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Anthranilic acid derivatives as novel ligands for farnesoid X receptor (FXR)

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ABSTRACT

Nuclear farnesoid X receptor (FXR) has important physiological roles in various metabolic pathways including bile acid, cholesterol and glucose homeostasis. The clinical use of known synthetic non-steroidal FXR ligands is restricted due to toxicity or poor bioavailability. Here we report the development, synthesis, in vitro activity and structure–activity relationship (SAR) of anthranilic acid derivatives as novel FXR ligands. Starting from a virtual screening hit we optimized the scaffold to a series of potent partial FXR agonists with appealing drug-like properties. The most potent derivative exhibited an EC_{50} value of 1.5 ± 0.2 μM and 37 ± 2% maximum relative FXR activation. We investigated its SAR regarding polar interactions with the receptor by generating derivatives and computational docking.

1. Introduction

The ligand-activated transcription factor farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily. It is predominantly expressed in liver, intestine and kidney and binds to specific DNA response elements as monomer or as a heterodimer with the retinoid X receptor (RXR). When physiologically activated by bile acids such as chenodeoxycholic acid (CDCA, 1a) FXR regulates a large number of target genes affecting metabolism and homeostasis of bile acids, lipids and glucose.[1,2]

Several pathophysiological conditions have been discovered in which FXR is involved. Both in vitro and in vivo models suggest a possible use of FXR ligands for treatment of metabolic[3–6] and inflammatory[7–11] diseases as well as a role of FXR in the development and growth of certain cancer cells.[12] FXR ligands might be beneficial for the treatment of primary biliary cirrhosis (PBC), diabetes, dyslipidemia, cancer and other disorders.[2]

Intensive research on FXR ligands has yielded several potent steroidal and non-steroidal compounds (reviewed in Ref. [13]). CDCA (**1a**) is the most potent bile acid physiologically activating FXR. Medicinal chemistry efforts have optimized **1a** to obtain 6-ethyl-CDCA[14] (INT-747, **1b**) and **1c**[15] which contains an elongated side chain in addition to the 6α-ethyl moiety. Among the non-steroidal FXR agonists GW4064[16] (**2**) and its derivatives are most potent so far.

Compounds **1b**, **1c** and **2** constitute full FXR agonists with low nanomolar EC_{50} values. Compound **1b** was effective in a co-activator recruitment assay (EC_{50} = 99 nM) and a reporter gene assay (EC_{50} = 85 nM). Compound **1c** is characterized as very potent FXR agonist with an EC_{50} value of 15 nM (290% of 20 μM CDCA) in a co-activator recruitment assay.[15] GW4064 (**2**) showed low nanomolar EC_{50} values in co-activator recruitment (15 nM and 37 nM respectively) and a binding affinity of 64 nM in a scintillation proximity assay (SPA) while its EC_{50} value in reporter gene assays was higher with around 0.9 μM (reviewed in Ref. 17).

However, several of the existing non-steroidal FXR agonists either do not possess acceptable bioavailability, are non-selective or exhibit toxicity.[13,18–21] Consequently their clinical utility is limited and novel FXR ligands are required. So far the only FXR agonists in clinical development are **1b**,[14] which has reached phase IIb (NCT01265498) for the treatment of non-alcoholic

Abbreviations: SAR, structure–activity-relationship; FXR, farnesoid X receptor; RXR, retinoid X receptor; DMEM, Dulbecco's modified eagle medium; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cirrhosis; PCC, pyridinium chlorochromate; SP, sodium pyruvate; PS, penicillin/streptomycin; RLU, relative light units; AF2, activation function 2.

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steatohepatitis (NASH), and primary biliary cirrhosis (PBC) as well as a derivative of **2** that has recently entered early clinical development (NCT01899703). Compound **1b** was also investigated in a phase II trial (NCT00501592) in patients with type **2** diabetes and non-alcoholic fatty liver disease (NAFLD) where it showed promising results on insulin sensitivity and liver fibrosis and was generally well tolerated.[22] Hence, INT-747 (**1b**) appears to be effective and non-toxic and may be the first marketed FXR-targeting drug (for preclinical and clinical data see Ref. 5). As a general consideration, it is however questionable whether full agonism on FXR may actually be beneficial for all conditions that might be treatable by FXR ligands. In vivo data indicates that full agonism is not crucial for in vivo efficacy[23] and may even have undesirable effects.[24] Therefore our approach was to develop novel FXR ligands that only partially and moderately activate this nuclear receptor.

