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FULL PAPER

Discovery and structure–activity relationship of plastoquinone analogs as anticancer agents against chronic myelogenous leukemia cells

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Abstract

Two series of amino-1,4-benzoquinones (**AQ1–18**) based on the structural analogs of plastoquinones were synthesized and the structure–activity relationship against chronic myelogenous leukemia activity was examined. All of the synthesized compounds were tested for their cytotoxic effects on different leukemic cell lines. Of interest, **AQ15** exhibited a better selectivity than the reference drug imatinib on cancer cells. Owing to this, **AQ15** was selected for a further apoptosis/necrosis evaluation where **AQ15**-treated K562 cells demonstrated similar apoptotic effects like imatinib-treated cells at their IC_{50} values. The inhibitory effects of **AQ15** and the other three compounds with various activities against eight tyrosine kinases, including ABL1, were investigated. **AQ15** showed weak activity against ABL1, and a correlation was observed between the anti-K562 and anti-ABL1 activities. The binding mode of **AQ15** into the ATP binding pocket of ABL1 kinase was predicted in silico, showing the formation of some key interactions. In addition, **AQ15** was shown to suppress the downstream signaling of BCR-ABL in K562 cells. Finally, **AQ15** obviously cleaved DNA in the presence of an iron(II) complex system, indicating that this can be the major mechanism of its antiproliferative action, whereas the mild inhibition of ABL kinase is just in-part mechanism of its overall outstanding cellular activity.

KEYWORDS
aminoquinone, apoptosis, chronic myelogenous leukemia, DNA cleavage, kinase inhibitor, plastoquinone, structure–activity relationship

1 | INTRODUCTION

Quinone structure is found in many medicinally important compounds possessing anticancer, anti-inflammatory, antimicrobial, and antiviral activities. In particular, 1,4-quinones constitute an important group of anticancer drugs.[1] DNA often becomes a major target for anticancer quinones, that is, mitomycin C—which crosslinks DNA[2]; streptonigrin—which cleaves DNA[3]; and quinone enediyne antibiotic dynemicin

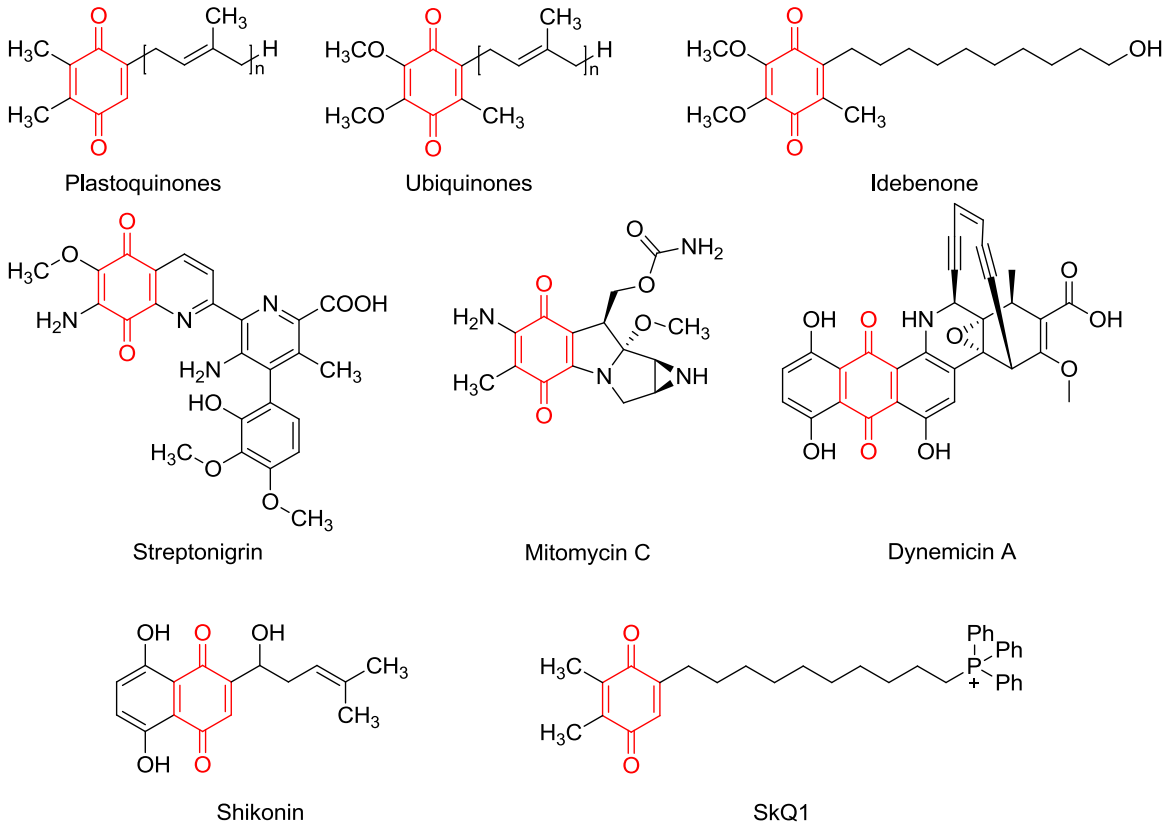


FIGURE 1 Biologically important molecules containing the 1,4-quinone moiety

A—which cleaves DNA, as shown in Figure 1.[4] Natural or synthetic 1,4-quinones take part in a wide range of redox processes in organisms and plants, such as plastoquinones and ubiquinones, which are two important prenylquinones.[5] Idebenone, a structural analog of plastoquinones and ubiquinones (Figure 1), is a drug for the treatment of emotional disturbances associated with cerebrovascular diseases.[6] Further investigation about the synthesis and in vitro studies has shown that cationic plastoquinones serve as powerful inhibitors of ROS-induced apoptosis and necrosis in HeLa cells and human fibroblasts.[7] The recent development in the design of a novel agent is SkQ1 (Figure 1), which suppresses the growth of fibrosarcoma HT1080 and rhabdomyosarcoma RD tumor cells in vitro.[8] In 2010, novel analogs containing 1,4-quinone moiety were reported as effective in combination with chemotherapeutic agents against pancreatic cancer cell lines.[9] 1,4-Quinone compounds are also reported as potential anticancer agents against ovarian cancer cell lines.[10] Recently, a new series of 1,4-quinones designed on the basis of the natural product hybrid approach was proven to be active against two different leukemia cell lines (CCRF-CEM and CEM/ADR5000).[11] In addition, a quinone-containing natural product shikonin is currently a center of interest due to its potent effect for multiple myeloma owing to its unique dual activities, proteasome inhibition and necroptosis induction.[12]

In our ongoing studies on the chemical modification of 1,4-quinones aiming at biologically useful compounds, our research group is working on the synthesis of dimethyl-1,4-benzoquinone analogs by using different pathways reported previously in the literature.[13] Previously, we synthesized some sulfanyl 1,4-naphthoquinone compounds containing aryl amines with different substituents to investigate antimicrobial activities against Gram-positive and -negative bacteria and anticancer activities against human tumor cell lines.[14] Some compounds having strong antibacterial efficiency were reported as promising antibacterial agents.[14a] Furthermore, some tested compounds showed important selectivity on peripheral blood mononuclear cells (PBMCs), though they exhibited considerable anticancer activities against colon and leukemia cancer cell lines.[14b] Herein, we focus on chronic myelogenous leukemia (CML) among various cancers.

CML is caused by a reciprocal translocation between chromosome 9 and chromosome 22, known as the Philadelphia chromosome or Philadelphia translocation.[15] This translocation gives rise to a BCR-ABL kinase made up of two genes BCR and ABL.[16] The ABL kinase family has been reported to play important roles in normal cells, for example, cell adhesion and motility, DNA damage response, and microbial pathogen response that may occur in the cytosol and cell membranes. ABL kinase is also present in the nucleus.[17] Deregulation and aberrant expression of ABL kinases are associated with many types of cancer, such as breast[18] and colon cancers.[19] Okabe et al.[20] presented the investigation of the combination therapy with an ABL tyrosine kinase inhibitor (TKI) and alisertib against Ph+ cells; thus, a strategy for the treatment of Ph+ leukemia patients could be Aurora A inhibition. Since the overactivity of the protein tyrosine kinase (PTK) has been implicated in a number of diseases, a variety of PTKs has been targeted for the screening of

antitumor drugs. TKIs, as rivals of ATP for the ATP-binding site of PTK reducing tyrosine kinase phosphorylation, have made progress although resistance has been restricted by the treatment of cancer.[21] Imatinib is a highly specific inhibitor of BCR-ABL binding to the ATP-binding site of the protein.[22] More recently, highly potent inhibitors dasatinib and nilotinib have been developed and used as a standard remedy together with imatinib. Although these anti-CML drugs have a combinatorial structure comprising various components, we considered a possibility of 1,4-benzoquinone variants to serve as anti-CML drugs.

In this study, a new series of 1,4-quinone compounds was synthesized, and its activity against CML cell lines was examined. Finally, some compounds were found to have unique activity based on the dual mechanism of kinase inhibition and DNA cleavage.

