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Positioning of an unprecedented spiro[5.5]undeca ring system into kinase inhibitor space

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In-house 1,5-oxaza spiroquinone 1, with spiro[5.5]undeca ring system, was announced as an unprecedented anti-inflammatory scaffold through chemistry-oriented synthesis (ChOS), a chemocentric approach. Herein, we studied how to best position the spiro[5.5]undeca ring system in kinase inhibitor space. Notably, late-stage modification of the scaffold 1 into compounds 2a-r enhanced kinase-likeness of the scaffold 1. The improvement could be depicted with (1) selectivity with target shift (from JNK-1 into GSK-3) and (2) potency (> 20-fold). In addition, ATP independent IC₅₀ of compound 2j suggested a unique binding mode of this scaffold between ATP site and substrate site, which was explained by docking based optimal site selection and molecular dynamic simulations of the optimal binding site. Despite the shift of kinase profiling, the anti-inflammatory activity of compounds 2a-r could be retained in hyperactivated microglial cells.

Chemistry-oriented synthesis of 1,5-oxaza spiroquinone with a spiro[5.5]undeca ring system. It is challenging for researchers to increase the occupation of artificial drug space in the enormity of chemical space1–4. How many structurally diverse molecules are synthesizable for both selective and effective targeting in drug discovery? Because current approaches (of informatics and medicinal chemistry) commonly use the target as their query, this question cannot be the top priority for drug discovery or cannot be considered without coupling from SAR. Therefore, it is inefficient for a researcher to focus on structural novelty without selecting a target molecule or a target disease. However, neither rational drug design nor virtual screening can consider (or predict) structural novelty beyond the existing dataset. Notably, unprecedented scaffolds, frameworks or ring systems will remain unidentified treasures for drug discovery3,4. Our research group has developed organic synthetic methodologies for increasing synthesizability of rare drug scaffolds5–7 and has synthesized unprecedented drug scaffolds using the developed methodologies without an initial target9,10. We named the strategy chemistry-oriented synthesis (ChOS)9,10. An unprecedented drug scaffold, 1,5-oxaza spiroquinone 1, was also acquired through the ChOS approach10. The target deconvolution of scaffold 1 in kinase space showed weak inhibitory activity but suggested its potential as a selective inhibitor (Fig. 1)10. In addition, the spiro[5.5] undeca ring system of the scaffold 1, which showed in vitro and in vivo efficacy, was first in kinase inhibitor space. Therefore, the results encouraged us to further investigate better positions of the scaffold in kinase inhibitor space.

Explored molecules in kinase inhibitor space. High throughput screenings for protein kinases have found notable hit compounds similar to the ATP adenine group, so that most approved kinase inhibitors possess heteroaromatic rings such as quinazoline, indolinone, pyrrolopyrimidine, pyrazolepyrimidine, or quinolone group (Fig. 2A,B)11–14. Furthermore, the inhibitors occupy ATP binding sites, such as binding to the adenosine group15, and achieve high selectivity and nanomolar affinity through structure-based rational design, which was used to control the the delicate interactions with the hinge, the gate keeper, and the sites near the p-loop and the activation loop (especially the DFG residue of the activation loop)15–17. Following these orthosteric inhibitors (type I), type II kinase inhibitors have also been developed through additional DFG motif occupancy (as an allosteric site) as well as the binding sites of type I inhibitors9,10. The structures of type II kinase inhibitors typically...

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Figure 1. The positioning of 1,5-oxaza spiroquinone 1 in kinase inhibitor space.

Figure 2. Classification of kinase inhibitors11–27. (A) Typical ATP competitive inhibitors, (B) the binding mode of ATP competitive inhibitors, (C) example kinase inhibitors of each class, and (D) the binding mode of non-ATP competitive inhibitors. The left of (D) is p38 complex with GW434756X (type IV inhibitor in the C-terminal domain). The right of (D) shows the MEK complex with PD318088 (a type III inhibitor between the P-loop and activation loop). The gate keeper (green), hinge (green), DFG residues of the A-loop (violet), P-loop (red), helix (yellow), and C-terminal domain (blue) are shown.
such as oxyindoles: spiro[2.4]hepta (e.g., PLK-4 inhibitor)40 and spiro[4.4]nonane ring systems (e.g., ZSET1446). The whole kinase inhibitor space, spiro ring systems are of limited use for constructing adenine mimetic rings binding sites (Fig. 2C,D)19,20. While a part of the inhibitors are peptides or peptidomimetics similar to kinase inhibitors suggested the introduction of arylamine or heteroaromatic groups into the R1 position17. Therefore, stage diversification compared to other substitution positions. Privileged Bemis Murcko frameworks of kinase inhibitors, glycopyran synthase kinase 3 (GSK-3) inhibitors are notable examples showing such structural diversity because GSK-3 has seven binding sites that have been kinetically characterized46. The multiple binding sites are relevant to how GSK-3 can have diverse binding mechanisms with over 100 known endogenous substrates27. Several non-ATP competitive inhibitors with diverse ring systems have been reported30–39. However, despite the diversity, a GSK-3 inhibitor with a spiro ring system had not been reported until our study. In the whole kinase inhibitor space, spiro ring systems are of limited use for constructing adenine mimetic rings such as oxyindoles: spiro[2.4]hepta (e.g., PLK-4 inhibitor)40 and spiro[4.4]nonane ring systems (e.g., ZSET1446 as a CaMKII/ PKC inhibitor)46 as shown in Fig. 1. Obviously, the spiro[5.5]undeca core can introduce an additional plane into the two planes of 1,5-oxaza spiroquinone 1. The angles between the three planes (rings) are (1) 79.6° between the quinone and oxaza ring, (2) 66.5° between the quinone and N-phenyl ring, and (3) 49.3° between the oxaza ring and N-phenyl ring. Such a spatial arrangement with a rigid conformation is unlike to the known inhibitors shown in Fig. 2 and is rarely observed in kinase inhibitors, although some inhibitors derived from natural products show such spatial arrangements through congested quaternary carbon centers. In the case of the quinone group, it is not a privileged scaffold of kinase inhibitors, but approximately 100 tests against 22 kinases include a 1,4-quinone substructure. Specifically, the quinone group is useful for designing covalent inhibitors such as pyranonaphthoquinone natural products showing irreversible AKT1 inhibition (type 3 inhibitor)42.

