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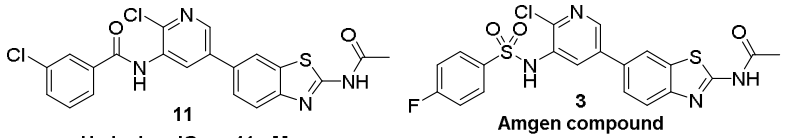
PII: S0960-894X(15)00642-3
DOI: http://dx.doi.org/10.1016/j.bmcl.2015.06.049
Reference: BMCL 22839

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 6 April 2015
Revised Date: 28 May 2015
Accepted Date: 12 June 2015

Please cite this article as: Yang, Z., Ma, H., Sun, Z., Luo, L., Tian, S., Zheng, J., Zhang, X., Discovery of a 6-(pyridin-3-yl)benzo[*d*]thiazole Template for Optimization of Hedgehog and PI3K/AKT/mTOR Dual Inhibitors, *Bioorganic & Medicinal Chemistry Letters* (2015), doi: http://dx.doi.org/10.1016/j.bmcl.2015.06.049

Graphical Abstract



Vismodegib is the first FDA approved cancer therapy based on inhibition of aberrant hedgehog signaling. Like most cancer therapies, vismodegib suffered from resistance, even during clinical development. Numerous reports demonstrated that simultaneous blockage of hedgehog and PI3K/AKT/mTOR pathways resulted in significantly superior outcomes compared with single agent alone in a number of animal disease models. The dual hedgehog and PI3K/AKT/mTOR inhibition represented a promising approach not only to overcoming the resistance but also to delaying its onset. Here we report a series of compounds based on a 6-(pyridin-3-yl)benzo[d]thiazole template which have demonstrated significant inhibition of both hedgehog and PI3K/AKT/mTOR signaling pathways. This new scaffold can serve as a lead for further optimization.

Discovery of a 6-(pyridin-3-yl)benzo[*d*]thiazole Template for Optimization of Hedgehog and PI3K/AKT/mTOR Dual Inhibitors

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Abstract: Vismodegib is the first FDA approved cancer therapy based on inhibition of aberrant hedgehog signaling. Like most cancer therapies, vismodegib suffered from resistance, even during clinical development. Numerous reports demonstrated that simultaneous blockage of hedgehog and PI3K/AKT/mTOR pathways resulted in significantly superior outcomes compared with single agent alone in a number of animal disease models. The dual hedgehog and PI3K/AKT/mTOR inhibition represented a promising approach not only to overcoming the resistance but also to delaying its onset. Here we report a series of compounds based on a 6-(pyridin-3-yl)benzo[*d*]thiazole template which have demonstrated significant inhibition of both hedgehog and PI3K/AKT/mTOR signaling pathways. This new scaffold can serve as a lead for further optimization.

Keywords: hedgehog pathway, PI3K/AKT/mTOR pathway, polypharmacology, GPCR, kinase, cancer therapy

Abbreviations

AKT, protein kinase B; BCC, basal cell carcinoma; CNS, central nervous system; GPCR, G protein-coupled receptor; HBA, hydrogen bond acceptor; Hh, Hedgehog; MB, modulloblastoma; mTOR, mammalian target of rapamycin; N-G-L, NIH3T3-GRE-Luc reporter gene assay; PI3K, phosphoinositide 3-kinase; Ptch, Patched; SAR, structure-activity relationship; Smo, Smoothened; SMO-BCB, BODIPY-Cyclopamine binding assay; SUFU, suppressor of fused.

Hedgehog signaling pathway is a pivotal developmental pathway responsible for patterning and organogenesis in early embryonic development. Hedgehog (Hh) signaling pathway is relatively dormant in adults, with limited functions in tissue repair and maintenance. In canonical hedgehog signaling, the secreted proteins Sonic hedgehog, Indian hedgehog and Desert hedgehog bind to the negative regulator Patched (Ptch), relieving the suppression of Ptch to a GPCR protein Smoothened (Smo). Activated Smo orchestrates a signaling cascade which involves the glioma-associated oncogene transcription factors' (Gli1, Gli2, and Gli3) dissociation from suppressor of fused (SUFU) and entrance to the nucleus, ultimately leading to specific gene expressions mediated by the Gli family transcription factors.[1] Aberrant hedgehog signaling is connected to numerous cancers. Mutational activation (ligand independent) of Hh from inactivation of the negative components (Patched, SUFU) or activation of the positive component (smoothened) leads to cancers such as basal cell carcinoma (BCC), modulloblastoma (MB), and rhabdomyosarcoma.[2] Ligand dependent activation was observed in colorectal, gastric, pancreatic, prostate, lung, haemotalogical cancers, and so on.[3] Inhibition of the abnormal Hh activity represents a promising strategy for the treatment of cancers.[4]

