MG-MID (μM) is considered as a measure of compound selectivity (Table 3). Ratios between 3 and 6 refer to moderate selectivity, ratios >6 indicate high selectivity toward the corresponding cell line, while compounds meeting neither of these criteria are rated non-selective.[23] Accordingly, compound **9a** was proven to be non-selective.

**2.2.2. Antimicrobial screening.** All newly synthesized compounds were evaluated for their *in vitro* antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* as Gram-positive bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* as Gram-negative bacteria. They were also evaluated for their *in vitro* antifungal potential against *Candida albicans*. Their inhibition zones (IZ) using the cup-diffusion technique were measured.[26] Further evaluation was carried out to determine their minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the twofold serial dilution method.[27] Ampicillin was used as standard antibacterial while clotrimazole was used as the antifungal reference. Dimethylsulfoxide (DMSO) was used as blank and showed no antimicrobial activity.

As revealed from Tables 4 and 5, regarding the antimicrobial activity against *S. aureus*, the tested compounds showed weak activity IZ (12–17 mm). Only compound **16** showed one fifth the activity of ampicillin. However, the tested compound demonstrated better activity against *B. subtilis* IZ (12–16 mm). Compound **16** was the most active compound in this respect. It was equipotent to ampicillin (MIC = 12.5 μg $mL^{-1}$). Compounds **9a** and **10a** exhibited half the potency of ampicillin (MIC = 25 μg $mL^{-1}$). The inhibition zone of the tested compounds against *P. aeruginosa* was 11–14 mm, two compounds **10a** and **16** were double as active as ampicillin (MIC = 25 μg $mL^{-1}$), and five compounds **9a**, **b**, **10b**, **13**, and **14** were equipotent to ampicillin (MIC = 50 μg $mL^{-1}$). The tested compounds displayed activity against *E. coli;* the inhibition zones ranging 12–16 mm, only compound **16** was nearly equipotent to ampicillin (MIC = 12.5 μg $mL^{-1}$). On the other hand, the inhibition zone against *C. albicans* was 12–17 mm. Compound **10a** showed nearly half the potency of clotrimazole and compounds **9b**, **10b**, **17**, **18**, and **19** showed one fifth the activity of clotrimazole.

It can be concluded that the tested compounds were weakly active against *S. aureus* and *C. albicans*. Compounds **10a** and **16** were double as active as ampicillin against *P. aeruginosa* and five compounds **9a**, **b**, **10b**, **13**, and **14** had similar activity to ampicillin, *i.e.* the tested compounds were very active against *P. aeruginosa*. The tested compounds showed medium activity against *B. subtilis* and *E. coli*. It is worth mentioning that compound **16** possesses a broad spectrum antimicrobial activity.

Table 4 The inhibition zones (IZ) in mm diameter of the tested compounds

| Cpd | *S. aureus* | *B. subtilis* | *P. aeruginosa* | *E. coli* | *C. albicans* |
|---|---|---|---|---|---|
| **7** | 12 | 13 | 11 | 12 | 14 |
| **9a** | 12 | 12 | 14 | 10 | 16 |
| **9b** | 14 | 12 | 12 | 14 | 15 |
| **10a** | 13 | 14 | 12 | 16 | 14 |
| **10b** | 15 | 16 | 14 | 15 | 15 |
| **10c** | 12 | 12 | 12 | 14 | 14 |
| **11** | 15 | 12 | 12 | 14 | 14 |
| **13** | 14 | 15 | 12 | 16 | 16 |
| **14** | 17 | 12 | 14 | 12 | 17 |
| **15** | 16 | 16 | 12 | 10 | 14 |
| **16** | 13 | 12 | 12 | 15 | 16 |
| **17** | 14 | 16 | 12 | 15 | 14 |
| **18** | 14 | 13 | 10 | 12 | 15 |
| **19** | 14 | 14 | 14 | 15 | 12 |

Table 5 Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the tested compounds in μg $mL^{-1}$

