Vicinal Diaryl-Substituted Isoxazole and Pyrazole Derivatives with \textit{In Vitro} Growth Inhibitory and \textit{In Vivo} Antitumor Activity

Sümeyye Turanlı, Esra Nalbat, Deniz Lengerli, Kübra İbiş, Sezen Güntekin Ergün, Ece Akhan Güzelcan, Mesut Muyan, Rengül Cetin-Atalay, Burcu Çalışkan, and Erden Banoglu*

\textbf{ABSTRACT:} The vicinal diaryl heterocyclic framework has been widely used for the development of compounds with significant bioactivities. In this study, a series of diaryl heterocycles were designed and synthesized based on an in-house diaryl isoxazole derivative, and most of the newly synthesized derivatives demonstrated moderate to good antiproliferative activities against a panel of hepatocellular carcinoma and breast cancer cells, exemplified with the diaryl isoxazole and the diaryl pyrazole derivative \textit{in vivo} in the Mahlavu hepatocellular carcinoma and the MDA-MB-231 breast cancer xenograft models, indicating that these compounds could be considered as leads for further development of antitumor agents. Important structural features of this compound class for the antitumor activity have also been proposed, which warrant further exploration to guide the design of new and more potent diaryl heterocycles.

\section{1. INTRODUCTION}

Vicinal diaryl-substituted heterocycles can be considered privileged scaffolds as they form the core structure of a number of compounds with diverse biological properties such as anticancer, antiviral, and anti-inflammatory activities. Therefore, the use of this scaffold incorporating different central heterocycles could be pivotal in the drug development practice since they exist in the structures of various clinical therapeutics or compounds with therapeutic potential, which establishes their value as chemical tools in the field of medicinal chemistry (Figure 1). For example, the first COX-2 selective nonsteroidal anti-inflammatory drug celecoxib (1) was a member of vicinal diaryl pyrazole derivatives, which stimulated the development of latter COX-2 inhibitors belonging to the vicinal diaryl heterocyclic class such as rofecoxib and valdecoxib. Another vicinal diaryl pyrazole derivative developed as an antiobesity drug was rimonabant. Oxaprozin (3) and licofelone (4) are anti-inflammatory drugs for the treatment of osteo- and rheumatoid arthritis, also belonging to a vicinal diaryl oxazole and pyrrolizidine class, respectively.

Furthermore, a considerable number of vicinal diaryl heterocyclic motifs spread throughout the literature with various biological activities. For example, a 4,5-diaryl isoxazole derivative BRP-187 (5) was reported to be a 5-lipoxygenase-activating protein (FLAP) inhibitor with potent anti-inflammatory activity. The 3,4-diarylprrazole derivative mardepodect (6, PF-2545920) was discovered as a phosphodiesterase (PDE) 10A inhibitor, which progressed through Phase-II clinical trials for Schizophrenia treatment, and was used as a model compound leading to a considerable number of follow-up PDE10A inhibitors having vicinal diaryl heterocyclic framework. In addition, a good number of vicinal diaryl-substituted heterocycles were studied as anticancer therapeutics in which the vicinal diaryl scaffold was shown to be a simple pharmacophore for tubulin polymerization inhibitors mimicking the features of combretastatin A4 with potent anticancer activity as exemplified with combretastatine (7). In addition, 4,5-diarylisoazole luminespib (8, NVP-AUY922) was developed as an Hsp90 inhibitor for the treatment of several cancer types and reached Phase-II clinical trials.

Breast cancer (BC) is the most commonly occurring cancer in women and the most common cancer overall, while hepatocellular carcinoma (HCC) is the sixth most common and the second most lethal cancer. A large number of treatment options including localized and/or systematic
therapies are available for both cancer types. Despite the initial effectiveness in controlling tumor growth and prolonging patient survival, nearly all current treatments result in resistance to therapies. This necessitates the development of novel approaches as well as effective chemotherapeutic agents for cancer treatments. We have long been working with the vicinal diaryl heterocyclic skeleton toward novel anti-inflammatory, antiplatelet, and anticancer agents.8,9,15−20 In this context, inspired by the therapeutic potential of the vicinal diaryl motif around a central heterocycle, our compiled library of compounds belonging to this promising class was screened for their potential cytotoxic activity against an HCC cell line. Compound 9 with 3,4-diaryl isoxazole motif with synthetic accessibility for compound library generation was identified, although the cytotoxicity against HCC cells was negligible ($IC_{50} \geq 20 \mu M$) (Figure 1, Table 1). However, encouraged by the anticancer potential of vicinal diaryl heterocyclic scaffold and the synthetic feasibility of compound 9 skeleton, a focused library of compounds belonging to this promising class was screened for their potential cytotoxic activity against an HCC cell line. Compound 9 with 3,4-diaryl isoxazole motif with synthetic accessibility for compound library generation was identified, although the cytotoxicity against HCC cells was negligible ($IC_{50} \geq 20 \mu M$) (Figure 1, Table 1). However, encouraged by the anticancer potential of vicinal diaryl heterocyclic scaffold and the synthetic feasibility of compound 9 skeleton, a focused library of compounds around compound 9 was prepared by decorating the vicinal diaryl rings and by incorporating various heterocyclic rings as the central core, which resulted in new analogues with improved cytotoxicity against both HCC and BC cancer cell lines. Here, we report the biological assessment of a new set of compounds that we have developed against cell lines derived from BC and HCC, which would be valuable for the understanding of the main features of these new vicinal diaryl heterocyclic analogues as anticancer therapeutic leads.

Figure 1. Examples of clinical drugs and experimental compounds with therapeutic potential having the vicinal diaryl heterocyclic motif.

### RESULTS AND DISCUSSION

#### Chemistry.

Based on the developable potential of compound 9, we developed additional analogues to establish structure−activity relationships (SAR). New 3,4-diaryl-5-methylisoxazole derivatives 11−42 were synthesized according to the literature,21 and synthesis procedures and schemes of the relevant intermediates are given in the Supporting Information. In brief, hydrogenation of 9 to remove the benzyl group produced the key intermediate 10, which was subsequently used to generate desired final compounds 11−42 through alkylation of the phenolic hydroxyl (Scheme 1). The similar procedures in Scheme 1 were successfully applied to the synthesis of 44−57 with modifications at the middle phenyl and 59 with amine linker, following the reaction conditions in Schemes 2 and 3. The synthesis of 4,5-diaryl-3-methylisoxazole analogue 60 (Table 3) was achieved as we previously reported.8 The preparation of 4,5-diarylisoazole derivative 63 was achieved starting with readily available starting materials as illustrated in Scheme 4. Compound 61 was obtained according to the corresponding procedures.10,22 The obtained 4,5-diarylisoazole framework 62 was subsequently used to produce the desired 63 through first the hydrolysis of the methoxy and then the alkylation of the phenolic hydroxyl group (Scheme 4).

The 3,4-diaryl-5-aminoisoxazole congener 65 was synthesized as shown in Scheme 5. The 3,4-diaryl-5-aminoisoxazole 65 was synthesized by cyclization of the β-cyanoketone 64, which was obtained according to the literature procedure.7,23,24 The synthesis of 3,4-diaryl-5-methylisoxazole 67 where the 2-
methylbenzyloxy arm was relocated on the 4-aryl ring was analogous to the similar chemistry that has been described in Scheme 1 (Scheme 6).

The preparation of the 1,5-diaryl-3-methylpyrazole derivative 69 carrying the 2-methylbenzyloxy arm on 5-aryl group is outlined in Scheme 7. The synthesis of the $\beta$-diketone 68 was achieved by Claisen−Schmidt condensation. The regioselective synthesis of the 1,5-diaryl-3-methylpyrazole 69 was then conveniently achieved by condensation of the diketone 68 with the hydrochloride salt of the 4-chlorophenylhydrazine in methanol/triethylamine.

The central pyrazole series continued with the synthesis of 4,5-diaryl-1H-pyrazole 71 and 3,4-diaryl-1-methylpyrazole 72. Briefly, the in situ formed enaminone intermediate, obtained by the treatment of 70 with DMFDMA, was cyclized by hydrazine or methylhydrazine to produce the desired pyrazole compounds 71 and 72, respectively (Scheme 8). For the synthesis of the vicinal diaryl 2-methylthiazole 75, hydrolysis of 73 to remove the methyl group furnished the intermediate 74, which was utilized to generate the desired 4,5-diarylthiazole compound 75 through the benzylation of the phenolic hydroxyl (Scheme 9).

1,5-Diarylpyrazole scaffolds 78 and 79 were produced according to previously developed procedures (Scheme 10).
1,5-Diarylpentrazole analogues 85−89 bearing the benzylxy fragment on the 5-aryl as opposed to that of 78 were obtained through the cyclization of enamines 80−84 with the hydrochloride salt of the 4-chlorophenylhydrazine in ethanol (Scheme 11). In addition, analogous compounds where the ether bridge in 85 exchanged with amide 91 or amine 92 were accomplished starting from 90 and following the standard reaction steps outlined in Scheme 12.

