Pyranonaphthoquinone Lactones: A New Class of AKT Selective Kinase Inhibitors Alkylate a Regulatory Loop Cysteine

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Abstract: The naturally occurring pyranonaphthoquinone (PNQ) antibiotic lactoquinomycin and related aglycones were found to be selective inhibitors of the serine-threonine kinase AKT. A set of synthetic PNQs were prepared and a minimum active feature set and preliminary SAR were determined. PNQ lactones inhibit the proliferation of human tumor cell lines containing constitutively activated AKT and show expected effects on cellular biomarkers. Biochemical data are presented supporting a proposed bioreductive alkylation mechanism of action.

The serine/threonine kinase AKT[1] is a central player in the signaling pathway that begins with the activation of phosphoinositide 3′-kinase (PI3K[a]), which generates phosphatidylinositol (3,4,5) triphosphate (PIP3) that binds and activates AKT. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) dephosphorylates PIP3, thus antagonizing AKT activation. Constitutively activated AKT has been found in a wide variety of human tumor types, often due to a mutational loss of PTEN activity, and its presence has been correlated with poorer prognoses.[2]

AKT has attracted considerable attention as the target of small molecule inhibitors, including analogues or mimics of PIP3, ATP-competitive active site binders, peptide or peptidomimetic pseudosubstrates, and allosteric site binders.[3] As part of a high-throughput screening effort to find new inhibitors of AKT, we discovered that the known antibiotic natural product lactoquinomycin (**1**), an amino-C-glycoside- pyranonaphthoquinone (PNQ) lactone also known as medermycin[4] (Chart 1) inhibited AKT1 with an IC_{50} of 149 nM. In addition, the related PNQ lactones kalafungin[5] (**2**) and frenolicin B[6] (**3**) were found to have similar inhibitory potencies (Table 1).

One intriguing aspect of the PNQ lactones is their novel structure compared to the known classes of kinase inhibitors. We recently disclosed evidence of their interaction with cysteine residues 310 and 296 found in the T-loop region of AKT1.[7] Single mutants C310A, C296A, and the double mutant C296A/C310A of AKT1 were prepared. The IC_{50} values of **1** vs the single mutants were 38.5 and 30.4 μM, respectively, and it did not inhibit the double mutant at concentrations up to 40 μM. In contrast the ATP-competitive active-site binding inhibitor staurosporine was equipotent against wild type and all three mutant forms of AKT. Exploration of the SAR of the PNQs could shed further light on their mechanism of action.

Chart 1. AKT Inhibitory Natural and Semisynthetic PNQs

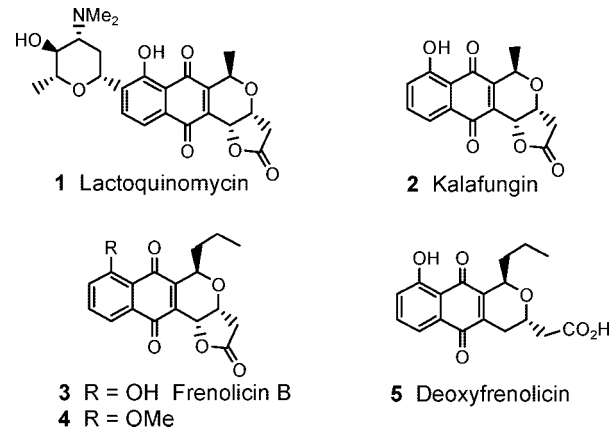

Table 1. Enzyme and Cell Proliferation Assay Results for Natural and Semisynthetic PNQs

compd	AKT IC_{50} μM	MDA468 IC_{50} μM
1	0.149	0.05
2	0.313	0.07
3	0.198	0.06
4	0.497	1.11
5	0.424	0.33