2 | RESULTS AND DISCUSSION

2.1 | Chemical synthesis

We designed our molecules based on the plastoquinone scaffold, replacing either prenyl from plastoquinone or decyl-triphenylphosphonium cation appendage from SkQ1 by aliphatic and aromatic amines, and named them AQ analogs (Figure 2). We also introduced the chloro group that appears to be favorable for biological activity.[23]

The reaction of primary and secondary amines with 1,4-quinones to give AQs has already been reported.[24] As shown in Scheme 1, initially, the 2,3-dimethyl hydroquinone (**1**) was smoothly oxidized to the corresponding 1,4-benzoquinones (**2** and **3**). The 2,3-dimethyl-1,4-benzoquinone (**2**) was obtained according to the reported methods using $KBrO_3$[25] or MnO_2[26] in 98% or 86% yield, respectively. Another precursor, 2,3-dichloro-5,6-dimethyl-1,4-benzoquinone (**3**) was synthesized by oxidizing with the HNO_3/HCl variation of the reported method.[27] Subsequently, AQ analogs (**AQ1–18**) were prepared by the reactions of the key 1,4-benzoquinones (**2** and **3**) with the corresponding substituted amines according to the literature procedure but omitting the use of $CeCl_3$.[13a] Heating the mixture in refluxing absolute ethanol at higher temperature gave the target compounds (**AQ1–18**). Among these compounds, **AQ1**, **AQ6**,[28a] **AQ8**,[29] and **AQ10**[30] have been reported in the literature, while the other AQ analogs are novel molecules. Structural characterization of the AQ analogs (**AQ1–18**, Table 1) was complemented by Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR), and high-resolution mass spectroscopy (HRMS). In addition, the structures of **AQ3** (1892191), **AQ9** (1892192), **AQ11** (1892193), **AQ17** (1892194), and **AQ18** (1892197) were further elucidated by single-crystal X-ray diffraction analysis (Figure S56).

2.2 | Evaluation of anticancer activity

The cytotoxicity and selectivity of the synthesized 18 AQ analogs (**AQ1–18**) against three cancer cell lines, K562 (chronic myelogenous leukemia), Jurkat, and MT-2 (other two human T-cell leukemias), were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using imatinib as a control drug.

IC_{50} values are summarized in Table 1. The compounds **AQ1–10** showed IC_{50} values higher than 15 μM against K562, MT-2, and Jurkat cells. Among them, **AQ7** was the most effective against the chronic myelogenous leukemia K562 cell line. Introduction of a trifluoromethyl group at R_1 (**AQ2**), R_2 (**AQ3**), or R_3 (**AQ4**) resulted in a decreased activity. The activity was retained by the introduction of the methyl group at R_2 (**AQ5**), R_3 (**AQ6**), and isopropyl group at R_1 (**AQ7**).

On the contrary, the introduction of a chloro group into the quinone moiety led to an increase in anticancer activity against all three cell lines. Thus, for the chloro-substituted series of AQ analogs (**AQ11–18**), **AQ11**, **AQ12**, **AQ14–15**, and **AQ17** exerted the best cytotoxic activity against K562 cell line better than imatinib. These compounds also exhibited strong anticancer activities against other two leukemia cell lines (Figure S1). Introduction of a trifluoromethyl group at R_2 (**AQ12**) retained the activity, but introduction at R_3 (**AQ13**) led to a decrease in activity. Introduction of the methyl group at R_2 (**AQ14**) or R_3 (**AQ15**) retained the activity. **AQ10** and **AQ18** with 2-picolylamine led to a decrease in activity in all cell lines.

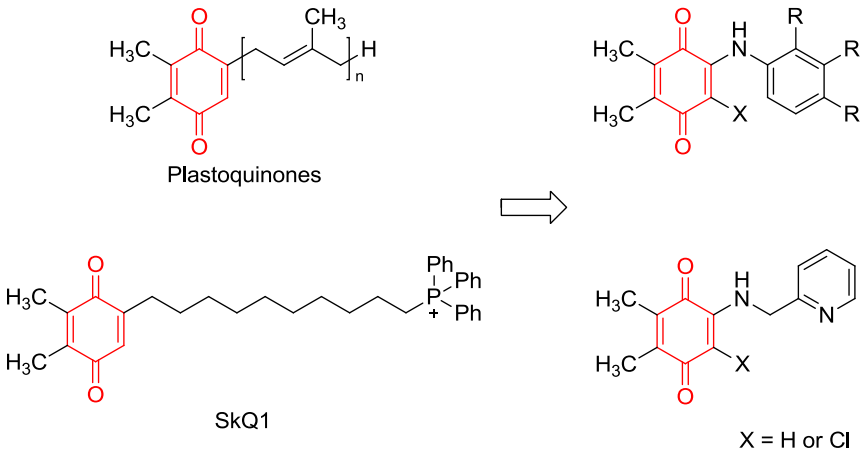
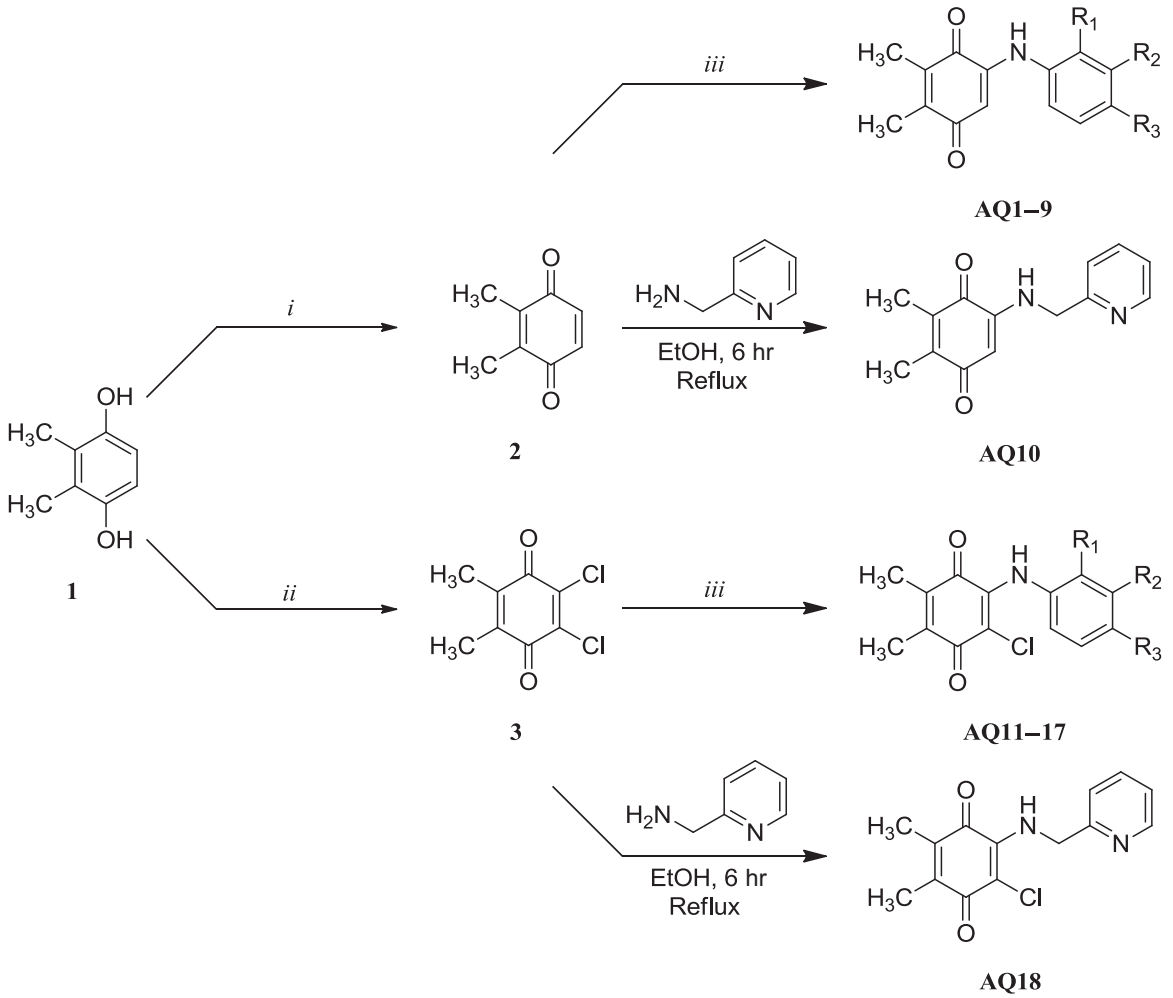


FIGURE 2 Design of new amino-1,4-benzoquinones based on plastoquinones and SkQ1, AQ analogs



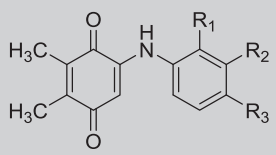
SCHEME 1 Synthesis of AQ analogs (**AQ1–18**): (i) $KBrO_3$, H_2SO_4/H_2O, 10 min, 80°C; (ii) HNO_3/HCl, 10 min, 90°C; (iii) substituted amines (1.2 equiv), EtOH, reflux, 6–12 hr

Table 2 shows the cytotoxicity results of selected AQ analogs (**AQ11**, **AQ12**, and **AQ15**), with submicromolar IC_{50} against K562, compared with PBMC. As the selectivity index (SI) is calculated as the ratio of cytotoxicity of the IC_{50} between the PBMC and K562 cells, the greater the SI value, the more selective it is for cancer cells. Among them, **AQ15** exhibited a better selectivity than imatinib against K562 cell line.

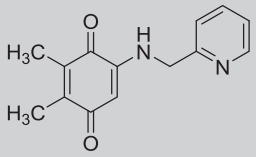
Among this series, **AQ15**, the most selective anticancer agent, was chosen to investigate its apoptotic activity in CML. Thus, the annexin V/ethidium homodimer III and Hoechst 33342 staining methods were carried out with K562 cell line treated with **AQ15** at IC_{50} concentration and then observed by a fluorescence microscope (Figure 3). In the control experiment, all cells were stained with blue (healthy cells) at 6 hr after treatment of dimethyl sulfoxide (DMSO; Figure 3a). On the contrary, K562 cells treated with **AQ15** and imatinib were stained mostly with healthy cells (blue), then with apoptotic cells (green), late apoptotic or necrotic cells (both green and red), and necrotic cells (red; Figure 3a), suggesting that **AQ15** and imatinib induced apoptosis mainly in an earlier time. The results indicated that **AQ15** had 66% apoptotic, 22% late apoptotic/necrotic, and 12% necrotic effects at 6 hr, as shown in Figure 3b. The response of K562 cells upon 6-hr imatinib treatment was 58% apoptosis, 23% late apoptosis/necrosis, and 19% necrosis (Figure 3b). The results revealed that **AQ15** induced cell apoptosis similar to imatinib in CML (Figure 3c).

