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Identification of novel GLUT inhibitors

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ABSTRACT

The compound class of 1*H*-pyrazolo[3,4-*d*]pyrimidines was identified using HTS as very potent inhibitors of facilitated glucose transporter 1 (GLUT1). Extensive structure–activity relationship studies (SAR) of each ring system of the molecular framework was established revealing essential structural motives (i.e., *ortho*-methoxy substituted benzene, piperazine and pyrimidine). The selectivity against GLUT2 was excellent and initial in vitro and in vivo pharmacokinetic (PK) studies are encouraging.

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Already in 1930 Otto Warburg observed a phenomenon in which cancer cells often metabolically switch from oxidative phosphorylation to glycolysis even under normal oxygen supply.[1–7] At first sight, the contradictory behavior of cancer cells with regard to their increased demand on energy supply is of crucial importance to feed the essential biochemical anabolic pathways with higher amounts of central intermediates.[6] To fulfill the increased demand on building blocks the glycolytic rate is often up-regulated in tumor entities.[8] As the cellular uptake of glucose is the first rate-limiting step in the glycolytic process it is not surprising that the transporters responsible for this uptake were found to be up-regulated in both solid and hematological malignancies.[9]

Of the two different classes of hexose transporters (SGLT: sodium-dependent glucose transporter, GLUT: facilitative glucose transporter) the GLUTs were found to be overexpressed in many tumors.[10] In particular, GLUT1 overexpression has been reported in many types of human cancers, including those of brain,[11] breast,[12,13] colon,[14,15] kidney,[16] lung,[17] ovary[18] and prostate,[19,20] and is correlated with advanced cancer stages and poor clinical outcomes. It was demonstrated that the activation of certain oncogenes like c-myc,[21] KRAS,[17] BRAF[22] and p53,[23] and transcription factors like hypoxia inducible factor-1a[24,25] can induce the GLUT1 overexpression. Additionally, there is a widely clinically applied diagnostic modality PET imaging, which makes use of increased glucose uptake in some types of cancer with [^{18}F]fluoro-2-deoxyglucose (FDG).[26,27] All these factors demonstrate the importance of GLUT1 function for cancer cell viability and lead to the therapeutic options that an inhibition of this transporter might be beneficial in the treatment of tumors with high glucose turnover.

Whereas GLUT1 is nearly ubiquitously expressed in all normal tissues to maintain the basal glucose supply[28] the expression of some members of the GLUT family is more specific. Those GLUTs can be involved in central processes like insulin secretion of pancreas (GLUT2)[29] and neuronal glucose uptake (GLUT3).[30] To enable a therapeutic window with a potential GLUT1 inhibitor selectivity within the GLUT family is decisive for any possible cancer treatment with this approach.

Several small-molecule GLUT1 inhibitors have already been described in literature including resveratrol,[31] naringenin,[32] phloretin,[33] WZB117,[34] thiazolidinedione,[35] and STF-31.[36] With their thiazolidinediones Wang et al. demonstrated the inhibition of [^{3}H]-2-deoxy-D-glucose ([^{3}H]-2-DG) uptake in LNCaP cells and the suppressive effects on the viability of LNCaP cells in MTT assays.[35] Chan et al. used the principle of chemical synthetic lethality to demonstrate the sensitivity of VHL deficient renal cancer cells to glucose uptake inhibition by STF-31.[36] The compound WZB117 was able to inhibit the glucose uptake in A549 cancer cells and their cell proliferation in a dose-dependent manner.[34] All these results underline the feasibility of GLUT1 inhibition as cancer treatment.