A consistent feature of the natural PNQ lactones is that the IC_{50} for inhibition of proliferation of the PTEN deleted breast tumor cell line MDA468 is 3−5-fold lower than that for inhibition of the isolated enzyme. This raises the concern that these compounds possess additional, off-target activities. Previous in-house screening[7] of lactoquinomycin against a panel of 14 kinases showed a high degree of selectivity for AKT. This selectivity was confirmed in an Invitrogen SelectScreen panel of 45 kinases at a concentration of 1 μM (data in Supporting Information). Notably, in both assays, **1** did not significantly inhibit PKA or PKCα, the kinases whose catalytic domains are most homologous to AKT. The activity of **2** and **3** show that the amino sugar of **1** is not required for AKT inhibition and the modest loss of potency for the methyl ether **4**[8] compared to frenolicin B reveals that the phenolic hydroxyl group is also not necessary. The activity of deoxyfrenolicin (**5**) is attributed to its known solution phase air-oxidation to **3** because the methyl ester[9] derived from **5** shows no AKT inhibition up to 20 μM. Given the degree of AKT inhibition by these less functionalized PNQs and in light of their greater synthetic accessibility, we decided to focus on the preparation of deshydroxy-PNQs in order to explore the SAR of this compound class. Numerous synthetic approaches to PNQ natural products and their analogues have been described.[10] We chose to utilize an oxa-Pictet−Spengler reaction to prepare the pyran ring from an appropriately substituted 2-naphthylethanol. This strategy has been used to prepare several PNQs including frenolicin B.[11]

The preparation of racemic PNQ lactones (Scheme 1) was accomplished via the alkylation of 1,4-naphthoquinone with the

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[a] Abbreviations: BSA, bovine serum albumin; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5) triphosphate; PKA, protein kinase A; PKC, protein kinase C; PNQ, pyranonaphthoquinone; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

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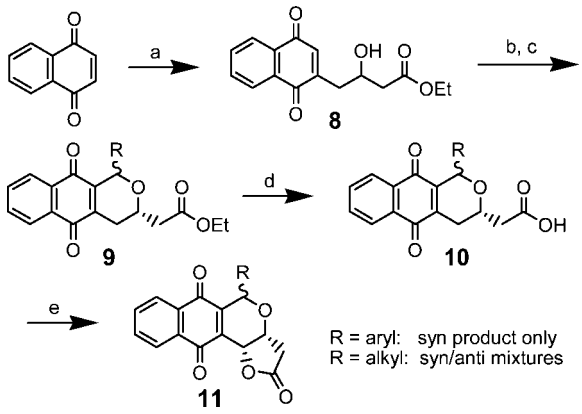
Scheme 1. Preparation of Racemic PNQs[a]



[a] Reagents and conditions: (a) $HO_2CCH_2CH(OH)CO_2Et$, $(NH_4)_2S_2O_8$, $AgNO_3$; (b) Pd/C; (c) RCHO, HCl_g, Et_2O; (d) 4 N HCl, THF; (e) MnO_2, CH_3CN.

Table 2. Enzyme and Cell Proliferation Assay Results for PNQ Lactones **11**

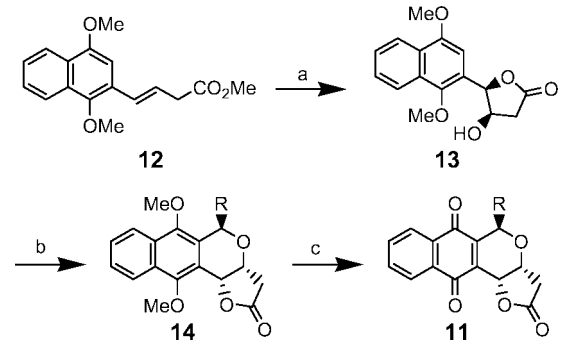
compd	R	stereo	AKT IC_{50} μM	MDA468 IC_{50} μM
11a	H	(+)	0.044	0.80
11b	2-thienyl	(+)-*anti*	0.057	0.60
11c	CH_2OBn	(±)-*anti*	0.072	1.00
11d	4-hydroxyphenyl	(±)-*syn*	0.080	0.55
11e	3-thienyl	(-)-*anti*	0.099	0.41
11f	3-thienyl	(+)-*anti*	0.122	0.30
11g	methyl	(+)-*anti*	0.150	0.48
11h	*n*-propyl	(±)-*anti*	0.163	0.60
11i	$(CH_2)_4$	(±)	0.295	1.60
11j	3-hydroxyphenyl	(±)-*syn*	0.350	1.00
11k	*n*-propyl	(±)-*syn*	0.383	1.30
11l	4-aminophenyl	(±)-*syn*	0.850	0.55
11m	*gem*-dimethyl	(±)	1.440	0.23

