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Novel Pyrrolo-quinoline Derivatives as Potent Inhibitors for PI3-Kinase Related Kinases

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Abstract—Several pyrrolo-quinoline γ-lactones were found as novel inhibitors for two members of the PI3-kinase related kinase (PIKK) family, Ataxia-Telangiectasia-*mutated* (ATM) protein and the mammalian Target of Rapamycin (mTOR). Preliminary structure–activity relationship studies indicated that an electrophilic exocyclic double bond conjugated to the carbonyl group of the γ-lactone ring was crucial for the PIKK inhibitory potency. One of the best ATM inhibitors in this series, DK8G557, showed IC_{50} values of 0.6 and 7.0 μM for ATM and mTOR, respectively. This compound exhibited potent and selective growth inhibition activities in the NCI 60 human tumor cell line screen with a GI_{50} MG-MID value of 2.69 μM. The best mTOR inhibitor in this series, HP9912, exhibited IC_{50} values of 0.5 and 6.5 μM for mTOR and ATM, respectively. These compounds suggest novel leads for the discovery of potent small molecule inhibitors of PIKKs as potential anticancer drugs, with therapeutic activities as either single, or as sensitizing agents to conventional radio-, or chemo-therapeutic strategies.

Introduction

PI3-kinase related kinases (PIKKs) are a novel family of signaling proteins that share a common COOH-terminal region similar to that of phosphatidylinositol 3-kinase (PI3-kinase).[1] In mammalian cells, the PIKK family comprises Ataxia-Telangiectasia-*mutated* (ATM) protein, the mammalian target of rapamycin (mTOR),[2] Ataxia-Telangiectasia-*mutated* and Rad3-related (ATR) protein,[3] and DNA-dependent protein kinase (DNA-PK),[4] which are different from PI3-kinase in the sense that they do not possess lipid kinase activity, but are serine/threonine protein kinases.

The ATM protein functions in multiple signal-transduction pathways that coordinate cell cycle arrest and DNA repair in response to DNA damage. The gene that encodes this protein is responsible for the human genetic disorder Ataxia Telangiectasia (A-T), which is characterized by cancer proneness and extreme radiosensitivity. In light of the radiosensitive phenotype exhibited by A-T cells, it is possible that small molecule inhibitors of the ATM protein might sensitize tumor cells to the cytotoxic effects of ionizing radiation or DNA-damaging chemo-therapeutic agents.[4] Recent reports[4–7] that wortmannin, an irreversible inhibitor of the PIKK family members is a highly effective radiosensitizer, support this prediction.

The mTOR protein is a critical enzyme in the transmission of mitogenic signals from cytokine receptors. It is known that mTOR functions as a key component in signaling pathways that regulate synthesis of proteins required for cell-cycle progression in both lymphoid and nonlympoid cells.[2] Anticancer activity has been found to be associated with two different types of mTOR inhibitors: rapamycin and wortmannin. Therefore small molecule mTOR inhibitors may have potential applications as anticancer agents.

As shown in Figure 1, only a few PIKK inhibitors have been reported. Wortmannin, a fungal metabolite, was identified as a potent inhibitor of PI3-kinase ($IC_{50} = 4.2$ nM).[8] The mechanism by which wortmannin inhibits PI3-kinase has been recently confirmed, in the crystal structure of wortmannin bound PI3Kγ,[9] as irreversible modification of the primary amine, Lys-833, in

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the active site. Wortmannin also showed differential inhibitory activities for members of the PIKK family (ATM, $IC_{50}=150$ nM; mTOR, $IC_{50}=40$ nM; DNA-PK, $IC_{50}=16$ nM; ATR, $IC_{50}=1.8$ μM), which likely result from inactivation of the PI3-kinase related catalytic domains in these enzymes by a similar mechanism. The radio-sensitizing effects of wortmannin correlated with its ATM, DNA-PK inhibitory activities in intact A549 lung cancer cells.[4] The competitive inhibitor LY294002 has been confirmed to occupy the ATP-binding site of PI3-kinase[9] and was reported to inhibit both PI3K ($IC_{50}=1.4$ μM)[10] and DNA PK ($K_i=6.0$ μM).[11] Dual ligands that combined a LY294002-like kinase inhibitor with geldanamycin, a high affinity binder of hsp90, through different linkers, were reported as selective inhibitors of the PI3K and DNA PK recently.[12]

Wortmannin and LY294002 have been invaluable tools in the studying the biological roles of members of the PIKK family in the intracellular signaling events. However, the significant toxicity and narrow therapeutic indices associated with these two compounds have made it difficult to develop them as drug candidates. Therefore, there exists a need for the development of PIKK inhibitors of distinct chemical identities that may possess more optimal in vitro and in vivo activities. We report here the discovery a group of novel pyrroloquinoline derivatives, as exemplified by DK8G557 and HP9912 in Figure 1, as potent irreversible inhibitors for PI3-kinase related kinases, specifically the ATM and mTOR protein kinases.

Results and Discussion

There had been no structural information for the catalytic domain of any member of the PIKK family until Williams and coworkers reported the crystal structures of PI3-kinase recently.[9,13,14] The starting point of our search for direct inhibitors of the PIKK catalytic domain was the observation that wortmannin, a potent inhibitor of the catalytic activities of most members of the PIKK family, possesses a near planar structure and a highly electron-deficient center that is prone to reacting with nucleophiles through Michael addition[15] (Fig. 2). In reviewing our in-house compound library, pyrrolo-quinoline γ-lactone **13** (DK8G557), a by product obtained in an earlier effort of synthesizing camptothecin in this group,[16] appears to provide a planar scaffold with its pyrrolo-quinoline moiety and a Michael acceptor from the exocyclic double bond on the γ-lactone ring.