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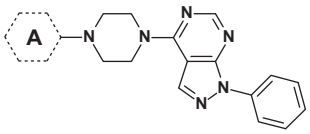
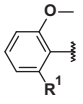
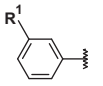
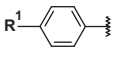
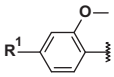
Therefore, an HTS screen was performed using a cell-based assay with CellTiter-Glo™,[37] readout for ATP production. In a pairwise chemical screen, DLD1 cells were co-incubated with the potential GLUT1 inhibiting test compounds and with rotenone as inhibitor for the oxidative phosphorylation.[38] With the rotenone co-incubation, the cells could only produce ATP via glycolysis and the amount of produced ATP could be linked to the amount of glucose taken up. Subsequent assays regarding cell uptake of radioactive [^{3}H]-2-DG, consumption of glucose and generation of lactate in the presence of the test compounds confirmed their competitive GLUT1 inhibitory behavior. In total 120,303 compounds were tested in this HTS and 435 hits were identified inhibiting the GLUT1 transporter while being selective against GLUT2 (CHO-hGLUT2 cells) and comparably active on GLUT3 (DLD1Glut1$^{-/-}$ cells, Horizon discovery).[39] From the originally found 6-(piperazin-1-yl)pyrimidin-4-aniline hits a conjunctive approach at the central pyrimidine quickly led to a 1*H*-pyrazolo[3,4-*d*]pyrimidine core.[40] This compound class was shown to be reversible to the glucose transporter by glucose competition experiments.[41] The SAR of each of the four elements of the molecular framework, named as ring systems A–D, was explored independently. Starting with the aryl ring connected to the piperazine (ring A, Table 1 - most interesting compounds are highlighted in bold) it was found that an *ortho*-methoxy group (**3**) at the aryl ring resulted in very potent GLUT1 inhibition and excellent selectivity against GLUT2 and a selectivity factor of around 10 against GLUT3. Omission of the methyl group of the methoxy group in **3** in comparison to **2** markedly deteriorated the excellent GLUT2 selectivity, while substitution by the metabolically more stable OCF_3 group (**4**) led to a completely inactive compound which was also observed for the corresponding *ortho*-CF_3 moiety (**6**). A methoxy monosubstitution in *meta*- (**8**) and *para*-position (**10**) at the aryl ring A decreased the GLUT1 potency by a factor of approximately 7 and 70, respectively, in comparison to **3**.

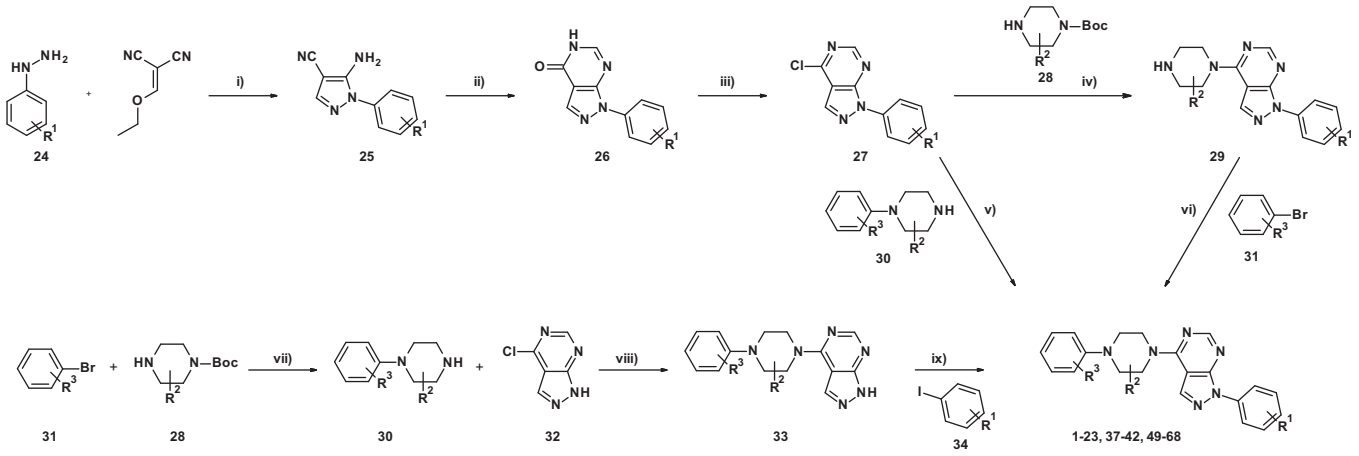
Keeping the *ortho*-methoxy substitution fixed while exploring the introduction of a second functional group in the ring revealed that only a substituent in *para*-position towards the methoxy group was beneficial regarding the GLUT1 potency. In this position not only a small fluorine atom (compound **15**) can be incorporated but also a carboxamide (compound **17**) with clear reduction of GLUT2 selectivity, though. The same observation could be made when a pyridine nitrogen was introduced in the *ortho*-methoxy substituted compounds **19**–**22**. Insertion of a pyridine nitrogen again gave a very potent GLUT1 inhibitor when it was located at *para*-position towards the methoxy group in the aryl ring. Introduction of other heteroaromatic systems at the piperazine like pyrimidine only led to inhibitors in the higher three-digit nM range. **23**, having a non aromatic ring A system, revealed to be a completely inactive compound.