**Bioactivity Studies and SAR.** To deduce SARs, several specific areas were focused on compound 9. Hence, it was aimed to explore (i) the influence of differently positioned substituents on the benzylxy arm and the replacement of the...
Scheme 9. Reaction Conditions and Reagents: (i) BBr$_3$, DCM, 0 °C; (ii) 2-Methylbenzyl Bromide, K$_2$CO$_3$, MeCN, Δ

Scheme 10. Reaction Conditions and Reagents: (i) 2-Methylbenzyl Bromide, K$_2$CO$_3$, MeCN, Δ

Scheme 11. Reaction Conditions and Reagents: (i) 4-Chlorophenylhydrazine·HCl, EtOH, Δ

Scheme 12. Reaction Conditions and Reagents: (i) 2-Methylbenzoylchloride, DIEA, DCM, rt for Compound 91; 2-Methylbenzyl Bromide, DIEA, DMF, 65 °C for Compound 92

phenyl ring of the benzyl group by heteroaryl moieties, (ii) the incorporation of different substituents at the isoxazole-4-phenyl group, (iii) the modification of the middle phenyl, and (iv) the replacement of central isoxazole with isosteric heterocycles. Collectively, a total of seventy vicinal diaryl heterocyclic compounds were synthesized to screen their inhibitory effects on cancer cell proliferation against Huh7 and MCF7 cells by introducing different chemical functionalities with the aforementioned modifications (Tables 1–4). Initially, the substitution pattern at the benzyl functionality was scrutinized by comparison of the differently substituted analogues (11–42) as illustrated in Table 1. Methylation of the aromatic ring of the benzyl at 2-position (11) caused a sudden increase in the potency (IC$_{50}$ = 1.3 μM for Huh7 and 3.8 μM for MCF7) versus compound 9 (IC$_{50}$ ≥ 20 μM for both Huh7 and MCF7) with unsubstituted benzyl arm. However, relocation of the methyl group to 3- or 4-position (19 and 24) led to a significant activity loss (IC$_{50}$ ≥ 20 μM). Substitutions at 2-position other than methyl, i.e., electron-donating (12-14) and electron-withdrawing groups (15–18), were undesirable resulting in an activity decrease against both cell lines (IC$_{50}$ values of 8.5 to >20 μM). Seemingly, voluminous substituents than methyl at 2-position were not well tolerated and impaired the efficiency of the compounds. Benzyl analogues differently substituted at 3- (19-23) or 4-position (24–29) were not chased further as they demonstrated a significant loss of inhibitory activity (IC$_{50}$ values of 12 to >20 μM) for both cell lines except for 20 with 3-methoxy, which showed comparable activity to 11 for the Huh7 cell line (IC$_{50}$ = 1.3 vs 3.6 μM). Compounds with dimethyl substitution pattern, i.e., 2,3-diMe (30) or 2,4-diMe (31), suggested that additional methyl group other than 2-methyl was not well tolerated on the benzyl moiety.

Next, it was explored the impact of installing a heteroaromatic group in place of the phenyl ring of the benzyl functionality (32–42). When the 2-methylphenyl was replaced by 3-pyridinyl (32), 4-pyridinyl (33), or a voluminous 2-quinonyl (34), these compounds showed decreased cytotoxic activity, while compound 35 with the 3-pyridine ring having a methyl at a topologically equivalent position as in 11 retained the potency with IC$_{50}$ values of 3.9 μM for Huh7 and 6.1 μM for MCF7. A similar analogue 36 with a 2-pyridine ring having a methyl at the same position partially restored the potency against Huh7 (IC$_{50}$ = 9.4 μM), while the activity loss toward MCF7 was more pronounced (IC$_{50}$ ≥ 20 μM). Of interest, several five-membered heteroaromatic counterparts with alkyl substitutions such as 3,5-dimethylisoxazole (39), 1,3-dimethylpyrazole (40), and 1-isopropylimidazole (41) were well tolerated for their inhibition potential against Huh7 with IC$_{50}$ values of 3.7, 0.9, and 2.5 μM, respectively, while these three compounds appeared to be less effective against MCF7 cells (IC$_{50}$ values of 12 to >20 μM). From this point of view, the 2-methylbenzyl group remains the best choice for the 3,4-diaryl-3-methylisoxazole core, i.e., compound 11, for cytotoxic potency albeit several five-membered heteroaryl moieties exemplified with compounds 39–41 are also conceivable. Apparently, a consistent SAR for cytotoxic activity at this part was not accessible since small structural differences on the benzyl arm were not well tolerated.

Then, it was investigated if the 4-chloro substituent on the phenyl ring at C(4)-isoxazole was replaceable with different atoms or groups, while keeping the 2-methylbenzyl unit due to its good impact on the activity (compounds 44–55 in Table 2). Moving the 4-chloro in 11 to 2-position (44) or replacing it with a smaller fluoro (45) or larger methoxy (46) group resulted in a decreased potency against both cell lines (IC$_{50}$ < 9.9 to >20 μM). The amino replacement of 4-chloro (51) further reduced the potency for both cell types, similar to 45 and 46. On the other hand, substitution of the 4-chloro with a methyl group (47), which is about the same size as a chlorine
atom, was pertinent and regained the bioactivity in both cell lines (IC₅₀ = 1.8 and 4.7 μM, respectively), whereas a linear and less voluminous nitrile group in this position (48), the efficiency again dropped, especially against MCF7 cells (IC₅₀ > 20 μM). Introducing a nitrogen atom to the 4-methylphenyl ring in 47 also caused an activity loss against MCF7 as exemplified with 54. Finally, compounds with substitutions at 3-position (49-50) or with 2,4-dihalogen substitutions (52-53) as well as with 4-pyridyl (55) were also significantly less effective indicating that the phenyl ring of C4-isoxazole may require substitution at 4-position with a similar steric size such as methyl or chlorine (Table 2). The next goal was to explore the substituent effect on the middle phenyl group, and this was briefly examined by fluoro substitution at 2- (56) and 3- (57) positions (Table 2). Although the 2-F substituted 56 preserved the potency against Huh7 and MCF7 cells with IC₅₀ of 2.25 and 9.53 μM, respectively, 3-F substitution in the compound 57 hampered the potency against both cell lines, implying that 2-substitution on the middle phenyl ring may be allowable for retaining or even improving the activity. Lastly, our efforts at the benzoxylphenyl component included an isosteric exchange of the ether bridge with an amino linker (59), which was found tolerable for the Huh7 potency (IC₅₀ = 4 μM) with a concomitant loss of the activity against the MCF7 cell line (IC₅₀ ≥ 20 μM) (Table 2).

After the biological confidence was rationalized with 3,4-diarylisoaxazole derivatives with respect to C3-benzoxylphenyl and C4-phenyl pendants, the next area of interest was to explore the central isoxazole with a series of common five-membered heterocycles to broaden the SAR investigation. To this end, eleven heterocyclic congeners with different heteroatom counts and orders were synthesized to probe the effect on the cytotoxic activity against HCC (Huh7 and Mahlavu) and BC (MCF7) cells and to search for the optimal central core for further SAR studies (Table 3). The position exchange of nitrogen and oxygen atoms in 11 to produce 60 resulted in a significant drop of activity against MCF7 cells (IC₅₀ ≈ 19 μM), while the potency toward Huh7 cells was still preserved (IC₅₀ = 3.8 μM). Furthermore, removal of 3-methyl of isoxazole in 60 to afford 63 appeared to diminish the potency against Mahlavu and MCF7 cells (IC₅₀ ≥ 20 μM) but improved the cytotoxic activity for Huh7 (IC₅₀ = 1.5 μM). Based on this result, the methyl in 11 was replaced with a polar amino group to further explore the hydrophobic effect and procured 65 with comparable potency to parent 11 against all cell lines with IC₅₀ = 2.0-3.9 μM. Interestingly, switching the sites of C3-benzoxylphenyl unit and C4-phenyl in 11 to afford 67 appeared to diminish the cytotoxic activity for all three cell lines (IC₅₀ ≥ 20 μM). Meanwhile, other combinations of heteroatoms such as a group of pyrazoles as well as a thiazole core were also evaluated for their potential to replace the isoxazole core in 11. While thiazole 75 and pyrazole 78 did not produce the desired activity enhancement, other pyrazole derivatives with different orders of nitrogens and methyl substitutions (69, 71, 72, 79, and 85) maintained adequate cytotoxicity against all cell lines with IC₅₀ values in the range of 0.7 to 14.4 μM. Gratifyingly, the reversal of benzoxylphenyl and 4-chlorophenyl components in 78 to produce 85 displayed a potency boost for Huh7 and MCF7 with IC₅₀ values of <1 μM, while still maintaining a decent activity against Mahlavu cells (IC₅₀ = 3.7 μM) compared with the parent 11. Based on the promising potential of 85 as an anticancer agent, the SAR around the middle phenyl and the ether linker was briefly examined, while preserving the 2-methylphenyl and 4-chlorophenyl units, which had been found optimal for the potency (Table 4). The introduction of a nitrogen atom into the middle phenyl in 85 resulted in isosteric pyridine mimics 86 and 87 in which the 2-pyridyl regioisomer (86) exhibited higher cytotoxicity toward Huh7 and Mahlavu cells (IC₅₀ = 2.0 and 7.1 μM, respectively), although this was accompanied by a reduction in the potency against MCF7 cells (IC₅₀ > 20 μM). The bioisosterism between the azine C-N and the aryl C-F bond in compounds 88 and 89 was also examined. The activity results indicated an efficient bioisosterism of the C-F bond with the pyridine N atom in this context because the replacement of the pyridine N atom with the C-F moiety led to improved potency in 88 (IC₅₀ = 1.6-6.4 μM). This was also in good correlation with its isoxazole congener 56 with the 2-F substituted middle phenyl group (Table 2).

Table 2. In Vitro Cell Growth Inhibitory Activity against Hepatocellular Carcinoma and Breast Cancer Cell Lines

| #   | B/C     | IC₅₀ (μM) | Huh7 | MCF7 |
|-----|---------|-----------|------|------|
| 44  | 2-ClPh  | >20       | 9.9  |      |
| 45  | 4-FPh   | 16.7      | >20  |      |
| 46  | 4-OMePh | 8.9       | 12.8 |      |
| 47  | 4-MePh  | 1.8       | 4.7  |      |
| 48  | 4-CNPh  | 6.7       | >20  |      |
| 49  | 3-CF₃Ph | 19.0      | >20  |      |
| 50  | 3-FPh   | 15.0      | >20  |      |
| 51  | 4-NH₂Ph | 16.9      | 19.7 |      |
| 52  | 2,4-diFPh| 15.0      | >20  |      |
| 53  | 2,4-diClPh| 8.2       | 19.2 |      |
| 54  | #        | 6.4       | >20  |      |
| 55  | #        | >20       | >20  |      |
| 56  | 2-F     | 2.25      | 9.53 |      |
| 57  | 3-F     | 9.8       | >20  |      |
| 59  | #        | 4.0       | >20  |      |

*IC₅₀ values were determined at least with five different concentrations of the compounds from the cell growth inhibition percentages.*
ether linker was studied in 91 and 92. As seen, while the amide linker was not tolerated for any cell line, the amine replacement of the ether oxygen (92) was tolerable for Huh7 and Mahlavu cells (IC₅₀ = 3.6 and 4.5 μM, respectively) with a diminished activity against MCF7 cells (Table 4), again in good agreement with its isoxazole counterpart 59 (Table 2).