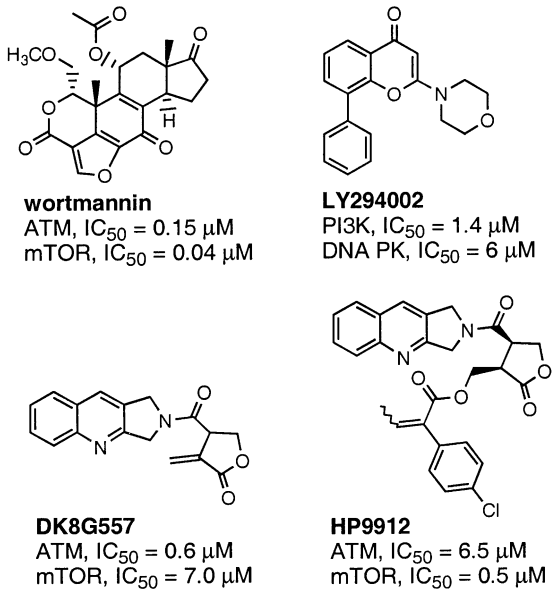


Figure 1. Chemical structures of wortmannin, LY29004, DK8G557, and HP9912.

Chemistry

Scheme 1 illustrates the synthesis of compound **12** as previously documented.[16,17] Briefly, bis-γ-lactone **7**, a racemic mixture, was synthesized in 4 steps using the method of Piskov,[18] which involved acid hydrolysis of α-hydroxymethyl-β-carboethoxyparaconate (**3**) and subsequent decarboxylation of the bis-γ-lactone-α-carboxylic acid **4**. In the acid catalyzed hydrolysis of **3**, compounds **5** and **6**[19] were obtained as by products. Freidlander condensation of *o*-amino benzaldehyde (**8**) and *N*-carbethoxy-3-pyrrolide (**9**) at 240 °C gave compound **10**,[20] which was hydrolyzed to 1,3-dihydro-2H-pyrrolo(3, 4-b)quinoline (**11**).[20] Coupling of **11** with bis-γ-lactone **7** in ethanol gave alcohol **12** as a racemic mixture. However, delocalization of the amide bond, and the bulky and asymmetrical pyrrolo-quinoline moiety render chiral feature to the amide nitrogen, which generally broadened and complicated the NMR signals.

As shown in Scheme 2, an improved procedure employing treatment of alcohol **12** with triethyl amine and oxalyl chloride/methacrylic acid (1:1) in chloroform at 0 °C for 1 h gave DK8G557 (**13**) as a racemic mixture in 57% yield. The effect of the bulky amide bond in this compound was evident from the split signals of one of the exocyclic methylene protons, δ 5.72 (d, $J=2.1$ Hz, 0.7H, H-3′), 5.71 (d, $J=2.7$ Hz, 0.3H, H-3′), indicating existence of two rotamers. Conversion of **13** to **14** was accomplished in 64% yield by stirring **13** with 1 equiv of DMAP at rt for 1 day. Oxidation of compound **13** using *m*-CPBA gave *N*-oxide derivative **15**, while epoxide derivative **16** was synthesized using urea/hydrogen peroxide complex.

As shown in Scheme 3, HP9912 (**17**) was synthesized by treatment of compound **12** with the optically pure acid chloride made from (*S*)-(+)-2-(4-chloro-phenyl)-2-hydroxy-butyric acid.[21] Formation of the ester was confirmed by the extra ^{1}H NMR signals at 4.42 ppm. These signals result from protons on C-3′, which was located at δ 3.97 (brs, 2H, H-3′) in alcohol **12**. However, the hydroxyl group from the 2-(4-chloro-phenyl)-2-hydroxy-butyric acid appeared to be eliminated during the preparation of either the acid chloride or the ester. In the ^{1}H NMR spectrum of compound **17**, extra peaks at δ 7.03 (q, $J=7.2$ Hz, H-3″), δ 1.53 (d, $J=7.2$ Hz, Me-4″) and δ 1.58 (d, $J=7.2$ Hz, Me-4″) may account for the highly conjugated olefinic proton (H-3″) and Me-4″