| | *S. aureus* | | *B. subtilis* | | *P. aeruginosa* | | *E. coli* | | *C. albicans* | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cpd | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| **7** | 50 | 100 | 50 | 100 | 100 | 100 | 50 | 50 | 100 | 200 |
| **9a** | 100 | 200 | 25 | 50 | 50 | 100 | 25 | 50 | 100 | 200 |
| **9b** | 50 | 100 | 100 | 100 | 50 | 50 | 100 | 100 | 25 | 50 |
| **10a** | 50 | 50 | 25 | 50 | 25 | 50 | 50 | 50 | 12.5 | 25 |
| **10b** | 50 | 100 | 100 | 200 | 50 | 100 | 50 | 50 | 25 | 25 |
| **10c** | 50 | 50 | 50 | 50 | 100 | 100 | 50 | 50 | 50 | 50 |
| **11** | 100 | 100 | 50 | 50 | 100 | 100 | 50 | 100 | 50 | 100 |
| **13** | 100 | 100 | 100 | 100 | 50 | 50 | 100 | 100 | 50 | 50 |
| **14** | 50 | 100 | 50 | 50 | 50 | 100 | 25 | 25 | 100 | 200 |
| **15** | 100 | 200 | 100 | 200 | 100 | 100 | 100 | 100 | 50 | 100 |
| **16** | 25 | 50 | 12.5 | 25 | 25 | 25 | 12.5 | 25 | 50 | 100 |
| **17** | 100 | 200 | 100 | 100 | 100 | 100 | 25 | 50 | 25 | 50 |
| **18** | 50 | 50 | 50 | 100 | 100 | 100 | 50 | 50 | 25 | 50 |
| **19** | 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 25 | 25 |
| Ampicillin | 5 | | 12.5 | | 50 | | 10 | | | |
| Clotrimazole | | | | | | | | | 5 | |

# 3. Conclusions

Preliminary *in vitro* anticancer screening revealed that compound **9a** was the most active. It was proven to possess the highest broad spectrum anticancer activity after its further evaluation for *in vitro* five dose assay against 60 human cell lines. It showed particular effectiveness towards leukemia SR, non-small cell lung cancer HOP-92, NCI-H460, colon cancer HCT-116, HCT-15, CNS cancer U251, melanoma LOX IMVI, renal cancer A498, prostate cancer PC-3, and breast cancer MDA-MB-468 cell lines ($GI_{50}$ = 3.91, 3.45, 3.49, 3.21, 1.96, 5.18, 3.69, 1.80, 5.19, and 5.55 μM, respectively). From the antimicrobial screening it was found that the most active compounds were **10a** and **16**. They showed twice the activity of ampicillin against *P. aeruginosa.* Moreover five compounds, namely **9a**, **b**, **10b**, **13** and **14** were equipotent to ampicillin against *P. aeruginosa*.

In conclusion, the compound 4-benzyl-1-(2-chlorophenyl)-1,2,4-triazolo[4,3-*a*]quinoxaline **9a** proved to possess dual effects as a broad spectrum anticancer and antimicrobial agent against *P. aeruginosa*.

# 4. Experimental section

## 4.1. Chemistry

All reagents and solvents were purchased from commercial suppliers and were dried and purified when necessary by standard techniques. All melting points were determined in open glass capillaries on a Gallenkamp melting point apparatus and are uncorrected. The IR spectra were recorded using KBr discs on a Perkin-Elmer 1430 spectrophotometer. $^1$H-NMR ($\delta$ ppm) spectra were recorded on a JNM-LA 400 FT NMR system (400 MHz) and on a Jeol (500 MHz) spectrometer (both JEOL, Tokyo, Japan). $^{13}$C-NMR spectra were run on Jeol spectrometer using TMS as the internal standard and DMSO-$d_6$ as a solvent. Mass spectra were run on a Finnigan mass spectrometer model SSQ/7000 (70 eV). The microanalyses were performed at the Microanalytical Laboratory, National Research Center, Cairo, Egypt and the data were within ±0.4% of the theoretical values. Following up of the reactions and checking the homogeneity of the compounds were made by TLC on silica gel aluminum sheets (Type 60 GF254, Merck, Darmstadt, Germany) and the spots were detected by exposure to a UV-lamp at $\lambda$ 254 nm for few seconds.

### 4.1.1. 4-Benzyl-1-methyl-1,2,4-triazolo[4,3-*a*]quinoxaline (7)

*Method A.* The title compound was prepared by refluxing a solution of 3-benzyl-2-hydrazinoquinoxaline **6** (0.5 g, 2 mmol) in acetic anhydride (2.5 mL) for 2 h. The reaction mixture was poured onto ice water under stirring and the solid obtained was collected by filtration, washed with water and recrystallized from ethanol as yellowish needles (0.42 g, 76.6%), m.p. 182–183 °C; reported m.p.176–178 °C.[15]