Virtual screening of a compound collection using an in silico model of FXR[25] yielded several hits with low potency that were confirmed in our cell-based FXR full-length transactivation assay. We selected one of these hits, the anthranilic acid derivative **3** (Fig. 1), for optimization by medicinal chemistry and automated computational docking studies. The optimization yielded a set of FXR agonists with considerable in vitro potency in the FXR transactivation assay and favorable physicochemical properties. Here we describe the synthesis, SAR and in vitro pharmacology of anthranilic acid derivatives as FXR ligands.

2. Results

Lead structure **3** possesses partial agonistic activity at FXR with 11% activity at 30 μM in a cell-based FXR full-length transactivation assay compared to the activity of **2** (3 μM, 100%).

An initial docking analysis of **3** (Fig. 2) indicated that the butyric acid side chain might be suitable for FXR activation and placed the acidic head group near Arg_{268} and Arg_{335}. Docking also suggested that enlargement of the lipophilic acyl substituent (4-methylbenzoyl moiety in **3**) might improve binding to FXR since the relatively small 4-methylbenzoyl residue did not fill the large lipophilic pocket. Therefore our optimization study started with a variation of the lipophilic acyl substituent.

2.1. Chemistry

Anthranilic acid derivatives were generated in a two-step synthesis using isatoic anhydride (**4a**) as origin of the anthranilic acid core. Compound **4a** was reacted with various amino acids (**5a**, **c**–**h**) and ester **5b** to introduce the acidic side chains by nucleophilic substitution (Scheme 1). To improve the yield of the required *o*-aminobenzoyl derivatives **6a**–**i** 4-(dimethylamino)pyridine (**7**)

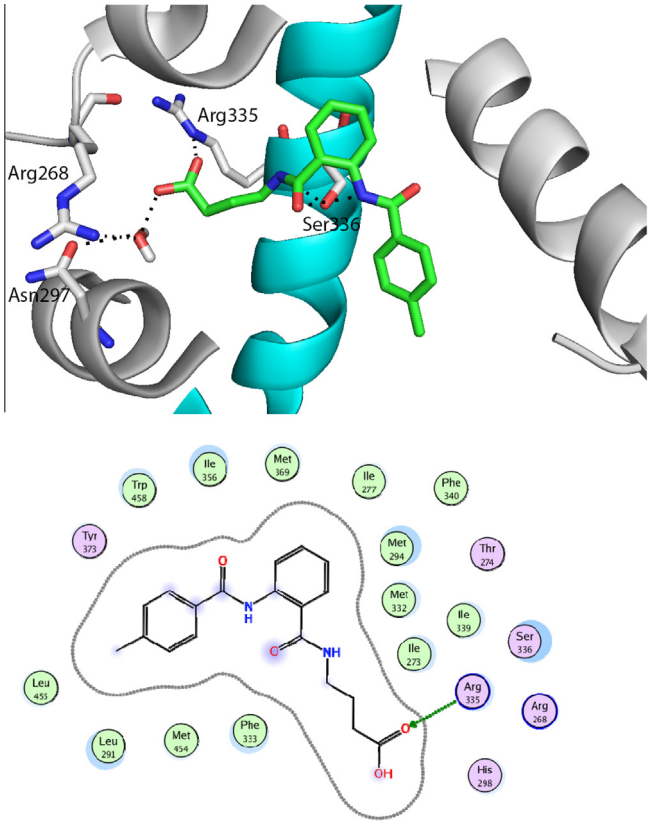


Figure 2. Docking analysis of lead structure **3**.

served as catalyst with an amount of 10 mol%, as described by Venuti.[26] We optimized the reaction by varying solvent, reaction time and temperature (Supporting information Table 1). The highest yield was achieved with a mixture of pyridine/DMF/NEt_3 at 80 °C over 16 h. With aliphatic amino acids as nucleophiles, the optimized conditions lead to yields of around 72%.

Anthranilic acid derivatives **6a**–**j** were subsequently reacted with suitable acyl chlorides (**8a**–**p**) to introduce the mostly lipophilic acyl substituents of the test compounds **15a**–**p**, **16**–**24** (Supporting information Scheme 1).