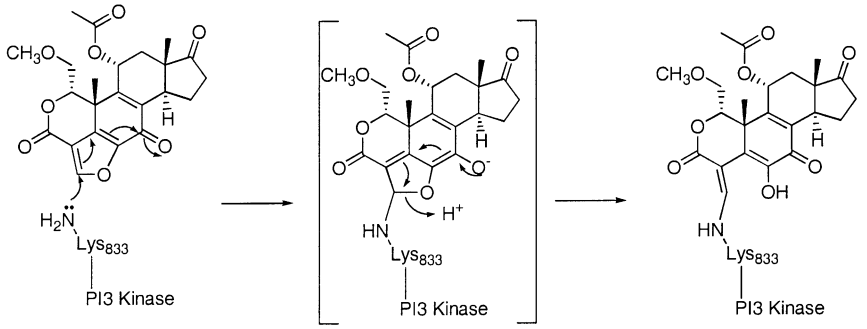
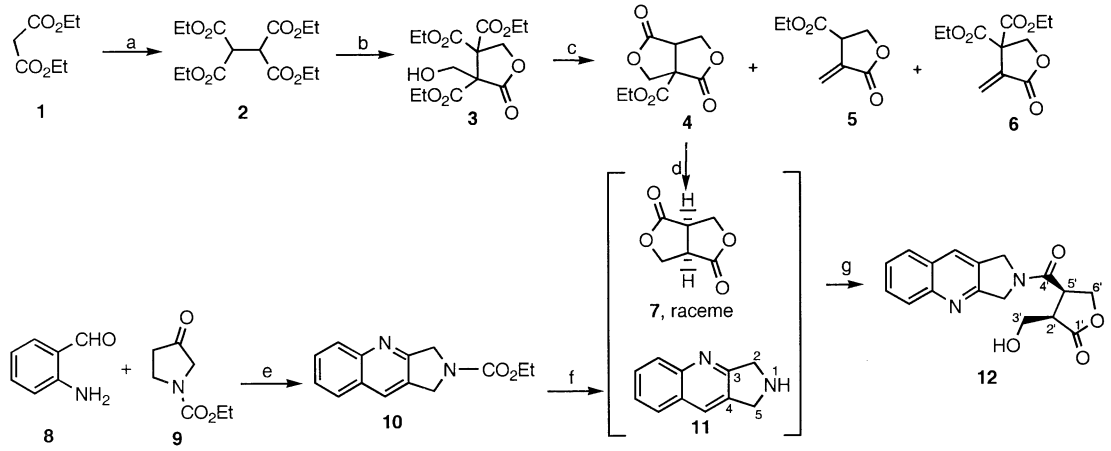
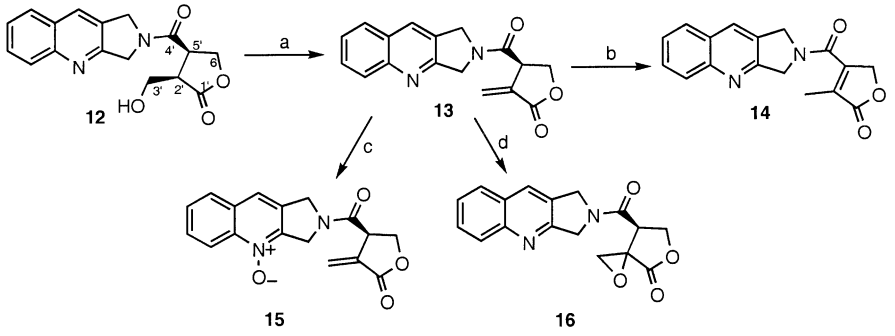


Figure 2. Proposed mechanism for irreversible inactivation of PI3 kinase by Wortmannin.



Scheme 1. Reagents and conditions: (a) Na/EtOH; I_2/ether, rt, 3 h; (b) HCHO, Na/EtOH, 55 °C, 2 h; (c) concd HCl, reflux, 3 h; (d) 185 °C, nitrogen, 15 min; (e) TsOH/240 °C, 5 min; (f) KOH/aq EtOH, overnight; (g) EtOH, reflux, 2 h.



Scheme 2. Reagents: (a) $EtN_3/(COCl)_2$–methacrylic acid (1:1), $CHCl_3$, 0 °C, 1 h; (b) DMAP/$CHCl_3$, 1 day; (c) *m*-CPBA/$CHCl_3$, rt, 2 h; (d) urea/H_2O_2, trifluoroacetic anhydride, 40–50 °C, 24 h.

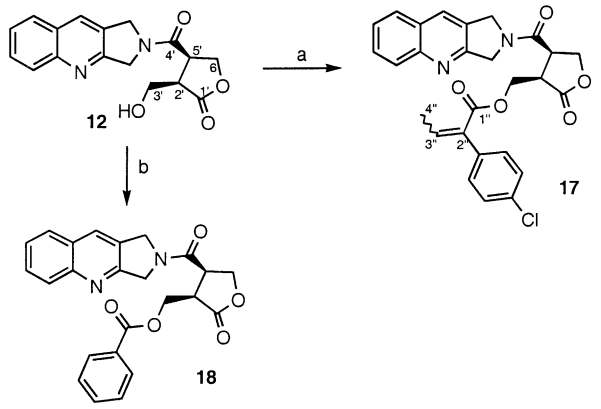
(differentiated for *Z* or *E* configurations of the double bonds, *Z*/*E* = 1:1). Ester **18** was prepared in similar condition using benzoyl chloride.

PIKK inhibitory activities

The inhibitory activities of the compounds described above were evaluated in the immune complex kinase assays for PI3-kinase related kinases as previously described.[4] Active compounds exhibited irreversible binding to the PIKKs tested. The results are listed in Table 1. Representative dose–response curve for DK8G557 is shown in Figure 3. DK8G557 (**13**), which contains an exocyclic double bond on the γ-lactone ring, inhibited the ATM protein with an IC_{50} value of 0.6 μM. The mechanism by which this compound inhibits ATM appears to be similar to that employed by wortmannin. The exocyclic double bond, like the one in the furan ring of wortmannin, could well serve as an excellent receptor for Michael addition of nucleophilic groups. In addition to being conjugated to the lactone carbonyl group, reaction of the double bond with a nucleophile releases ring strain by conversion of the sp^2 carbon on the five-membered ring into a sp^3 center. The activity of this moiety has been exemplified by the well-known toxicities associated with the exocyclic double bonds of sesquiterpenoid lactones. The irreversible binding of DK8G557 (**13**) to ATM was confirmed by the fact that extensive wash of the inhibited, protein A-Sepharose beads-bound ATM against buffer did not

recover the enzymatic activity. Compound **13** showed significantly lower potency in inhibiting the other two members of the PIKK family, mTOR ($IC_{50} = 7.0\,\mu M$) and DNA-PK (<50% inhibition at 10 μM).