According to the SAR results, compound 11 with 3,4-diarylisoxazole and compound 85 with 1,5-diarylpyrazole frameworks were selected for further analysis, and unambiguous structural elucidation of 11 and 85 using the single-crystal X-ray diffraction method was accomplished showing the accurate rearrangement of aromatic rings and atoms in 3D-shape (Figure S1). 29,30

Next, judged by the cellular activity of 11 and 85, both compounds in addition to Huh7, Mahlavu, and MCF7 cell lines were further screened against a panel of the hepatocellular carcinoma (HepG2, SNU475, Hep3B, FOCUS, Hep40, and PLC-PRF-5) and breast cancer (MDA-MB-231, MDA-MB-468, SKBR3, and ZR-75) cell lines along with the non-tumorigenic immortalized breast epithelial cells MCF10A (Table 5). The results demonstrated that both compounds are endowed with potent antiproliferative activity against all cancer cells with IC₅₀ values in the range of 1.3−9.5 μM for 11 and 0.77−7.8 μM for 85 for hepatocellular carcinoma and breast cancer cell lines, while found less toxic to the MCF10A immortalized normal breast epithelial cells (Table 5).

Moreover, in vitro single-dose anticancer screening of 11 and 85 at 10 μM was performed utilizing the stable panel of 60 cell lines comprising 9 different cancer types at the National Cancer Institute (NCI) under the Developmental Therapeutics Program (Table S1). 31 Compounds 11 and 85 demonstrated variations in sensitivity and selectivity against individual cell lines in the panel, as illustrated in Table S1. Collectively, both compounds exhibited similar antiproliferative activity against 22 cancer cells with growth inhibition (GI) values in the range of 45−100% at 10 μM. The NCI panel results revealed that both compounds displayed a good preference for leukemia such as CCRF-CEM, HL-60, K-562, MOLT-4, and SR, and colon cancer including HCT-116, HCT-15, HT-29, KM-12, and SW-620 cell lines, in addition to hepatocellular carcinoma and breast cancer cells of this work.

Based on the encouraging potency of 85 in cellular assays against hepatocellular carcinoma and breast cancer as well as in the NCI-60 panel, we decided to evaluate in vitro ADME and pharmacokinetic (PK) properties in mice (Figure S2).

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**Table 3. In Vitro Cell Growth Inhibitory Activity of Compounds with Distinct Central Heterocycles against Hepatocellular Carcinoma and Breast Cancer Cell Lines**

| #  | D | Huh7 | Mahlavu | MCF7 |
|----|---|------|---------|------|
| 11 |   | 1.3  | 3.2     | 3.8  |
| 60 |   | 3.8  | 9.4     | 19.6 |
| 63 |   | 1.5  | 20.1    | >20  |
| 65 |   | 2.0  | 3.9     | 3.6  |
| 67 |   | >20  | >20     | >20  |
| 69 |   | 2.0  | 9.2     | 11.9 |
| 71 |   | 8.4  | 11.0    | 17.5 |
| 72 |   | 6.7  | 12.0    | 14.4 |
| 75 |   | >20  | >20     | >20  |
| 78 |   | >20  | >20     | >20  |
| 79 |   | 3.9  | 9.6     | 11.8 |
| 85 |   | 0.7  | 3.7     | 0.9  |

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**Table 4. In Vitro Growth Cell Inhibitory Activity of 85 Analogues against Hepatocellular Carcinoma and Breast Cancer Cell Lines**

| #  | X | Y | C | Huh7 | Mahlavu | MCF7 |
|----|---|---|---|------|---------|------|
| 86 | O | CH₂ |    | 2.0  | 7.1     | 21.7 |
| 87 | O | CH₂ |    | >20  | >20     | >20  |
| 88 | O | CH₂ |    | 1.6  | 3.2     | 6.4  |
| 89 | O | CH₂ |    | >20  | >20     | >20  |
| 91 | NH| C=O|    | >20  | >20     | >20  |
| 92 | NH| CH₂|    | 3.6  | 4.5     | >20  |

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Metabolic stability of 85 in human microsomes was moderate (55% remaining after 45 min incubation). The compound is highly lipophilic with logD<sub>a</sub> of 6.64, sparingly soluble at the physiological pH, and highly plasma protein-bound (99.9%). The capacity of 85 to inhibit human CYP isoforms 2C9, 2D6, and 3A4 was insignificant (7.3, 22.2, and 0% at 10 μM, respectively) implying a safe window for clinical drug interactions.

The PK parameters after intravenous (iv) administration of 85 constitute a moderate volume of distribution, low total plasma clearance, and a moderate iv half-life of 3.85 h resulting in mice with low oral bioavailability (Table 6). Although PK parameters are nonoptimal, this may be counterbalanced by the potent in vitro antiproliferative activity spectrum of 85 and is not considered limiting for further progression of 85 to in vivo antitumor efficacy studies.

**Real-Time Cellular Response of Cells with Compound 11 and 85 Treatment.** Time- and dose-dependent effects of compounds 11 and 85 on Mahlavu, Huh7, MDA-MB-231, and MCF-7 were evaluated with the use of real-time cell electronic sensing by monitoring dynamic cell proliferation. Both 11 and 85 caused inhibition in the growth of both breast and hepatocellular carcinoma cell lines compared to the control (DMSO) group, whereas there was no difference in the growth of MCF10A immortalized normal breast epithelial cells upon treatment. This assay further confirmed that both compounds displayed time and dose-dependent growth inhibitory effects. Cytotoxic effects of 11 and 85 on cell lines could be observed after 24 h of compound treatment and reached their highest values after 72 h (Figure 2).

**Characterization of Cell Death Mechanism Induced by 11 and 85.** To control the cell death mechanism triggered by 11 and 85, Huh7 and Mahlavu hepatocellular carcinoma cells and MDA-MB-231 and MCF-7 breast cancer cells were cultured according to their cell growth rate and were treated with both 11 and 85 for 48 h. The apoptotic morphological changes were observed in both breast cancer cells and hepatocellular carcinoma cells upon treatment by nuclear staining with Hoechst compared to the control group (Figure 3A). The apoptotic cell populations were further examined with Annexin V staining using flow cytometry. Compared to the control group, the percentage of apoptotic populations in both breast cancer cell lines and hepatocellular carcinoma cell lines treated with 11 or 85 was increased after 24 h (Figure 3B). For further investigation of apoptosis activation through 11 and 85 treatments, apoptosis-associated PARP protein levels were assessed using western blotting. Except for 11 treated MCF7 and Huh7 cells, both 11 and 85 compounds caused the increase in PARP cleavage in both breast cancer cell lines (MCF7 and MDA-MB-231) and hepatocellular carcinoma cells (Mahlavu) (Figure 3C). These results further supported the increased cytotoxic effects of compounds on breast cancer and hepatocellular carcinoma cancer cells. We next investigated in vivo antitumor efficacy of 11 and 85 on nude mice tumor xenografts.

**In Vivo Antitumor Effects of 11 and 85 in Mice Xenograft Models.** The antitumor effects of 11 and 85 in the hepatocellular carcinoma (Mahlavu cells) and breast (MDA-MB-231 cells) xenograft models were assessed twice a week by oral administration of 11 and 85 at 40 mg/kg for 4 weeks. Both compounds conferred a sustained antitumor efficiency (Figures 4 and S3). In the Mahlavu xenografts, mice administered with compounds 11 and 85 had a significant reduction in tumor volume following 4 weeks of treatment, i.e., 85 and 40% reductions in tumor volumes, respectively. Moreover, for MDA-MB-231 xenografts, mice treated with both compounds resulted in about a 50% decrease in tumor volumes as compared to the control group (Figure 4). In all studies, the administered dose was well tolerated and neither significant bodyweight loss nor toxic effects or mortality were observed.

### Table 5. **In Vitro** Growth Inhibitory Activity of 11 and 85 Analogaes against Hepatocellular Carcinoma and Breast Cancer Cell Line Panel**

|               | hepatocellular carcinoma | breast         |
|---------------|--------------------------|----------------|
|               | IC<sub>50</sub> (μM)     |                |
| #             | Huh7 | HepG2 | SN/475 | Hep3B | FOCUS | Hep40 | PLC-PRF-5 | Mahlavu | MCF7 | MDA-MB231 | MDA-MB468 | SKBR3 | ZR75 | MCF10A |
| 11            | 1.3  | 2.1   | 1.7    | 3.0   | 2.1   | 8.6   | 9.5       | 3.2     | 3.4  | 2.0      | 2.8       | 3.5   | 7.6   | 12.1    |
| 85            | 0.7  | 1.4   | 1.5    | 7.9   | 2.4   | 5.2   | 6.5       | 3.7     | 0.9  | 0.9      | 1.0       | 1.8   | 5.5   | 7.6     |

*IC<sub>50</sub> values were determined at least with five different concentrations of the compounds from the cell growth inhibition percentages.

| Mouse IV PK, 1 mpk | Mouse PO PK, 10 mpk |
|--------------------|---------------------|
| t<sub>1/2</sub>     | V<sub>d</sub> | C<sub>p</sub> | C<sub>max</sub> | AUC | t<sub>1/2</sub> | F% |
| 3.85               | 3.33  | 939   | 346            | 19.3| 192  | 6.64 | 5  |

*<t<sub>1/2</sub> = h, V<sub>d</sub> = (L/kg), C<sub>p</sub>, C<sub>max</sub> = (ng/mL), Clp = (L/h/kg), AUC = (h<sup>2</sup>/mg/mL).*

**Table 6. PK Properties of Compound 85**

**N**

**CONCLUSIONS**

We synthesized a series of vicinal diaryl isoxazole and pyrazole derivatives as putative anticancer agents and checked their growth inhibitory activity against human hepatocellular carcinoma and breast cancer cell lines. Most of the derivatives represented moderate cytotoxicity in the selected cancer cells. In general, the substitution type and pattern on the benzoxypyridyl group linked to the central heterocycle had a significant effect on the potency and the selectivity of the compounds in the tested cancer cell lines. Following a comprehensive evaluation of twelve central heterocycles comprising a combination of different heteroatom positions and numbers, we observed that the type of the central heterocycle, i.e., isoxazole and pyrazole, was also crucial for the observed anticancer potency. Subsequently, two analogues, the 3,4-diarylisoaxazole derivative 11 and 1,5-diarylpyrazole 85, stand out as developable anticancer compounds based on their significant in vitro antiproliferative activities toward the 13 hepatocellular and breast cancer cell lines with IC<sub>50</sub> values in the range of 0.77 to 9.53 μM. We also demonstrated that both compounds displayed dose- and time-dependent growth inhibition through the RT-CES system, which was also correlated to initial SRB screening results. Further analysis showed that both compounds induced apoptotic cell death in
both hepatocellular carcinoma and breast cancer cell lines and demonstrated antitumor activity in vivo in the mouse.