To investigate the necessity of the amide hydrogens in the anthranilic acid core for interaction with FXR, monomethylated derivatives **23** and **24** were generated with the same synthetic strategy using either *N*-methyl isatoic anhydride (**4b**) or 4-(methylamino)butyric acid (**5i**) as methylated starting material

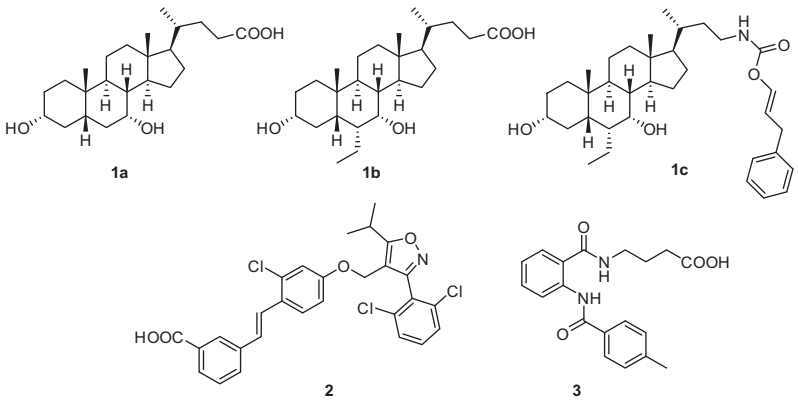
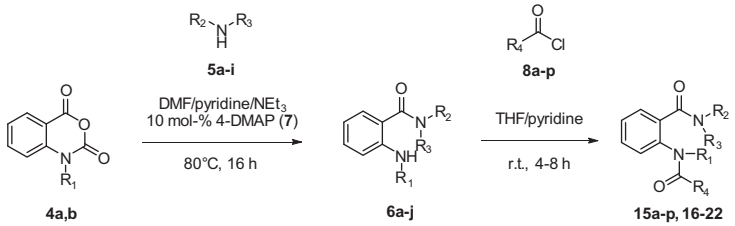


Figure 1. Important FXR ligands CDCA (**1a**) 6-ECDCA (**1b**) and GW4064 (**2**), and lead structure **3**.



Scheme 1. General synthetic procedure: *Ortho*-aminobenzoylation of amino acids **5a**–**i** with isatoic anhydride derivatives **4a** and **b** and acylation with acyl chlorides **8a**–**p**.

(Scheme 2). We also examined the requirement of the amide carbonyl groups for interaction with the receptor by replacing the amides with a sulfonic amide (**25**) or secondary amines (**26**, and **27**). Sulfonic amide **25** was synthetized using sulfonic acid chloride **9** instead of an acyl chloride (Supporting information Scheme 2).

Secondary amine **26** was generated by reductive amination using 2-naphthaldehyde **10** and anthranilic acid derivative **6a** (Scheme 2).

For the preparation of amine **27** a different synthetic strategy was required. The secondary amine of the acidic side chain had to be introduced in the last step since it would exhibit higher nucleophilicity than the aromatic amine of the anthranilic acid core, which was supposed to react with an acyl chloride. For the generation of **27** 2-aminobenzyl alcohol (**11**) was therefore used as starting material, which we selectively oxidized to 2-aminobenzaldehyde (**12**) using pyridinium chlorochromate (PCC, **13**). **12** subsequently reacted with acyl chloride **8j** to form aldehyde **14** which was then suitable for a reductive amination with 4-aminobutyric acid (**5a**) yielding amine **27** (Scheme 3).

2.2. Biological evaluation

FXR activation by the described compounds was tested in a HeLa cell-based full-length FXR transactivation assay with a firefly luciferase as reporter gene and a constitutively expressed renilla luciferase as control. Maximal relative FXR activation of the compounds refers to the activity of GW4064 (**2**) at 3 μM, which we defined as 100%.

In the first structural optimization study the acyl substituent in the lipophilic backbone of the anthranilic acid derivatives was varied (**15a**–**p**, Table 1). The more polar 3,5-dinitrobenzoyl derivative **15a** showed no activity at FXR as well as **15b** with a 4-methoxybenzoyl moiety, all biphenyl derivatives (**15e**–**g**) and the highly lipophilic and sterically demanding diphenylacetyl (**15l**) and diphenylpropanoyl (**15m**) derivatives. Introduction of an aromatic heterocycle as lipophilic substituent in **15h** and **15i** also led to lack of activity.