In this study, ring systems that have been neither synthesized nor tested as kinase inhibitors are referred to as ‘NE ring system’ (not yet existing ring systems): we introduce a spiro[5.5]undeca ring system as an NE ring system, describe how to modify the initial scaffold 1 into compounds 2 to achieve the best positioning, and elucidate the shifted kinase selectivity with its unique binding mode between the ATP and substrate binding sites. In addition, the in vitro potencies of compounds 2 are described by a hyperactivated microglia model.

Results
Late-stage modification of 1,5-oxaza spiroquinone 1. In fragment based drug design, nonpolar atoms are typically used instead of polar heteroatoms to grow fragments41. Such a strategy cannot disrupt the potential binding of a pharmacophore to a target, so this fragment growing strategy was benchmarked to modify 1,5-oxaza spiroquinone 1. For this purpose, we should also consider late-stage functionalization for efficient introduction of diverse substituents in the very last steps of the synthesis44,45. As shown in Fig. 1 and Scheme 1, the α-position of tertiary amide, which is the starting point for fragment growth, is the most suitable for late stage diversification compared to other substitution positions. Privileged Bemis Murcko frameworks of kinase inhibitors suggested the introduction of arylamine or heteroaromatic groups into the R1 position17. Therefore, compounds 2, obtained by merging oxaza spiroquinone 1 with N-benzyl- or N-aryl-1,2,3-triazole derivatives, could be designed based on the kinase-likeness concept. Compound 7 was synthesized from malonamide ester 3 and used conditions identical to those we reported previously except for a modification of the oxidative cyclization10. The general conditions of the hypervalent iodine (III)-mediated oxidative cyclization (PIFA, anhydrous K2CO3, dry ACN, 0–25 °C) rely on the synthetic skill and maintaining rigorously anhydrous conditions, or else the isolated yields can decrease less than 10%. Undesirable side reactions include (1) oxidative removal of the quinone groups and (2) hydride or hydroxyl group transfer into the quinone iminium intermediate instead of the beta hydroxyl group. 1,1,1,3,3,3-Hexafluoro-2-propanol, a preferred solvent for hypervalent iodine chemistry, did not influence the inefficiency of the reaction. A base (e.g., K2CO3) was essential to prevent acid catalytic C-H functionalization of trivalent iodine7,8, nitromethane presented consistently the product of 60% isolated yield without any base. After improving the cost-effectiveness and synthetic efficiency of the reaction, aromatic azides 8a-r were synthesized according to the literature protocol16. The Huisgen 1,3 dipolar cycloaddition reaction of the two click partners, 7 and 8a-r regioselectively produced desirable products 2a-r in good yields with CuSO4 as the catalyst and in the presence of sodium ascorbate (see Supplementary S1)17. All of the synthesized compounds were purified by column chromatography, and the structure of all synthesized compounds was characterized using MS, IR, 1H NMR, and 13C NMR techniques.

Kinase specificity and inhibitory effect of 1,5-oxaza spiroquinone 2. After synthesizing compounds 2a-r, compound 2d was randomly chosen for the comparison with compound 1c in kinome space. To navigate kinome space, among all typical protein kinases (No. of kinases: 369), compound 2d was first tested for kinases that were inhibited by compound 1c. Specifically, the classes of human protein kinases that were inhibited by compound 1c led to the selection of thirty-three kinases from eight classes (TK, CMGC, CAMK,
AGC, CK1, STE, TKL, and others). For example, CDK family and JNKs were chosen from CMGC. PKC, PKN and ROCK were selected from AGC, and DAPKs and NUAK1 also were selected from CAMK. Secondly, atypical kinases screening was conducted, and this step included seventeen lipid kinases and twenty non-eukaryotic kinases, as shown in Fig. 3 and Supplementary S7. Surprisingly, among a total of 70 kinases, compound \(2d\) perfectly inhibited only GSK-3β (more than 90% activity), as shown in Fig. 3 and Table 1. Obviously, while compound \(1c\) did not show any inhibitory activity on GSK-3β, compound \(2d\) fully inhibited GSK-3β. In the case of CMGC, AGC, and CAMK kinase, the two compounds showed slightly different inhibition for DAPK1 and JNK1, and CDK family members were similarly inhibited by the two compounds. Based on these promising results, the inhibitory activities of eighteen late-stage synthetic compounds (\(2a-r\)) were tested on GSK-3β, as shown in Table 2. The steric effects of the substituents, the electronic effects of the substituents, and the substitution positions were considered in the investigation. First, compounds with an \(N\)-benzyl group (compound \(2r\)) and an \(N\)-phenyl group (compound \(2a\)) showed similar activities. Thus, flexible benzyl derivatives were not considered for further study. Second, the ortho, meta, and para positions of the \(N\)-benzene group did not show consistent results. For example, a meta-benzyl group (compound \(2j\)) was superior to compound \(2a\), but the para-methoxy (compound \(2h\)) and para-thiomethoxy (compound \(2i\)) groups did not cause enhanced activity compared to that of compound \(2a\). Small substituents (compound \(2c\) or electron-withdrawing groups (compound