*Method B.* A mixture of **6** (0.5 g, 2 mmol) and 2-acetylbutyrolactone (0.28 g, 2.2 mmol) in dry xylene (5 mL) was refluxed for 3 h. The reaction mixture was cooled; the obtained crystalline product was filtered, dried, and recrystallized from ethanol to yield the desired compound (0.3 g, 54.8%). The products obtained from method A and B were identical in IR (KBr, $cm^{-1}$): 1677 (C=N); 1605, 1495 (C=C). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 3.08 (s, 3H, $CH_3$), 4.54 (s, 2H, $CH_2$–$C_6H_5$), 7.20 (t, 1H, $J$ = 7.4 Hz, $C_6H_5$–$C_4$–H), 7.31 (t, 2H, $J$ = 7.4 Hz, $C_6H_5$–$C_{3,5}$–H), 7.46 (d, 2H, $J$ = 7.4 Hz, $C_6H_5$–$C_{2,6}$–H), 7.59–7.78 (m, 2H, triazoloquinox. $C_{7,8}$–H), 8.04 (ddd, 1H, $J$ = 7.2, 4.65, 2.1 Hz, triazoloquinox. $C_6$–H), 8.30 (dd, 1H, $J$ = 9, 4.5 Hz, triazoloquinox. C9–H). Anal. calcd for $C_{17}H_{14}N_4$ (274.32): C, 74.43; H, 5.14; N, 20.42. Found: C, 74.29; H, 5.27; N, 20.74.

**4.1.2. 4-Benzyl-1-(substituted phenyl)-1,2,4-triazolo[4,3-*a*]-quinoxalines (9).** A mixture of **6** (0.5 g, 2 mmol) and the appropriate substituted benzoic acid (2 mmol) in $POCl_3$ (2 mL) was refluxed for 2 h in an oil bath at 100 °C. The reaction mixture was cooled to room temperature, poured onto crushed ice and neutralized with sodium bicarbonate solution. The

resulting solid was filtered, dried and recrystallized from ethanol.

*4.1.2.1. 4-Benzyl-1-(2-chlorophenyl)-1,2,4-triazolo[4,3-a]quinoxaline (**9a**).* Reddish-orange crystals (0.7 g, 94.5%), m.p. 200–201 °C; reported m.p. 194–195 °C.[22] IR (KBr, $cm^{-1}$): 1643 (C=N); 1597, 1489 (C=C); 767 (C–Cl). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.61, 4.70 (2d, each 1H, $J$ = 14.1 Hz, C$H_2$–$C_6H_5$), 7.10 (d, 1H, $J$ = 7.5 Hz, $C_6H_5$–$C_2$–H), 7.26 (d, 1H, $J$ = 7.5 Hz, $C_6H_5$–$C_6$–H), 7.34 (t, 2H, $J$ = 7.5 Hz, $C_6H_5$–$C_{3,5}$–H), 7.49–7.55 (m, 3H, $C_6H_5$–$C_4$–H and chlorophenyl $C_{4,5}$–H), 7.62–7.71 (m, 2H, triazoloquinox. $C_{7,8}$–H), 7.78–7.86 (m, 3H, chlorophenyl $C_{3,6}$–H and triazoloquinox. $C_6$–H), 8.09 (dd, 1H, $J$ = 8.1, 1.5 Hz triazoloquinox. $C_9$–H). $^{13}$C-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 42.3 (*C*$H_2$–$C_6H_5$), 73.2 (triazoloquinox. $C_1$), 114.6 (triazoloquinox. $C_9$), 115 (triazoloquinox. $C_7$), 125 (triazoloquinox. $C_6$), 126.7 (benz. $C_1$), 128.0, 128.1, 129.0, 129.1, 129.7, 129.9, 130.1, 132.4, 133.2, 133.8, 135.7, 136.5, 153.6, 209.6. Anal. calcd for $C_{22}H_{15}ClN_4$ (370.84): C, 71.25; H, 4.08; N, 15.11. Found: C, 71.42; H, 4.04; N, 14.86.

*4.1.2.2. 4-Benzyl-1-(4-nitrophenyl)-1,2,4-triazolo[4,3-a]quinoxaline (**9b**).* Brownish clusters of needles (0.7 g, 91.9%), m.p. 212–213 °C. IR (KBr, $cm^{-1}$): 1630 (sh C=N); 1601 (C=C); 1527, 1348 ($NO_2$). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.64 (s, 2H, C$H_2$–$C_6H_5$), 7.23 (t, 1H, $J$ = 7.65 Hz, $C_6H_5$–$C_4$–H), 7.33 (t, 2H, $J$ = 7.65 Hz, $C_6H_5$–$C_{3,5}$–H), 7.36 (d, 1H, $J$ = 7.65 Hz, $C_6H_5$–$C_2$–H), 7.47 (d, 1H, $J$ = 7.65 Hz, $C_6H_5$–$C_6$–H), 7.51–7.60 (m, 2H, triazoloquinox. $C_{7,8}$–H), 7.64 (dd, 1H, $J$ = 7.8, 1.2 Hz, triazoloquinox. $C_6$–H), 8.1 (dd, 1H, $J$ = 8.6, 1.5 Hz triazoloquinox. $C_9$–H), 8.12 (d, 2H, $J$ = 8.7 Hz, nitrophenyl $C_{2,6}$–H), 8.51 (d, 2H, $J$ = 8.7 Hz, nitrophenyl $C_{3,5}$–H). Mass spectrum $m/z$ (%): 381 ($M^{+\bullet}$) (100), 380 (98), 334 (25), 233 (61), 232 (95), 205 (25), 102 (35), 91(87), 77 (25), 65 (45). Anal. calcd for $C_{22}H_{15}N_5O_2$ (381.39): C, 69.28; H, 3.96; N, 8.39. Found: C, 68.99; H, 4.09; N, 8.21.