A 2-naphthoyl moiety (**15j**) as lipophilic substituent produced partial FXR transactivation of 37 ± 2% with an EC_{50} value of 8.6 ± 1.3 μM. Interestingly, both its 1-naphthoyl (**15k**) and 2-naphthylacetyl (**15p**) derivatives were inactive.

Lower EC_{50} values with reduced partial transactivation of FXR were measured for the 4-ethylbenzoyl (**15c**) and the 4-*t*-butylbenzoyl moiety (**15d**). Compound **15c** exhibited partial FXR activation of 28 ± 2% with an EC_{50} value of 5.8 ± 1.0 μM while **15d** activated FXR to an extent of 19 ± 1% with EC_{50} = 2.5 ± 0.4 μM.

The lowest EC_{50} value of 0.72 ± 0.01 μM within this series was observed for cinnamoyl derivative **15n** combined with a FXR transactivation of 12.39 ± 0.02%. The saturated dihydrocinnamoyl derivative **15o** was inactive.

After optimization of the acyl substituent, the acidic head group was varied (**16**–**22**, Table 1). For this SAR investigation the 2-naphthoyl moiety of **15j** was selected as lipophilic substituent since it produced the highest relative FXR activation although **15j** had the poorest EC_{50} value among the series **15c**, **15d**, **15j** and **15n**. The higher relative FXR activation of **15j** is preferred since in a cell-based assay there is the risk of false-negatives when the relative activation is too low.

The methyl ester **16** of **15j** turned out to be still active with a comparable EC_{50} value but led to lower maximum relative activation of 19.0 ± 0.6%. Elongation of the acidic side chain from C4 (**15j**) over C5 (**17**) to C6 (**18**) led to a moderate improvement in the EC_{50} value with 8.3 ± 1.0 μM for **17** and 4.4 ± 0.6 μM for **18** but at the same time the relative activation considerably dropped to 11.4 ± 0.4% and 10.4 ± 0.4%, respectively.

Introduction of aromatic moieties in the acidic head group improved the EC_{50} value compared to **15j**. Within the series of aromatic carboxylic acids, the *p*-benzoic acid derivative **19** showed the best EC_{50} value of 1.0 ± 0.2 μM with 23 ± 1% maximum relative FXR activation. The *m*-benzoic acid derivative **20** relatively activated FXR to an extent of 37 ± 1% with a slightly higher EC_{50} value of 1.5 ± 0.2 μM. Compound **21** with an additional methylene group between aromatic ring and carboxylic acid and phenylacetic acid derivative **22** yielded EC_{50} values in the same range with 1.3 ± 0.1 μM for **21** and 3.1 ± 0.3 μM for **22** but their maximum relative FXR activation activities were lower (10.02 ± 0.04% for **21** and 9.8 ± 0.4% for **22**).

Then we blocked selected polar functions in compounds **23**–**27** to investigate hypothetical interactions of the anthranilic acid derivatives with the FXR ligand binding site (**23**–**27**, Table 2). For this purpose amide nitrogen atoms were methylated (**23** and **24**) and amides were reduced to secondary amines (**26** and **27**). In addition, sulfonic amide **25** was investigated which has a different acidity and geometry than the respective amide **15j**.

All derivatives **23**–**27** were inactive up to concentrations of 30 μM suggesting that the polar functions and the geometry of the anthranilic acid core might be important for ligand-FXR interaction.

Compounds **15j** and **20** turned out as the most potent derivatives. Therefore we investigated their activity on common *off*-targets and physicochemical properties. Since anthranilic acid derivatives such as the NSAIDs mefenamic acid and flufenamic acid are known to interact with the arachidonic acid cascade we studied **15j** on several enzymes of this pathway. The compound did not inhibit cyclooxygenases I and II, 5-lipoxygenase and mPGES-1 up to a concentration of 10 μM. Additionally, **15j** and **20** were soluble in various solvents including water at alkaline pH. For both compounds **15j** an **20** no toxic effects occurred in the HeLa cell based reporter gene assay up to the highest used concentration of 60 μM. In a cell proliferation assay (WST-1) in HepG2 cells **15j** showed no significant toxicity up to 100 μM while **20** slightly inhibited proliferation starting from 30 μM which increased with higher concentrations (60 μM and 100 μM; for values see experimental section). **15j** and **20** furthermore passed computational tests on ADME and toxicological properties (FAF*Drugs*2,[27] standard algorithms) which confirms the drug-likeness of the compounds and indicates that no known toxic pharmacophores are included. With the optimization of **15j** yielding **20** ligand efficiency (LE)