**Scheme 1.** Reagents and conditions: (a) NaH, propargyl bromide, dry DMF, 0 °C-rt, 4 h, 92%; (b) LiAl(O(Bu))\(_3\)H, dry THF, −15 °C-rt, 4 h, 82%; (c) BF\(_3\).Et\(_2\)O, DMS, 0–15 °C, 10–20 min, 88%; (d) PhI(CF\(_3\)CO\(_2\))\(_2\), anhydrous K\(_2\)CO\(_3\), NO\(_2\)Me, 0 °C–25 °C, 4 h, 60%; (e) CuSO\(_4\), Sodium Ascorbate, 0 °C–rt, THF:H\(_2\)O, 12 h.
Meta-substituted compounds 2d (meta-Cl) and 2g (meta-CN) showed better activity than compound 2a, which encouraged us to further study the meta position. Heteroaromatic rings did not offer any improvement over compound 2b.

When the electrostatic effects of substituents at the same position were compared, electron withdrawing groups at the para position did not improve the activity, except for compound 2k. Notably, a bulky adamantyl group (compound 2q) showed activity similar to those of compounds with benzene group (compound 1a).

The comparison between compounds 2k and 2l showed the complexity of the noncovalent interactions involving the para position. Activity of symmetrical di-meta substituted compound 2m suggested different environments for the two meta positions. To further elucidate the inhibitory activity at the atomic level, a molecular docking simulation of compound 2a-r was essential. After selecting the most potent compound 2j, IC50 values were measured under five ATP concentrations (1, 10, 50, 100, and 200 µM). Surprisingly, while staurosporine showed a steep decrease in IC50 (5 nM vs 140 nM) corresponding to increased ATP concentration, compound 2j retained its IC50 activity regardless of the ATP concentration (Table 3). It means that potency of compound 2j didn't depend on ATP concentration.

### Molecular binding mode of selected compounds 2 on GSK-3β

Molecular docking simulations of the chosen compounds (2d, 2h, 2j-m, and 2p) were conducted with GSK-3β crystal structures (PDB ID: 1J1B, 3I4B, 1Q3W and 1Q4L) (Tables 4–5). The logic of PDB selection was identical to Palomo et al. study. These chosen compounds were used to explain the environment near to the meta-substituent (2d and 2m) and the stereo-electronic effects of the para position (2h, 2j, 2k, and 2l). Palomo et al. proposed six binding sites, which were reproduced for our docking models (except for axin binding site, site3). Sites 1 and 2 were partially overlapped between the ATP binding region (site 1) and the substrate binding region (site 2), so that sites 1 and 2 were merged for an additional docking model to investigate binding on the interface of both binding sites. After validating the models, docking was performed to identify which of the six sites is more likely to be favored by these compounds. The binding modes of more active (2j, 2d, 2k and 2p) and less active compounds (2h, 2l and 2m) were iteratively compared for all probable binding sites to explore the most relevant binding site and orientation. The percentage probabilities for each binding site of each compound were calculated by counting the number of poses in each binding site among the top 1000 poses of a respective compound. The data in Table 4 suggest that the probable binding sites are site 1, site 2 and site 1/2, but the data in Table 5 suggest that site 1 and site 1/2 have better docking score. The data in both tables emphasize that the scaffold binds to the hinge region, which is present in two sites (site 1 and site 1/2), and suggest that the most likely binding site is the merged site, which
| Entry | R Group | % GSK3β Residual Activity\(^a\) (IC50 on GSK3β)\(^b\) | NO Inhibition IC50 (μM)\(^c\) | Cell Viability (%)\(^d\) |
|-------|---------|-------------------------------------------------|----------------------------|-----------------|
| 1a    | ![Chemical Structure] | 85.8 ± 16.1 | 0.54 ± 0.0 | 74 ± 7 |
| 1b    | ![Chemical Structure] | 91.0 ± 12.6 | 0.98 ± 0.03 | 84 ± 3 |
| 1c    | ![Chemical Structure] | 107.5 ± 1.9 | 0.07 ± 0.01 | 92 ± 13 |
| 7     | ![Chemical Structure] | 55.7 ± 8.4 | 0.68 ± 0.07 | 100 ± 24 |
| 2a    | ![Chemical Structure] | 62.3 ± 2.3 | 0.67 ± 0.05 | 81 ± 3 |
| 2b    | ![Chemical Structure] | 87.5 ± 0.6 | 0.87 ± 0.11 | 120 ± 11 |
| 2c    | ![Chemical Structure] | 84.8 ± 5.3 | 0.78 ± 0.04 | 109 ± 13 |
| 2d    | ![Chemical Structure] | **51.1 ± 6.1** (7.04 μM) | **0.66 ± 0.00** | **90 ± 6** |
| 2e    | ![Chemical Structure] | 69.6 ± 5.1 | 0.67 ± 0.01 | 93 ± 6 |
| 2f    | ![Chemical Structure] | 96.3 ± 16.5 | 0.77 ± 0.02 | 115 ± 3 |
| 2g    | ![Chemical Structure] | 41.8 ± 0.9 | 0.80 ± 0.13 | 128 ± 17 |
| 2h    | ![Chemical Structure] | 111.7 ± 4.5 | 0.76 ± 0.01 | 124 ± 7 |
| 2i    | ![Chemical Structure] | 66.9 ± 11.4 | 0.98 ± 0.04 | 121 ± 2 |
| 2j    | ![Chemical Structure] | **10.5 ± 2.0** (1.53 μM) | **0.63 ± 0.01** | **74 ± 4** |