We speculated that significant anticancer activity of **AQ15** against CML (BCR-ABL positive leukemia) may be due to its potential inhibitory activity on ABL (the kinase portion of BCR-ABL). Thus, a panel of kinases including ABL1 was selected. In this activity-based kinase system, the inhibitory effects of **AQ11**, **AQ15**, **AQ16**, and **AQ18** with various activities were tested against eight kinases (ABL1, BRK, BTK, CSK, FYN A, LCK, LYN B, and SRC) using multipoint dose–response experiments and the results are shown in Table 3. In this series, **AQ15** was found as the most potent ABL1 kinase inhibitor with the IC_{50} value of 17.92 ± 2.45 μM, followed by **AQ11** with the IC_{50} value of 23.60 ± 1.94 μM when compared with imatinib (0.27 ± 0.04 μM). On the contrary, **AQ16** and **AQ18** were found to be inactive against ABL1 at 100 μM concentration. A correlation was observed between anti-K562 and anti-ABL1 activities. Notably, **AQ15** significantly inhibited BRK, BTK, CSK, and SRC stronger than compounds **AQ11**, **AQ16**, **AQ18**, and imatinib with IC_{50} values in the micromolar range. Furthermore, all compounds were found to be

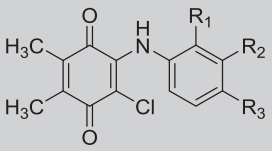
TABLE 1 Structure and cytotoxicity of AQ analogs (**AQ1–18**) in K562, Jurkat, and MT-2 cell lines by MTT assay



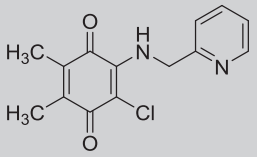
AQ1–9



AQ10



AQ11–17



AQ18

	Substitution groups			Cell type (IC_{50}, μM)[a]		
ID	R_1	R_2	R_3	K562[b]	Jurkat[b]	MT-2[b]
AQ1	H	H	H	20.07 ± 2.95	13.38 ± 1.02	>30
AQ2	CF_3	H	H	15.30 ± 3.21	15.89 ± 4.03	>30
AQ3	H	CF_3	H	>30	>30	>30
AQ4	H	H	CF_3	>30	>30	>30
AQ5	H	CH_3	H	19.08 ± 1.17	>30	>30
AQ6	H	H	CH_3	20.49 ± 2.45	16.55 ± 1.56	>30
AQ7	$CH(CH_3)_2$	H	H	15.07 ± 0.89	15.59 ± 3.21	>30
AQ8	H	H	$CH(CH_3)_2$	>30	>30	>30
AQ9	H	H	$N(CH_2CH_3)_2$	24.84 ± 1.72	17.53 ± 2.19	>30
AQ10	Shown above			>30	>30	>30
AQ11	H	H	H	0.75 ± 0.05	3.01 ± 0.62	2.99 ± 1.02
AQ12	H	CF_3	H	0.88 ± 0.06	2.92 ± 0.41	2.55 ± 0.83
AQ13	H	H	CF_3	17.82 ± 1.87	5.48 ± 0.95	13.84 ± 2.44
AQ14	H	CH_3	H	1.85 ± 0.11	2.29 ± 0.53	3.17 ± 0.95
AQ15	H	H	CH_3	0.76 ± 0.04	2.34 ± 0.88	5.09 ± 1.11
AQ16	$CH(CH_3)_2$	H	H	7.38 ± 0.57	8.58 ± 1.34	11.77 ± 1.47
AQ17	H	H	$CH(CH_3)_2$	1.89 ± 0.09	2.46 ± 0.38	3.51 ± 0.12
AQ18	Shown above			22.15 ± 2.19	>30	>30
Imatinib[c]				5.58 ± 1.83	9.65 ± 2.17	20.75 ± 1.55

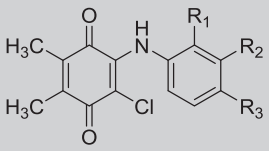
Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation.

[a]The reported values represent the mean ± SD for each compound based on three independent experiments.

[b]Cell lines include chronic myelogenous leukemia (K562) and other leukemias (Jurkat and MT-2).

[c]Used as the reference.

TABLE 2 Cytotoxicity of selected AQ analogs (**AQ11**, **AQ12**, and **AQ15**) and selectivity index (SI)



Selected AQ Analogs

	Substitution group			Cell type (IC_{50}, μM)		
ID	R_1	R_2	R_3	K562[a]	PBMC[a]	SI[b]
AQ11	H	H	H	0.75 ± 0.05	5.14 ± 1.76	6.85
AQ12	H	CF_3	H	0.88 ± 0.06	3.00 ± 1.22	3.41
AQ15	H	H	CH_3	0.76 ± 0.04	7.64 ± 1.58	10.05
Imatinib[c]				5.58 ± 1.83	33.92 ± 4.19	6.08

[a]Cell lines include chronic myelogenous leukemia (K562) and peripheral blood mononuclear cells (PBMCs).

[b]The SI values are calculated as the ratio of the IC_{50} between PBMC and chronic myelogenous leukemia (K562) cells.

[c]Used as the reference.

inactive against FYN A and LYN B. These results pointed out that **AQ15** could be defined as a promising lead multitargeted kinase inhibitor with different kinase inhibitory profile than imatinib.

Afterward, we tried to explore the potential binding mode of **AQ15** into the ATP-binding site of ABL kinase by computational approaches using MOE software. Despite the distinct stereoelectronic features and a molecular weight from imatinib, **AQ15** could form some critical interactions that granted it an acceptable inhibition effect (IC_{50} = 17.92 ± 2.45 μM). **AQ15** NH forges a key H-bond with the gatekeeper amino acid Thr315. Moreover, the dimethyl benzoquinone is sandwiched between Val256 and Leu370 forming two CH–π interactions. The compound is further anchored by another CH–π interaction with Phe317. The chlorine atom does not seem to contribute to the interaction through hydrogen bond or

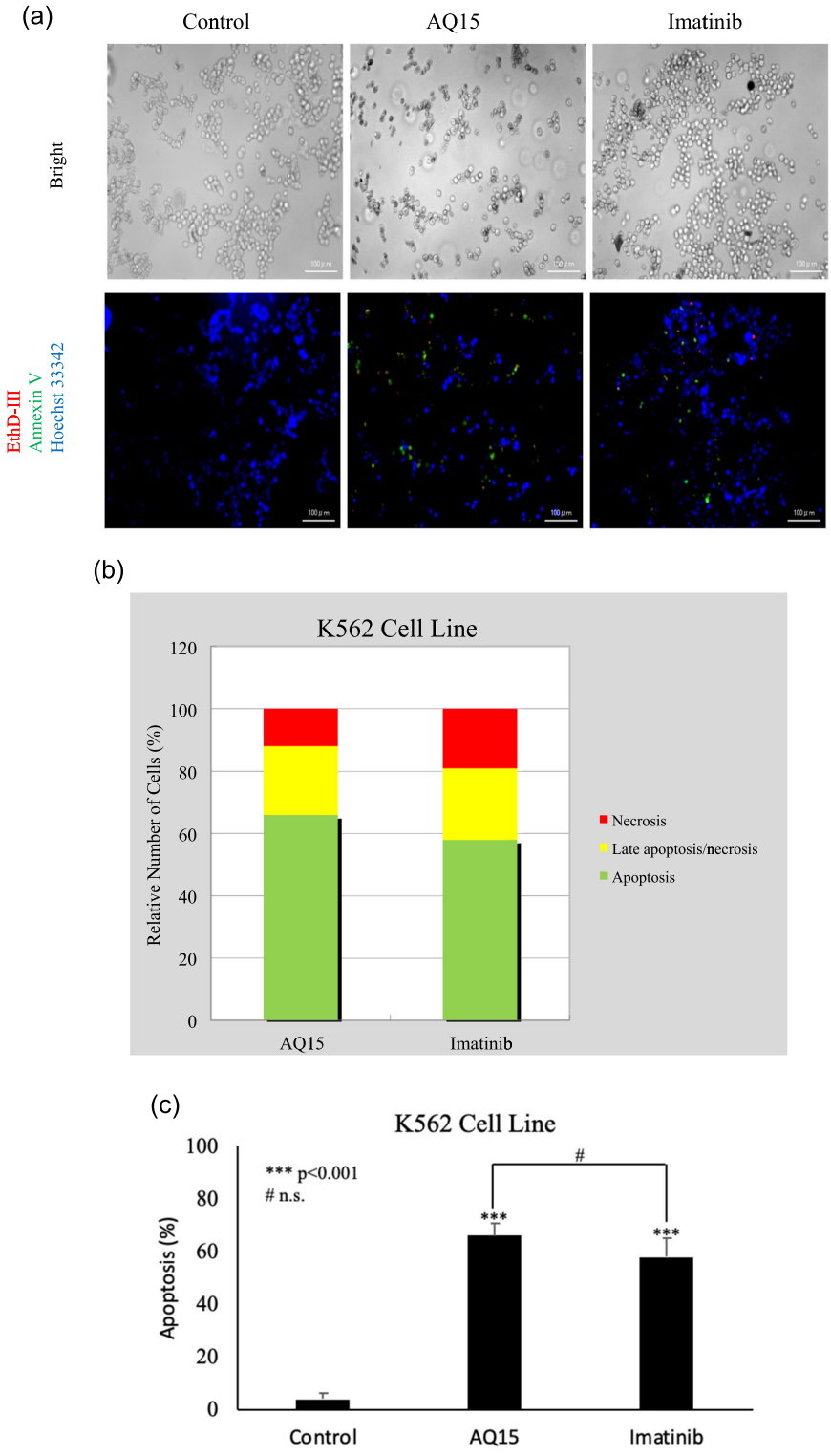


FIGURE 3 Alteration in K562 cells at IC_{50} concentrations of **AQ15** and imatinib (a) for 6 hr. (b) A total of approximately 100 stained cells was selected randomly in each experiment of (a) and was classified into three types "apoptosis" (green), "necrosis or late apoptosis" (both green and red), and "necrosis" (red). (c) Quantification of the effect of **AQ15** and imatinib on apoptosis. Data from three independent experiments are shown as means ± standard deviations and *p* values were determined using Student's test