Table 1
In vitro activities of compounds **15a–p** and **16–22**

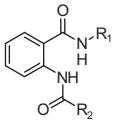
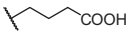
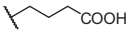
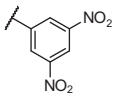
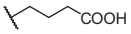
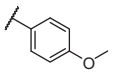
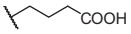
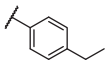
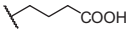
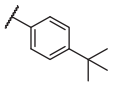
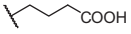
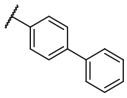
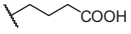
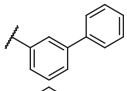
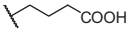
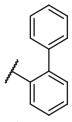
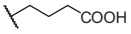
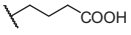
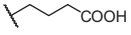
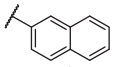
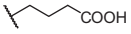
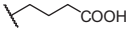
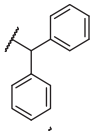
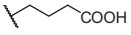
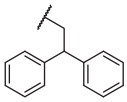
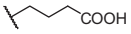
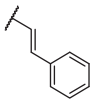
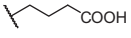
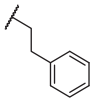
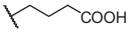
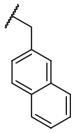
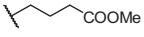
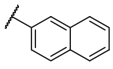
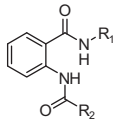
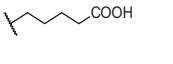
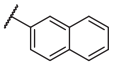
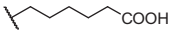
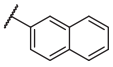
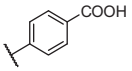
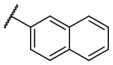
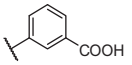
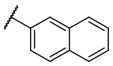
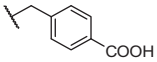
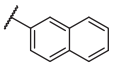
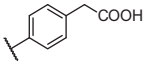
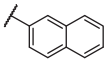
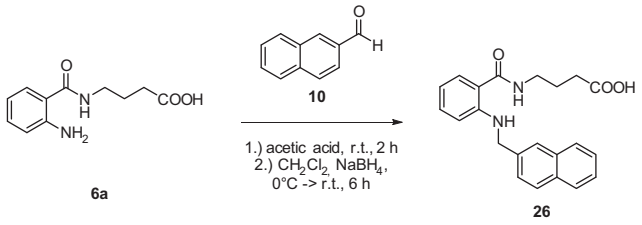
#			**EC_{50}** [μM] (max. rel activation [%])
	R_1	R_2	
3			11% at 30 μM
15a			i.a. at 30 μM
15b			i.a. at 3 μM*
15c			**5.8 ± 1.0** (28 ± 2)
15d			**2.5 ± 0.4** (19 ± 1)
15e			i.a. at 3 μM*
15f			i.a. at 30 μM
15g			i.a. at 30 μM
15h			i.a. at 30 μM
15i			i.a. at 3 μM*
15j			**8.6 ± 1.3** (37 ± 2)
15k			i.a. at 30 μM
15l			i.a. at 3 μM*
15m			i.a. at 30 μM
15n			**0.72 ± 0.01** (12.39 ± 0.02)
15o			i.a. at 30 μM
15p			i.a. at 30 μM
16			**7.1 ± 0.6** (19.0 ± 0.6)

Table 1 (*continued*)

#			**EC$_{50}$** [μM] (max. rel activation [%])
	R_1	R_2	
17			**8.3 ± 1.0** (11.4 ± 0.4)
18			**4.4 ± 0.6** (10.4 ± 0.4)
19			**1.0 ± 0.2** (23 ± 1)
20			**1.5 ± 0.2** (37 ± 1)
21			**1.3 ± 0.1** (10.02 ± 0.04)
22			**3.1 ± 0.3** (9.8 ± 0.4)

Values are expressed as mean ± SEM. n = 3–6. i.a. inactive.

* Compounds **15b**, **15e**, **15i**, **15l** showed toxicity at concentrations ⩾10 μM. Therefore no statistically significant relative FXR activation at higher concentrations could be determined.