Table 2. (continued)
starts from site1 and extend up to site2. In addition, site 1 cannot explain these experimental results of Table 3. In general, docking scores and experimental activity do not show complete correlation. In our case, the activity order of tested compounds except for compound \(2j\) roughly matched the predicted order (\(2p > 2j > 2k > 2m, 2l\), and \(2h\)). When the docking pose of compound \(2j\) (or \(2k\)) is compared with the pose of compound \(2l\), the triazole group commonly has a pi-cation interaction with Lys85 and the amide group (C=O, oxygen) of spiroquinone has additional hydrogen bonding with Lys85 in sites 1 and 2 in Fig. 4. Molecular docking calculations indicated

### Table 2. Inhibitory effect on the % GSK3β activity of 1,5-oxaza spiroquinone compounds (1a-c, 2a-r) and anti-inflammatory effect on LPS-induced BV-2 cells.

| Compound | ATP concentration (µM) | 1 | 10 | 50 | 100 | 200 |
|----------|-------------------------|---|----|----|-----|-----|
| \(2k\)  | 35.1 ± 4.2               | 0.77 ± 0.06 | 71 ± 3 |
| \(2l\)  | 122.3 ± 2.3              | 0.93 ± 0.06 | 109 ± 16 |
| \(2m\)  | 114.4 ± 4.5              | 1.22 ± 0.30 | 108 ± 5 |
| \(2n\)  | 85.6 ± 2.0               | 0.85 ± 0.04 | 102 ± 8 |
| \(2o\)  | 75.0 ± 0.7               | 0.74 ± 0.08 | 89 ± 8 |
| \(2p\)  | 35.7 ± 3.7 (11.4 µM)     | 0.85 ± 0.19 | 97 ± 2 |
| \(2q\)  | 71.6 ± 1.4               | 0.78 ± 0.10 | 87 ± 7 |
| \(2r\)  | 61.7 ± 13.8              | 0.65 ± 0.06 | 75 ± 11 |
| L-NMMA *  | 19.65 ± 0.42             | 105 ± 4 |

### Table 3. IC\(_{50}\) Measurement of the GSK3β activity of 1,5-oxaza spiroquinone compound \(2j\) in several ATP concentrations.

| Compound | ATP concentration (µM) | 1 | 10 | 50 | 100 | 200 |
|----------|-------------------------|---|----|----|-----|-----|
| \(2j\)  | 2.65 3.53 3.79 4.04 3.45 |
| Staurosporine | 5.05 6.49 19.44 32.24 140.20 |
The higher selectivity of compounds 2j, 2k, 2p, and 2d for the interface between sites 1 and 2 (Fig. 4, Table 4 and Table 5). The docking results of the more and less actives compounds suggested the binding preference to site

### Table 4. Binding site population of the Top 1000 docking poses of the selected compounds 2. aPDB: 1J1B was used for building docking models (sites 1, 4, 5, and 7). bPDB: 3I4B was used for building docking models (site 1 and sites 1 and 2). cPDB: 1Q4L was used for building a docking model (site 6).

| Compound | Site 1 | Site 2 | Site 1/2 | Site 4 | Site 5 | Site 6 | Site 7 |
|----------|--------|--------|----------|--------|--------|--------|--------|
| 2d       | 256    | 192    | 320      | 0      | 168    | 0      | 64     |
| 2h       | 128    | 192    | 256      | 0      | 128    | 0      | 296    |
| 2j       | 192    | 360    | 384      | 64     | 0      | 0      | 0      |
| 2k       | 320    | 360    | 320      | 0      | 0      | 0      | 0      |
| 2l       | 64     | 360    | 384      | 0      | 0      | 0      | 192    |
| 2m       | 384    | 232    | 320      | 0      | 0      | 0      | 64     |
| 2p       | 384    | 128    | 320      | 168    | 0      | 0      | 0      |

### Table 5. Best docking score* of the selected compounds 2 on each annotated binding site. *Glide XP was selected as a scoring function with epic penalty.

| Compound | Site 1 | Site 2 | Site 1/2 | Site 4 | Site 5 | Site 6 | Site 7 |
|----------|--------|--------|----------|--------|--------|--------|--------|
| 2d       | −5.373 | −4.008 | −4.761   | 0      | −3.614 | 0      | −3.119 |
| 2h       | −4.489 | −3.928 | −5.438   | 0      | −3.576 | 0      | −3.606 |
| 2j       | −5.546 | −4.019 | −6.41    | −3.133 | 0      | 0      | 0      |
| 2k       | −4.731 | −3.371 | −6.144   | 0      | 0      | 0      | 0      |
| 2l       | −5.617 | −4.925 | −5.395   | 0      | 0      | 0      | −5.798 |
| 2m       | −4.245 | −4.06  | −5.423   | 0      | 0      | 0      | −3.659 |
| 2p       | −5.712 | −3.165 | −7.362   | −3.488 | 0      | 0      | 0      |

**Figure 4.** Docking simulations of selected compounds 2 at site 1 and 2 interface in 3I4B (Important interaction residues are represented with orange color text); (A) Interaction of compound 2d, (B) Interaction of compound 2j, (C) Interaction of compound 2k, (D) Interaction of compound 2p.
1/2 but were unable to differentiate between their bioactivity profile, which is a drawback of docking because it cannot not consider the dynamic nature of the protein.