**4.1.3. General procedure for the synthesis of 2-aryl-5-benzyl-1*H*-[1,2,4]triazino[4,3-*a*]quinoxalines (10a–c).** To a solution of **6** (0.5 g, 2 mmol) in dry dioxane (10 mL), the appropriate phenacyl bromide (2 mmol) was added. The reaction mixture was heated under reflux; while a reddish precipitate separated out during the first 5 minutes. The reflux was continued for 1 h, the reaction mixture was cooled, and the product was filtered, dried, and recrystallized from the proper solvent.

*4.1.3.1. 5-Benzyl-2-phenyl-1H-[1,2,4]triazino[4,3-a]quinoxaline (**10a**).* Yellow clusters of needles (0.51 g, 72.9%), m.p. 239–241 °C (ethanol/water). IR (KBr, $cm^{-1}$): 3430 (NH); 1635 (C=N); 1592 (C=C); 1546 ($\delta$NH). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.49 (s, 2H, C$H_2$–$C_6H_5$), 5.60 (s, 2H, triazinoquinox. $C_1$–$H_2$), 7.27–7.08 (m, 10H, Ar–H), 8.02–8.08 (m, 2H, triazinoquinox. $C_{8,9}$–H), 8.17 (dd, 1H, $J$ = 7.8, 2 Hz, triazinoquinox. $C_7$–H), 8.35 (d, 1H, $J$ = 6.3 Hz, triazinoquinox. $C_{10}$–H). Mass spectrum $m/z$ (%): 351 (12), 350 ($M^{+\bullet}$) (50), 349 (24), 247 (86), 246 (72), 219 (79), 218 (32), 116 (18), 103 (67), 102 (28), 91 (68), 77 (100), 65 (34), 51 (43). Anal. calcd for $C_{23}H_{18}N_4$ (350.42): C, 78.83; H, 5.18; N, 15.99. Found: C, 78.67; H, 4.96; N, 16.15.

*4.1.3.2. 5-Benzyl-2-(4-chlorophenyl)-1H-[1,2,4]triazino[4,3-a]quinoxaline (**10b**).* Orange-yellow fine needles (0.49 g, 63.7%), m.p. 244–245 °C (Ethanol). IR (KBr, $cm^{-1}$): 3431 (NH); 1632 (C=N); 1592 (C=C); 1541 ($\delta$NH); 830 (C–Cl). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.49 (s, 2H, C$H_2$–$C_6H_5$), 5.58 (s, 2H, triazinoquinox. $C_1$–$H_2$), 7.29–7.40 (m, 5H, $CH_2$–$C_6H_5$), 7.67–7.86 (m, 2H, triazinoquinox. $C_{8,9}$–H), 7.72 (d, 2H, $J$ = 8.9 Hz, chlorophenyl $C_{2,6}$–H), 8.05 (dd, 1H, $J$ = 8.25, 7.8 Hz triazinoquinox. $C_7$–H), 8.19 (d, 2H, $J$ = 8.9 Hz, chlorophenyl $C_{3,5}$–H), 8.34 (d, 1H, $J$ = 8.4 Hz, triazinoquinox. $C_{10}$–H). $^{13}$C-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 38.9 (*C*$H_2$–$C_6H_5$), 62.3 (triazinoquinox. $C_1$), 114.2 (triazinoquinox. $C_{10}$), 117.8 (triazinoquinox. $C_8$), 122.9 (triazinoquinox. $C_7$), 125.2 (phenyl $C_4$), 127.1 (chlorophenyl $C_4$), 127.8 (phenyl $C_{3,5}$), 128.5 (chlorophenyl $C_{3,5}$), 129.0 (phenyl $C_{2,6}$), 129.3 (chlorophenyl $C_{2,6}$), 130.4, 132.5, 134.8, 136.2, 137.1, 138.2, 138.9, 147.0, 164.5, 172.5, 177.5. Anal. calcd for $C_{23}H_{17}ClN_4$ (384.87): C, 71.78; H, 4.45; N, 14.56. Found: C, 72.06; H, 4.32; N, 14.68.