TABLE 3 The inhibition profile of tested compounds and imatinib in the panel of eight kinases

	IC_{50} (μM)				
Kinase	**AQ11**	**AQ15**	**AQ16**	**AQ18**	**Imatinib**
ABL1	23.60 ± 1.94	17.92 ± 2.45	>100	>100	0.27 ± 0.04
BRK	>100	18.53 ± 2.13	>100	>100	20.56 ± 1.41
BTK	26.46 ± 3.12	12.01 ± 0.94	29.27 ± 3.85	>100	>100
CSK	>100	24.00 ± 1.78	>100	>100	25.45 ± 2.30
FYN A	>100	>100	>100	>100	13.34 ± 0.93
LCK	82.52 ± 5.91	>100	49.70 ± 6.43	87.61 ± 9.72	0.68 ± 0.06
LYN B	>100	>100	>100	>100	7.16 ± 0.83
SRC	39.85 ± 5.68	13.82 ± 1.29	>100	>100	>100

halogen bond formation.[31] Less affinity of the **AQ15**, compared to the native ligand imatinib, may be attributed to the missing crucial bonding with Met318, Asp381, and Glu286 (Figure S58).

As shown, **AQ15** could be roughly superimposed on the pyridine-pyrimidine-amino-benzene segment of imatinib and lacks the piperazine-benzamide fragment (Figure 4). Presumably, **AQ15** is less active against ABL1, FYN A, LCK, and LYN B due to the lack of the piperazine-benzamide fragment and more potent against BTK and SRC owing to the lack of the piperazine-benzamide fragment.

The BCR-ABL and its downstream signaling pathway such as rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) play important roles in CML K562 cells. The K562 cells treated with **AQ15** or imatinib were evaluated for their inhibitory effects at 20 μM concentration after 6 hr of drug treatment (Figure 5). **AQ15** showed stronger inhibitory activity on phosphorylation of ERK than imatinib. It can be concluded that **AQ15** suppresses signaling downstream of tyrosine kinases.

Overall, the docking output is matched with the experimental data and both confirm that the mild inhibition of ABL kinase by **AQ15** is not the sole underlying mechanism for its pronounced cytotoxicity. As described in the Introduction, anticancer quinones target DNA.[1–4] To explain high toxicity of **AQ15** on CML, we next examined another mechanism of its action with some other compounds (**AQ11**, **AQ16**, and **AQ18**), involving metal-binding/oxygen-activating site of quinones, that is, oxidative cleavage of DNA strand, which was reported by many papers.[32] Thus, the DNA-cleaving capability of **AQ15** at 1 and 3 μM and **AQ11**, **AQ16**, and **AQ18** at 3 μM concentrations was studied using pUC19 DNA with and without the iron(II), hydrogen peroxide (H_2O_2), and ascorbic acid complex as shown in Figure 6. The reaction solution was incubated at 37°C for 2 hr and electrophoresis was performed at 100 V for 30 min. The DNA was stained with ethidium bromide and the gel image was captured by an electronic camera under ultraviolet radiation (UV). **AQ15** exhibits the strongest DNA-cleaving activity at 3 μM (Figure 6a), followed by **AQ11**, **AQ16**, and **AQ18**, respectively (Figure 6b). These results demonstrated that tested compounds may generate activated oxygen and cleaved DNA in a cell. In addition, these compounds may activate oxygen in the cytoplasm. It also points out that the induction of DNA may correlate with the cytotoxic activity against K562.

To assess the drug-likeness of **AQ15**, we used computational calculations of ADMET Predictor software to predict its potential risks and physicochemical properties. **AQ15** toxicity risk (TOX-Risk) value is 1 (acceptable value is up to 3.3). Mutagenic risk (MUT-Risk)

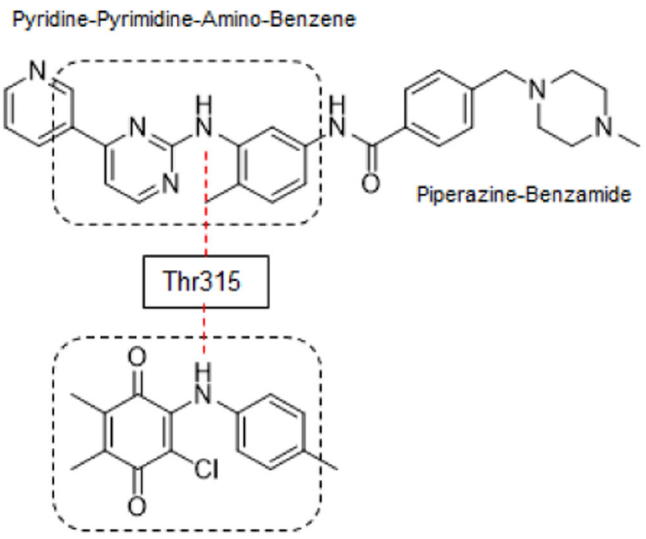


FIGURE 4 Comparison of the structure between imatinib and **AQ15** is shown below

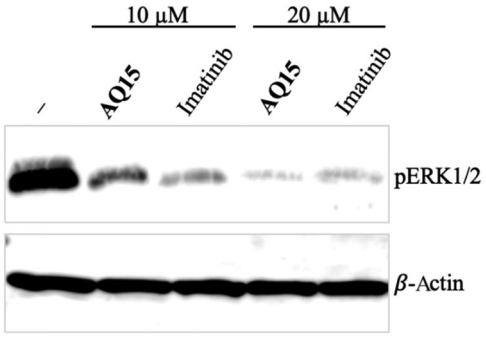


FIGURE 5 The effect of **AQ15** and imatinib on ERK signaling. K562 cells were incubated with tested compounds at 10 and 20 μM for 6 hr, and then immunoblot analysis was conducted. ERK, extracellular signal-regulated kinase

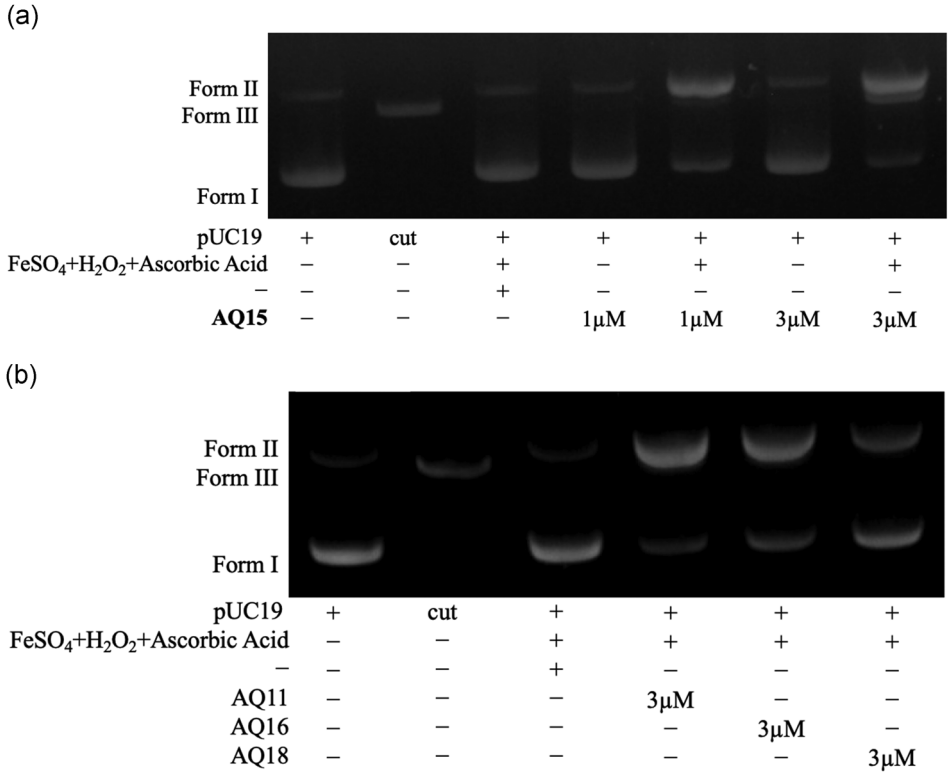


FIGURE 6 The DNA-cleaving activity of **AQ15** in the presence and absence of $FeSO_4$, H_2O_2, and ascorbic acid system (a) and comparison experiments using **AQ11**, **AQ16**, and **AQ18** (b)

value is 1.5, slightly exceeding the standard value 1. Risk related to metabolism by or inhibition of major cytochrome P450s (CYP-Risk) is 0.0 (standard value does not exceed 2.5). Noticeably, **AQ15** has an adequate safety profile. **AQ15** exhibits zero violation of Lipinski rule of 5 in terms of molecular weight, Log P, tPSA, number of H-bond donors and acceptors, and number of rotatable bonds. Moreover, absorption risk (Absn-Risk) is 1.0 (desirable value does not exceed 3.5) reflecting a high likelihood of good oral activity. In general, the overall ADMET-Risk value is 2.0 (acceptable value is up to 7.5). In conclusion, **AQ15** is predicted to have both favorable pharmacokinetic and safety profiles (Figure S57).