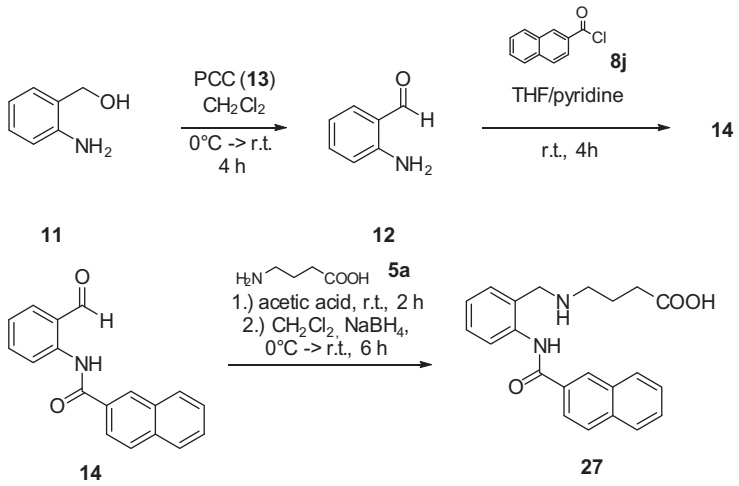
Scheme 2. Synthesis of amine **26** from anthranilic acid derivative **6a** and aldehyde **10** by reductive amination.

and size independent ligand efficiency (SILE) were slightly improved (**15j**: LE = 0.25; SILE = 1.87; **20**: LE = 0.26; SILE = 2.08). We also determined the aqueous solubility of **15j** and **20** and investigated their metabolic stability by incubation with liver microsomes of Sprague–Dawley rats. For **15j** an aqueous solubility of 45 mg/L was measured and the compound was highly stable against metabolic degradation by liver microsomes with 92 ± 2% of **15j** being detectable after 60 min. Compound **20** showed an aqueous solubility of 0.3 mg/L and moderate metabolic stability. After 60 min incubation with liver microsomes 61 ± 2% of the compound were detectable. (Fig. 3)

2.3. Receptor-ligand docking

Docking (Fig. 4A) of **15j** into the ligand binding site of FXR (model derived from PDB ID: 3OLF,[23] full agonist conformation) suggested prominent polar interactions between the carboxylic acid head group and Arg_{335} in activation function 2 (AF2) as well as Arg_{268}.

In the model, three water molecules were found to participate in this cluster of interactions. A further polar interaction was formed between the benzamide nitrogen and Ser_{336}, and an



Scheme 3. Synthesis of amine **27**. Alcohol **11** was oxidized to aldehyde **12** which was acylated with **8j** to yield **14**. Compound **27** was generated from **14** and **5a** by reductive amination.

Table 2
In vitro activities of compounds **23–27**

#	R_1	R_2	X	Y	**EC_{50}** [μM] (max. rel activation [%])
15j	H	H	C=O	C=O	**8.6 ± 1.3** (37 ± 2)
23	CH_3	H	C=O	C=O	i.a. at 30 μM
24	H	CH_3	C=O	C=O	i.a. at 30 μM
25	H	H	C=O	SO_2	i.a. at 30 μM
26	H	H	C=O	CH_2	i.a. at 30 μM
27	H	H	CH_2	C=O	i.a. at 30 μM

Values are mean ± SEM. n = 3–6. i.a. inactive.

intramolecular hydrogen bridge between benzamide carbonyl oxygen and the second amide nitrogen. The lipophilic 2-naphthoyl substituent was buried in a deep apolar pocket lined by Ile_{277}, Leu_{291}, Ile_{356}, Ile_{361} and Trp_{458}.

Compound **20** showed a similar binding pose in the docking experiment (Fig. 4B) with the exception that the interaction with Ser_{336} was not visible. Additionally, its lipophilic tail was placed deeper into the hydrophobic subpocket (Fig. 4).

Cinnamoyl derivative **15n** had a similar docking pose (Fig. 4C) as **15j** with the cinnamic amide residue placed in the same pocket as the 2-naphthoyl moiety. Docking revealed no additional interactions that could explain the significant difference in the activities of **15n** and **15j** but shows Ser_{336} and Tyr_{373} in a range of 3.9 and 4.6 Å around the double bond of **15n**.