*4.1.3.3. 5-Benzyl-2-(4-bromophenyl)-1H-[1,2,4]triazino[4,3-a]quinoxaline (**10c**).* Yellowish crystals (0.58 g, 67.6%), m.p. 248–249 °C (Ethanol). IR (KBr, $cm^{-1}$): 3430 (NH); 1631 (C=N); 1587 (C=C); 1539 ($\delta$NH); 762 (C–Br). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.49 (s, 2H, C$H_2$–$C_6H_5$), 5.57 (s, 2H, triazinoquinox. $C_1$–$H_2$), 7.31–7.42 (m, 5H, $CH_2$–$C_6H_5$), 7.77–8.05 (m, 2H, triazinoquinox. $C_{8,9}$–H), 7.86 (d, 2H, $J$ = 8.3 Hz, bromophenyl $C_{2,6}$–H), 8.08–8.14 (m, 1H, triazinoquinox. $C_7$–H), 8.10 (d, 2H, $J$ = 8.3 Hz, bromophenyl $C_{3,5}$–H), 8.33 (d, 1H, $J$ = 8.4 Hz, triazinoquinox. $C_{10}$–H). Anal. calcd for $C_{23}H_{17}BrN_4$ (429.32): C, 64.35; H, 3.99; N, 13.05. Found: C, 64.17; H, 3.72; N, 13.26.

**4.1.4. Ethyl 3-[(3-benzylquinoxalin-2-yl)hydrazono]butyrate (11).** A mixture of **6** (0.5 g, 2 mmol) and ethyl acetoacetate (0.29 g, 2.2 mmol) was heated for 1 h in an oil bath at 160–170 °C, triturated with petroleum ether (60–80 °C), filtered dried and recrystallized from ethanol/water. This compound was obtained as white crystals (0.5 g, 69.1%), m.p. 115–116 °C. IR (KBr, $cm^{-1}$): 1720 (C=O ester); 1630 (C=N); 1599, 1510 (C=C); 1287, 1193, 1090 (C–O–C). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 1.43 (t, 3H, $J$ = 6.6 Hz, $CH_2CH_3$), 2.50 (s, 3H, N=C–C$H_3$), 3.34 (s, 2H, $CH_2$), 4.57 (q, 2H, $J$ = 6.6 Hz, C$H_2$$CH_3$), 4.65 (s, 2H, C$H_2$–$C_6H_5$), 7.22 (t, 1H, $J$ = 7.4 Hz, $C_6H_5$–$C_4$–H), 7.30 (t, 2H, $J$ = 7.4 Hz, $C_6H_5$–$C_{3,5}$–H), 7.47 (d, 2H, $J$ = 7.4 Hz, $C_6H_5$–$C_{2,6}$–H), 7.74–7.78 (m, 2H, quinox. $C_{6,7}$–H), 8.09 (ddd, 1H, $J$ = 7.4, 3.8, 2.1 Hz, quinox. $C_8$–H), 8.75 (ddd, 1H, $J$ = 7.5, 3.8, 2.1 Hz quinox. $C_5$–H). $^{13}$C-NMR (500 MHz, $\delta$ ppm): 13.80 (2 × $CH_3$), 40 (*C*$H_2$–$C_6H_5$), 63.11(COO*C*$H_2$$CH_3$), 118.9 (quinox-$C_6$), 124.85 (quinox-$C_8$), 126.69 (phenyl-$C_4$), 128.43 (phenyl-$C_{3,5}$), 128.59 (quinox-$C_5$), 129.03 (quinox-$C_7$), 129.21 (phenyl-$C_{2,6}$), 129.61 (phenyl-$C_1$), 136.3 (quinox-$C_{8a}$), 136.59 (quinox-$C_{4a}$), 142.52 (quinox-$C_3$), 144.53 (quinox-$C_2$), 153.23 (N=C), 158.61(*C*OOCH$_2$CH$_3$). Anal. calcd for $C_{21}H_{22}N_4O_2$ (362.17): C, 69.59; H, 6.12; N, 15.46. Found: C, 69.28; H, 6.20; N, 15.29.

**4.1.5. 2-[5-Amino-3-(4-chlorophenyl)pyrazol-1-yl]-3-benzylquinoxaline (13).** To a solution of **6** (0.5 g, 2 mmol) in ethanol (8 mL) and acetic acid (2 mL), 4-chlorophenyl-ω-cyanoacetophenone (2 mmol) was added. The reaction mixture was heated under reflux for 2 h, cooled, and the separated product was filtered, dried, and recrystallized from acetonitrile as white-greyish crystals (0.8 g, 97.2%), m.p. 174–175 °C. IR (KBr, $cm^{-1}$): 3462, 3368 ($NH_2$); 1609, 1578, 1559 (C=N, C=C); 761 (C–Cl).