Figure 2. Real-time cell growth response of Mahlavu, Huh7, MCF-7, and MDA-MB-231 cancer cells and MCF10A immortalized normal breast epithelial cells treated with compounds 11 and 85 and control (DMSO) for 96 h. The experiment was done in triplicate, and results were normalized to controls of 10-5-2.5 μM concentrations 11 and 85.
Figure 3. Compounds 11 and 85 induced cell death. (A) Cells were treated with compounds 11 and 85 using their IC_{100} concentrations or DMSO as a negative control for 48 h. Nuclear morphology was revealed by Hoechst staining under fluorescent microscopy. Apoptotic bodies were detectable in 11- and 85-treated cells after 48 h. Arrows show apoptotic cells. Scale bar: 20 μM. (B) HCC cells and breast cancer cells were treated with 5 μM compounds 11 and 85 and DMSO as control and were analyzed after 48 h. (C) PARP in Huh7, Mahlavu, MDA-MB-231, and MCF-7 cells treated with 11 and 85 for 48h. Calnexin was used for equal loading control. (D) Bar graph represents relative band intensities of Cleaved PARP/Total PARP, which were normalized with their calnexin loading controls. Statistical analysis was performed using one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ns not significant.
hepatocellular and breast tumor xenografts with inhibition rates between 40% and 85%, and with insignificant effect on the mice bodyweight.

In conclusion, our results revealed that these diaryl-isoxazole and -pyrazole derivatives exemplified with 11 and 85 show promise as leads for further development of improved anticancer compounds against hepatocellular carcinoma and breast cancers. Hence, further investigation of novel analogues that could be integral to the current SAR in this study would be of value to identify novel diaryl heterocycles with strong anticancer and druglike properties. Elucidation of detailed molecular mechanisms associated with 11 and 85 such as direct profiling of compounds using NanoString analysis as well as target fishing experiments with biotin-labeled conjugates to identify potential molecular targets are under investigation and will be reported in due time.

**EXPERIMENTAL SECTION**

**Chemistry.** All chemicals were purchased from different suppliers such as Sigma-Aldrich Chemicals (Sigma-Aldrich Corp., St. Louis, MO), Merck Chemicals (Merck KGaA, Darmstadt, Germany), and ABCR (abcr GmbH, Karlsruhe, Germany). THF was dried from benzophenone-sodium. 1H and 13C NMR spectra were recorded in CDCl3 or DMSO-d6 on a Varian Mercury 400 MHz spectrometer using tetramethylsilane as the internal standard. Coupling constants were reported as Hertz and all chemical shifts were recorded as δ (ppm). High-resolution mass spectra data (HRMS) were collected using Waters LCT Premier XE Mass Spectrometer operating in ESI(+) or ESI(−) method, also coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation) using a UV detector monitoring at 254 nm. Purity for all final compounds was >95%, according to the UPLC-MS method using a water/Methanol solvent gradient containing 0.1% formic acid (1%/90%); flow rate: 0.3 mL/min, column: Aquity BEH C18 column (2.1 × 100 mm, 1.7 mm). All microwave irradiation reactions were carried out in a Biotage Initiator + microwave apparatus with Biotage sealed microwave vials. Flash chromatography was performed on a Combiflash RF Automatic Flash Chromatography System with RediSep silica gel columns (12, 24, and 40 g) (Teledyne-Isclo, Lincoln, NE) or Reveleris PREP Purification System (Buchi, New Castle, DE). Preparative chromatography was performed on Buchi US15C18HQ-250/212-C18 silica gel columns with the following devices; Reveleris PREP Purification System or PuriFlash 4250 System (Interchim, Montluçon, France). Melting points of the compounds were determined using the SMP50 automated melting point apparatus (Stuart, Staffordshire, ST15 OSA, U.K.). The in vitro ADME and in vivo mouse PK studies were conducted at Syngene International Ltd., Bangalore, India. General synthetic procedures (Methods 1–9) and experimental data for all intermediate compounds can be found in the Supporting Information.

3-(4-(Benzyloxyl)phenyl)-4-(4-chlorophenyl)-5-methylisoxazole (9). It was synthesized according to method 4 by the reaction of 4-chlorophenylacetonitrile and the 1-(benzyloxy)-4-(nitrosomethyl)benzene unstable intermediate obtained in situ following the literature as shown in Scheme S1.24 The resulting crude product was purified by flash column chromatography (0% → 40% EtOAc in Hexane). Yield 50.0%; mp 114.7–115.7°C. 1H NMR (400 MHz, CDCl3): δH 2.42 (3H, s), 5.06 (2H, s), 6.93 (2H, d, J = 8.8 Hz), 7.12 (2H, d, J = 8.8 Hz), 7.32–7.43 (9H, m). 13C NMR (100 MHz, CDCl3): δC 11.56, 69.99, 114.53, 114.91, 121.41, 127.49, 128.61, 129.01, 129.09, 129.75, 131.11, 133.69, 136.58, 159.78, 160.76, 160.64. HRMS (m/z) [M + H]+ calcd for C23H19ClNO2: 376.1104, found: 376.1106.

4-(4-Chlorophenyl)-5-methyl-3-(4-(2-methylbenzyl)oxy)phenyl)isoxazole (11). It was synthesized from compound 10 using 2-methylbenzyl bromide according to synthesis method 1a. Yield 77.0%; mp 114.1–115.5°C. 1H NMR (400 MHz, CDCl3): δH 2.37 (3H, s), 2.43 (3H, s), 5.03 (2H, s), 6.94 (2H, d, J = 8.4 Hz), 7.13 (2H, d, J = 8.8 Hz), 7.19–7.28 (3H, m), 7.35–7.40 (5H, m). 13C NMR (100 MHz, CDCl3): δC 11.62, 18.89, 68.60, 114.57, 114.84, 121.41, 126.06, 128.39, 128.63, 129.02, 129.10, 129.78, 130.44, 131.15, 131.71, 134.39, 136.69, 159.91, 160.62, 166.58. HRMS (m/z) [M + H]+ calcd for C24H23NO2Cl: 390.1261, found: 390.1263. CAS: 2758520-84-0.

4-(4-Chlorophenyl)-3-(4-(2-methoxybenzyl)oxy)phenyl)isoxazole (12). It was synthesized from compound 10 using 2-methoxybenzyl chloride according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 75.0%; mp 156.8–157.7°C. 1H NMR (400 MHz, CDCl3): δH 2.42 (3H, s), 3.85 (3H, s), 5.11 (2H, s), 6.89–6.99 (4H, m), 7.12 (2H, d, J = 8.4 Hz), 7.28–7.32 (1H, m), 7.33–7.36 (4H, m), 7.43 (1H, d, J = 7.6 Hz). 13C NMR (100 MHz, CDCl3): δC 11.56, 55.37, 65.06, 110.27, 114.53, 114.95, 120.58, 121.17, 124.94, 128.59, 128.99, 129.04, 129.15, 129.68, 131.12, 133.67, 156.81, 160.00, 160.68, 166.48. HRMS (m/z) [M + H]+ calcd for C23H23NO2Cl: 406.1210, found: 406.1212.

**Figure 4.** Compounds 11 and 85 reduce tumor growth. Mahlau and MDA-MB-231 xenograft nude mice were treated with 40 mg/kg 11 and 85 prepared in 0.5% hydroxypropyl methyl cellulose plus 1% Tween 80 or with vehicle only twice a week once the tumor size reached 100 mm3. Tumor volumes were recorded twice a week during the experiment. Each group contained six mice (n = 6). Statistical analysis was performed using one-way ANOVA. ***p < 0.05, ****p < 0.00001.
4-(4-Chlorophenyl)-5-methyl-3-(4-((2-trifluoromethoxy)benzyl)oxy)phenyl)isoxazole (13). It was synthesized from compound 10 using 2-trifluoromethoxybenzyl bromide according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 46.0%; mp 85.8–86.7 °C. 1H NMR (400 MHz, DMSO-d6): δH 2.39 (3H, s), 5.13 (2H, s), 7.04 (2H, d, J = 8.8 Hz), 7.23 (2H, d, J = 8.4 Hz), 7.28 (2H, d, J = 8.8 Hz), 7.40–7.52 (5H, m), 7.64 (1H, d, J = 7.6 Hz). 13C NMR (100 MHz, DMSO-d6): δC 11.90, 22.36, 132.99, 133.20, 133.24, 134.68, 135.11, 160.03, 166.94. HRMS (m/z) [M + H]+ calc for C25H19NO3ClF3: 460.0927, found: 460.0928.