Compared to the binding mode of GW4064 (**2**) in its co-crystal structure (Fig. 4D) on which the docking analysis is based, the docking suggests a similar polar interaction of the acidic head groups of compounds **3**, **15j**, **15n** and **20** with Arg_{335} as it is present in the co-crystal structure of **2**. Additionally the lipophilic tails were placed in the same pocket as the dichlorobenzene moiety of GW4064 (**2**). However, there is no polar interaction between Ser_{336} and GW4064 (**2**) that is comparable to the interaction of the benzamide nitrogen of **15j** with Ser_{336} in the docking.

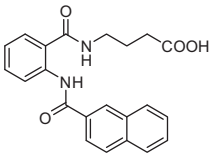
The Gibbs energies of the docking poses were −6.9 J/mol for **3**, −7.1 J/mol for **15j**, −8.1 J/mol for **20** and −21.6 J/mol for **15n** which is in congruence with the rank order of potency of the compounds concerning their EC_{50} values.

Our initial docking study suggested additional space for larger lipophilic substituents might be available in the hydrophobic subpocket where the 4-methylbenzoyl moiety of lead structure **3** was placed automatically. This hypothesis was not confirmed since large rigid substituents such as biphenyl residues are not tolerated. The compounds' rank order according to potency and the beneficial introduction of an aromatic moiety in the acidic head group however, are in agreement with the docking model.

3. Discussion

To explore the SAR of anthranilic acid derivatives derived from **3** as FXR ligands we varied the two substituents of the anthranilic acid core. First we investigated the acyl substituent at the aniline nitrogen. By replacing the 4-methylbenzoyl moiety of **3** with more polar (**15a** and **15b**) or small heteroaromatic substituents such as thiophene (**15i**) or pyridine (**15h**) activity was lost. Similarly, all large and rigid biphenyl derivatives (**15e–g**) as well as the sterically even more demanding diphenylacetyl (**15l**) and diphenylpropanoyl (**15m**) derivatives were also inactive. In contrast to more polar residues at the aromatic acyl substituent larger lipophilic substituents in the 4-position of the aromatic ring strongly improved the potency of compound **3**. The 4-ethylbenzoyl derivative **15c** and the 4-*t*-butylbenzoyl derivative **15d** showed the desired moderate FXR transactivation at low micromolar concentrations. Introduction of a cinnamoyl moiety in **15n** lead to a favorable EC_{50} value but the maximum relative FXR activation turned out to be low with ~12%. We found the best overall characteristics for further optimization in the 2-naphthoyl derivative **15j** with a low micromolar EC_{50} value, moderate FXR transactivation, lack of toxicity in our cell-based assay and in HepG2 cells, good aqueous solubility and low molecular weight.

The fact that 1-naphthoyl derivative **15k**, 2-naphtylacetyl derivative **15p** and dihydrocinnamoyl derivative **15o** were inactive indicates that the SAR is steep. The results suggest that π-electrons



15j

FXR transactivation: EC_{50} = 8.55±1.33 μM (37±2% max.)

molecular weight = 376

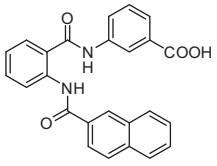
H-bond donors: 3; H-bond acceptors: 6

clogP = 3.35, aqueous solubility: 45 mg/L

LE = 0.25; SILE = 1.87

inactive at COX, 5-LO, mPGES-1 up to 10 μM (for details see supporting information)

not toxic up to 60 μM in HeLa and HepG2 cells



20

FXR transactivation: EC_{50} = 1.54±0.19 μM (37±1% max.)

molecular weight = 410

H-bond donors: 3; H-bond acceptors: 6

clogP = 4.96, aqueous solubility: 0.3 mg/L

LE = 0.26; SILE = 2.08

not toxic up to 60 μM in HeLa cells and up to 30 μM in HepG2 cells

Figure 3. Properties of optimized structures **15j** and **20**.