radical derived from silver nitrate/ammonium peroxydisulfate treatment of monoethyl 3-hydroxypentanedioate.[12] Although the yield of **8** was modest (29%), this transformation could be conveniently run on a multigram scale. Pyran ring formation was effected in one pot by a palladium catalyzed reduction to the hydroquinone followed by the addition of an aldehyde and 2 N HCl in ether. The resulting tricyclic hydroquinones reoxidize upon workup in air to give the pyranoquinones **9**. For aromatic aldehydes, only *syn* diastereomers were isolated, while aliphatic aldehydes gave ~3:1 mixtures of *syn* and *anti* diastereomers. The stereochemical outcome of these cyclizations has been found[13] to vary with the exact conditions used as the initially formed *syn* isomers are subject to equilibration. Acid hydrolysis gave the deoxyfrenolicin analogues **10**, which are known to oxidatively cyclize to **11** upon exposure to air in solution (Table 2).[14] We found this transformation to be more reproducible when using activated manganese dioxide in acetonitrile.

While the use of enantiomerically pure 3-hydroxypentanedioates (**8**) in Scheme 1 can provide homochiral PNQ lactones, we found that the route shown in Scheme 2 was better suited to our targets. The enantioselective synthesis of hydroxylactone **13** and its enantiomer were accomplished by asymmetric dihydroxylation of the β,γ-unsaturated ester **12**.[15] Treatment of **13** with boron trifluoride etherate and an aldehyde in dichloromethane then gave the pyranolactones **14** exclusively as *anti* diastereomers for all aldehydes examined.[16] The final targets were then obtained after CAN oxidation. This sequence complements the previous, *syn*-selective route.

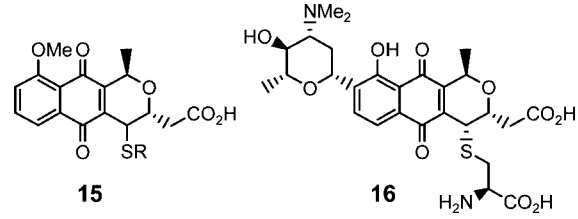
The unsubstituted PNQ lactone **11a** appears to represent the minimum required structural set for AKT inhibition. Quinone

Scheme 2. Preparation of Homochiral PNQs[a]



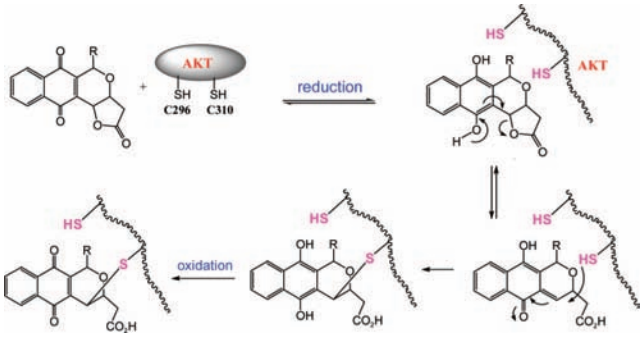
[a] Reagents and conditions: (a) asymmetric dihydroxylation; (b) RCHO, $BF_3 \cdot OEt_2$, CH_2Cl_2; (c) CAN, CH_3CN, H_2O.