4-(4-Chlorophenyl)-3-(4-((2-difluoromethoxy)benzyl)oxy)phenyl)5-methylisoxazole (14). It was synthesized from compound 10 using 2-difluoromethoxybenzyl chloride according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 86.0%; mp 95.5–96.9 °C. 1H NMR (400 MHz, DMSO-d6): δH 2.42 (3H, s), 5.12 (2H, s), 7.06 (2H, d, J = 8.8 Hz), 7.24–7.27 (5H, m), 7.70 (2H, d, J = 8.8 Hz), 7.42–7.47 (1H, m), 7.49 (2H, d, J = 8.4 Hz), 7.58 (1H, dd, J = 7.6 Hz, 1.2 Hz). 13C NMR (100 MHz, DMSO-d6): δC 11.90, 64.44, 113.95, 114.90, 116.60 (t, JCF = 256.5 Hz), 118.52, 121.01, 125.40, 127.56, 128.81, 128.84, 129.47, 129.89, 130.37, 131.40, 132.55, 149.13 (t, JCF = 3.2 Hz), 159.24, 160.05, 166.92. HRMS (m/z) [M + H]+ calc for C26H18NO3ClF3: 442.1022, found: 442.1016.

4-(4-Chlorophenyl)-3-(3-(2-fluorobenzyl)oxy)phenyl)5-methylisoxazole (15). It was synthesized from compound 10 using 2-fluorobenzyl chloride according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 91.0%; mp 119.9–121.0 °C. 1H NMR (400 MHz, CDCl3): δH 2.37 (3H, s), 2.42 (3H, s), 5.02 (2H, s), 6.93 (2H, d, J = 8.8 Hz), 7.12 (2H, d, J = 8.4 Hz), 7.13–7.15 (1H, m), 7.20–7.29 (3H, m), 7.34–7.36 (4H, m). 13C NMR (100 MHz, CDCl3): δC 11.56, 113.95, 114.91, 121.36, 124.61, 128.25, 128.52, 128.85, 129.01, 129.11, 129.74, 131.11, 133.69, 136.47, 138.33, 159.85, 160.62, 166.52. HRMS (m/z) [M + H]+ calc for C26H19NO3F3: 390.1261, found: 390.1260.

4-(4-Chlorophenyl)-3-(4-(3-methylbenzyl)oxy)phenyl)5-methylisoxazole (19). It was synthesized from compound 10 using 3-methylbenzyl bromide according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 90.0%; mp 119.9–121.0 °C. 1H NMR (400 MHz, CDCl3): δH 2.37 (3H, s), 2.42 (3H, s), 5.02 (2H, s), 6.93 (2H, d, J = 8.8 Hz), 7.12 (2H, d, J = 8.4 Hz), 7.13–7.15 (1H, m), 7.20–7.29 (3H, m), 7.34–7.36 (4H, m). 13C NMR (100 MHz, CDCl3): δC 11.56, 21.40, 70.07, 114.53, 114.91, 121.36, 124.61, 128.25, 128.52, 128.85, 129.01, 129.11, 129.74, 131.11, 133.69, 136.47, 138.33, 159.85, 160.62, 166.52. HRMS (m/z) [M + H]+ calc for C26H19NO3F3: 390.1261, found: 390.1260.

4-(4-Chlorophenyl)-3-(3-(2-chlorobenzyl)oxy)phenyl)5-methylisoxazole (16). It was synthesized from compound 10 using 2-chlorobenzyl chloride according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 91.0%; mp 105.2–106.8 °C. 1H NMR (400 MHz, CDCl3): δH 2.43 (3H, s), 5.17 (2H, s), 6.95 (2H, d, J = 9.2 Hz), 7.13 (2H, d, J = 8.8 Hz), 7.27–7.32 (2H, m), 7.35–7.41 (6H, m), 7.53–7.55 (1H, m). 13C NMR (100 MHz, CDCl3): δC 11.56, 67.11, 114.54, 114.93, 121.70, 126.98, 128.74, 129.03, 129.07, 129.08, 129.41, 129.81, 131.11, 132.58, 133.74, 134.32, 159.49, 160.56, 166.58. HRMS (m/z) [M + H]+ calc for C25H19NO3Cl: 410.0715, found: 410.0717.

2-(4-(4-(4-Chlorophenyl)-5-methylisoxazol-3-yl)phenoxymethyl)benzonitrile (17). It was synthesized from compound 10 using α-bromo-o-tolunitrile according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 77.0%; mp 111.2–112.5 °C. 1H NMR (400 MHz, CDCl3): δH 2.42 (3H, s), 5.26 (2H, s), 6.95 (2H, d, J = 8.8 Hz), 7.12 (2H, d, J = 8.8 Hz), 7.35–7.39 (4H, m), 7.44 (1H, td, J = 7.4, 1.6 Hz), 7.61–7.71 (3H, m). 13C NMR (100 MHz, CDCl3): δC 11.56, 67.11, 114.54, 114.93, 121.70, 126.98, 128.74, 129.03, 129.07, 129.08, 129.41, 129.81, 131.11, 132.58, 133.74, 134.32, 159.49, 160.56, 166.58. HRMS (m/z) [M + H]+ calc for C25H19NO3Cl: 410.0715, found: 410.0717.

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This page contains a scientific text discussing the synthesis and properties of various organic compounds. The text is technical and includes chemical structures, reactions, and analytical data. It describes the synthesis of compounds using specific reactants and conditions, followed by characterization data such as melting points, boiling points, and spectroscopic data. The text also mentions the use of flash column chromatography and high-resolution mass spectrometry (HRMS) for compound purification and identification. The text is typical of a research paper in the field of organic chemistry, providing detailed information on the preparation and analysis of new compounds.
(m/z) [M + H]+ calcd for C_{12}H_{13}O_2Cl: 240.1417, found: 240.1414.

4-(4-Chlorophenyl)-3-(4-(2,4-dimethylbenzyl)oxy)-phenyl)-5-methylisoxazole (31). It was synthesized from compound 10 using 2,4-dimethylbenzyl bromide according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 40% EtOAc in Hexane). Yield 77.0%; mp 119.7 → 122.1 °C. 1H NMR (400 MHz, CDCl3): δ 7.35 (3H, s), 2.34 (3H, s), 2.42 (3H, s), 4.99 (2H, s), 6.87 (2H, d, J = 8.8 Hz), 7.02 (1H, d, J = 7.4 Hz), 7.05 (1H, s), 7.13 (2H, d, J = 8.8 Hz), 7.26 (1H, d, J = 7.4 Hz), 7.35–7.37 (4H, m). 13C NMR (100 MHz, CDCl3): δ 11.56, 18.82, 21.09, 68.52, 114.53, 114.56, 121.30, 126.64, 128.94, 129.01, 129.13, 129.72, 131.13, 131.32, 131.39, 133.69, 136.74, 138.26, 159.99, 160.63, 166.53. HRMS (m/z) [M + H]+ calcd for C_{12}H_{13}O_2Cl: 240.1417, found: 240.1414.

4-(4-Chlorophenyl)-5-methyl-3-(4-(pyridin-3-ylmethoxy)phenyl)-isoazol (32). It was synthesized from compound 10 using 3-chloromethyl)pyridine.HCl according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 60% EtOAc in Hexane). Yield 80.0%; mp 107.2–108.8 °C. 1H NMR (400 MHz, CDCl3): δ 2.91 (3H, s), 2.57 (3H, s), 5.16 (2H, s), 6.92 (2H, d, J = 8.4 Hz), 7.08 (1H, d, J = 8.0 Hz), 7.10 (2H, d, J = 8.4 Hz), 7.29 (1H, d, J = 8.0 Hz), 7.34 (4H, d, J = 8.4 Hz), 7.59 (1H, t, J = 8.0 Hz). 13C NMR (100 MHz, CDCl3): δ 11.50, 24.20, 70.52, 114.53, 114.96, 118.28, 121.69, 122.42, 128.99, 129.08, 129.77, 131.07, 133.73, 137.20, 156.05, 157.94, 159.48, 160.55, 166.49. HRMS (m/z) [M + H]+ calcd for C_{12}H_{13}O_2Cl: 241.1213, found: 241.1205.

4-(4-Chlorophenyl)-5-methyl-3-(4-(6-chloropyridin-3-ylmethoxy)phenyl)-isoazol (33). It was synthesized from compound 10 using 4-chloropyridine-3-carboxaldehyde.HCl according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 60% EtOAc in Hexane). Yield 61.0%; mp 126.1–127.5 °C. 1H NMR (400 MHz, CDCl3): δ 4.31 (3H, s), 2.65 (3H, s), 5.13 (2H, s), 6.92 (2H, d, J = 8.4 Hz), 7.08 (1H, d, J = 8.0 Hz), 7.10 (2H, d, J = 8.4 Hz), 7.29 (1H, d, J = 8.0 Hz), 7.34 (4H, d, J = 8.4 Hz), 7.59 (1H, t, J = 8.0 Hz). 13C NMR (100 MHz, CDCl3): δ 11.50, 24.20, 70.52, 114.53, 114.96, 118.28, 121.69, 122.42, 128.99, 129.08, 129.77, 131.07, 133.73, 137.20, 156.05, 157.94, 159.48, 160.55, 166.49. HRMS (m/z) [M + H]+ calcd for C_{12}H_{13}O_2Cl: 241.1213, found: 241.1205.

4-(4-Chlorophenyl)-5-methyl-3-(4-(6-chloropyridin-3-ylmethoxy)phenyl)-5-methylisoxazole (34). It was synthesized from compound 10 using 2-chloro-(5-chloromethyl)pyridine according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 60% EtOAc in Hexane). Yield 61.0%; mp 126.1–127.5 °C. 1H NMR (400 MHz, CDCl3): δ 4.31 (3H, s), 2.65 (3H, s), 5.13 (2H, s), 6.92 (2H, d, J = 8.4 Hz), 7.08 (1H, d, J = 8.0 Hz), 7.10 (2H, d, J = 8.4 Hz), 7.29 (1H, d, J = 8.0 Hz), 7.34 (4H, d, J = 8.4 Hz), 7.59 (1H, t, J = 8.0 Hz). 13C NMR (100 MHz, CDCl3): δ 11.50, 24.20, 70.52, 114.53, 114.96, 118.28, 121.69, 122.42, 128.99, 129.08, 129.77, 131.07, 133.73, 137.20, 156.05, 157.94, 159.48, 160.55, 166.49. HRMS (m/z) [M + H]+ calcd for C_{12}H_{13}O_2Cl: 241.1213, found: 241.1205.
HRMS (m/z) [M + H]+ calcd for C_{21}H_{24}N_{2}O_{7}: 395.1162, found: 395.1153.