3 | CONCLUSION

In this study, we found a new amino-1,4-benzoquinone compound **AQ15** with high antiproliferative activity against CML cell line K562 among various plastoquinone analogs. The activity of **AQ15** (IC_{50} = 0.76 μM) surpassed imatinib (IC_{50} = 5.58 μM), and its selectivity index between K562 and PBMC of **AQ15** (10.05) was higher than imatinib (6.08), indicating a potentially enhanced safety profile. The results proved that the main mechanism of AQs anticancer activity is DNA cleavage but not inhibition of BCR-ABL kinase. DNA cleavage causes apoptosis of K562 via downstream signaling of the kinase, showing the unique activity of **AQ15** distinct from imatinib. Further investigation of plastoquinone analogs and their potential anti-CML activity is in progress.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

Melting points (mp) were uncorrected and recorded on a Buchi B-540 melting point apparatus. Thin-layer chromatograph (TLC) was purchased from Merck KGaA (Silica gel 60 F254) based on Merck DC-plates (aluminum-based). Compound visualization for TLC was achieved by UV light (254 nm). For column chromatography, silica gel 60 (63–200-μm particle-sized, 60–230 mesh; Merck) was used as the stationary phase. FTIR spectra were recorded as ATR on either a Thermo Scientific Nicolet 6700 spectrometer, an Alpha T FTIR spectrometer or a Perkin Elmer Spectrum 100 Optical FTIR spectrometer. NMR spectra were obtained as $CDCl_3$ solutions using either Bruker spectrometers with 400-MHz frequency for ^1H and 100-MHz frequency for ^{13}C NMR in ppm (δ) or Varian^{UNITY} INOVA spectrometers with 500 MHz frequency for ^1H and 125 MHz frequency for ^{13}C NMR in ppm (δ). ^1H NMR and ^{13}C NMR signals were reported relative to the solvent signal at δ 7.19 and δ 76.0 ppm, respectively. Chemical shifts (δ) were reported in parts per million (ppm). Coupling constants (J) are reported in Hz. Multiplicities were described using the following abbreviations: *s* (singlet), *br s* (broad singlet), *d* (doublet), *t* (triplet), *q* (quartet), *hept* (heptet), *m* (multiplet), and *td* (triplet of doublets). Mass spectra were recorded using a Thermo Finnigan LCQ Advantage MAX MS/MS spectrometer equipped with an ESI (electrospray ionization) source. HRMS were recorded by a JEOL JMSDX303HF using positive fast atom bombardment (FAB) with

3-nitrobenzyl alcohol as a matrix. The purity of the AQ analogs was analyzed by HPLC (Shimadzu/DGU-20A$_5$ HPLC apparatus fitted with a 25-cm Chiralpac AD-H chiral column) using hexane/2-propanol = 95:5 as the mobile phase with a flow rate of 1.0 ml/min. The purity of all analogs was ≥95%. Data for the single-crystal compounds were obtained with a Bruker APEX II QUAZAR three-circle diffractometer. Indexing was performed using APEX2.[33] Data integration and reduction were carried out with SAINT.[34] Absorption correction was performed by a multiscan method implemented in SADABS.[35] The Bruker SHELXTL[36] software package was used for structures solution and structures refinement. Aromatic C- and N-bound hydrogen atoms were positioned geometrically and refined using a riding mode. Crystal structure validations and geometrical calculations were performed using the Platon software.[37] Mercury software[38] was used for visualization of the .cif files. The precursors (**2**[25] and **3**[27]) were synthesized using the reported method in the literature. All substituted amines were commercially obtained from the commercial supplier and used without further purification unless specified otherwise.

The InChI codes of the investigated compounds are provided as Supporting Information.

4.1.2 | General procedure for the preparation of the AQ analogs[13a]

A mixture of the corresponding substituted amines (1.2 equiv) and quinone ([**2**, 0.50 g, 3.67 mmol] or [**3**, 0.50 g, 2.44 mmol]) in ethanol (25 ml) was stirred at reflux for 6–12 hr until consumption of the quinone. The reaction mixture was cooled to ambient temperature. After ethanol was evaporated under reduced pressure, the residue was dissolved with CH_2Cl_2 (50 ml), and the solution was washed sequentially with water (3 × 30 ml). The organic layer was dried over $CaCl_2$, filtered, and concentrated under reduced pressure, and the residue was purified by means of column chromatography on silica gel to give AQ analogs.

*2,3-Dimethyl-5-(phenylamino)-1,4-benzoquinone (**AQ1**)*

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and aniline (0.410 g, 1.2 equiv, 4.40 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ1** as a dark red solid. Yield: 191 mg, 23%, mp 109–110°C. FTIR (ATR) υ (cm^{-1}): 3,304 (NH), 3,058 ($CH_{aromatic}$), 2,962, 2,922 ($CH_{aliphatic}$), 1,642 (>C═O). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.95 (q, J = 1.5 Hz, 3H, CH_3), 1.96 (q, J = 1.0 Hz, 3H, CH_3), 6.05 (s, 1H, CH), 7.06 (t, J = 7.8 Hz, 1H, $CH_{aromatic}$), 7.10 (d, J = 7.8 Hz, 2H, $CH_{aromatic}$), 7.26–7.29 (m, 3H, [$CH_{aromatic}$ and NH]). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.0, 11.7 (CH_3), 99.8, 120.9, 124.1, 128.5, 135.6, 136.9, 141.6, 142.8 ($C_{aromatic}$ and C_q), 183.0, 185.4 (>C═O). MS (+ESI) m/z (%): 229 (14, $[M+2H]^+$), 228 (100, $[M+H]^+$). Anal. calcd. for $C_{14}H_{13}NO_2$ (227.26).

*2,3-Dimethyl-5-((2-(trifluoromethyl)phenyl)amino)-1,4-benzoquinone (**AQ2**)*

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and 2-trifluoromethylaniline (0.710 g, 1.2 equiv, 4.41 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ2** as a red solid. Yield: 324 mg, 30%, mp 111–112°C. FTIR (ATR) υ (cm^{-1}): 3,330 (NH), 2,959, 2,926 ($CH_{aliphatic}$), 1,639 (>C═O). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.98 (q, J = 1.1 Hz, 3H, CH_3), 1.99 (q, J = 1.1 Hz, 3H, CH_3), 5.91 (s, 1H, CH), 7.22 (t, J = 7.7 Hz, 1H, $CH_{aromatic}$), 7.38–7.42 (m, 2H, [NH and $CH_{aromatic}$]), 7.50 (t, J = 7.7 Hz, 1H, $CH_{aromatic}$), 7.62 (d, J = 7.8 Hz, 1H, $CH_{aromatic}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.1, 12.7 (CH_3), 101.6, 123.6 (q, $^1J_{CF}$ = 273.0 Hz, CF_3), 123.9 (q, $^2J_{CF}$ = 30.0 Hz, C_q), 125.0, 125.4, 127.2 (q, $^3J_{CF}$ = 5.2 Hz), 132.9, 136.0, 137.0, 143.0, 143.6 ($C_{aromatic}$ and C_q), 183.4, 186.6 (>C═O). MS (+ESI) m/z (%): 297 (17, $[M+2H]^+$), 296 (100, $[M+H]^+$). HRFABMS: Calcd. for $C_{15}H_{13}F_3NO_2$ $[M+H]^+$: 296.0898; Found: 296.0889.

*2,3-Dimethyl-5-((3-(trifluoromethyl)phenyl)amino)-1,4-benzoquinone (**AQ3**)*

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and 3-trifluoromethylaniline (0.710 g, 1.2 equiv, 4.41 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ3** as a red solid. Yield: 271 mg, 25%, mp 142–144°C. FTIR (ATR) υ (cm^{-1}): 3,297 (NH), 3,054 ($CH_{aromatic}$), 2,963 ($CH_{aliphatic}$), 1,644 (>C═O). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.97 (q, J = 1.0 Hz, 3H, CH_3), 1.98 (q, J = 1.0 Hz, 3H, CH_3), 6.07 (s, 1H, CH), 7.32 (d, J = 7.8 Hz, 2H, $CH_{aromatic}$), 7.36 (s, 1H, $CH_{aromatic}$), 7.38 (br s, 1H, NH), 7.42 (t, J = 7.8 Hz, 1H, $CH_{aromatic}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.0, 11.7 (CH_3), 100.6, 117.6, 120.4, 122.6 (q, $^1J_{CF}$ = 272.7 Hz, CF_3), 123.7, 129.2, 131.2 (q, $^2J_{CF}$ = 33.0 Hz, C_q), 135.9, 137.7, 141.1, 142.9 ($C_{aromatic}$ and C_q), 182.7, 185.4 (>C═O). MS (−ESI) m/z (%): 295 (15, $[M]^-$), 294 (100, $[M-H]^-$). HRFABMS: Calcd. for $C_{15}H_{13}F_3NO_2$ $[M+H]^+$: 296.0898; Found: 296.0894.