The drawback of docking was complemented by molecular dynamics (MD) simulations, and it allowed us to further explore the interaction patterns in solvated dynamic conditions at the interface of sites 1 and 2, as suggested by docking (supplementary S3 to S5). The MD simulations of the chosen docking complex (site 1 and site 1/2) provided a sample of all possible interactions in dynamic stages and validated the above binding mode indicated by molecular docking (Fig. 5). In addition, our MD results were compared with the crystallographic interaction information of ATP/substrate and their competitive inhibitors reported in the PDB. In the case of ATP competitive inhibitors (site 1), a substantial amount of X-ray data was available for comparison. Notably, site 2 is reported to bind with primed substrates at the catalytic core consisting of Phe 67 (Gly 63 to Val 69 loop) and Arg96 (Gln 89 to Arg96 loop) at the interface of the primed phosphate binding pocket (red region of Fig. 5A)48, 49. In contrast to the substrates, the simulations suggested that our 1,5-oxazaspiroquinone scaffold interacts partially with the catalytic core (compound 2p of Fig. 5A)48,50. By comparing these results to those in the literature, we determined that the occupied regions of the chosen compounds could partially overlap with the site 1 (ATP binding site) and site 2 (substrate binding site) regions. It was repetitively observed that the triazole intermittently switched between interactions with Lys85 and Asp200 in the active site. Lys85 formed the strongest interactions and was able to maintain those interactions throughout the MD simulation (Fig. 5B–C). Based on this observation, it was expected that Lys85 plays a key role in the stabilization of the binding complex through π-cation interactions with the triazole rings of compound 2 and the aromatic rings present on both sides of the triazole (Fig. 5C–D), to facilitate other noncovalent interactions at site 1 (H-bonding interactions with carbonyl groups of compounds 2) and site 2 (hydrophobic interactions with Phe67, Val88, and Phe93).
Anti-inflammatory effects of compounds 2. To investigate the pharmacological potential of compounds 2a-r, they and compound 7, a click partner, were administered to lipopolysaccharide (LPS) treated BV-2 cells as an inflammation model of hyperactivated microglia. First, nitric oxide (NO) production and cell viability were measured according to our previous study of compound 1. The observed IC50 values of NO production are given in Table 2. Every tested compound showed IC50 values (from 0.63 to 1.22 µM) superior to (2S)-N-methylarginine (L-NMMA), a well-known NOS inhibitor. The scaffold extension through click chemistry did not influence on NO production, and the IC50 values of the compounds 2a-r were similar to those of compound 1a-b. In the case of cell viability, the cell viabilities of some compounds slightly decreased at 5 µM but the decrease were recovered at 1 µM to solve the cell toxicity issue (Supplementary S8).

The promising results of compounds 2a-r encouraged us to check the regulation of pro-inflammatory cytokines and mediators related to NO production. Fortunately, the tested compounds regulated the expression levels of iNOS and COX-2. Compounds 2a and 2d (0.01 to 1 µM) showed efficacies similar to 20 µM level L-NMMA (Fig. 6). While compound 2a showed limited potency and efficacy, compound 2d downregulated iNOS and COX-2 more efficiently than compound 2a, and the regulation of COX-2 showed concentration dependency. Compound 2d presented 20-fold higher potency than L-NMMA, and the efficacy of the compound was retained at 0.01 µM. Finally, pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and PGE2, another pro-inflammatory mediator, were studied with respect to compounds 2a and 2d (Fig. 7). The study also confirmed the superiority of compound 2d with respect to compound 2a or L-NMMA. Interestingly, compound 2d also regulated the expression levels of PGE2, TNF-α, IL-6, and IL-1β in a concentration-dependent manner, confirming their pharmacological potential on inflammation.

Discussion and conclusions
Kinase-like shape of 1,5-oxaza spiroquinone as an NE ring system. Ideally, if researchers can find unprecedented inhibitors in kinase space, the success rates of kinase therapies can be enhanced due to these enriched pools. In particular, biologically active ‘NE ring systems’ (not yet existing ring systems) can help researchers update the definition of what structures are kinase-like. NE ring systems can provide unique molecular shapes and pharmacophore features, different from known inhibitors. Although a pocket of a kinase cannot be occupied with any known inhibitor due to dissimilar shape or improper electrostatic property between the pocket and the inhibitor, NE ring systems are eligible for the pocket. Thus, unprecedented features of active NE ring systems can be useful for investigating novel binding modes (using known sites), as well as new binding sites of kinases. In the case of our spiro[5.5]undeca ring system, the core structure cannot exist within one flat space, and the quinone group and the oxaza ring are perpendicular to each other. The three rings of 1,5-oxazaquinone cannot exist on approximately one plane. In other words, one ring forces another ring out of the plane. At a
Molecular insight into the spiro[5.5]undeca ring system for targeting kinases. As a spiro[5.5]undeca ring system, our 1,5-oxaza spiroquinone did not satisfy the “2–0” rules, N(aromatic) + NH(aromatic) > 2 or Ar–NH + R-CN > 0.5. Experimentally, compound 1c also showed selective weak potency against 5 kinases (JNK1, CDK1/2, DAPK1, and PKCa) among the 379 protein kinases tested. After obtaining data on spiroquinone 1, the optimization of the kinase-likeness of unprecedented scaffold 1 could be achieved by merging our scaffold 1 with a privileged Bemis Murcko framework: (1) an aniline (Ar−NH), (2) a bisarylaniline (Ar1−NH−Ar2), (3) a heteroaromatic group having nitrogens (Naro), and (4) a heteroaromatic group having NH (NHaro). Based on the efficiency of the synthetic route, R1 group modification can provide facile derivatization during the late-stage development of the scaffold 1 (Fig. 1). After the cyclization of the 1,5-oxaza ring, click chemistry for triazole formation (compounds 2a-r) was the most atom- and step- economical method that did not interfere with other functionalities. The introduction of the triazole group is an example of a heteroaromatic group having nitrogens (Naro) to satisfy the “2–0” rules. As a result, dramatic changes in the kinase profiles were observed between JNK-1 and GSK-3. Notably, the potencies were also enhanced from compound 1c to compound 2d. During further SAR studies through the merging strategy, an introduction of a second privileged Bemis Murcko framework, such as a 3-pyridine group (2b), a cyano group (2g), and an acetamido group (2l), was considered. However, the introduction of this second group did not result in an impressive change in potency. The potencies of compounds 2j and 2p, as well as the specificity of compound 2d, verified the kinase-like potential of scaffold 1. To explore the cause of the selective bioactivity against GSK-3β, we performed docking followed by