Table 1
SAR at piperazine aryl head[39,a]



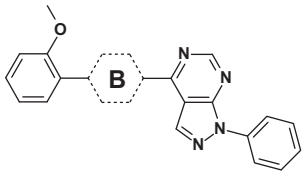
Compd	A	R1	GLUT1[b] IC_{50} (μM)	GLUT2[c] IC_{50} (μM)	GLUT3[d] IC_{50} (μM)	Compd	A	R1	GLUT1[b] IC_{50} (μM)	GLUT2[c] IC_{50} (μM)	GLUT3[d] IC_{50} (μM)
1		F	0.27	1.12	0.68						
2		OH	0.007	0.06	0.06						
3		**OMe**	**0.025**	**>10**	**0.25**	**18**		F	0.11	1.2	1.6
4		OCF_3	>10	>10	>10						
5		CN	1.81	5.99	2.05	**19**		–	0.18	1.36	0.68
6		CF_3	>10	>10	>10						
7		F	0.29	1.21	0.54						
8		OMe	0.18	1.48	0.32	**20**		–	0.23	ND	1.51
9		CN	0.039	0.22	0.069						
10		OMe	1.77	8.7	1.77	**21**		–	0.009	0.51	0.06
11		F	2.4	>10	10						
12		CN	1.2	>10	0.54	**22**		–	0.06	0.95	0.07
13		F	0.01	>10	0.04						
14		CN	0.15	1.7	0.17	**23**		–	>10	>10	>10
15		**F**	**0.001**	**>10**	**0.01**						
16		CN	0.02	>10	0.05						
17		$CONH_2$	0.005	0.10	0.037						

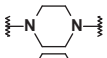
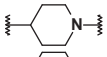
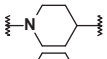
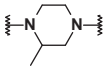
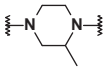
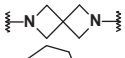
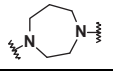
ND = not determined.
[a] All mean data values reported are derived from at least two experiments.
[b] Cell-based assay using DLD1 cell line mainly expressing hGLUT1 (co-incubation of test compound with 100 mM glucose and 1 μM rotenone for 15 min).
[c] Cell-based assay using CHO cell line transfected with hGLUT2 (co-incubation of test compound with 30 mM fructose and 1 μM rotenone for 15 min).
[d] Cell-based assay using hGLUT1 knock-out DLD1 cell line (DLD1GLUT1$^{-/-}$) mainly expressing hGLUT3 (co-incubation of test compound with 300 mM glucose and 1 μM rotenone for 15 min).



Scheme 1. Reagents and conditions: (i) EtOH, Et_3N, rt; (ii) HCOOH, 110 °C; (iii) $POCl_3$, 130 °C; (iv) (a) Et_3N, THF, rt–60 °C, (b) 4 M HCl, dioxane, rt; (v) DBU, DMF, 120 °C or THF, Et_3N, rt–60 °C; (vi) $Pd(OAc)_2$, XPhos, NaO^{t}Bu, toluene, 90 °C; (vii) (a) $Pd(OAc)_2$, XPhos, NaO^{t}Bu, toluene, 90 °C, (b) 4 M HCl, dioxane, rt; (viii) Et_3N, THF, 60 °C or DBU, DMF, 120 °C; (ix) CuI, K_3PO_4, *trans*-*N*,*N*′-dimethylcylcohexane-1,2-diamine, NMP, microwave irradiation, 180 °C.