*2,3-Dimethyl-5-((4-(trifluoromethyl)phenyl)amino)-1,4-benzoquinone (**AQ4**)*

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and 4-trifluoromethylaniline (0.710 g, 1.2 equiv, 4.41 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:1) eluent to furnish **AQ4** as a red solid. Yield: 311 mg, 29%, mp 179–181°C. FTIR (ATR) υ (cm^{-1}): 3,243 (NH), 3,058 ($CH_{aromatic}$), 2,962 ($CH_{aliphatic}$), 1,645 (>C═O). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.98–2.01 (m, 6H, CH_3), 6.18 (s, 1H, CH), 7.22 (d, J = 8.8 Hz, 2H, $CH_{aromatic}$), 7.39 (br s, 1H, NH), 7.56 (d, J = 8.3 Hz, 2H, $CH_{aromatic}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.1, 11.7 (CH_3), 101.5, 119.9, 122.9 (q, $^1J_{CF}$ = 271.6 Hz, CF_3), 125.4 (q, $^3J_{CF}$ = 33.1 Hz, C_q), 125.8 (q, $^2J_{CF}$ = 3.8 Hz, CH), 136.0, 140.5, 142.8 ($C_{aromatic}$ and C_q), 182.6, 185.5 (>C═O). MS (−ESI) m/z (%): 295 (15, $[M]^-$), 294 (100, $[M-H]^-$). HRFABMS: Calcd. for $C_{15}H_{13}F_3NO_2$ $[M+H]^+$: 296.0898; Found: 296.0901.

*2,3-Dimethyl-5-(m-tolylamino)-1,4-benzoquinone (**AQ5**)*

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and *m*-toluidine (0.472 g, 1.2

equiv, 4.40 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ5** as a red solid. Yield: 227 mg, 26%, mp 98–100°C. FTIR (ATR) υ (cm^{-1}): 3,311 (NH), 3,047 ($CH_{aromatic}$), 2,920 ($CH_{aliphatic}$), 1,641 (>C=O). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.96 (q, J = 1.0 Hz, 3H, CH_3), 1.98 (q, J = 1.0 Hz, 3H, CH_3), 2.27 (s, 3H, CH_3), 6.06 (s, 1H, CH), 6.88–693 (m, 3H, $CH_{aromatic}$), 7.17 (t, J = 7.8 Hz, 1H, $CH_{aromatic}$), 7.23 (br s, 1H, NH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.1, 11.7, 20.4 (CH_3), 99.6, 117.9, 121.4, 124.8, 128.3, 135.6, 136.8, 138.6, 141.6, 142.9 ($C_{aromatic}$ and C_q), 183.0, 185.5 (>C=O). MS (+ESI) m/z (%): 243 (14, $[M+2H]^+$), 242 (100, $[M+H]^+$). HRFABMS: Calcd. for $C_{15}H_{16}NO_2$ $[M+H]^+$: 242.1181; Found: 242.1121.

*2,3-Dimethyl-5-(p-tolylamino)-1,4-benzoquinone (**AQ6**)*[28a]

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and *m*-toluidine (0.472 g, 1.2 equiv, 4.40 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ6** as a dark purple solid. Yield: 185 mg, 21%, mp 110–111°C. FTIR (ATR) υ (cm^{-1}): 3,301 (NH), 3,054 ($CH_{aromatic}$), 2,962, 2,919 ($CH_{aliphatic}$), 1,641 (>C=O). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.98 (q, J = 1.0 Hz, 3H, CH_3), 1.99 (q, J = 1.0 Hz, 3H, CH_3), 2.27 (s, 3H, CH_3), 6.00 (s, 1H, CH), 7.01 (d, J = 8.8 Hz, 2H, $CH_{aromatic}$), 7.11 (d, J = 8.3 Hz, 2H, $CH_{aromatic}$), 7.19 (br s, 1H, NH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.1, 11.8, 20.0 (CH_3), 99.3, 121.1, 129.1, 134.0, 134.2, 135.5, 141.9, 143.0 ($C_{aromatic}$ and C_q), 183.1, 185.4 (>C=O). MS (+ESI) m/z (%): 243 (16, $[M+2H]^+$), 242 (100, $[M+H]^+$). Anal. calcd. for $C_{15}H_{15}NO_2$ (241.29).

*5-((2-Isopropylphenyl)amino)-2,3-dimethyl-1,4-benzoquinone (**AQ7**)*

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and 2-isopropylaniline (0.595 g, 1.2 equiv, 4.40 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ7** as a purple oil. Yield: 326 mg, 33%. FTIR (ATR) υ (cm^{-1}): 3,357 (NH), 3,063 ($CH_{aromatic}$), 2,963, 2,926, 2,870 ($CH_{aliphatic}$), 1,641 (>C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.23 (s, 3H, CH_3), 1.24 (s, 3H, CH_3), 2.08 (s, 6H, CH_3), 3.07 (hept, J = 6.8 Hz, 1H, CH), 5.70 (s, 1H, CH), 7.14 (br s, 1H, NH), 7.20–7.29 (m, 3H, $CH_{aromatic}$), 7.35–7.40 (m, 1H, $CH_{aromatic}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.1, 12.9, 23.2 (CH_3), 28.2 (CH), 99.9, 125.4, 126.6, 126.8, 127.2, 134.4, 136.4, 143.7, 144.1, 144.8 ($C_{aromatic}$ and C_q), 184.2, 186.3 (>C=O). MS (+ESI) m/z (%): 271 (20, $[M+2H]^+$), 270 (100, $[M+H]^+$). HRFABMS: Calcd. for $C_{17}H_{20}NO_2$ $[M+H]^+$: 270.1494; Found: 270.1467.

*5-((4-Isopropylphenyl)amino)-2,3-dimethyl-1,4-benzoquinone (**AQ8**)*[29]

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and 4-isopropylaniline (0.595 g, 1.2 equiv, 4.40 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ8** as a purple solid. Yield: 107 mg, 11%, mp 104–105°C. FTIR (ATR) υ (cm^{-1}): 3,337 (NH), 2,957, 2,922, 2,870 ($CH_{aliphatic}$), 1,646 (>C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.17 (s, 3H, CH_3), 1.19 (s, 3H, CH_3), 1.98 (q, J = 1.2 Hz, 3H, CH_3), 1.99 (q, J = 1.2 Hz, 3H, CH_3), 2.83 (hept, J = 6.9 Hz, 1H, CH), 6.03 (s, 1H, CH), 7.05 (d, J = 8.3 Hz, 2H, $CH_{aromatic}$), 7.16 (d, J = 8.4 Hz, 2H, $CH_{aromatic}$), 7.21 (br s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.1, 12.8, 24.0 (CH_3), 33.7 (CH), 100.3, 122.2, 127.5, 135.4, 136.5, 142.9, 144.0, 146.1 ($C_{aromatic}$ and C_q), 184.2, 186.5 (>C=O). MS (+ESI) m/z (%): 271 (27, $[M+2H]^+$), 270 (100, $[M+H]^+$). Anal. calcd. for $C_{17}H_{19}NO_2$ (269.34).

*5-((4-(Diethylamino)phenyl)amino)-2,3-dimethyl-1,4-benzoquinone (**AQ9**)*

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and *N,N*-diethyl-*p*-phenylenediamine (0.723 g, 1.2 equiv, 4.40 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ9** as a black solid. Yield: 155 mg, 14%, mp 125–126°C. FTIR (ATR) υ (cm^{-1}): 3,294 (NH), 2,967, 2,922 ($CH_{aliphatic}$), 1,640 (>C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.09 (t, J = 7.1 Hz, 6H, CH_3), 1.96 (q, J = 1.2 Hz, 3H, CH_3), 1.98 (q, J = 1.2 Hz, 3H, CH_3), 3.28 (q, J = 7.0 Hz, 4H, NCH_2), 5.88 (s, 1H, CH), 6.58 (d, J = 8.5 Hz, 2H, $CH_{aromatic}$), 6.98 (d, J = 8.8 Hz, 2H, $CH_{aromatic}$), 7.13 (br s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.0, 12.5, 12.9 (CH_3), 44.5 (NCH_2), 98.9, 112.2, 124.3, 125.5, 136.2, 143.7, 144.3, 145.8 ($C_{aromatic}$ and C_q), 184.4, 186.1 (>C=O). MS (+ESI) m/z (%): 300 (20, $[M+2H]^+$), 299 (100, $[M+H]^+$), 298 (4, $[M]^+$). HRFABMS: Calcd. for $C_{18}H_{22}N_2O_2$ $[M]^+$: 298.1681; Found: 298.1676.

*2,3-Dimethyl-5-((pyridin-2-ylmethyl)amino)-1,4-benzoquinone (**AQ10**)*[30]

The title compound was synthesized according to the general method from compound **2** (1 equiv, 3.67 mmol) and 2-picolylamine (0.477 g, 1.2 equiv, 4.41 mmol), the crude residue was purified by column chromatography using initially petroleum ether and subsequently petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ10** as a red solid. Yield: 89 mg, 10%, mp 126–128°C. FTIR (ATR) υ (cm^{-1}): 3,344 (NH), 3,055 ($CH_{aromatic}$), 2,918 ($CH_{aliphatic}$), 1,640 (>CυO). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.94 (q, J = 1.5 Hz, 3H, CH_3), 1.96 (q, J = 1.5 Hz, 3H, CH_3), 4.3 (d, J = 5.4 Hz, 2H, CH_2), 5.39 (s, 1H, CH), 6.71 (br s, 1H, NH), 7.14–7.17 (m, 2H, $CH_{aromatic}$), 7.60 (td, J = 7.8 and 2.0 Hz, 1H, $CH_{aromatic}$), 8.51–8.53 (m, 1H, $CH_{aromatic}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 10.9, 11.8 (CH_3), 46.1 (CH_2), 97.7, 120.5, 121.6, 135.4, 135.8, 143.0, 145.1, 148.4, 154.0 ($C_{aromatic}$ and C_q), 182.7, 184.6 (>C=O). MS (+ESI) m/z (%): 243 (100, $[M+H]^+$), 242 (3, $[M]^+$), 241 (12, $[M-H]^+$). Anal. calcd. for $C_{14}H_{14}N_2O_2$ (242.27).