$^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.75 (s, 2H, C$H_2$–$C_6H_5$), 5.94 (s, 1H, pyrazole $C_4$–H), 6.13 (s, 2H, $NH_2$, $D_2O$ exchangeable), 7.06–7.17 (m, 5H, $C_6H_5$), 7.48 (d, 2H, $J$ = 8.1 Hz, chlorophenyl $C_{2,6}$–H), 7.80–7.88 (m, 3H, quinox. $C_{5,6,7}$–H), 7.81 (d, 2H, $J$ = 8.1 Hz, chlorophenyl $C_{3,5}$–H), 8.11 (dd, 1H, $J$ = 8.4, 3.6 Hz, quinox. $C_8$–H). Mass spectrum $m/z$ (%): 412 (36), 411 ($M^{+\bullet}$) (100), 334 (10), 320 (15), 273 (50), 227 (19), 218 (21), 197 (15), 138 (15), 116 (25), 102 (16), 91(44). Anal. calcd for $C_{24}H_{18}ClN_5$ (411.89): C, 69.99; H, 4.40; N, 17.00. Found: C, 70.24; H, 4.38; N, 17.16.

**4.1.6. 4-Benzyl-1,2,4-triazolo[4,3-*a*]quinoxaline (14).** A solution of **6** (0.5 g, 2 mmol) in formic acid (3 mL) was refluxed for 3 h. After cooling, the reaction mixture was poured onto ice water with stirring and the precipitated solid was collected by filtration, washed with water and recrystallized from ethanol as yellow fine needles (0.3 g, 57.3%), m.p. 240–241 °C; reported m.p. >320 °C.[22] IR (KBr, cm$^{-1}$): 1681 (C=N); 1539 (C=C). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.60 (s, 2H, C$H_2$–$C_6H_5$), 7.60 (t, 2H, $J$ = 7.35 Hz, $C_6H_5$–$C_{3,5}$–H), 7.78 (ddd, 2H, $J$ = 7.35, 7.3, 1.5 Hz, $C_6H_5$–$C_{2,6}$–H), 7.95 (t, 1H, $J$ = 7.35 Hz, $C_6H_5$–$C_4$–H), 8.07–8.10 (m, 2H, triazoloquinox. $C_{7,8}$–H), 8.17 (dd, 1H, $J$ = 8.4, 1.5 Hz, triazoloquinox. $C_6$–H), 8.54 (dd, 1H, $J$ = 8.4, 1.5 Hz triazoloquinox. $C_9$–H), 10.27 (s, 1H, triazoloquinox. $C_1$–H). Anal. calcd for $C_{16}H_{12}N_4$ (260.30): C, 73.83; H, 4.65; N, 21.52. Found: C, 73.80; H, 4.59; N, 21.67.

**4.1.7. 4-Benzyl-1,2-dihydro-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (15).** Ethyl chloroformate (0.22 g, 2 moles) was added drop wise under stirring to a solution of **6** (0.5 g, 2 mmol) in absolute ethanol (10 mL). The reaction mixture was refluxed for 3 h. After cooling the separated crystals were filtered, dried, and recrystallized from ethanol as yellowish white crystals (0.38 g, 69.1%), m.p. 262–264 °C; reported m.p. 256–258 °C.[15] IR (KBr, cm$^{-1}$): 3272, 3248, 3199, 3184 (NH); 1682 (C=O); 1658 (C=N); 1629 (C=C); 1450, 1372 (C–N lactam). Anal. calcd for $C_{16}H_{11}N_4O$, (275.29): C, 69.81; H, 4.03; N, 20.35. Found: C, 69.63; H, 4.16; N, 20.09.

**4.1.8. 3-(4-Benzyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-yl)propanoic acid (16).** A mixture of **6** (0.5 g, 2 mmol) and succinic anhydride (0.18 g, 2 mmol) in glacial acetic acid (5 mL) was heated under reflux for 5 h. The reaction mixture was cooled to ambient temperature. The obtained crystalline product was filtered, dried, and recrystallized from ethanol as yellow fine needles (0.38 g, 57.2%), m.p. 256–258 °C. IR (KBr, cm$^{-1}$): 3500–2543 (br OH); 1693 (C=O); 1665 (C=N). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 1.05 (t, 2H, $J$ = 6.6 Hz, $CH_2$, 3-propanoic), 3.38 (t, 2H, $J$ = 6.6 Hz, $CH_2$, 2-propanoic), 4.41, 4.51 (2d, 2H, $J$ = 14 Hz, $CH_2$–$C_6H_5$), 7.35–7.42 (m, 3H, $C_6H_5$–$C_{2,4,6}$–H), 7.44 (t, 1H, $J$ = 7.2 Hz, triazoloquinox. $C_8$–H), 7.57 (t, 2H, $J$ = 7.8 Hz, $C_6H_5$–$C_{3,5}$–H), 7.66 (ddd, 1H, $J$ = 10, 7.4, 1.5 Hz triazoloquinox. $C_7$–H), 7.83 (dd, 1H, $J$ = 8.1, 1.2 Hz, triazoloquinox. $C_6$–H), 7.97 (d, 1H, $J$ = 7.2 Hz, triazoloquinox. $C_9$–H), 12.87 (s, 1H, OH, $D_2O$ exchangeable). Anal. calcd for $C_{19}H_{16}N_4O_2$ (332.36): C, 68.66; H, 4.85; N, 16.86. Found: C, 68.50; H, 4.93; N, 17.04.