analogues without the lactone functionality such as the PNQ esters **9** did not generally inhibit AKT at concentrations below 10 μM. Lactones with the quinone reduced and protected such as **14** were similarly inactive. Consistent with the observed activity of deoxyfrenolicin (**5**), the acids **10** which can lactonize in situ, displayed somewhat lower inhibitory potency than the corresponding lactones. Interestingly, the absolute configuration of the PNQ does not affect its inhibitory activity as shown by the essentially equal potency of the enantiomeric 3-thienyl analogues **11e** and **11f**. This same equipotency of enantiomeric pairs has been observed[17] for the antibiotic activity of both **1** and **2**. The *n*-propyl isomers **11h** and **11k** reveal a 2−3-fold greater potency for the *anti* compared to the *syn* diastereomer. Relatively bulky R groups are tolerated as seen for the benzyloxymethyl compound **11c** and the various aryl and heteroaryl analogues, but disubstitution at R leads to a loss of activity (**11i** and **11m**). There appears to be no clear correlation between the level of enzyme inhibition and the cell antiproliferative activity in this series of analogues. The least potent AKT inhibitors **11l** and **11m** have antiproliferative activities equal to or greater than some of the more active compounds. The stability of the synthetic analogues under cell proliferation conditions vs those of the enzyme assay may be a factor. The unsubstituted PNQ lactone **11a** was seen to decompose over time in air-exposed DMSO solution. Inhibition of AKT activity in cells was confirmed by immunoblot assay of phosphorylation of downstream targets of AKT.[7] Sample data are given in the Supporting Information. Despite the high degree of kinase selectivity of the PNQs for AKT, the possibility of off-target activity remains. However, the fact that various nonlactone PNQs fail to inhibit AKT suggests a simple oxidative deactivation mechanism is not responsible for the observed enzyme or cell activities.



One possible explanation for AKT inhibition by the PNQs is the bioreductive alkylation mechanism proposed by Moore[18] for quinones, including the PNQ lactones, that upon reduction in vivo to the hydroquinone can subsequently form a quinone methide. This reactive intermediate could then alkylate a nucleophilic site on a protein. The first experimental evidence

Scheme 3. Possible Bioreductive Alkylation Mechanism for PNQ Lactones



to support this mechanism was Brimble's description[19] of adducts (**15**) between the dithionite reduced methyl ether of **2** and simple thiols. Given our earlier AKT1 mutant data,[7] we therefore considered alkylation of a cysteine in the activation loop (T-loop) of the kinase domain by a PNQ lactone-derived quinone methide as a plausible mechanism for the inhibition of AKT (Scheme 3). In fact, treating **1** with a 10-fold excess of cysteine in water for 24 h resulted in its nearly complete conversion to a 1:1 adduct which proved to be **16**, as expected from the proposed mechanism. This adduct has minimal AKT inhibitory activity, presumably due to a greatly diminished ability to form the quinone methide.

We then sought additional, direct biochemical evidence of the formation of an adduct between the synthetic PNQ **11f** and AKT2. This isoform of AKT can be more easily obtained in a nonaggregated state amenable to biochemical analysis compared to AKT1 and it was also sensitive to the PNQ inhibitors.

AKT2 contains four cysteine residues with C297 and C311 in the T-loop corresponding to C296 and C310 in AKT1. To assess the accessibility of these cysteine residues, AKT2 was treated in both its activated (phosphorylated) and unactivated states with iodoacetic acid;[20] the resulting reaction mixtures were analyzed using mass spectrometric methods. For the inactive form of AKT2, iodoacetic acid reacted equally with all four cysteine residues. In the activated enzyme, half of the product was monoadduct formed by alkylation at C297 and half was bis-adduct formed by reaction at both C297 and C311. The remaining cysteine residues at positions 226 and 345 were much less reactive (<6%), suggesting that those two residues are relatively inaccessible, while residues 297 and 311 are solvent exposed in the active form of the enzyme. This alkylation of active AKT2 resulted in a >90% loss of activity with respect to phosphorylation of the substrate peptide used in the biochemical assays.

We next investigated the reaction of activated AKT2 with varying concentrations of **11f**. Mass spectral analyses indicated that the enzyme of molecular weight 39553 daltons formed a monoadduct of 39908 daltons when treated with a two-molar excess of the PNQ (Figure 1).