*2-Chloro-5,6-dimethyl-3-(phenylamino)-1,4-benzoquinone (**AQ11**)*

The title compound was synthesized according to the general method from compound **3** (1 equiv, 2.44 mmol) and aniline (0.273 g, 1.2 equiv, 2.93 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ11** as a claret red oil. Yield: 451 mg, 71%. FTIR (ATR) υ (cm^{-1}): 3,237 (NH), 3,064 ($CH_{aromatic}$), 2,961, 2,923 ($CH_{aliphatic}$), 1,660 (>C=O). ^{1}H NMR

(500 MHz, $CDCl_3$) δ (ppm): 2.00 (q, J = 1.5 Hz, 3H, CH_3), 2.06 (q, J = 1.5 Hz, 3H, CH_3), 6.95 (d, J = 7.8 Hz, 2H, $CH_{aromatic}$), 7.10 (t, J = 7.8 Hz, 1H, $CH_{aromatic}$), 7.25 (t, J = 7.8 Hz, 2H, $CH_{aromatic}$), 7.30 (br s, 1H, NH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.3, 12.4 (CH_3), 110.4, 123.1, 124.2, 127.4, 135.9, 136.5, 138.4, 142.9 ($C_{aromatic}$ and C_q), 178.7, 181.7 (>C═O). MS (−ESI) m/z (%): 262 (30, $[M+H]^-$), 261 (10, $[M]^-$), 260 (100, $[M-H]^-$). HRFABMS: Calcd. for $C_{14}H_{12}ClN_2O_2$ $[M]^+$: 262.0635; Found: 262.0634.

*2-Chloro-5,6-dimethyl-3-((3-(trifluoromethyl)phenyl)amino)-1,4-benzoquinone (**AQ12**)*

The title compound was synthesized according to the general method from compound **3** (1 equiv, 2.44 mmol) and 3-trifluoromethylaniline (0.472 g, 1.2 equiv, 2.93 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ12** as a claret red solid. Yield: 449 mg, 56%, mp 141–142°C. FTIR (ATR) υ (cm^{-1}): 3,230 (NH), 3,050 ($CH_{aromatic}$), 2,918 ($CH_{aliphatic}$), 1,661 (>C═O). ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 2.01–2.02 (m, 3H, CH_3), 2.07–2.08 (m, 3H, CH_3), 7.09 (d, J = 7.3 Hz, 1H, $CH_{aromatic}$), 7.17 (s, 1H, $CH_{aromatic}$), 7.30 (br s, 1H, NH), 7.33–7.39 (m, 2H, $CH_{aromatic}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.3, 12.4 (CH_3), 112.1, 119.3, 120.5 (q, $^3J_{CF}$ = 3.9 Hz), 122.7 (q, $^1J_{CF}$ = 272.4 Hz), 125.5, 127.8 (q, $^3J_{CF}$ = 5.3 Hz), 130.0 (q, $^2J_{CF}$ = 32.7 Hz), 136.3, 137.1, 137.9, 142.8 ($C_{aromatic}$ and C_q), 178.6, 181.4 (>C═O). MS (−ESI) m/z (%): 331 (69, $[M+2H]^-$), 330 (58, $[M+H]^-$), 329 (100, $[M]^-$). HRFABMS: Calcd. for $C_{15}H_{12}ClF_3NO_2$ $[M+H]^+$: 330.0509; Found: 330.0507.

*2-Chloro-5,6-dimethyl-3-((4-(trifluoromethyl)phenyl)amino)-1,4-benzoquinone (**AQ13**)*

The title compound was synthesized according to the general method from compound **3** (1 equiv, 2.44 mmol) and 4-trifluoromethylaniline (0.472 g, 1.2 equiv, 2.93 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ13** as a claret red oil. Yield: 331 mg, 41%. FTIR (ATR) υ (cm^{-1}): 3,233 (NH), 2,962, 2,918, 2,849 ($CH_{aliphatic}$), 1,661 (>C═O). ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 2.02 (q, J = 1.5 Hz, 3H, CH_3), 2.07 (q, J = 1.5 Hz, 3H, CH_3), 6.97 (d, J = 8.3 Hz, 2H, $CH_{aromatic}$), 7.29 (br s, 1H, NH), 7.50 (d, J = 8.8 Hz, 2H, $CH_{aromatic}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.3, 12.4 (CH_3), 113.0, 118.0, 121.7, 120.0 (q, $^1J_{CF}$ = 271.7 Hz), 124.6 (q, $^3J_{CF}$ = 3.7 Hz), 124.6 (q, $^2J_{CF}$ = 32.7 Hz), 136.5, 137.7, 139.6, 142.7 ($C_{aromatic}$ and C_q), 178.6, 181.4 (>C═O). MS (−ESI) m/z (%): 330 (35, $[M+H]^-$), 329 (29, $[M]^-$), 328 (100, $[M-H]^-$). HRFABMS: Anal. calcd. for $C_{15}H_{11}ClF_3NO_2$ $[M+Na]^+$: 352.0328; Found: 352.0335.

*2-Chloro-5,6-dimethyl-3-(m-tolylamino)-1,4-benzoquinone (**AQ14**)*

The title compound was synthesized according to the general method from compound **3** (1 equiv, 2.44 mmol) and *m*-toluidine (0.314 g, 1.2 equiv, 2.93 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ14** as a purple solid. Yield: 277 mg, 41%, mp 141–143°C. FTIR (ATR) υ (cm^{-1}): 3,236 (NH), 3,022 ($CH_{aromatic}$), 2,922, 2,857 ($CH_{aliphatic}$), 1,661 (>C═O). ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.98 (q, J = 1.5 Hz, 3H, CH_3), 2.04 (q, J = 1.5 Hz, 3H, CH_3), 2.26 (s, 3H, CH_3), 6.73–6.75 (m, 2H, $CH_{aromatic}$), 6.90 (d, J = 7.8 Hz, 1H, $CH_{aromatic}$), 7.11 (t, J = 7.8 Hz, 1H, $CH_{aromatic}$), 7.28 (br s, 1H, NH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.2, 12.4, 20.4 (CH_3), 110.2, 120.1, 123.6, 125.0, 127.0, 135.9, 136.3, 137.3, 138.4, 142.8 ($C_{aromatic}$ and C_q), 178.6, 181.7 (>C═O). MS (−ESI) m/z (%): 277 (4, $[M+2H]^-$), 276 (31, $[M+H]^-$), 275 (13, $[M]^-$), 274 (100, $[M-H]^-$); MS (+ESI) m/z (%): 276 (100, $[M+H]^+$). HRFABMS: Calcd. for $C_{15}H_{15}ClNO_2$ $[M+H]^+$: 276.0791; Found: 276.0787.

*2-Chloro-5,6-dimethyl-3-(p-tolylamino)-1,4-benzoquinone (**AQ15**)*

The title compound was synthesized according to the general method from compound **3** (1 equiv, 2.44 mmol) and *p*-toluidine (0.314 g, 1.2 equiv, 2.93 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ15** as a dark purple solid. Yield: 251 mg, 37%, mp 148–149°C. FTIR (ATR) υ (cm^{-1}): 3,239 (NH), 3,023 ($CH_{aromatic}$), 2,961, 2,923 ($CH_{aliphatic}$), 1,664 (>C═O). ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.97 (q, J = 1.0 Hz, 3H, CH_3), 2.04 (q, J = 1.0 Hz, 3H, CH_3), 2.26 (s, 3H, CH_3), 6.83 (d, J = 8.3 Hz, 2H, $CH_{aromatic}$), 7.03 (d, J = 8.3 Hz, 2H, $CH_{aromatic}$), 7.27 (br s, 1H, NH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.2, 12.4, 20.0 (CH_3), 109.6, 123.2, 127.8, 133.9, 134.2, 135.8, 138.5, 142.9 ($C_{aromatic}$ and C_q), 178.6, 181.7 (>C═O). MS (−ESI) m/z (%): 276 (40, $[M+H]^-$), 275 (15, $[M]^-$), 274 (100, $[M-H]^-$). HRFABMS: Calcd. for $C_{15}H_{15}ClNO_2$ $[M+H]^+$: 276.0791; Found: 276.0781.

*2-Chloro-3-((2-isopropylphenyl)amino)-5,6-dimethyl-1,4-benzoquinone (**AQ16**)*

The title compound was synthesized according to the general method from compound **3** (1 equiv, 2.44 mmol) and 2-isopropylaniline (0.396 g, 1.2 equiv, 2.93 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ16** as a dark red oil. Yield: 403 mg, 54%. FTIR (ATR) υ (cm^{-1}): 3,325 (NH), 2,962, 2,926, 2,867 ($CH_{aliphatic}$), 1,656 (>C═O). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.15 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 2.00 (q, J = 1.2 Hz, 3H, CH_3), 2.06 (q, J = 1.2 Hz, 3H, CH_3), 3.08 (hept, J = 6.8 Hz, 1H, CH), 6.89 (d, J = 7.8 Hz, 1H, $CH_{aromatic}$), 7.07 (td, J = 7.6, 1.7 Hz, 1H, $CH_{aromatic}$), 7.12–7.17 (m, 3H, $CH_{aromatic}$ and NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.3, 13.5, 22.9 (CH_3), 28.5 (CH), 109.5, 125.5, 125.8, 126.9, 127.1, 135.1, 136.6, 140.3, 143.8, 144.2 ($C_{aromatic}$ and C_q), 179.7, 182.7 (>C═O). MS (+ESI) m/z (%): 305 (18, $[M+2H]^+$), 304 (100, $[M+H]^+$). HRFABMS: Calcd. for $C_{17}H_{19}ClNO_2$ $[M+H]^+$: 304.1104; Found: 304.1090.