**4.1.9. 2-(4-Benzyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-yl)benzoic acid (17).** This compound was prepared analogous to **16** from **6** (0.5 g, 2 mmol) and phthalic anhydride (0.3 g, 2 mmol) in glacial acetic acid (5 mL). The obtained crystalline product was filtered, dried, and recrystallized from ethanol/water as white crystalline flakes (0.45 g, 59.2%), m.p. 223–224 °C. IR (KBr, cm$^{-1}$): 3500–2919 (br-OH); 1717 (C=O); 1689 (C=N); 1521 (C=C). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 5.60 (s, 2H, $CH_2$–$C_6H_5$), 7.37–8.10 (m, 11H, Ar–H, triazoloquinox. $C_{7,8}$–H), 8.08 (dd, 1H, $J$ = 6.6, 5.1 Hz, triazoloquinox. $C_6$–H), 8.14 (ddd, 1H, $J$ = 9.6, 6.9, 1.8 Hz, triazoloquinox. $C_9$–H), 10.74 (s, 1H, OH, $D_2O$ exchangeable). Mass spectrum $m/z$ (%): 382 ($M^{+\bullet}$ + 2), 380 ($M^{+\bullet}$) (5), 247 (20), 235 (10), 219 (13), 206 (31), 205 (100), 102 (35), 90 (10), 77 (47). Anal. calcd for $C_{23}H_{16}N_4O_2$ (380.41): C, 72.62; H, 4.24; N, 14.73. Found: C, 72.47; H, 4.09; N, 14.61.

**4.1.10. 1-Ethoxycarbonyl-4-benzyl-1,2,4-triazolo[4,3-*a*]quinoxaline (18).** A mixture of **6** (0.5 g, 2 mmol) and diethyl oxalate (0.32 g, 2.2 mmol) in dry xylene (5 mL) was refluxed for 3 h. The reaction mixture was cooled; the crystalline formed product was filtered, dried, and recrystallized from ethanol to yield **18** (0.45 g, 67.8%), m.p. 117–118 °C. IR (KBr, cm$^{-1}$): 1720 (C=O ester); 1599, 1510 (C=N,C=C); 1278, 1193, 1091 (C–O–C). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 1.42 (t, 3H, $J$ = 7.4 Hz, $CH_2CH_3$), 4.58 (q, 2H, $J$ = 7.4 Hz, $CH_2CH_3$), 4.65 (s, 2H, C$H_2$–$C_6H_5$), 7.22 (t, 1H, $J$ = 7.33 Hz, $C_6H_5$–$C_4$–H), 7.30 (t, 2H, $J$ = 7.33 Hz, $C_6H_5$–$C_{3,5}$–H), 7.46 (d, 2H, $J$ = 7.33 Hz, $C_6H_5$–$C_{2,6}$–H), 7.74–7.79 (m, 2H, triazoloquinox. $C_{7,8}$–H), 8.10 (ddd, 1H, $J$ = 7.5, 3.8, 2.4 Hz, triazoloquinox. $C_6$–H), 8.76 (ddd, 1H, $J$ = 7.5, 3.8, 2.4 Hz triazoloquinox. $C_9$–H). Anal. calcd for $C_{19}H_{16}N_4O_2$ (332.36): C, 68.66; H, 4.85; N, 16.86. Found: C, 68.82; H, 4.68; N, 16.63.