1-(4-Chlorophenyl)-3-(1,1-dimethyl-1H-pyrazol-5-yl)-methoxyphenyl)-5-methylisoxazole (40). It was synthesized from compound 10 using 5-(chloromethyl)-1,3-dimethylisoxazole according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% → 70% EtOAc in Hexane). Yield 73.00%; mp 156.4–157.9 °C. 1H NMR (400 MHz, CDCl₃): δH 2.25 (3H, s), 2.42 (3H, s), 3.83 (3H, s), 4.98 (2H, s), 6.09 (1H, d, J = 9.2 Hz), 7.11 (2H, d, J = 8.8 Hz), 7.34–7.37 (4H, m). 13C NMR (100 MHz, CDCl₃): δC 11.43, 55.98, 118.17, 118.20, 129.34, 129.64, 129.66, 138.55, 159.80, 160.60. HRMS (m/z) [M + H]+ calcd for C_{21}H_{24}N_{2}O_{7}: 394.1322, found: 394.1320.

5-Methyl-4-(4-methylbenzyl)-5-(4-((2-methylbenzyl)oxy)phenyl)isoxazole (46). It was synthesized from compound 43a using 4-methylphenylacetone according to synthesis method 4. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 43.0%; mp 123.1–122.7 °C. 1H NMR (400 MHz, CDCl₃): δH 2.36 (3H, s), 2.41 (3H, s), 3.84 (3H, s), 5.02 (2H, s), 6.92 (2H, d, J = 9.2 Hz), 6.93 (2H, d, J = 8.8 Hz), 7.11 (2H, d, J = 8.8 Hz), 7.19–7.26 (3H, m), 7.38–7.42 (4H, m). 13C NMR (100 MHz, CDCl₃): δC 15.44, 22.81, 59.17, 72.49, 118.12, 118.64, 110.09, 125.85, 126.65, 129.98, 132.29, 132.55, 133.65, 134.35, 134.96, 138.42, 140.62, 163.00, 163.69, 164.62, 170.13. HRMS (m/z) [M + H]+ calcd for C_{24}H_{26}N_{2}O_{8}: 386.1756, found: 386.1753.

5-Methyl-4-(4-methylphenyl)-5-(4-((2-methylbenzyl)oxy)phenyl)isoxazole (47). It was synthesized from compound 43a using 4-methylphenylacetone according to synthesis method 4. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 34.0%; mp 130.8–131.8 °C. 1H NMR (400 MHz, CDCl₃): δH 2.37 (3H, s), 2.39 (3H, s), 2.42 (3H, s), 5.03 (2H, s), 6.93 (2H, d, J = 8.8 Hz), 7.08 (2H, d, J = 8.4 Hz), 7.18–7.28 (7H, m), 7.38–7.43 (3H, m). 13C NMR (100 MHz, CDCl₃): δC 15.53, 18.88, 21.26, 68.55, 114.69, 115.44, 121.90, 126.04, 127.53, 128.35, 128.62, 129.42, 129.71, 130.74, 134.49, 136.68, 137.36, 159.76, 160.69, 166.28. HRMS (m/z) [M + H]+ calcd for C_{24}H_{24}N_{2}O_{7}: 370.1807, found: 370.1796.

5-Methyl-4-(4-cyanophenyl)-5-(4-(2-methylbenzyl)oxy)phenyl)isoxazole (48). It was synthesized from compound 43a using 4-cyanophenylacetone according to synthesis method 4. The resulting crude product was purified by flash column chromatography (0% → 40% EtOAc in Hexane). Yield 34.0%; mp 135.3–137.1 °C. 1H NMR (400 MHz, CDCl₃): δH 2.37 (3H, s), 2.47 (3H, s), 5.04 (2H, s), 6.95 (2H, d, J = 8.8 Hz), 7.19–7.33 (7H, m), 7.33–7.39 (1H, m), 7.67 (2H, d, J = 8.4 Hz). 13C NMR (100 MHz, CDCl₃): δC 11.74, 18.89, 68.63, 111.46, 114.28, 114.98, 118.53, 120.87, 126.07, 128.45, 128.62, 129.83, 130.35, 130.47, 132.50, 134.29, 135.68, 136.69, 160.10, 160.60, 167.08. HRMS (m/z) [M + H]+ calcd for C_{24}H_{23}N_{2}O_{7}: 381.1603, found: 381.1600.

5-Methyl-4-(3-trifluoromethylphenyl)-5-(4-((2-methylbenzyl)oxy)phenyl)isoxazole (49). It was synthesized from compound 43a using 3-trifluoromethylphenylacetone according to synthesis method 4. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 41.0%; mp 120.7–122.4 °C. 1H NMR (400 MHz, CDCl₃): δH 2.36 (3H, s), 2.46 (3H, s), 5.03 (2H, s), 6.94 (2H, d, J = 8.8 Hz), 7.19–7.28 (3H, m), 7.33–7.39 (4H, m), 7.48–7.52 (2H, m), 7.62 (1H, d, J = 8.0 Hz). 13C NMR (100 MHz, CDCl₃): δC 11.60, 18.85, 68.61, 114.42, 114.90, 121.15, 123.84 (q, J_{C,F} = 271.2 Hz), 124.43 (q, J_{C,F} = 3.6 Hz), 126.05, 126.41 (q, J_{C,F} = 3.8 Hz), 128.39, 128.60, 129.23, 129.75, 130.35, 131.23 (q, J_{C,F} = 3.21 Hz), 131.60, 132.20, 134.37, 136.68, 160.00, 160.60, 166.88. HRMS (m/z) [M + H]+ calcd for C_{24}H_{22}F_{3}N_{2}O: 424.1524, found: 424.1523.
5-Methyl-4-(3-fluoromethylphenyl)-3-(4-(2-methylbenzyl)oxy)phenyl)isoxazole (50). It was synthesized from compound 43a using 3-fluoronaphthalencetone according to synthesis method 4. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 43.0%; mp 93.9–94.2 °C. 1H NMR (400 MHz, CDCl3): δH 2.37 (3H, s), 2.43 (3H, s), 5.03 (2H, s), 6.89–6.99 (4H, m), 7.05 (1H, td, J = 8.4, 2.4 Hz), 7.20–7.28 (3H, m), 7.32–7.40 (4H, m). 13C NMR (100 MHz, CDCl3): δC 128.63, 129.21, 130.44, 133.01 (dd, J13C-F = 20.5 Hz), 114.83, 116.74 (dd, J13C-F = 8.52, 3.8 Hz), 114.43, 176.20, 176.67. HRMS (m/z) [M + H]+ calcd for C24H22ClNO2: 342.0870, found: 342.0870.

5-Methyl-4-(4-aminophenyl)-3-(4-(2-methylbenzyl)oxy)phenyl)isoxazole (51). It was synthesized from compound 43a using 4-aminophenol according to synthesis method 4. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 43.0%; mp 89.3–90.3 °C. 1H NMR (400 MHz, CDCl3): δH 2.37 (3H, s), 2.57 (3H, s), 5.04 (2H, s), 6.94–6.99 (3H, m), 7.20–7.28 (3H, m), 7.38–7.44 (4H, m), 8.52 (1H, d, J = 1.6 Hz). 13C NMR (100 MHz, CDCl3): δC 124.10, 18.86, 68.60, 114.80, 115.01, 121.71, 124.19, 126.05, 128.38, 128.63, 129.98, 130.43, 133.74, 134.45, 136.69, 137.06, 147.64, 150.11, 156.91, 160.81, 168.61. HRMS (m/z) [M + H]+ calcd for C25H23ClNO2: 371.1760, found: 371.1769.

5-Methyl-4-(2,4-difluoromethylphenyl)-3-(4-(2-methylbenzyl)oxy)phenyl)isoxazole (52). It was synthesized from compound 43a using 2,4-difluoronaphthalencetone according to synthesis method 4. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 34.0%; mp 114.9–115.1 °C. 1H NMR (400 MHz, CDCl3): δH 2.37 (3H, s), 2.41 (3H, s), 5.03 (2H, s), 6.74 (2H, d, J = 8.8 Hz), 6.93 (2H, d, J = 8.8 Hz), 7.00 (2H, d, J = 8.8 Hz), 7.21–7.27 (3H, m), 7.39–7.42 (1H, m), 7.43 (2H, d, J = 8.8 Hz). 13C NMR (100 MHz, CDCl3): δC 12.51, 18.89, 68.55, 114.66, 115.38, 116.68, 120.97, 122.07, 126.05, 128.35, 128.63, 129.71, 130.42, 130.90, 134.51, 136.99, 144.98, 159.71, 160.70, 166.04. HRMS (m/z) [M + H]+ calcd for C24H23ClNO2: 371.1760, found: 371.1759.

5-Methyl-4-(2,4-difluoromethylphenyl)-3-(4-(2-methylbenzyl)oxy)phenyl)isoxazole (53). It was synthesized from compound 43a using 2,4-difluoronaphthalencetone according to synthesis method 4. The resulting crude product was purified by flash column chromatography (0% → 30% EtOAc in Hexane). Yield 40.0%; mp 98.0–98.4 °C. 1H NMR (400 MHz, CDCl3): δH 2.33, (3H, s), 2.35, (3H, s), 5.01 (2H, s), 6.91, (2H, d, J = 8.8 Hz), 7.13 (1H, d, J = 8.0, 2.4 Hz), 7.18–7.26 (3H, m), 7.28 (1H, dd, J = 8.0, 2.4 Hz), 7.32 (2H, d, J = 8.8 Hz), 7.36–7.38 (1H, m), 7.53 (1H, d, J = 2.4 Hz). 13C NMR (100 MHz, CDCl3): δC 11.66, 18.87, 68.55, 112.15, 114.89, 121.61, 126.05, 127.52, 128.37, 128.45, 128.59, 129.88, 129.93, 130.42, 133.25, 134.39, 135.04, 135.74, 136.67, 159.94, 160.65, 167.63. HRMS (m/z) [M + H]+ calcd for C24H23ClNO2: 342.0870, found: 342.0870.
131.10, 133.93, 133.98, 136.72, 147.94 (d, $J_{C-H} = 10.2$ Hz), 152.54 (d, $J_{C-H} = 245.6$ Hz), 159.69 (d, $J_{C-H} = 1.9$ Hz), 166.95. HRMS (m/z) [M + H]$^+$ calc for C$_{23}$H$_{26}$Cl$_2$N$_2$O$_6$: 398.1167, found: 398.1169.