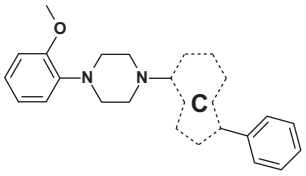
Table 2
Substitution of central piperazine moiety[39,a]

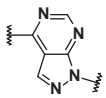
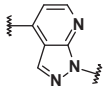
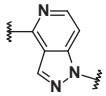
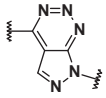
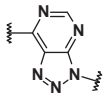
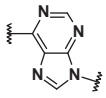
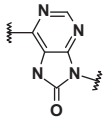


Compd	B	GLUT1[b] IC_{50} (μM)	GLUT2[b] IC_{50} (μM)	GLUT3[b] IC_{50} (μM)
3		**0.025**	**>10**	**0.25**
35		1.9	>10	>10
36		0.47	ND	1.20
37		**0.014**	**1.4**	**0.12**
38		0.12	2.5	0.48
39		2.3	>10	4.1
40		>10	>10	>10
41		4.4	ND	2.2
42		**0.004**	**0.021**	**0.008**

ND = not determined.
[a] All mean data values reported are derived from at least two experiments.
[b] For details of the biological assays, see Ref. 39.

Table 3
Variation of pyrazolopyrimidine core[39,a]



Compd	C	GLUT1[b] IC_{50} (μM)	GLUT2[b] IC_{50} (μM)	GLUT3[b] IC_{50} (μM)
3		**0.025**	**>10**	**0.25**
43		0.3	0.6	4.0
44		4.0	5.0	2.0
45		>10	>10	>10
46		0.049	3.3	0.43
47		0.027	3.5	0.064
48		**0.053**	**>10**	**0.63**

ND = not determined.
[a] All mean data values reported are derived from at least two experiments.
[b] For details of the biological assays, see Ref. 39.

Several synthetic approaches were employed to build the desired 1*H*-pyrazolo[3,4-*d*]pyrimidines (see Scheme 1). One way started with conversion of appropriately substituted hydrazines **24** with (ethoxymethylene)malononitrile to obtain the 5-amino-pyrazole-4-carbonitriles **25**.[42,43] These were heated with formic acid to obtain pyrazolo[3,4-*d*]pyrimidin-4-ones **26**.[44] After activation of the pyrimidin-4-one with $POCl_3$[44] the required piperazine aryl head **30** can be added in a one pot procedure with DBU as base.[45] Alternatively, a mono-Boc-protected piperazine **28** can be added to the 4-chloro-1*H*-pyrazolo[3,4-*d*]pyrimidines **27**[46] followed by deprotection[47] and cross-coupling with the desired aryl bromides in a Buchwald–Hartwig reaction[48–51] using $Pd(OAc)_2$, XPhos and NaO^{t}Bu. Furthermore, the piperazine aryl head

30 could be synthesized first via Buchwald–Hartwig and then coupled to 4-chloro-1*H*-pyrazolo[3,4-*d*]pyrimidine **32** to receive intermediates **33**. A final Goldberg reaction[52] using K_3PO_4 and *trans*-*N*,*N*′-dimethylcylcohexane-1,2-diamine as ligand was employed to couple the aryl moiety to the pyrazole.

Within the lead structure it was found that the central piperazine played a very decisive part for the excellent GLUT1 potency (ring B, Table 2). Trials to omit one of the piperazine nitrogens (**35** and **36**) resulted in less potent test compounds. Substitution adjacent to the ring A attachment point (**37**) was tolerated whereas the corresponding regioisomer (**38**) led to a decrease in GLUT1 potency by a factor of ~10. Methylene bridges across the piperazine ring were not tolerated (**39** and **40**). Switching to a piperazine mimic, that is, the spiro-compound **41**, was not successful. Ring enlargement to the corresponding 1,4-diazepane (**42**) resulted in a markedly improved potency however the selectivity over the other GLUT members was deteriorated.