Smoothened is the most studied Hh pathway component as a drug target.[4] The most advanced Smo antagonist, GDC-0449 (vismodegib), was approved by the FDA in 2012 for the treatment of basal cell carcinoma which was not suitable for operation.[5] There are numerous Smo antagonists such as NVP-LDE225, BMS-833923, LY2940680, PF-04449913, NVP-LEQ506 (Figure 1) in advanced developmental stages.[6]

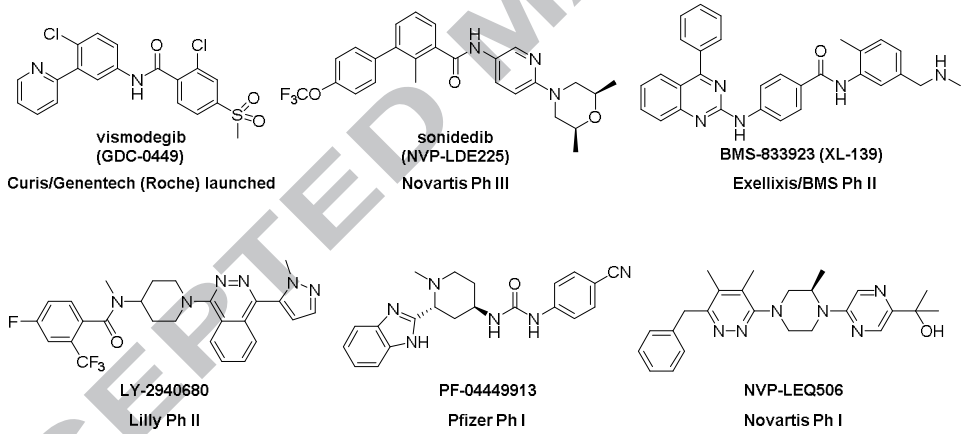


Figure 1. Chemical structures of clinical Hh signaling pathway inhibitors.

Like most cancer therapies, vismodegib suffered from resistance even during clinical development. A patient with metastatic MB went into remission after initial treatment with vismodegib; however, the cancer reoccurred shortly after, and the patient succumbed to the disease.[7] Later analysis demonstrated that a mutation in Smo, codon 473 from Asp to His (D473H) led to the diminished binding of vismodegib to the mutated Smo.[8] Similar resistance mutations had also been observed for NVP-LDE225 in a mouse MB model driven by a Ptch-mutant, albeit the identified mutations were different from the vismodegib induced mutation.[9] Numerous work aiming to overcome the resistance developed by the D473H mutation had been reported. Allosteric inhibitors ALLO-1 and ALLO-2 identified by Norvatis scientists demonstrated a distinctive binding mode and can inhibit vismodegib resistant Smo.[10] It appeared that antagonists which bind

to the same site as vismodegib may also block the mutant Smo with different, usual larger substitutions on the core structure (exemplified by compound 1, Figure 2). [6d,11] This may be due to the extended interactions between Smo and the antagonists away from the mutant site. Most recently, more human mutations have been identified,[12] which may complicate approaches targeting mutational Smo. Nevertheless, the potential success of blocking the mutant Smo could be limited once the Gli2 is up-regulated, as demonstrated by both vismodegib and NVP-LDE225 in the Ptch-mutant MB mouse model.[9,11a] Gli2 up-regulation represents another mechanism of resistance for Smo antagonists. There are reports of Gli inhibitors, however, the current Gli inhibitors could be hampered by safety and developability hurdles.[13] Alternative pathway activation represents a third mechanism of resistance. For example, treatment with vismodegib and NVP-LDE225 both led to an activated PI3K/AKT/mTOR signal pathway in a MB mouse model. [9,11a] Simultaneous blockage of Hh and PI3K/AKT/mTOR pathways resulted in significant tumor regression compared with single agent alone in both cases. The dual hedgehog and PI3K/AKT/mTOR inhibition represented a promising approach not only to overcoming the resistance but also to delaying its onset.