Figure 7. The control of LPS-induced inflammation by compounds 2a and 2d in BV-2 cells: (A) the secreted level of PGE2 as a pro-inflammatory mediator, (B) TNF-α, (C) IL-6, and (D) IL-1β as pro-inflammatory cytokines.
molecular dynamics to investigate the interaction pattern (Supplementary S3). At this moment, the origin of current improvement and the points for further improvements are not perfect. However, notably, our simulations indicate that the spiroquinone ring system could not perfectly occupy the ATP binding site of GSK-3 as do typical ATP competitive inhibitors. The molecular dynamics results of active compound 2j or 2p support that the key differential inhibitory effect of these compounds arises from hydrophobic interactions with the substrate binding pocket, particularly with residues Phe93/Phe67. Although most interactions are common for the main core of compounds 2, the difference in the inhibitory activities can be explained by the residues surrounding the N-aryl substituent at the triazole ring. When a polar group is present near the triazole ring substituent (2h, 2l and 2m), the hydrophobicity of the 63 to 69 loop can decrease, supporting the decrease in inhibitory activity. The 3-benzylphenyl substituent (compound 2p) could reach the Leu88 backbone to form a hydrogen bonding interaction as well as a π–π stacking interaction with Phe93/Phe67 to maximize the binding potential. The length of the substituent was also critical for reaching the edge of the 89 to 95 loop at site 2 and forming a hydrogen bond with the protein backbone, as well as a π–π stacking interaction with Phe93/Phe67. Our simulations showed that a substituent on the triazole matches well with the protein if the abovementioned condition is satisfied. A comparison between the more and less active compounds showed differences in the synchronization through the RMSF values (Supplementary S4). In particular, the simulation results suggest that the ligand is shuffling between sites 1 and 2 and stabilized by the Lys85 anchor. The ligand movement towards site 2 provides an anchor for the water bridge interaction with Tyr134 instead of a hydrogen bonding and Phe67 interaction (Fig. 5A). The ligand forms a hydrogen bond with the phenolic OH group of Tyr134 (site 1) and then destabilizes the interaction with Phe67, which is compensated by the hydrophobic region of the 63 to 69 loop in site 2.

Anti-inflammatory effect & target deconvolution. Well-known literature56,57 described that the inhibition of GSK-3 can result in the downregulation of microglial migration (activation) and the control of pro-inflammatory factors. Notably, GSK-3 has been proposed as a crucial regulator to balance pro- and anti-
inflammatory cytokine production\textsuperscript{56}. LPS is a known ligand of TLRs, a pathogen recognition receptor (PRR), so LPS binds to TLRs on the cell surface (including microglia) to induce inflammatory cytokines and nitric oxide production from NO\textsubscript{S}\textsuperscript{5}. During this process, active GSK-3 can amplify the neuroinflammatory process\textsuperscript{56,58}. In other words, induced pro-inflammatory cytokines were diminished by GSK-3 inhibitors (through the control of transcriptional factors) and NO production is also dependent on GSK-3\textsuperscript{56–59}. On the other hand, the JNK signaling pathway mediates apoptosis and is critical for neuronal cytotoxicity, and JNK can be activated by nitric oxide stimulation\textsuperscript{10}. Although studies on the role of GSK-3\textbeta in JNK activation are controversial, it was reported that GSK3 inhibition also promotes LPS-stimulated IFN\textbeta production via its ability to regulate c-Jun\textsuperscript{60}. When GSK3 inhibition also promotes LPS-stimulated IFN\textbeta production via its ability to regulate c-Jun\textsuperscript{60}. When 

Experimental section

Chemistry. Every general chemistry procedure of this study is identical to our previous study on the scaffold\textsuperscript{10}. All of the reagents and solvents were used without further purification. General reactions were performed under an atmosphere nitrogen with magnetically stirring and reaction monitoring was conducted by analytical thin layer chromatography (TLC) in the visualized condition of UV light (254 nm) and staining (Ninhydrine & PMA solutions) with heating. Flash column chromatography was performed on silica gel (230–400 mesh). \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were analyzed using Brucker 600 MHz and chemical shifts were quoted in parts per million (ppm) with the calibration through the residual proton and carbon resonance of the solvent (CDCl\textsubscript{3} for sites 2 and 5, in addition to the merged model of sites 1 and 2. Furthermore, 1Q3W was selected for stimulation\textsuperscript{10}. Although studies on the role of GSK-3\textbeta in JNK activation are controversial, it was reported that GSK3 inhibition also promotes LPS-stimulated IFN\textbeta production via its ability to regulate c-Jun\textsuperscript{60}. When GSK3 inhibition also promotes LPS-stimulated IFN\textbeta production via its ability to regulate c-Jun\textsuperscript{60}. When