In contrast, alcohol **12** and the compound with an endocyclic double bond, **14**, were essentially inactive for both ATM and mTOR. In addition, when the eletrophilic center was changed to an epoxide, as in compound **16**, the inhibitory activity was significantly reduced, confirming the importance of the exocyclic double bond in inhibiting these enzymes. One exception, however, was found that benzoate derivative **18** exhibited moderate inhibitory activity ($IC_{50} = 10\,\mu M$) without the presence of the exocyclic double bond. The activity might arise from an effective fit in the active binding cleft, or it might result from partial elimination of the benzoate, under the assay condition, to give the active compound **13**.



Scheme 3. Reagents: (a) (*S*)-(+)-2-(4-chloro-phenyl)-2-hydroxy-butyric acid chloride, Et_3N, DMAP, $CHCl_3$; (b) benzoyl chloride, Et_3N, DMAP, $CHCl_3$.

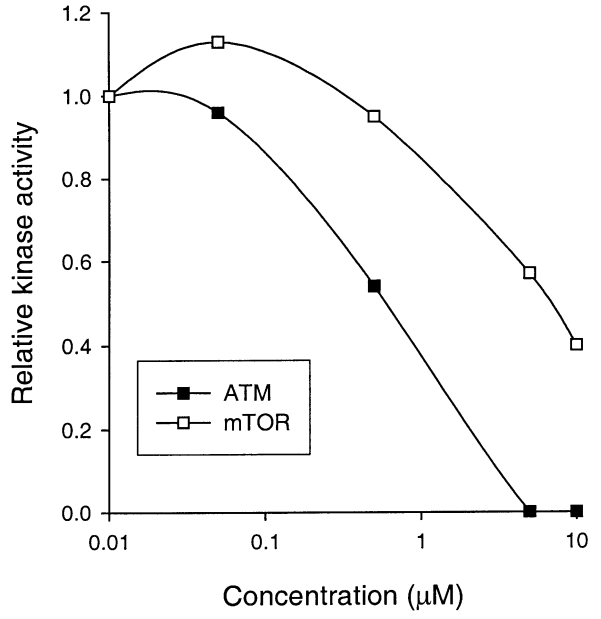


Figure 3. Inhibition of ATM and mTOR by DK8G557. ATM and mTOR were immunoprecipitaed from A549 cell extract and rat brain extract, respectively. The immune complexes were treated with DK8G557 for 30 min at rt, and kinase activity were assayed under linear conditions. The kinase activities were normalized to those measured in the no-drugs controls

Oxidation of the pyrrolo-quinoline moiety, as in compound **15** ($IC_{50} = 10\,\mu M$), led to reduced ATM inhibitory activity. However, in the absence of the pyrrolo-quinoline moiety, simple compounds with just the exocyclic double bond-containing γ-lactone also showed medium (compound **5**, $IC_{50} = 6.0\,\mu M$) to excellent (compound **6**, $IC_{50} = 0.25\,\mu M$) inhibitory activity against ATM. The potency of compound **6** was similar to that of wortmannin, which was used as a control to monitor the assay. Surprisingly, simple lactones **5** and **6** exhibited less than 50% inhibition of mTOR at 10 μM. The excellent selectivity of **6** observed between the two highly homologous members within the PIKK family was unexpected, since simple electrophilc species like these are usually predicted to exhibit non-specific inhibitions.

It appears that inhibition of mTOR requires more than simply a Michael addition acceptor such as those exocyclic double bonds in compounds **13** and **6**. While the pyrrolo-quinoline derivative **13** showed moderate mTOR inhibitory activity, simple lactone **6** was inactive at 10 μM for mTOR even though it showed excellent ATM inhibition. It is likely that inhibition of mTOR requires finer compound–enzyme interactions that could be obtained from the pyrrolo-quinoline moiety. The best mTOR inhibitor in this series is HP9912 (**17**), which inhibited mTOR with an IC_{50} of approximately 0.5 μM. This compound inhibited ATM with about 10 times less potency (Table 1). The superior inhibitory activity of **17** toward mTOR may arise from the simultaneous presence of a Michael acceptor (the electrophilic double bond activated by the adjacent carbonyl), the pyrrolo-quinoline and the *p*-chloro phenyl moieties. The structure might be too bulky for the ATM active site to produce excellent inhibition.

NCI 60-human tumor cell lines screen

The growth inhibition activity of compounds **13**, **15**, and **18** was evaluated at the National Cancer Institute against a panel of 60 human tumor cell lines representing nine different cancer types. The GI_{50}, TGI and LC_{50} values are shown in Table 2 for sensitive subpanels.

Table 1. PIKK inhibitory activities of compounds **5**, **6**, **12**–**18** and Wortmannin

Compound no.	**5**	**6**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	Wortmannin[4,22]
ATM IC_{50} (μM)[a]	6.0	0.25	>10	0.6	>10	10	>10	6.5	10	0.15
mTOR IC_{50} (μM)[a]	>10	>10	ND	7.0	>10	>10	>10	0.5	ND	0.04
DNA PK IC_{50} (μM)[a]	ND	ND	ND	>10	ND	ND	ND	>10	ND	0.016

[a]IC_{50} values were determined from at least two independent determinations.