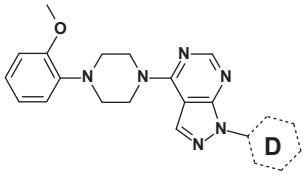
Regarding GLUT1 potency some regions of the 1*H*-pyrazolo[3,4-*d*]pyrimidine core (ring C, Table 3) allowed for more flexibility than the piperazine moiety. For example, the pyrazole ring could be exchanged for a triazole (**46**), imidazole (**47**) or imidazole-2-one (**48**) without a loss in activity. However, in the case of the imidazole **47**, the GLUT3 selectivity deteriorated. On the other hand, the presence of the pyrimidine moiety revealed to be more crucial for a good GLUT1 inhibition.

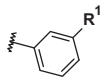
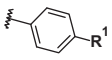
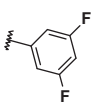
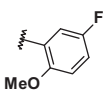
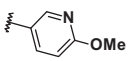
Removing a nitrogen from 5- or 7-position (**43** and **44**) or adding a nitrogen at the 6-position (**45**) gave only compounds of low activity or no activity at all.

The aryl ring connected to the pyrazole (ring D, Table 4) can be substituted with small functional groups in the *ortho*-, *meta*- and *para*-position without significant loss of GLUT1 potency. Regarding the *ortho*-position a fluorine atom (**49**) has even beneficial effect in comparison to compound **3**. In case of a *para*-substituent an electron-withdrawing group like fluorine (**56**) or cyanide (**57**) revealed to be superior to an electron-donating function such as methoxy (**58**) or methyl (**59**). Double substitution at the aryl ring was also feasible but the electronic behavior and the relative positioning of the substituents was important to keep the excellent GLUT1 potency (**60** and **61**). Introduction of a pyridine nitrogen to the benzene ring D with (**66** and **67**) or without further substitution (**63**, **64**, and **65**) decreased the GLUT1 activity significantly. The same effect was observed when a substituted methylene was inserted between rings C and D (compound **62**). A complete switch to a saturated cyclopentyl replacing ring D (compound **68**) was also feasible without losing potency and selectivity, but this substituent proved to be metabolically too labile (Table 5) already in in vitro PK assays with human liver microsomes (F_{max}: 37% h) and rat hepatocytes (F_{max}: 11% r). Consistent with prediction, for aromatic ring D analogs the in vitro PK data looked more promising than for compound **68**.

The in vitro PK assays with human liver microsomes and rat hepatocytes (Table 5) revealed a moderate to good metabolic stability of the GLUT1 inhibitor compounds with exception to compound **68** containing the liable cyclopentyl moiety. Comparing the human liver microsome data of compounds **3** and **15** the additional substitution with fluorine at ring A did not further increase

Table 4
SAR at pyrazoloaryl tail[39,a]



Compd	D	R^1	GLUT1[b] IC_{50} (μM)	GLUT2[b] IC_{50} (μM)	GLUT3[b] IC_{50} (μM)	Compd	D	GLUT1[b] IC_{50} (μM)	GLUT2[b] IC_{50} (μM)	GLUT3[b] IC_{50} (μM)
3		H	**0.025**	**>10**	**0.25**	**63**		0.51	8.0	0.94
49		F	**0.007**	**1.1**	**0.04**					
50		OMe	0.05	1.2	0.11					
51		Me	0.01	1.0	0.25					
52		F	**0.035**	**>10**	**0.12**	**64**		0.75	6.0	1.5
53		CN	0.033	nd	0.35					
54		OMe	0.2	0.9	0.17					
55		Me	0.05	10	0.096					
56		F	0.017	>10	0.046	**65**		1.2	4.4	1.6
57		CN	0.017	>10	0.2					
58		OMe	0.23	2.6	0.17					
59		Me	1.5	10	3.0					
60		—	0.008	>10	0.09	**66**		0.08	7.0	ND
61		—	0.03	0.88	0.32	**67**		0.1	ND	0.34
62		—	0.41	2.1	0.95	**68**		0.04	2.0	0.12

ND = not determined.
[a] All mean data values reported are derived from at least two experiments.
[b] For details of the biological assays, see Ref. 39.