To a solution of compound 7 (1 eq.) in 1:1 THF and H\textsubscript{2}O mixture, aromatic azide (2 eq.) was added, and then followed by addition of CuSO\textsubscript{4} (2 eq.) and sodium ascorbate (1 eq.) at room temperature. The reaction mixture was stirred 12–15 h and reaction was monitored by TLC. After completion of reaction, the reaction mixture was filtered through celite. The filtrate was washed with water. The organic layer was washed with brine solution and organic layers was dried over sodium sulphate and evaporated under reduced pressure to afford the crude residue, which was purified by silica gel column chromatography by eluting with ethyl acetate/hexane to yield corresponding triazole derivatives. All synthesized triazole derivatives were confirmed by the spectral analysis. Brown gummy liquid; 85% yield; IR (neat): 2988, 2862, 2718, 1740, 1710, 1689, 1489, 1270, 1098, 733 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (600 MHz, CDCl\textsubscript{3}) \(\delta\) 7.96 (s, 1H), 7.78–7.73 (m, 2H), 7.71 (d, \(J=1.1\) Hz, 1H), 7.33–7.27 (m, 3H), 7.19 (dd, \(J=10.3, 3.2\) Hz, 1H), 7.02–6.96 (m, 2H), 6.83 (dd, \(J=10.2, 3.2\) Hz, 1H), 6.37 (d, \(J=1.1\) Hz, 1H), 6.12 (dd, \(J=10.3, 2.0\) Hz, 1H), 6.03 (dd, \(J=10.2, 2.0\) Hz, 1H), 4.62 (dd, \(J=12.1, 9.5\) Hz, 1H), 4.40 (dd, \(J=12.2, 6.4\) Hz, 1H), 3.43 (dd, \(J=15.0, 6.8\) Hz, 1H), 3.38–3.32 (m, 1H), 3.20 (dd, \(J=15.0, 3.9\) Hz, 1H), 2.49 (d, \(J=1.1\) Hz, 3H); \textsuperscript{13}C NMR (150 MHz, CDCl\textsubscript{3}) \(\delta\) 184.00, 169.59, 159.96, 151.51, 144.68, 142.89, 136.41, 133.40, 129.94, 129.90, 129.81 (3 C), 129.17 (4 C), 129.11, 126.29, 120.81, 115.78, 115.74, 108.40, 83.53, 63.80, 41.51, 23.67, 18.76 ppm; HRMS (EI, m/z): M\textsuperscript{+} calculated for C\textsubscript{28}H\textsubscript{22}N\textsubscript{4}O\textsubscript{5} 494.1590, Found 494.1575.

Molecular docking simulations. As the best PDB, 1J1B was selected for sites 1 and 7 and 3I4B was chosen for sites 2 and 5, in addition to the merged model of sites 1 and 2. Furthermore, 1Q3W was selected for
site 4 and 1Q4L for site 6 modeling. The reliability of the docking protocol for GSK3 was validated through redocking of respective binding modes (RMSD between redocking pose and crystal pose: BDBM8269-ligand of 1Q4L = 0.6475 Å, BDBM35641-ligand of 3I4B = 0.8745 Å, see also supplementary S2). The ligands were sketched and subjected to ligand preparation using the LigPrep module of the Schrodinger software suite (2018). The resulting ligands are subjected to ConGen for generating 100 minimized conformations for each ligand for preparation of molecular docking. Structures of GSK-3 were retrieved from the PDB with the identification numbers 2j2b, 3I4B and 1Q4L. The initial structure of the protein was refined and subjected to energy minimization in order to ensure correct starting structures. All heteroatoms (except inhibitor and metal ion) were removed from protein files. All water molecules (beyond 3 Å from the inhibitor) and the rest of the chains (except A) were removed from the complex, and the protein was minimized using the OPLS3e force field. Furthermore, H atoms were added to the protein to correct ionization and tautomeric states of amino acid residues. A receptor grid was generated with a 30 Å size for each site by selecting centroid amino acids using receptor grid generation in the Glide application based on published probable sites (7 sites). The ligands were docked to the protein using the extra precision mode (XP) Glide docking protocol after respective receptor grid generation. Ten poses were written for each ligand. OPLS3e was used to calculate the binding energy and protein–ligand binding poses for each compound. All of the docking poses of selected ligands at six binding sites were collected, sorted by docking score, and filtered by top 1000 scores. Docking scores of two different sites could not be normalized due to insufficient data for describing the probability density function of the docking score in each site. The percent probability was calculated by shorting the ligand based on the docking score after clubbing all selected receptor results.