Table 2. Inhibition growth of in vitro human cancer cell lines by the pyrrolo-quinoline derived compounds

	13			15	18		
Disease type and cell lines	GI_{50}[a]	TGI[b]	LC_{50}[c]	GI_{50}[a]	GI_{50}[a]	TGI[b]	LC_{50}[c]
Leukemia							
HL60TB	1.06	4.90	21.6	0.0036	5.31	14.1	>25
RPMI-8226	2.02	>25	>25	7.37	2.24	9.50	>25
Non-Small Cell Lung Cancer							
EKVX	3.69	7.39	14.8	>25	11.7	>25	>25
NCI-H226	0.38	0.71	1.32	>25	0.95	3.62	9.51
NCI-H522	0.35	0.83	2.00	4.62	1.05	3.62	10.1
Colon Cancer							
HCT116	1.06	4.05	10.1	>25	3.88	8.34	17.9
HT29	4.01	7.58	14.3	>25	9.58	>25	>25
SW620	0.50	1.64	7.42	9.07	1.51	5.85	18.9
CNS Cancer							
SF-268	3.58	8.15	18.5	>25	16.3	>25	>25
SF-539	0.56	1.70	8.62	6.27	2.69	7.09	18.7
Melanoma							
LOXIMVI	1.73	4.86	11.0	>25	6.16	>25	>25
MALME-3M	0.82	2.96	9.68	9.11	3.16	6.58	13.7
M14	1.36	4.70	11.4	>25	4.25	7.93	14.8
Ovarian Cancer							
IGR-OV1	2.92	5.99	12.3	>25	7.22	13.9	>25
OVCAR3	2.99	7.72	19.9	>25	6.73	>25	>25
OVCAR4	1.31	5.46	23.2	>25	3.69	8.13	17.9
Renal Cancer							
786-0	1.20	4.17	10.2	>25	3.77	7.47	14.8
ACHN	0.79	3.45	9.28	11.5	3.11	6.63	14.2
CAKI-1	0.92	3.66	10.6	15.9	3.36	7.40	16.3
TK-10	7.73	3.00	8.77	>25	4.21	9.17	20.0
UO-31	1.32	4.77	11.4	>25	4.50	14.3	>25
Prostate Cancer							
PC-3	3.45	6.81	13.4	>25	13.5	>25	>25
DU-145	3.12	6.89	15.2	>25	8.35	>25	>25
Breast Cancer							
MDA-MB-435	1.00	4.27	14.1	>25	4.47	10.8	>25
MDA-N	2.74	5.84	12.5	>25	6.58	>25	>25
T-47D	1.18	6.40	>25	12.6	4.84	13.9	>25
MG-MID	2.69	5.49	14.1	17.5	7.76	15.8	22.4

[a]GI_{50} represent the micromolar compound concentration required to achieve 50% inhibition of tumor cell growth.
[b]TGI represents the micromolar concentration required to achieve total growth inhibition of tumor cells.
[c]LC_{50} represent the micromolar concentration that is required to achieve 50% tumor cell killing.

As shown in Table 2, DK8G557 (**13**) exhibited potent and selective growth inhibition activities, with a mean GI_{50} value of 2.69 μM. The two most sensitive cell lines to this compound are the NCI-H226 and NCI-H522 non-small cell lung cancer cell lines, where high potency and consistent selectivity in GI_{50} (0.38 and 0.35 μM, respectively), TGI (0.71 and 0.83 μM, respectively) and LC_{50} (1.32 and 2.00 μM, respectively) were obtained. The HCT-116 and SW-260 colon cancer cell lines also exhibited sensitivity to this compound, with GI_{50}s of 1.06 and 0.50 μM, respectively; the LC_{50} concentrations for these two tumor cell lines were at about 10 μM. It is noteworthy that DK8G557 showed selectivity in inhibiting growth of several renal cancer cell lines (including ACHN, CAKI-1 and UO-31), and the selectivity was consistent through the LC_{50}s. Other sensitive subpanels included the SF539 CNS cancer cell line (GI_{50} = 0.56 μM), the MALME-3M melanoma cell line (GI_{50} = 0.82 μM), and the MDA-MB-435 breast cancer cell line (GI_{50} = 1.00 μM).

The benzoate derivative **18** showed sensitivity pattern similar to that of DK8G557 (**13**), but the potencies for different tumor cell lines are generally lower. The *N*-oxide derivative **15** only showed limited growth inhibition effect in a few cancer cell lines (Table 2), and the TGI and LC_{50} values are generally larger than 25 μM. This may result from the presence of charges on the molecule that could prevent it from crossing the plasma membrane. Compounds **13** and **18** have been referred to the Biological Evaluation Committee (BEC) at the National Cancer Institute.

Conclusion

In conclusion, a novel group of pyrrolo-quinoline derived γ-lactones were found to be potent inhibitors for two members of the PI3-kinase related kinase family, ATM protein and mTOR. Initial structure–activity relationships indicate that an electrophilic exocyclic double bond conjugated to the carbonyl group of the γ-lactone ring, as in DK8G557 (Fig. 1), is crucial for the ATM inhibitory activity. Furthermore, simpler γ-lactones (**5**, **6**) also exhibited potent and even more selective ATM inhibitory activities. The inhibition appeared to be irreversible based on the

washout experiments. It is observed that compounds **6** and **13** showed inhibitory selectivity for ATM over mTOR, with a reversed selective preference and higher selectivity ratio as compared to wortmannin (Table 1).