Despite encouraging results from combination treatment in animal disease models, a combination therapy may face problems from patients' compliance, drug-drug interaction risks etc.[14] Alternatively, a compound which can block the Hh and the PI3K pathways simultaneously could offer significant advantages. Polypharmacology was very successful in the CNS drug development and kinase development.[15] However, it is challenging to develop compounds cross different classes of drug targets. In the current case, Smo is a GPCR and PI3K/AKT/mTOR are kinases.

In our pursuit of novel Hh inhibitors,[18] we studied numerous templates under the guidance of the recently published Smo crystal structure[16] and a pharmocophore model.[17] We have previously reported two novel templates based on tetrahydroimidazo[1,2-*a*]pyrazine[18a] and tetrahydrothiazolo[5,4-*c*]pyridine.[18b] Compounds from these templates demonstrated potent Smo inhibition, improved physical-chemical properties, and good pharmacokinetic profiles (exemplified by compound **2**, Figure 2). Alternative ring closure by hybridization of compounds **1** and **2** led to a new template based on 6-(pyridin-3-yl)benzo[*d*]thiazole, exemplified by compound **11** (Figure 2). Compounds from the 6-(pyridin-3-yl)benzo[*d*]thiazole template also demonstrated potent inhibition of Smo. Based on the aforementioned rationale, we also surveyed a number of PI3K/AKT/mTOR inhibitors in the literature in an effort to dial in kinase activity. We found a series of pan PI3K inhibitors developed by scientists from Amgen which showed great similarity to our template.[19] The leading compound, Amgen compound **3**, looked exactly like compound **11**, except that an amide linkage was replaced by a sulphonamide (Figure 2). In theory, the sulphonamide could provide the same key hydrogen bond acceptor (HBA) interaction, as demonstrated by the extensive SAR investigation in the development of vismodegib.[5a] Therefore we hypothesized that compound **3** should be an active Hh inhibitor as well. Here we report the identification of the 6-(pyridin-3-yl)benzo[d]thiazole template as a valuable lead for the optimization of dual Hh and PI3K inhibitors.

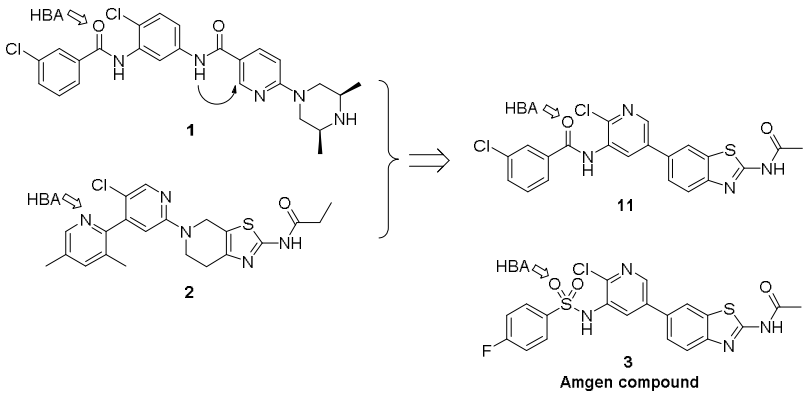
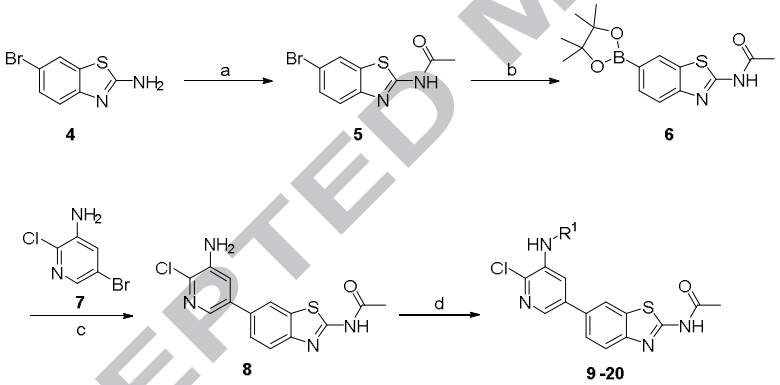


Figure 2. Scaffold morphing strategy and molecular similarity of Hh inhibitor **11** and Amgen pan PI3K inhibitor **3**.