4-(4-(4-Chlorophenyl)-5-methylisoxazol-3-yl)-N-(2-methylbenzyl)aniline (59). It was synthesized from compound 58 according to synthesis method 1c. The resulting crude product was purified by flash column chromatography (0% → 10% EtOAc in Hexane). Yield 76.0%; mp 84.5–86.5 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta_{H}$ 2.37 (3H, s), 2.43 (3H, s), 2.50 (2H, m), 2.70 (2H, d, $J = 8.8$ Hz), 2.72–7.32 (3H, m), 7.38–7.44 (3H, m).

4-(4-(4-Chlorophenyl)-5-methylisoxazol-3-yl)-N-(2-methylbenzyl)aniline (59). It was synthesized from compound 58 according to synthesis method 1c. The resulting crude product was purified by flash column chromatography (0% → 10% EtOAc in Hexane). Yield 76.0%; mp 84.5–86.5 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta_{H}$ 2.37 (3H, s), 2.43 (3H, s), 2.50 (2H, m), 2.70 (2H, d, $J = 8.8$ Hz), 2.72–7.32 (3H, m), 7.38–7.44 (3H, m).

1-(4-Chlorophenyl)-3-methyl-5-(4-((2-methylbenzyl)oxy)-phenyl)-1H-pyrazole (69). It was synthesized from compound 68 using 4-chlorophenylhydrazine.HCl according to synthesis method 8c. The resulting crude product was purified by preparative column chromatography (0% → 60% ACN in Water). Yield 54.0%; mp 111.8–113.6 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta_{H}$ 2.38 (3H, s), 2.43 (3H, s), 2.50 (2H, m), 2.70 (2H, d, $J = 8.8$ Hz), 2.72–7.32 (3H, m), 7.38–7.41 (1H, m).

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8.4 Hz), 7.21 (1H, d, J = 1.6 Hz), 7.13 (1H, d, J = 8.8, 8.4 Hz), 7.22-7.32 (7H, m), 7.40 (1H, m), 7.75 (1H, d, J = 2.0 Hz). 13C NMR (100 MHz, CDCl3): δc 19.07, 69.23, 103.11 (d, JCF = 25.1 Hz), 109.51 (d, JCF = 1.5 Hz), 111.12 (d, JCF = 15.3 Hz), 111.24 (d, JCF = 3.1 Hz), 125.57, 126.52, 128.84, 128.89, 129.22, 130.74, 133.24, 134.32, 134.68, 139.11, 141.81, 158.31. HRMS (m/z) [M + H]+ calc for C23H23ClNO2: 375.1264, found: 375.1262.

5-(4-Chlorophenyl)-3-methyl-1-(4-(2-methylbenzyl)oxy)-1H-pyrazole (79). It was synthesized from compound 77 using 2-methylbenzyl bromide according to synthesis method 1a. The resulting crude product was purified by flash column chromatography (0% 40% EtOAc in Hexane). Yield 83.0%; mp 126.63, 128.38, 128.59, 128.68, 129.01, 129.74, 130.43, 133.24, 134.06, 134.38, 136.65, 142.49, 149.13, 158.10. HRMS (m/z) [M + H]+ calc for C30H32ClNO2: 389.1421, found: 389.1416.

1-(4-Chlorophenyl)-5-(4-(4-methylbenzyl)oxy)phenyl]-1H-pyrazole (80). It was synthesized from compound 80 using 4-chlorophenylhydrazine.HCl according to synthesis method 8a. The resulting crude product was purified by flash column chromatography (0% 40% EtOAc in Hexane). Yield 61.0%; mp 164.0-147.9 °C. 1H NMR (400 MHz, CDCl3): δh 2.38 (3H, s), 5.04 (2H, s), 6.45 (1H, d, J = 1.4 Hz), 6.29 (1H, d, J = 1.8 Hz). 13C NMR (100 MHz, CDCl3): δc 18.90, 68.85, 103.11, 125.92, 126.22, 128.45, 128.46, 129.05, 130.08, 130.47, 132.98, 134.36, 136.70, 138.68, 140.49, 142.91, 159.04. HRMS (m/z) [M + H]+ calc for C29H23ClNO2: 375.1264, found: 375.1263. CAS: 2750233-50-0.

2-(4-Chlorophenyl)-1H-pyrazol-5-yl)-5-(2-methylbenzyl)oxy)pyridine (86). It was synthesized from compound 81 using 4-chlorophenylhydrazine.HCl according to synthesis method 8a. The resulting crude product was purified by flash column chromatography (0% 30% EtOAc in Hexane). Yield 43.0%; mp 95.0-95.4 °C. 1H NMR (400 MHz, CDCl3): δh 2.40 (3H, s), 5.12 (2H, s), 6.46 (1H, d, J = 1.6 Hz), 6.88-6.91 (1H, m), 6.96-7.02 (2H, m), 7.20-7.27 (5H, m), 7.32-7.35 (2H, m), 7.40-7.41 (1H, m), 7.71 (1H, d, J = 2.0 Hz). 13C NMR (100 MHz, CDCl3): δc 19.07, 70.17, 108.23, 115.62 (d, JCF = 2.0 Hz), 116.93 (d, JCF = 19.2 Hz), 123.75 (d, JCF = 7.1 Hz), 125.02 (d, JCF = 3.8 Hz), 126.24, 126, 128.76, 128.82, 129.35, 130.68, 134.36, 134.09, 136.92, 138.62, 140.77, 141.91 (d, JCF = 1.9 Hz), 147.18 (d, JCF = 10.3 Hz), 152.67 (d, JCF = 246.2 Hz). HRMS (m/z) [M + H]+ calc for C28H23ClNO2: 393.1170, found: 393.1172.

1-(4-Chlorophenyl)-5-(3-(fluoro-4-(2-methylbenzyl)oxy)phenyl]-1H-pyrazole (89). It was synthesized from compound 84 using 4-chlorophenylhydrazine.HCl according to synthesis method 8a. The resulting crude product was purified by flash column chromatography (0% 30% EtOAc in Hexane). Yield 43.0%; mp 95.0-95.4 °C. 1H NMR (400 MHz, CDCl3): δh 2.40 (3H, s), 5.12 (2H, s), 6.46 (1H, d, J = 1.6 Hz), 6.88-6.91 (1H, m), 6.96-7.02 (2H, m), 7.20-7.27 (5H, m), 7.32-7.35 (2H, m), 7.40-7.41 (1H, m), 7.71 (1H, d, J = 1.6 Hz). 13C NMR (100 MHz, CDCl3): δc 19.07, 70.17, 108.23, 115.62 (d, JCF = 2.0 Hz), 116.93 (d, JCF = 19.2 Hz), 123.75 (d, JCF = 7.1 Hz), 125.02 (d, JCF = 3.8 Hz), 126.24, 126, 128.76, 128.82, 129.35, 130.68, 134.36, 134.09, 136.92, 138.62, 140.77, 141.91 (d, JCF = 1.9 Hz), 147.18 (d, JCF = 10.3 Hz), 152.67 (d, JCF = 246.2 Hz). HRMS (m/z) [M + H]+ calc for C28H23ClNO2: 393.1170, found: 393.1172.
(0% → 20% ACN in water). Yield 32.0%; mp 141.5–142.8 °C. 1H NMR (400 MHz, CDCl3); δ 2.36 (3H, s), 4.27 (2H, s), 6.39 (1H, d, J = 1.2 Hz), 6.56 (2H, d, J = 8.4 Hz), 7.03 (2H, d, J = 8.8 Hz), 7.18–7.32 (8H, m), 7.67 (1H, d, J = 1.2 Hz). 13C NMR (100 MHz, CDCl3): δc 18.93, 46.23, 107.13, 112.54, 119.15, 126.21, 126.25, 127.66, 128.29, 128.94, 129.85, 130.52, 132.69, 136.29, 136.35, 139.00, 140.49, 143.55, 148.07. HRMS (m/z) [M + H]+ calc'd for C23H21ClN2: 374.1424, found: 374.1425.

**Biological Studies.** Cell Culture. HepG2, FOCUS, Hep3B, Mahlau, Huh7, Hep40, and PLC-PRF-5 hepatocellular carcinoma cells; MCF-7, MDA-MB-231, MDA-MB-468, SKBR3, and ZR-75 breast cancer cells; and normal-like epithelial MCF-10A breast cells were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Biological Industries-BI) supplemented with 10% fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific), 2 mM l-Glutamine (Gibco/Thermo Fisher Scientific), 100 Units/mL Penicillin/Streptomycin, and 0.1 mM non-essential amino acid (Gibco/Thermo Fisher Scientific). SNU475 hepatocellular carcinoma cells were maintained in RPMI (Biological Industries-BI) supplemented with 10% fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific), 1 mM l-Glutamine (Gibco/Thermo Fisher Scientific), 100 Units/mL Penicillin/Streptomycin, while ZR-75 breast cancer cells were cultured RPMI (Biological Industries-BI) supplemented with 10% fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific), 1× sodium pyruvate, and 84.5 glucose. Normal-like MCF-10A breast cells were sustained in DMEM/HAM’S F12 (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific), 100 mg/mL EGF (20 ng/mL), Hydrocortisone (0.5 mg/mL), Cholera toxin (100 ng/mL), and 100 Units/mL Penicillin/Streptomycin. The cells were incubated at 37 °C in a humidified incubator under 5% CO2.