The significantly lower activity of lactones **5** and **6** toward mTOR suggest that in addition to a presence of a Michael acceptor, inhibition of mTOR may require more extended enzyme–inhibitor interaction than that of ATM. The best of the mTOR inhibitors discovered is pyrrolo-quinoline derived 2-(4-chloro-phenyl)-2-ene-butyric acid ester HP9912 (Fig. 1, 0.5 μM). It showed selectivity for mTOR over ATM, with the same selective preference as that of wortmannin, but higher selectivity ratio (Table 1). In the NCI 60 human tumor cell line screen, DK8G557 (**13**) showed potent and selective growth inhibition activities.

Experimental

Thin layer chromatography analysis (TLC) was performed on aluminum sheets precoated with 0.2 mm of silica gel containing 60F254 indicator. Spots were detected with shortwave UV light or Ceric sulfate spray. Flash chromatography was run using 230–400 mesh silica gel. The homogeneity of all the compounds was routinely checked by TLC on silica gel plates, and also by HPLC. Melting points were measured on a Kofler hot stage apparatus attached to a digital thermometer and were uncorrected. Fourier transformed infrared spectra were obtained on a Nicolet 520 FT-IR spectrometer. ^{1}H (300 or 400 MHz), ^{13}C (75 or 100 MHz) NMR and DEPT spectra were recorded on either a Varian Gemini-300 or on a Varian XL-400 spectrometer. Chemical shifts are reported relative to $CDCl_3$ (δ 7.24). High-resolution mass spectra (EI or FAB) were recorded on a VG Analytical 70-SE mass spectrometer equipped with a 11-250J data system. Elemental analyses were performed by Atlantic Microlab, Norcross, GA, USA.

Syntheses of precursors **2**–**11** have been previously documented.[16,17,20]

Synthesis of alcohol 12

To a solution of 1,3-dihydro-2H-pyrrolo[3, 4-*b*]quinoline **11** (4.0 g, 23.5 mmol) in absolute ethanol (200 mL) was added bis-γ-lactone **7** (3.45 g, 24.3 mmol). The mixture was refluxed in a nitrogen atmosphere for 2 h. Concentration of the solution followed by titration with chloroform precipitated alcohol **12** as white solid (4.7 g, 64%), mp 201–202 °C. FT-IR (KBr) 3300, 3200, 1760, 1625 cm^{-1}; ^{1}H NMR (300 MHz, CD_3COCD_3) δ 8.09 (s, 1H), 7.82 (d, J = 7.8 Hz, 1H), 7.74 (t, J = 8.4 Hz, 1H), 7.57 (t, J = 8.1 Hz, 1H), 5.07 (m, 4H, H-2, H-5), 4.72 (t, J = 8.7 Hz, 1H, H-6′), 4.52 (t, J = 8.4 Hz, 1H, H-6′), 3.97(brs, 2H, H-3′), 3.85(m, 1H, H-5′), 3.05(m, 2H, H-2′ and –O*H*); HRMS m/z calcd for $C_{17}H_{16}N_2O_4$ 312.1110, found 312.1091. Anal. calcd for $C_{17}H_{16}N_2O_4$: C, 45.38; H, 5.13. Found: C, 45.22; H, 5.22.

Synthesis of compound 13

To a suspension of **12** (26 mg, 0.096 mmol) and triethylamine (500 μL) in $CHCl_3$ (3 mL) was added a 1:1 mixture (100 μL) of methacrylic acid and oxalic chloride at 0 °C. After stirring for 1 h, the mixture was washed with water and $CHCl_3$. The combined organic layer was dried, concentrated and purified by silica gel column chromatography using methanol/ethyl acetate/chloroform (1:1:10) as mobile phase to give **13** as colorless solid (13 mg, 57%), mp 202–205 °C. FT-IR (KBr) 3271, 2928, 1777, 1646, 1449, 1412, 1266, 1121, 778 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (m, 2H), 7.83 (d, J = 8.4 Hz, 1H), 7.74 (t, J = 7.8 Hz, 1H), 7.58 (t, J = 7.8 Hz, 1H), 6.42 (d, J = 3 Hz, 1H, H-3′), 5.72 (d, J = 2.1 Hz, 0.7H, H-3′), 5.71 (d, J = 2.7 Hz, 0.3H, H-3′), 5.11 (m, 4H, H-2, H-5), 4.72 (t, J = 8.4 Hz, 1H, H-6′), 4.59 (t, J = 9.3 Hz, 1H, H-6′), 4.30 (m, 1H, H-5′); HRMS (m/z) calcd for $C_{17}H_{14}N_2O_3$ 294.1004, found 294.0994. Anal. calcd for $C_{17}H_{14}N_2O_3 \cdot 0.25H_2O$: C, 68.33; H, 4.89; N, 9.37. Found: C, 68.30; H, 4.89; N, 9.20.