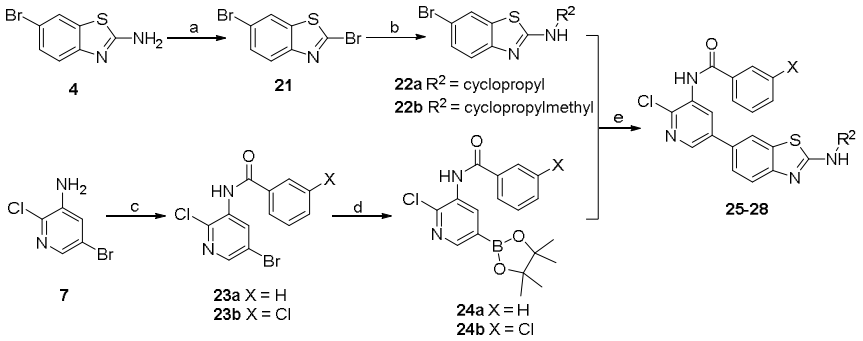
The synthesis of compounds **9**-**20** was outlined in Scheme 1. Commercially available 2-amino-6-bromobenzothiazole (**4**) was acetylated and followed by Miyaura borylation to give pinacol boronate **6**. Suzuki coupling of **6** and bromopyridine **7** provided the key intermediate **8**, which was acylated with corresponding acyl chloride to afford amides **9**-**18**. Treatment of intermediate **8** with benzaldehyde under reductive amination conditions using sodium triacetoxyborohydride afforded compound **19**, or under Strecker reaction conditions using trimethylsilyl cyanide afforded α-aminonitrile **20**.



Scheme 1. Reagents and conditions: (a) Ac_2O, DMAP, CH2Cl2, r. t., 12 h, 79%. (b) bis(pinacolato)diboron, $Pd(dppf)Cl_2$, KOAc, DMSO, 90 °C, 97%. (c) K_2CO_3, $Pd(PPh_3)_4$, 1,4-dioxane/H_2O, 80 °C. 8 h, 20%. (d) acyl chloride, pyridine, 0 °C to r. t., 12 h, 20 - 50% for **9**-**18**; TMSCl, $NaBH(AcO)_3$, AcOH, r. t., 12 h, 31% for **19**; TMSCN, $ZnCl_2$ in Et_2O, THF, r. t. to reflux, 12 h, 25% for **20**.

The synthesis of compounds **25**-**28** was outlined in Scheme 2. Dibromide derivative **21** was prepared from **4** through diazotization-bromination in 87% yield. Displacement of the bromine of intermediate **21** with cyclopropylamine or cyclopropylmethylamine provided compounds **22a** and **22b**. Our initial attempt to convert **22a**-**b** into corresponding pinacol boronates for the next step Suzuki coupling with bromide **7** failed, presumably due to the electron-donating nature of the amino functional group. Alternatively, acylation of **7** with benzoyl chloride or 3-chlorobenzoyl chloride, followed by Miyaura borylation furnished pinacol boronates **24a** and **24b**. Suzuki

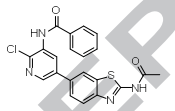
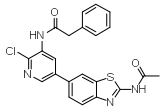
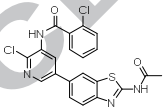
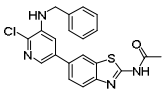
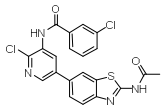
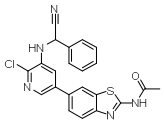
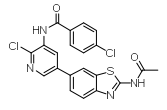
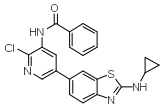
coupling between **24a**-**b** and aryl bromide **22a**-**b** led to the final compounds **25**-**28**, respectively.