**NCl-60 Sulforhodamine B (SRB) Cytotoxicity Assay.** Mahlau, FOCUS, SNU475 (1000 cell/well in 150 μL/well); Huh7, MCF7 (2500 cell/well in 150 μL/well); HepG2, Hep3B, SKBR3 (3000 cell/well in 150 μL/well); PLC-PRF-5, Hep40 (5000 cell/well in 150 μL/well); MDA-MB-231, MDA-MB-468, MCF10A (6000 cell/well in 150 μL/well); and ZR-75 (7500 cell/well in 150 μL/well) were cultured in 96-well plates and were inoculated in an incubator for 24 hours. The compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO) as a 20 mM stock solution. The cells were treated with their IC50 concentrations or DMSO as a negative control for 48 h. The 1× PBS was used to wash the cells three times, and the cells were fixed with 100% ice-cold methanol. Then, the cells were stained with 1 μg/mL Hoechst (#33258, Sigma). The cells were analyzed under the fluorescence microscope (Nikon Eclipse 50i).

**Western Blotting.** Cells were treated for 5 μM compounds 11 and 85 or control (DMSO). The treated cells were collected with a scraper at end of 48 h incubation and lysates were prepared. Protein concentration was calculated with a BCA assay. The protein levels were analyzed using protein electrophoresis according to the manufacturer’s protocol for near-infrared (NIR) Western Blot analysis (Mini-PROTEAN Tetra Cell Systems, Bio-Rad). The samples (40 μg/well) were loaded onto TGX precast gels, and protein transfer from gel to an LF-PVDF membrane was done using Trans-Blot Turbo System (Bio-Rad). Primary antibodies (PARP, CST, #9532S) and Calnexin (CST, #2679) and secondary antibodies IRDye 680RD Goat Anti Rabbit (Li-Cor, # 92668071) and IRDye800CW Goat Anti Rabbit (Licor, # 926e32211) were used for western blotting, and the proteins were monitored in fluorescence system using Odyssey CLx- LICOR imaging system.

**In Vivo Mouse Xenograft Experiments.** Mahlau cells (10 × 106 cells/mouse) prepared in 150 μL of DMEM were injected subcutaneously to the flank of 6–8 weeks old male athymic nude mice, while MDA-MB-231 cells (2 × 105 cells/mouse) in 1:1 DMEM and matrigel were injected into mammary fat pad (MFP) of 6–8 weeks old female athymic nude mice. Tumor volume and mouse weight were measured twice a week using calipers. When the tumor volume reached ~150 mm3, xenografts were randomized into groups (6 mice per group), and the mice were administered subsequently with 11 or 85 (40 mg/kg in 0.5% hydroxypropyl methylcellulose plus 1% Tween 80) or vehicle by gavage feeding (3–4 days/week) for 4 weeks. The mice were sacrificed 30 days after initiation of the compound treatment and tumors were collected. Two-way ANOVA was performed for statistical
significance by GraphPad Prism. All of the mice were maintained under a temperature-controlled environment with a 12 h light/dark cycle and received a standard diet and water ad libitum.

**Statistical Analysis.** All data were obtained from three independent experiments and standard deviation (S.D) values were accessed. All experiments except western blotting were done two times with n ≥ 3 biological replicates. One-way ANOVA and two-way ANOVA were applied using GraphPad (Prism) for statistical analysis. Results were shown as follows: ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03405.

Chemical procedures and experimental data of intermediates, NCI-60 cancer cell panel results for compounds 11 and 85, X-ray data, PK and in vivo test data, and copies of $^1$H and $^{13}$C NMR spectra of final compounds (PDF)

### AUTHOR INFORMATION

#### Corresponding Author

**Erden Banoglu** — Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, Ankara 06560, Turkey; orcid.org/0000-0003-4737-1733; Email: banoglu@gazi.edu.tr

#### Authors

**Sümeýye Turanlı** — Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, Ankara 06560, Turkey; Present Address: Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Adıyaman University, Adıyaman, Turkey

**Esra Nalbät** — Cancer Systems Biology Laboratory, Graduate School of Informatics, Middle East Technical University, Ankara 06800, Turkey

**Deniz Lengerli** — Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, Ankara 06560, Turkey; orcid.org/0000-0001-9838-8995

**Kübra İbiş** — Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, Ankara 06560, Turkey; orcid.org/0000-0002-6898-0258

**Sezen Güntekin Ergin** — Cancer Systems Biology Laboratory, Graduate School of Informatics, Middle East Technical University, Ankara 06800, Turkey; Present Address: Department of Medical Biology, Hacettepe University, Ankara 06800, Turkey.

**Ece Akhan Güzelcan** — Cancer Systems Biology Laboratory, Graduate School of Informatics, Middle East Technical University, Ankara 06800, Turkey; Present Address: Center for Genomics and Rare Diseases & Biobank for Rare Diseases, Hacettepe University, Ankara 06800, Turkey.

**Mesut Muyan** — Department of Biological Sciences, Middle East Technical University, Ankara 06800, Turkey

**Rengül Cetin-Atalay** — Cancer Systems Biology Laboratory, Graduate School of Informatics, Middle East Technical University, Ankara 06800, Turkey; Present Address: Section of Pulmonary and Critical Care Medicine, University of Chicago, Chicago, Illinois 60637, United States.

**Burcu Çalışkan** — Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, Ankara 06560, Turkey; orcid.org/0000-0003-2391-5644

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsomega.2c03405

#### Author Contributions

The manuscript was written with the contribution of all authors. All authors have approved the final version of the manuscript.

#### Notes

The authors declare no competing financial interest.

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### REFERENCES

1. Barnade, M. A.; Ghuge, R. B. Vincinal Diaryl Heterocyclic System: A Privileged Scaffold in the Discovery of Potential Therapeutic Agents. In Vincinal Diaryl Substituted Heterocycles. A Gold Mine for the Discovery of Novel Therapeutic Agents, Yadav, M. R.; Murumkar, P.; Ghuge, R., Eds.; Elsevier: 2018; pp 1–20.

2. Penning, T. D.; Talley, J. J.; Bertenshaw, S. R.; Carter, J. S.; Collins, P. W.; Docter, S.; Graneto, M. J.; Lee, L. F.; Malecha, J. W.; Miyashiro, J. M.; et al. Synthesis and biological evaluation of the 1,5-diarylpyrazole class of cyclooxygenase-2 inhibitors: identification of 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulfonylamide (SC-58635, celecoxib). J. Med. Chem. 1997, 40, 1347–1365.

3. Rayar, A. M.; Lagarde, N.; Ferroux, C.; Zagury, J. F.; Montes, M.; Sylla-Iyarreta Veitia, M. Update on COX-2 Selective Inhibitors: Chemical Classification, Side Effects and their Use in Cancers and Neurological Diseases. Curr. Top Med. Chem. 2017, 17, 2935–2956.

4. Boyd, M. J.; Freemantle, B. A. Ramelteon—a selective CBI antagonist. Ann. Pharmacother. 2005, 39, 684–690.

5. Rinaldi-Carmona, M.; Barth, F.; Congy, C.; Martinez, S.; Oustric, D.; Perio, A.; Poncelet, M.; Mariani, J.; Arnone, M.; Finance, O.; et al. SR147778 [5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-N-(1-piperidinyl)-1H-pyrazol-3 -carboxamide], a new potent and selective antagonist of the CB1 cannabinoid receptor: biochemical and pharmacological characterization. J. Pharmacol. Exp. Ther. 2004, 310, 905–914.

6. Fischer, L.; Hornig, M.; Pergola, C.; Meinld, N.; Franke, L.; Tanrikulu, Y.; Dodt, G.; Schneider, G.; Steinhiber, D.; Werz, O. The molecular mechanism of the inhibition by licofelone of the biosynthesis of 5-lipoxygenase products. Br. J. Pharmacol. 2007, 152, 471–480.

7. Weaver, A.; Rubin, B.; Caldwell, J.; McMahon, F. G.; Lee, D.; Makarowski, W.; Offenberg, H.; Sack, M.; Sikes, D.; Trapp, R.; et al. Comparison of the efficacy and safety of oxaprozin and nabumetone in the treatment of patients with osteoarthritis of the knee. Clin. Ther. 1995, 17, 735–745.

8. Banoglu, E.; Celikoglu, E.; Volker, S.; Olcag, A.; Gerstmeier, J.; Garscha, U.; Çalışkan, B.; Caliskan, B.; Schubert, U. S.; Carotti, A.; Macchiariulo, A. 4,5-Diarylsoxazol-3-carboxylic acids: A new class of leukotriene biosynthesis inhibitors potentially targeting 5-lipoxygenase-activating protein (FLAP). Eur. J. Med. Chem. 2016, 113, 1–10.
inhibitors with acid scaffold favorably modulates the activity as dual mPGES-1/5-LO.

Simple heteroaryl modifications in the 4,5-diarylisoxazol-3-carboxylic acid scaffold have been reported to modulate COX-2 activity. A high throughput assay was used to identify novel COX-2 inhibitors with acid scaffold. The predicted binding modes of the inhibitors with the COX-2 enzyme were compared using molecular dynamics simulations, which showed that the inhibitors interacted with the enzyme in a manner similar to the reference COX-2 inhibitor celecoxib. The inhibitors were evaluated in a biochemical assay and found to inhibit COX-2 activity. These results suggest that the inhibitors have potential as novel COX-2 inhibitors for the treatment of inflammation.

In addition, the inhibitors were evaluated for their ability to inhibit the growth of cancer cells. The results showed that some of the inhibitors inhibited the growth of cancer cells in a dose-dependent manner. The inhibitors were also evaluated for their ability to induce apoptosis in cancer cells. The results showed that some of the inhibitors induced apoptosis in cancer cells.

Overall, these results suggest that the inhibitors with acid scaffold have potential as novel COX-2 inhibitors for the treatment of inflammation and cancer. Further studies are needed to evaluate the inhibitors in vivo and to test their efficacy in clinical trials.