Synthesis of compound 14

A mixture of **13** (50 mg, 0.170 mmol) and DMAP (23 mg, 0.187 mmol) in chloroform (3 mL) was stirred for 1 day at room temperature. The mixture was directly concentrated and purified by silica gel chromatography using methanol/ethyl acetate/chloroform (1:1:15) as the mobile phase to give **14** (32 mg, 64%) as colorless solid: mp 235 °C. FT-IR (KBr) 3044, 2929, 2873, 1857, 1778, 1749, 1651, 1600, 1505, 1435, 1410, 1378, 1295, 1095, 1031, 927, 778, 759 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.05 (m, 2H), 7.84 (t, J = 7.2 Hz, 1H), 7.74 (m, 1H), 7.57 (t, J = 7.2 Hz, 1H), 5.11–5.00 (m, 4H, H-2, H-5), 4.94, 4.90 (s, 2H, H-6′), 2.04 (t, J = 2.1 Hz, 3H, H-3′); EIMS m/z (relative intensity) 294.0 (M^+, 13), 169.1 (20), 97.1 (10), 83.1 (12), 69.1 (21), 57.1 (21), 44.0 (100); HRMS (m/z) calcd for $C_{17}H_{14}N_2O_3$ 294.1004, found 294.0994. Anal. calcd for $C_{17}H_{14}N_2O_3 \cdot 0.25H_2O$: C, 68.33; H, 4.89; N, 9.37. Found: C, 68.41; H, 4.81; N, 9.24.

Synthesis of compound 15. To a solution of **13** (37 mg, 0.127 mmol) in chloroform (3 mL) was added 2 equiv of *m*-CPBA(73 mg) in three portions. The reaction was allowed to react at rt for 2 h, then the mixture was quenched with satd $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The combined organic layer was dried over $MgSO_4$, concentrated and purified by silica gel chromatography using methanol/ethyl acetate/chloroform (1:1:10) as mobile phase to give compound **15** (30 mg, 71%) as colorless solid, mp 190–192 °C. IR (KBr) 3087, 2987, 2922, 2864, 2246, 1765, 1666, 1650, 1580, 1457, 1427, 1401, 1118, 737 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.70 (d, J = 9.3 Hz, 1H), 7.90 (d, J = 8.4 Hz, 1H), 7.79 (t, J = 1.6 Hz, 1H), 7.69 (m, 2H), 6.43 (d, J = 2.7 Hz, 1H), 5.74 (d, J = 2.7 Hz, 0.7H), 5.70 (d, J = 2.7 Hz, 0.3H), 5.33–5.06 (m, 4H), 4.73–4.68 (m, 1H), 4.59 (t, J = 8.9 Hz, 1H), 4.32 (m, 1H); EIMS m/z (relative intensity) 310.1 (M^+, 28.7), 294.2 (36.4), 235.2 (9.6), 197.1 (10.8), 185.1 (10.1), 169.1 (100), 140.1 (15.1), 125.1 (32.4), 115.1 (12.5), 67.0 (15.1); HRMS (m/z) calcd for $C_{17}H_{14}N_2O_4$ 310.0954, found 310.0948. Anal.

calcd for $C_{17}H_{14}N_2O_4 \cdot 0.29H_2O$: C, 64.71; H, 4.66; N, 8.88. Found: C, 64.71; H, 4.51; N, 8.80.

Synthesis of compound 16. Trifluoroacetic anhydride (60 μL, 0.425 mmol) was added to a mixture of compound **13** (15 mg, 0.051 mmol), urea H_2O_2 complex (130 mg, 1.38 mmol) and $NaHCO_3$ (102 mg, 1.214 mmol) in chloroform (5 mL) at rt under Argon. The reaction was allowed to react at 40–50 °C for 24 h, then the mixture was poured into water and washed with chloroform. The combined organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The concentrate was purified by silica gel chromatography using methanol/ethyl acetate/chloroform (1:1:30) as mobile phase to afford compound **16** (2.2 mg, 14%) as a colorless solid: mp 171–174 °C. IR (film) 2928, 2856, 1793, 1650, 1446, 1413, 1325, 1234, 1101, 1019, 922, 726 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.06 (m, 2H), 7.84–7.81 (2s, 1H), 7.73 (tt, $J = 1.8$ Hz, 7.2 Hz, 1H), 7.56 (2d, $J = 7.2$ Hz, 1H), 5.16–4.90 (m, 5H), 4.73 (m, 1H), 4.09 (m, 1H), 3.23 (d, $J = 5.7$ Hz, 1H), 3.07 (dd, $J = 1.8$ Hz, 5.4 Hz, 1H); HRMS(FAB, m/z) calcd for $C_{17}H_{15}N_2O_4$ ($M^+ + 1$) 311.1032, observed 311.1060.

Synthesis of ester 17

To a solution of (*S*)-(+)-2-(4-chloro-phenyl)-2-hydroxy-butyric acid (100 mg, 0.5 mmol) in 10 mL CH_2Cl_2 was added DMF and then $SOCl_2$ (43 μL, 0.6 mmol) dropwise at room temperature. After the reaction was completed, methylene chloride, excess $SOCl_2$ and HCl were removed under reduced pressure. The residue was dissolved in 5 mL CH_2Cl_2 and the distillation repeated to give (*S*)-(+)-2-(4-chloro phenyl)-2-hydroxy-butyric acid chloride, which was used for next reaction without further purification.