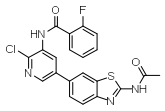
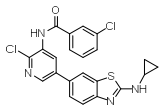
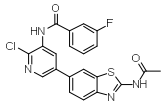
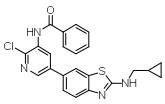
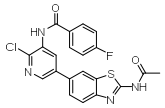
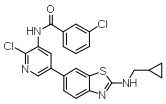
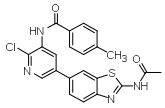
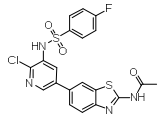
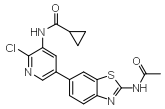


Scheme 2. Reagents and conditions: (a) *iso*-pentyl nitrite, $CuBr_2$, MeCN, 0 °C, 1 h, 87%. (b) corresponding amine, dioxane, 80 °C, 12 h, 57-67%. (c) ArCOCl, pyridine, 0 °C, 1 h, 88-95%. (d) bis(pinacolato)diboron, $Pd(dppf)Cl_2$, KOAc, 1,4-dioxane, 100 °C, 12 h, 70-90%. (e) Na_2CO_3, $Pd(dba)_2$, PCy_3, 1,4-dioxane/H_2O, 80 °C, 20-30%.

The Hh inhibition activity of the synthesized compounds was tested in a cell based NIH3T3-GRE-Luc reporter gene assay with 10 nM SAG as the Hh pathway agonist.[20] This assay can identify Smo inhibitors and inhibitors downstream of Smo such as Gli. However, this assay cannot identify inhibitors interacting with targets upstream of Smo, such as Hh protein inhibitors or Hh acyltransferase inhibitors.[21] The structure-activity-relationship (SAR) was summarized in Table 1.

Table 1. Structure Activity Relationship (SAR) of designed compounds

Compd	Structure	N-G-L IC_{50} (nM)[a] (SMO-BCB IC_{50} (nM)[b])	PI3Kα IC_{50} (nM) (% inhib. (1 μM)[c])	Compd	Structure	N-G-L IC_{50} (nM)[a] (SMO-BCB IC_{50} (nM)[b])	PI3Kα IC_{50} (nM) (% inhib. (1 μM)[c])
9		64 ± 21 (5.6[b])	9%	**18**		3400 ± 2200	
10		4100 ± 1400		**19**		940 ± 130	
11		41 ± 13 (10[b])	980 ± 280 (93%)	**20**		100 ± 27	490 ± 1.6 (85%)
12		>10000		**25**		210 ± 18	

13		>10000		**26**		150 ± 22	10%
14		5000 ± 2800		**27**		150 ± 1.2	
15		170 ± 50	48%	**28**		190 ± 32	3%
16		810 ± 230		**3**		120 ± 3.8	1.2 ± 0.9[d]
17		>10000			Vismodegib	16 ± 2.4[e]	

[a] Inhibition of luminescence signaling in NIH3T3-GRE-Luc reporter gene assay (N-G-L) with 10 nM SAG as the Hh pathway agonist. Data are expressed as geometric mean values of at least two runs ± the standard error measurement (SEM).

[b] Inhibition of BODIPY-cyclopamine fluorescence signaling in the competitive displacement experiment using U2OS cells over-express human Smo. Data are expressed from a single IC_{50} determination.

[c] Inhibition of luminescence signaling in Kinase-Glo Plus Luminescent Kinase Assay (Promega). Data are expressed as geometric mean values of two runs ± the standard deviation (SD) or percentage of inhibition at 1 μM concentration.

[d] Data is expressed as PI3Kα K_i (nM) which was reported in the literature.[19]

[e] Vismodegib was run as standard in each NIH3T3-GRE-Luc reporter gene assay. Data are expressed as geometric mean values of four runs ± the standard error measurement (SEM).