*2-Chloro-3-((4-isopropylphenyl)amino)-5,6-dimethyl-1,4-benzoquinone (**AQ17**)*

The title compound was synthesized according to the general method from compound **3** (1 equiv, 2.44 mmol) and 4-isopropylaniline (0.396 g, 1.2 equiv, 2.93 mmol), the crude residue was purified by column chromatography using petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ17** as a dark purple solid. Yield: 292 mg, 39%, mp 105–106°C. FTIR (ATR) υ (cm^{-1}): 3,224 (NH), 2,959, 2,922, 2,870 ($CH_{aliphatic}$), 1,668 (>C═O). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.16 (s, 3H, CH_3), 1.18 (s, 3H, CH_3), 1.97 (q, J = 1.2 Hz, 3H, CH_3), 2.04 (q, J = 1.2 Hz, 3H, CH_3),

2.82 (hept, J = 6.9 Hz, 1H, CH), 6.87 (d, J = 8.3 Hz, 2H, $CH_{aromatic}$), 7.09 (d, J = 8.3 Hz, 2H, $CH_{aromatic}$), 7.31 (br s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.3, 13.5, 24.0 (CH_3), 33.6 (CH), 110.6, 124.1, 126.3, 135.1, 136.8, 139.5, 143.9, 146.2 ($C_{aromatic}$ and C_q), 179.7, 182.8 (>C═O). MS (+ESI) m/z (%): 305 (19, $[M+2H]^+$), 304 (100, $[M+H]^+$). HRFABMS: Calcd. for $C_{17}H_{19}ClNO_2$ $[M+H]^+$: 304.1104; Found: 304.1064.

*2-Chloro-5,6-dimethyl-3-((pyridin-2-ylmethyl)amino)-1,4-benzoquinone (**AQ18**)*

The title compound was synthesized according to the general method from compound **3** (1 equiv, 2.44 mmol) and 2-picolylamine (0.317 g, 1.2 equiv, 2.93 mmol), the crude residue was purified by column chromatography using initially petroleum ether and subsequently petroleum ether/$CHCl_3$ (1:2) eluent to furnish **AQ18** as a dark purple solid. Yield: 287 mg, 35%, mp 143–144°C. FTIR (ATR) υ (cm^{-1}): 3,242 (NH), 3,055 ($CH_{aromatic}$), 2,962, 2,916 ($CH_{aliphatic}$), 1,667 (>C═O). ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.95 (q, J = 1.0 Hz, 3H, CH_3), 2.03 (q, J = 1.0 Hz, 3H, CH_3), 5.00 (d, J = 5.4 Hz, 2H, CH_2), 7.16–7.20 (m, 2H, $CH_{aromatic}$), 7.31 (br s, 1H, NH), 7.63 (td, J = 7.3 and 2.0 Hz, 1H, $CH_{aromatic}$), 8.55 (d, J = 4.4 Hz, 1H, $CH_{aromatic}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.1, 12.4 (CH_3), 47.4 (CH_2), 120.8, 121.6, 135.2, 135.9, 141.3, 142.8, 148.1, 154.5 ($C_{aromatic}$ and C_q), 178.1, 181.6 (>C═O). MS (−ESI) m/z (%): 278 (11, $[M+2H]^-$), 277 (24, $[M+H]^-$), 276 (43, $[M]^-$), 275 (100, $[M-H]^-$); MS (+ESI) m/z (%): 278 (14, $[M+2H]^+$), 277 (100, $[M+H]^+$). HRFABMS: Calcd. for $C_{14}H_{14}ClN_2O_2$ $[M+H]^+$: 277.0744; Found: 277.0769.

4.2 | Biological assays

4.2.1 | Cell culture and drug treatment

Briefly, the K562, Jurkat, and MT-2 leukemia cell lines were cultured in Rosewell Park Memorial Institute (RPMI) 1640 (Wako Pure Chemical Industries, Osaka, Japan) medium with 10% fetal bovine serum (FBS; Sigma-Aldrich, MO). PBMCs (Precision Bioservices, Frederic, MD) were incubated in RPMI 1640 medium with 10% FBS. All media were supplemented with 89 μM/ml streptomycin (Meiji Seika Pharma, Tokyo, Japan) in a humid atmosphere at 37°C and 5% CO_2. In experiments, the leukemia cells and PBMCs were incubated in 24-well and 96-well culture plates (Iwaki brand Asahi Glass Co., Chiba, Japan) at 2×10^4 and 1×10^6 cells/ml concentration, respectively, for 24 hr. The stock solution of compounds and Imatinib (Wako Pure Chemical Industries) in concentrations between 0.3–3 mM were prepared in DMSO (Wako Pure Chemical Industries) and then were added to the fresh culture medium. The concentration of DMSO in the final culture medium was 1%.[39]

4.2.2 | Cytotoxicity assay

The MTT test was performed as previously described in the literature with small modifications.[40] The tested compounds were cultured with cells in different concentrations (0.3–30 μM) for 24 hr and then, MTT (Dojindo Molecular Technologies, Kumamoto, Japan) was added to cells. After 4 hr of incubation, the medium was taken out and 100 μl DMSO was added to each well. The absorbance at 550 nm was measured using a microplate reader Infinitive M1000 (Tecan, Mannedorf, Switzerland) with background subtraction at 630 nm. All experiments were run in triplicate and IC_{50} values were estimated from the results of the MTT test described as the drug concentrations that reduced absorbance to 50% of control values.

4.2.3 | Detection of cell death

Apoptotic/necrotic/healthy detection kit (PromoKine, Heidelberg, Germany) was performed according to PromoKine's instructions with the modifications.[41] Briefly, K562 cells were treated with **AQ15** and imatinib at IC_{50} concentrations for 6 hr. Then, the cells were harvested and washed with phosphate-buffered saline (PBS) and stained with 4 μl of FITC–Annexin V, 4 μl of ethidium homodimer III and 4 μl of Hoechst 33342 in 1× binding buffer for 30 min at room temperature in the dark. The cells were analyzed by a fluorescence microscope Biorevo Fluorescence BZ-9000 (Keyence, Osaka, Japan). The number of apoptotic cells (Annexin V), late apoptotic or necrotic cells (Annexin V and ethidium homodimer III), and necrotic cells (ethidium homodimer III) were counted as previously described.[42]

4.2.4 | Tyrosine kinase assay

The kinase inhibition assay system (TK-2; Promega Corporation, Madison, WI) was performed as previously described with some modification.[43] In this system, eight kinase strips (ABL1, BRK, BTK, CSK, FYN A, LCK, LYN B, and SRC) and their substrates were diluted with 2.5× kinase reaction buffer (95 μl) and 100 μM ATP (15 μl) solution, respectively. The reaction of kinases was performed in the 384-well plate using 2 μl of the compound solution at multiple concentrations in a buffer, 4 μl of kinase working stock and 4 μl of ATP/substrate working stock. After 1 hr of incubation at room temperature, the ADP-Glo Kinase Assay (Promega Corporation) protocol was employed and inhibitory kinase activity of the test compounds was determined as previously described.[44]

4.2.5 | Immunoblot analysis

The K562 cells were incubated in the presence of 10 and 20 μM of **AQ15** and imatinib for 6 hr and then lysed in PBS-Laemmli sample buffer. Immunoblot analysis using phosphospecific-p44/42 MAPK (Erk1/2; Thr202/Tyr204; D13.14.4E) XP rabbit mAb (1:1,000; Cell Signaling Technology, Danvers, MA) or anti-β-actin clone AC-15 (Sigma-Aldrich) was conducted. For immunoreactivity detection, chemiluminescence method was performed.[45]

4.2.6 | DNA-cleaving activity

The DNA cleavage activities of the compounds on supercoiled plasmid pUC19 DNA were studied by gel electrophoresis. pUC19 DNA (2 μg) was dissolved in water and Tris/boric acid (Nacalai Tesque, Kyoto, Japan) buffer (10 mM, pH 8.5) in the presence and absence of iron(II)

sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$; 30 μM; Wako Pure Chemical Industries), H_2O_2 (30 μM; Tokyo Chemical Industry, Tokyo, Japan) and ascorbic acid (30 μM; Tokyo Chemical Industry) as an activator and mixed with the different concentration of compounds. The reaction was incubated at 37°C for 2 hr and then mixed with the loading buffer (Takara, Kyoto, Japan). Agarose gel electrophoresis was undertaken for 30 min at 100 V in Tris-acetate/ethylenediaminetetraacetic acid buffer. The gel (1% slab) was stained with ethidium bromide (Wako Pure Chemical Industries) and then DNA was visualized by photographing the fluorescence of intercalated ethidium bromide under a UV illuminator (Nippon Genetics, Tokyo, Japan).

4.3 | Molecular docking simulation and ADMET prediction

The crystal structure of the ABL kinase domain in complex with imatinib was retrieved from the RCSB Brookhaven Protein Data Bank (PDB: 1IEP); **AQ15** was built by ChemDraw Professional 15.1. Before docking simulations, **AQ15** and 1IEP were prepared as previously described.[46] MOE 2018.01 software (Chemical Computing Group, Montreal, Canada) was employed for preparation, interactive docking, visualization and analysis procedures using its default parameters.[47] The ADMET properties were calculated in silico using ADMET Predictor 9.0 from Simulation Plus, Inc.; the risk models were previously explained in details.[48]

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data can be obtained available free of charge from http://www.ccdc.cam.ac.uk/conts/retrieving.html or the Cambridge Crystallographic Data Centre (CCDC).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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