To a suspension of **12** (100 mg, 0.32 mmol), triethylamine (70 μL) and catalytic amount of DMAP in $CHCl_3$ was added the above acid chloride via syringe as a concentrated CH_2Cl_2 solution. The reaction mixture was stirred at 0 °C for 2 h. Then the solution was poured into ice water and extracted with CH_2Cl_2 twice. The combined organic phases were dried over Na_2SO_4, and concentrated and purified by silica gel column chromatography to give ester **17** as a colorless gum: ^1H NMR δ 8.08 (s, 1H), 8.04 (d, $J = 10.2$ Hz), 7.87 (d, $J = 8.4$ Hz, 1H), 7.76 (t, $J = 8.1$ Hz, 1H) 7.60 (t, $J = 8.4$ Hz, 1H), 7.13 (m, 2H), 7.03 (q, $J = 7.2$ Hz, 1H, H-3″), 6.95 (m, 2H), 4.70–4.92 (m, 4H, H-2, H-5), 4.40–4.60 (m, 4H, H-3′, H-6′), 3.69 (m, 1H, H-5′), 3.28 (m, 1H, H-2′), 1.58, 1.53 (d, $J = 7.2$ Hz, 3H, H-4″, *E* and *Z* isomers); EIMS m/z (relative intensity) 490 (M^+, 10), 294 (62), 263 (11), 235 (25), 196 (54), 169 (100), 115 (77); HRMS (FAB, m/z) calcd for $C_{27}H_{24}ClN_2O_5$ 491.13737, found 491.13748. Anal. calcd for $C_{27}H_{23}ClN_2O_5 \cdot 0.20H_2O$: C, 65.57; H, 4.77; N, 5.66. Found: C, 65.26; H, 4.72, N, 5.66.

Synthesis of compound 18. Benzoyl chloride (16 μL, 0.138 mmol) was added to a mixture of compound **12** (22 mg, 0.071 mmol), triethylamine (30 μL, 0.215 mmol) and a catalytic amount of DMAP in chloroform (3 mL) at 0 °C. After stirring overnight at room temperature, the mixture was poured into water and extracted with chloroform. The combined organic layer was dried over $MgSO_4$ and concentrated at reduced pressure. The concentrate was purified by silica gel column chromatography using methanol/ethyl acetate/chloroform (1:1:30) as mobile phase to afford compound **18** (12 mg, 41%) as colorless solid: mp 191–193 °C. IR (KBr) 3056, 2929, 1788, 1712, 1636, 1452, 1280, 1117, 1039, 756, 713 cm^{-1}; ^1H NMR (300 MHz, CD_3COCD_3) δ 8.22 (s, 1H), 8.05–7.85 (m, 4H), 7.75 (t, $J = 7.6$ Hz, 1H), 7.59 (m, 1H), 7.44 (m, 1H), 7.26 (t, $J = 7.8$ Hz, 2H), 5.24 (s, 1H), 5.14 (d, $J = 1.8$ Hz, 1H), 4.87 (s, 1H), 4.77 (s, 1H), 4.74–4.52 (m, 4H), 4.24 (m, 1H), 3.72 (m, 1H); HRMS (FAB, m/z) calcd 417.1450 for $C_{24}H_{21}N_2O_5$, observed 417.1447. Anal. calcd for $C_{24}H_{20}N_2O_5$: C, 69.22; H, 4.84; N, 6.73. Found: C, 69.24; H, 4.76; N, 6.56.

PIKK immune complex kinase assays

The detailed procedure of ATM immune complex kinase assays have been previously described.[4] Briefly, A549 cells were cultured and harvested during exponential growth. The cells were washed twice with PBS and then scraped on ice in lysis buffer (20 mM HEPES, 0.15 M NaCl, 1.5 mM $MgCl_2$, and 1 mM EGTA, pH 7.4) containing 10 μg/mL aprotinin, 1 mM DTT, 5 μg/mL leupeptin, 20 nM microcystin, 5 μg/mL pepstatin, and 0.2% Tween 20. Lysates were cleared of insoluble material by centrifugation, and equal amounts of lysate protein (0.5 mg) were incubated with ATM-specific antibody on ice for 2 h. The immune complexes were precipitated with protein A-Sepharose beads, and the immunoprecipitates were washed twice with the lysis buffer, once with high-salt buffer (0.6 M NaCl, 0.1 M Tris–HCl, pH 7.4) and once with kinase buffer (10 mM HEPES, 50 mM NaCl, and 10 mM $MgCl_2$, pH 7.4). The resulting immunoprecipitates were incubated for 30 min at room temperature with kinase buffer containing various concentrations of inhibitors. The kinase reaction mixture was then added to yield final concentration of 10 mM HEPES, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 μM [γ-^{32}P]-ATP (specific activity, 50 Ci/mmol, ICN), and 25 ng/μL recombinant PHAS-1 in a total volume of 40 μL. The reaction mixture was incubated for 20 min at 30 °C. The reactions were terminated with equal volume of 30% acetic acid, and duplicate aliquots were spotted on P81 phosphocellulose papers (Whatman). After four 5-min washes with 1% phosphoric acid containing 10 mM sodium PP_i, the radioactivity retained in the paper was measured by liquid scintillation counting. All kinase reactions were performed under linear reaction conditions. The kinase activities for each PIKK were normalized to that measured in the no-drug control. The IC_{50} values were determined from two independent determinations, each run in duplicate. The variation from the mean did not exceed ±30%.

For measurements of DNA-PK activity, cells were lysed as described above, with the exception that the lysates were sonicated prior clearing. Cell extracts (0.75 mg protein) were immunoprecipitated with anti-DNA-PK

antibodies. The immune complexes were washed twice with lysis buffer and twice with kinase base buffer prior to the kinase reaction. The kinase reaction conditions were identical to those described above, except that the reaction time was 15 min and the substrate used for DNA-PK was a p53-derived peptide added to a final concentration of 250 ng/μL in a total volume of 40 μL.

The mTOR immune complex assay was performed as described previously.[23] mTOR was immunoprecipitated from rat brain extract with anti-mTOR antibodies. The preincubation procedure and the kinase reactions were performed using conditions that were identical those used in the ATM assay.

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