In reverse phase HPLC analyses, the elution of the free PNQ was readily identified by the characteristic quinone absorption maximum at 340 nm. When the complex of AKT2 with a two molar excess of the inhibitor was resolved on a reverse phase C18 column, two elution peaks were observed, one due to the free inhibitor and the other overlapped with the elution of the free enzyme, at 10.5 min. However, the absorption spectrum of the peak eluting around 10.5 min consisted of the quinone absorption, suggesting it corresponded to the monoadduct, as half the concentration of the free inhibitor was consumed to

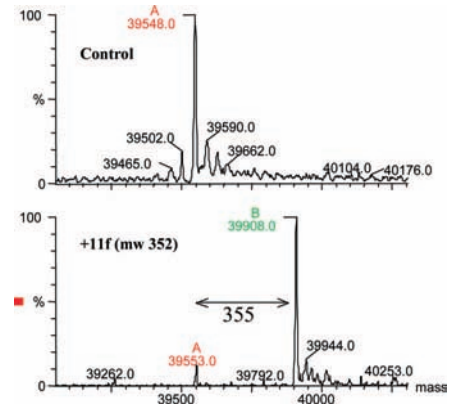


Figure 1. Alkylation of Akt2 to form the monoadduct with **11f**. Resolved LC-MS (ESI) results showing the increase in mass of 355 daltons of the Akt2 adduct peak.

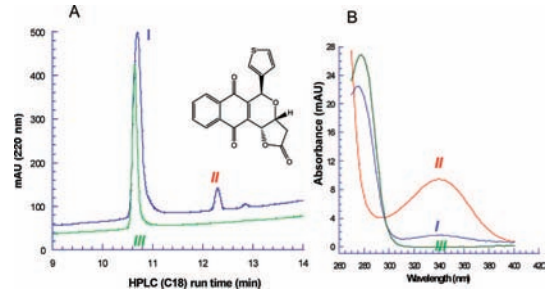


Figure 2. (A) Separation of AKT2 and free inhibitor, **11f**, on reverse phase HPLC. (B) Diode array UV−vis detection of covalent adduct. I: AKT2-(**11f**) adduct. II: (**11f**). III: AKT2. Column: Vydac C4 protein and peptide. Gradient: CH3CN/0.1%TFA (CH3CN%: 10−90 in 15 min).

form this adduct based upon quantitation of the elution peak of the free inhibitor (Figure 2). This observation suggests that the quinone ring is intact after the formation of the monoadduct.

In the inactive, unphosphorylated form of AKT2 both C297 and C311 are equally reactive with **11f** with the major product being a monoadduct. In support of our observed kinase inhibitory selectivity, the PNQ did not form a covalent complex with either p70s6 kinase or bovine serum albumin despite the presence of a similar T-loop cysteine pair in p70s6 kinase[21] and the known[22] reactivity of BSA cysteines with alkylating agents. Mass spectral analysis of the tryptic digest of the activated AKT2/**11f** adduct showed that C311 was selectively alkylated. Consistent with this observed selectivity, active AKT2 that had been previously alkylated with iodoacetic acid further reacted to give an adduct with **11f**. As expected for an interaction at the allosteric T-loop site, adduct formation is reduced but not eliminated in the presence of staurosporine. Furthermore, when AKT2 is treated with **11f** at concentrations sufficient to form adduct and inhibit enzyme activity, there is no formation of the disulfide resulting from oxidation of C297 and C311.

The pyranonaphthoquinone lactones are a new class of highly selective inhibitors of AKT kinase. Identification of a monoadduct between the PNQ **11f** and C311 of phosphorylated AKT2 coupled with the lack of C297/C311 disulfide formation supports the proposal that these compounds are the first examples of inhibitors that act via a previously unobserved bioreductive alkylation mechanism involving an activation loop cysteine.

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Supporting Information Available: Experimental details on the preparation and characterization of new PNQ lactones, MS and tryptic digest results for AKT-2 adducts, enzyme selectivity data for lactoquinomycin, and sample immunoblot assays for phosphorylation of AKT targets. This material is available free of charge via the Internet at http://pubs.acs.org.

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