**4.1.11. 1-Ethoxycarbonylmethyl-4-benzyl-1,2,4-triazolo[4,3-*a*]quinoxaline (19).** A mixture of **6** (0.5 g, 2 mmol) and diethyl malonate (0.35 g, 2.2 mmol) was heated for 1 h in an oil bath at 160–170 °C. After cooling, the product was triturated with petr. ether (60–80 °C), filtered dried and recrystallized from DMF as pure white crystals (0.6 g, 86.7%), m.p. above 300 °C. IR (KBr, cm$^{-1}$): 1719 (C=O ester); 1630 (C=N); 1606, 1516, 1489 (C=C); 1241, 1071 (C–O–C). $^1$H-NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 1.44 (t, 3H, $J$ = 7.4 Hz, $CH_2CH_3$), 4.53 (s, 2H, C$H_2$–$C_6H_5$), 5.12 (q, 2H, $J$ = 7.4 Hz, $CH_2CH_3$), 5.83 (s, 2H, C$H_2$–CO), 7.11 (t, 1H, $J$ = 7.33 Hz, $C_6H_5$–$C_4$–H), 7.17 (t, 2H, $J$ = 7.33 Hz, $C_6H_5$–$C_{3,5}$–H), 7.42 (d, 2H, $J$ = 7.33 Hz, $C_6H_5$–$C_{2,6}$–H), 7.45–7.63 (m, 2H, triazoloquinox. $C_{7,8}$–H), 8.04 (d, 1H, $J$ = 7.5 Hz, triazoloquinox. $C_6$–H), 8.18 (d, 1H, $J$ = 7.5 Hz triazoloquinox. $C_9$–H). Anal. calcd for $C_{20}H_{18}N_4O_2$ (346.39): C, 69.35; H, 5.24; N, 16.17. Found: C, 69.42; H, 5.28; N, 16.02.

### 4.2. Biological evaluation methodology

**4.2.1. Anticancer screening.** Ten of the prepared compounds were selected by the National Cancer Institute (NCI) Bethesda-Maryland (USA) and tested for their *in vitro* anticancer activity against 60 human tumor cell lines, derived from nine clinically isolated types of cancer (leukemia, lung, brain, melanoma, colon, ovarian, renal, prostate and breast). These cell lines were incubated with one concentration (10 μM) for each tested compound. Only compounds which satisfy predetermined threshold-inhibition criteria were tested at five tenfold dilutions (0.01 to 100 μM). A 48 h continuous drug-exposure protocol was used, and a sulforhodamine B (SRB)

protein assay was employed to estimate the cell viability or growth.[23,24] The results are presented in Tables 1–3.

#### 4.2.2. Antimicrobial screening

*4.2.2.1. Inhibition-zone measurements.* All synthesized compounds were evaluated for their antimicrobial activity by the agar cup diffusion technique using a 1 mg $mL^{-1}$ solution in DMSO.[26] The test organisms were *Staphylococcus aureus* (DSM 1104) and *Bacillus subtilis* (ATCC 6633) as Gram-positive bacteria; *Escherichia coli* (ATCC 11775) and *Pseudomonas aeruginosa* (ATCC 10145) as Gram-negative bacteria. *Candida albicans* (DSM 70014) was also used as a representative for fungi. Each 100 mL of sterile molten agar (at 45 °C) received 1 mL of 6 h-broth culture and then the seeded agar was poured into sterile Petri dishes. Cups (8 mm in diameter) were cut in the agar. Each cup received 0.1 mL of the 1 mg $mL^{-1}$ solution of the test compounds. The plates were then incubated at 37 °C for 24 h or, in case of *C. albicans*, for 48 h. A control using DMSO without the test compound was included for each organism. Ampicillin was used as the standard antibacterial, while clotrimazole was used as the antifungal reference. The resulting inhibition zones are recorded (Table 4).

*4.2.2.2. Minimal inhibitory concentration (MIC) measurement.* The minimal inhibitory concentrations (MIC) of the most active compounds were measured using the twofold serial broth dilution method.[27] The test organisms were grown in their suitable broth: 24 h for bacteria and 48 h for fungi at 37 °C. Two fold serial dilutions of solutions of the test compounds were prepared using 200, 100, 50, 25, and 12.5 μg $mL^{-1}$. The tubes were then inoculated with the test organisms; each 5 mL received 0.1 mL of the above inoculum and were incubated at 37 °C for 48 h. Then, the tubes were observed for the presence or absence of microbial growth. The MIC values of the prepared compounds are listed in Table 5.

*4.2.2.3. Minimal bactericidal concentration (MBC) measurement.* MIC tests were always extended to measure the MBC as follows: A loop-full from the tube not showing visible growth (MIC) was spread over a quarter of Müller–Hinton agar plate. After 18 h of incubation, the plates were examined for growth. Again, the tube containing the lowest concentration of the test compound that failed to yield growth on subculture plates was judged to contain the MBC of that compound for the respective test organism (Table 5).

## Acknowledgements

The authors are grateful to the staff of the department of Health and Human Services, National Cancer Institute, Bethesda, Maryland, USA for carrying out the anticancer screening of the selected synthesized compounds.

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