Molecular dynamics simulation. The MD calculations were performed with the help of Desmond to evaluate the protein–ligand interactions in solvated conditions. The modeled protein–ligand complexes were embedded through docking in the TIP3P water model. Orthorhombic periodic boundary conditions were selected to specify the shape and size of the repeating unit with minimization of the total volume. The protein–ligand complex system was neutralized with the counterions, and a physiological salt concentration was used at 0.15 M. The system was developed and minimized with default settings (except time and temperature). The relaxed system was subjected to 50 ns simulation time with a time step of 2 fs in the constant number, pressure, and temperature (NPT) ensemble using a Nose–Hoover thermostat at 310 K and Martyna–Tobias–Klein barostats at 1.01 bar pressure. Every trajectory was recorded with a time interval of 20 ps. Energy potential, root mean square deviation (RMSD), root mean square fluctuations (RMSF) and intermolecular hydrogen bond interactions were monitored for the stability of the protein–ligand complex and analyzed to understand the underpinning of the dynamic interaction. The MD simulations of the chosen docking complex (site 1 and sites 1/2) were conducted to sample all possible scenarios of the interaction in dynamic stages of the enzyme and to validate the above binding mode of molecular docking.

Reagents and cell culture. Lipopolysaccharide (LPS), were obtained from Sigma chemical (St. Louis, MO). Penicillin–streptomycin (PS), Fetal bovine serum (FBS), Dulbecco modified Eagle medium (DMEM) were brought from Invitrogen (Carlsbad, CA, USA). sulfanilamide and 0.1% N-1-naphthylethenediamine dihydrochloride were purchased from sigma chemicals. Prostaglandin E2 (PGE2), Interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and interleukin (IL-1β) elisa kit was purchased from R&D Systems (Minneapolis, MN, USA). Primary antibodies against various proteins such as inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), and a-tubulin were obtained from abcam, Santa Cruz technology and cell signaling technology. Secondary antibodies against rabbit, goat and mouse were obtained from sigma chemicals. Murine microglial cell BV2 were used to evaluate the effect of compound against LPS activated microglia. BV2 cell we obtained as a gift sample by Dr. E Choi from Korea University, Seoul South Korea. This cell was maintained with high glucose DMEM supplemented with10% heat-inactivated FBS and 1% of mixture of penicillin–streptomycin (penicillin (1 × 10^{-3} U/L), and streptomycin (100 mg/L)). Cultured cells were maintained by keeping the in a humidified incubator with 5% CO_{2} at 37 °C.

Kinase assays. Every inhibitory activity of tested compounds was measured by radiolabelled method of Reaction Biology Corp). The radiolabelled ATP ([γ-^{33}P] ATP) replaced a substrate with 33P-phosphorylated substrate so that the activity of a kinase was measured from the radiolabelled phosphorylated substrate. Kinase panel assay was tested at 30 μM testing compounds with an ATP concentration of 10 μM and a substrate concentration of 10 μM. % Residual activity against GSK3β was measured at 10 μM compounds 1 and 2. ATP competitive assays of compound 2j was measured in 10-dose IC_{50} mode with 3 or fourfold serial dilution starting at 10 μM. Curve fits of control compounds were performed where the enzyme activities at the highest concentration of compounds were less than 65%. The concentration of DMSO was controlled and raw data, % kinase activity was calculated relative to DMSO controls.

Cell treatment, nitric oxide and cell viability assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed to evaluate the cytotoxicity of the samples to the microglia. BV2 cells at the density of 4 × 10^4 cells /well were seeded in the 96 well plate and incubated for 24 h. Seeded cells were pre-treated with different concentration of the samples, and LPS (100 ng/ml) activation was performed after 30 min of compound treatment. Treated plate were incubated for 24 h in the incubator and nitric oxide assay and cell viability assay was performed. Treated cell's conditioned medium was used to evaluate the NO assay, and the attached cells were used to evaluate the cell viability assay. 50 μL of conditioned medium (CM) from treated cells were transferred to a new 96 well plate for NO assay. This CM was mixed with the equal volume of the mixture of
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< 0.05. Each experiment was performed in triplicate.

P by the Tukey post-hoc test using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical

Taglantidin E2 (PGE2), Tumor necrosis factor (TNF-α), Interleukin (IL-6), Interleukin (IL-1β), BV2 cells were

Quantitative analysis of the protein bands was

Western blot analysis. Western blot analysis was performed to see the protein expression of the iNOS and COX-2 in the BV2 cells. BV2 cells were seeded in a 6 well plate at the density of 6 × 10⁶ cells/well overnight and cells were treated as mentioned earlier in the cell treatment section and this time treatment was performed for 6 h only. After 6 h, cells were harvested and lysed with lysis buffer containing (RIPA, protease inhibitor & phosphatase inhibitor), protein estimation was performed using biorad assay and the 30 µg of total protein was separated by 8% SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes, and incubated with primary antibodies against tubulin, iNOS, COX-2, and α-tubulin. PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and protein bands were visualized using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Quantitative analysis of the protein bands was quantified using Image Master 2D Elite software (version 3.1, Amersham Pharmacia Biotech).

Statistical analysis. All results are expressed as mean ± standard error of the mean (SEM). Statistical signific-

ance between experimental groups were determined by using one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was set at P < 0.05. Each experiment was performed in triplicate.

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**Author contributions**
M.-h. K., S.Y.K. and S.Y. L. conceived and designed the study with research funding. Under M.-h.K’s design of drug scaffold, A.V., L.P.D., and B. G. N. synthesized the tested compounds and analysed every in vitro data. Under S.Y.K’s design, L.S. conducted cell based assay and wrote her experimental section. M.K.T carried out all the modeling and in silico work. M.-h. K., A.V., and M.K.T analysed the data and wrote the manuscript. M.-h. K. and M.K.T revised the manuscript. M.-h.K. and S.Y.K. provided the molecular modelling lab and in vitro research work facility. All the authors read and approved the final manuscript.

**Competing interests**
The authors declare no competing interests.

**Additional information**
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