Table 5
In vitro pharmacokinetic profiling of pyrazolopyrimidines

Compd	LM[a] stability $Cl_{b,LM}$[b] (L/h/kg); F_{max}[c] (%)	Hep[d] stability $Cl_{b,Hep}$ (L/h/kg); F_{max} (%)	Caco-2 permeability P_{app} A–B[e] (ER[f])
3	0.14; 89 (h) 2.2; 47 (r) 2.3; 57 (m)	0.84; 36 (h) 2.7; 36 (r)	59 (0.29)
15	0.37; 72 (h)	2.6; 39 (r)	ND
49	0.5; 62 (h) 1.7; 68 (m)	0.79; 40 (h) 2.2; 48 (r)	175 (0.42)
52	0.55; 58 (h) 1.9; 65 (m)	ND	ND
68	0.83; 37 (h)	3.7; 11 (r)	153 (0.33)

ND = not determined.
[a] Liver microsomes (LM) (h: human, r: rat, m: mouse).
[b] Blood clearance (Cl_b): $Cl_b = (QH \cdot Cl_{int})/(QH + Cl_{int})$, QH: liver blood flow (human: 1.3 L/h/kg, rat: 4.2 L/h/kg, mouse: 5.4 L/h/kg), Cl_{int}: intrinsic clearance (well-stirred model of hepatic clearance).
[c] Maximal predicted bioavailability after peroral administration (F_{max}): $F_{max} = (1 - Cl_b/QH) \cdot 100\%$.
[d] Hepatocytes (Hep).
[e] The apparent permeability (P_{app}) values (nm/s) are derived from the transport of the compounds (2 μM) over a 2 h period from the apical (A) to the basolateral (B) compartments.
[f] The letters ER refer to the efflux ratios and are calculated by dividing the P_{app} B–A values by the P_{app} A–B values.

Table 6
In vivo pharmacokinetic profile of compound **49** in male Wistar rats (0.3 mg/kg iv,[a] 5 mg/kg po[b])

Cl_b (L/h/kg)	V_{ss} (L/kg)	Terminal $t_{1/2}$ (h)	$C_{max,po}$ (μM)	F (%)
1.2	1.9	2.7	1.7	67

[a] Formulation: PEG400 50% + water 40% + EtOH 10%.
[b] Formulation: PEG300 90% + NMP 10%.

metabolic stability, neither did the introduction of a fluorine at ring D (**49** and **52**). The Caco-2 permeability was generally high in this compound class, only compound **3** showed moderate apical to basolateral transport. All compounds showed indication for uptake transport in Caco-2 cells, as the efflux-ratio was found to be less than 1.

In vivo PK results showed compound **49** to have a low blood clearance (29% of liver blood flow) in Wistar rat (Table 6). The volume of distribution was high and the terminal half-life of the compound was intermediate. Oral bioavailability was 67% which is in line with the total blood clearance indicating complete absorption.

In summary, optimization at each ring of the molecular framework of the 1*H*-pyrazolo[3,4-*d*]pyrimidines revealed that with an appropriate substitution pattern (e.g., **49**) a single-digit nanomolar inhibition at GLUT1 is feasible. Furthermore, excellent selectivity towards GLUT2 can be obtained within this compound class. The preliminary in vitro profile looked promising and first in vivo PK studies showed that good oral bioavailability is attainable. Further efforts are undertaken to increase GLUT3 selectivity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.02.050. These data include MOL files and InChiKeys of the most important compounds described in this article.

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