The simplest benzamide, compound **9**, was a potent Hh inhibitor (64 nM). It was within a few folds less active when compared with vismodegib (16 nM). Chlorine substitution led to different results: while meta-chloro substitution resulted in improved activity (compound **11**, 41 nM), ortho and para-chloro substitutions were detrimental to activity (compounds **10** and **12**). In contrast, para-fluoro gave the best result (compound **15**, 170 nM), while ortho and meta-fluoro substitution diminished activity (compounds **13** and **14**). Para-substituted methyl group was also detrimental to Hh inhibition. The overall trend indicated that the para position prefer small substitutions while the meta position can accommodate larger hydrophobic groups. This was consistent with other studies.[10] Attempt to replace the phenyl group with a smaller aliphatic cyclopropane led to diminished activity (compound **17**). Introducing flexibility by addition of a methylene or reduction of the amide bond both led to reduced Hh inhibition activity (compounds **18**, **19**). However, bioisosteric replacement of the amide led to sustained activity, as demonstrated by compound **20**

(100 nM). Replacement of the acetamide with amines was well tolerated, as compounds **25**, **26**, **27** and **28** all showed only small reduction of Hh inhibition activity.

In order to confirm that the hedgehog inhibition of these compounds is due to the inhibition of the Smo, we tested representative compounds **9** and **11** in the BODIPY-cyclopamine displacement experiment in the U2OS cells over-expressing human Smo. Both compounds demonstrated potent activities in displacing BODIPY-cyclopamine (5.6 and 10 nM, respectively, Table 1). Further, compounds **9** and **11** were tested in a counter screening Wnt pathway assay and were confirmed that their hedgehog inhibition activity was not due to non-specific cytotoxicity (see Supporting Information).

The Amgen compound **3** was synthesized and characterized according to the literature.[19] The pan PI3K inhibition activity was confirmed (data not shown). Given the molecular similarity, we decided to test compound **3** in the NIH3T3-GRE-Luc reporter gene assay. Compound **3** demonstrated significant Hh inhibition activity (120 nM).

We then tested compounds **9**, **11**, **15**, **20**, **26**, and **28** for their PI3Kα inhibition activity in a Kinase-Glo Plus Luminescent Kinase Assay (Promega). While compounds **9**, **15**, **26**, **28** were not active, compounds **11** and **20** demonstrated decent PI3Kα inhibition at 980 nM and 490 nM, respectively (Table 1. detailed experimental procedure and data analysis can be found in Supporting Information). It was reported that the acetamide participated in key interactions in the kinase hinge binding region.[19] It was therefore detrimental for the kinase activity to replace the acetamide with amines, albeit both acetamide and amine functional groups were well tolerated by Smo. The Amgen compound **3** was obtained by an intensive PI3K optimization campaign.[19] The sulfonamide functionality was found to contribute significantly to the PI3K activity. This is consistent with our modeling results (Supporting Information). While the sulfonamide functionality was tolerated in Smo, it led to poor physicochemical properties (e.g. extremely poor solubility), therefore hampered the enthusiasm to further pursue templates associated with this functional group.

Vismodegib as the first FDA approved cancer therapy based on inhibition of aberrant hedgehog signaling suffered from resistance. Numerous reports demonstrated that simultaneous blockage of hedgehog and PI3K/AKT/mTOR pathways resulted in significantly superior outcomes compared with single agent alone in a number of animal disease models. The dual hedgehog and PI3K/AKT/mTOR inhibition represented a promising approach not only to overcoming the resistance but also to delaying its onset. The data presented above demonstrated that compound **3** is a potent pan PI3K and Hh inhibitor. This may help to explain the broad in vivo anti tumor activities demonstrated by compound **3**.[19] Further, close analogues **11** and **20** also demonstrated potent Hh inhibition activity and decent PI3Kα inhibition activity. Therefore, the 6-(pyridin-3-yl)benzo[*d*]thiazole template can serve as a starting point for the development of dual Hh and PI3K/AKT/mTOR inhibitors.

In summary, during the process of design and synthesis of a dual Hh and PI3K/AKT/mTOR template, we discovered that compound **3**, a potent pan PI3K inhibitor developed by Amgen, also possessed potent Hh inhibition. This may help explain the broad in vivo anti tumor activity of this compound. We also showed that compounds **11** and **20** were potent Hh inhibitors as well as decent PI3Kα inhibitors, thus establishing that the 6-(pyridin-3-yl)benzo[*d*]thiazole template may serve as a lead to optimize dual Hh and PI3K inhibitors for cancer treatment.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No.81273372, 81473090), China Postdoctoral Science Foundation (2013M541729), Natural Science Foundation of Jiangsu Province (BK20140313), BM2013003, and PAPD (A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions).

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