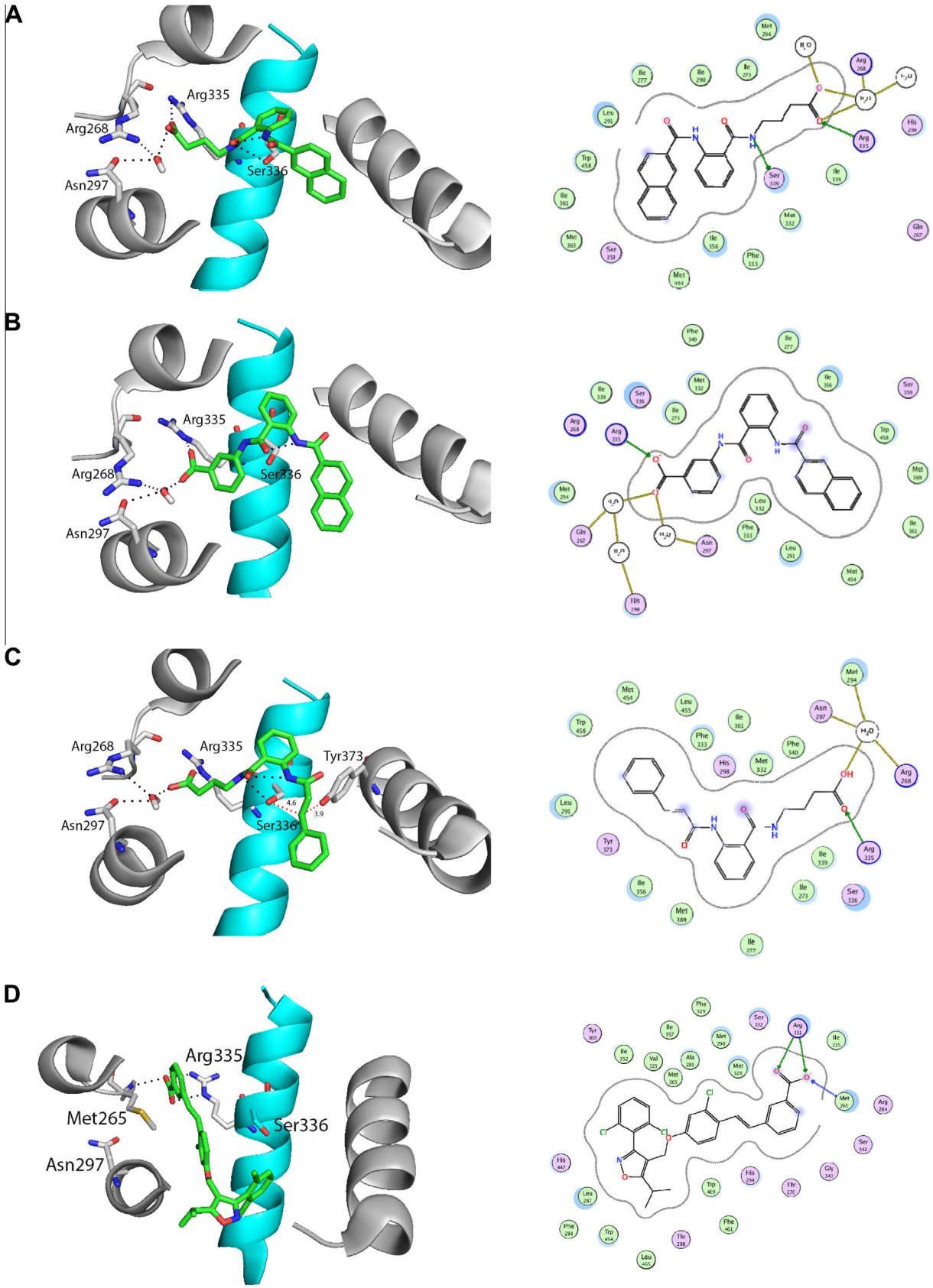


Figure 4. Docking poses of **15j** (A), **20** (B) and **15n** (C) and binding mode of **2** (D) in the co-crystal structure 3OLF[23] on which the docking is based for comparison. Helix 12 (AF2) in blue.

next to the acyl aniline moiety might be necessary since only derivatives with an aromatic moiety or a double bond in conjugation with the amide were active. In accordance with this hypothesis, cinnamoyl derivative **15n** was active while dihydrocinnamoyl derivative **15o** was inactive. Another possible but speculative explanation for the low EC_{50} value of **15n** and the inactivity of the saturated analog **15o** might be a covalent bond to the target. The cinnamoyl moiety of **15n** constitutes a Michael acceptor system that is lost when its double bond is hydrated. It could react with nucleophilic centers within the FXR binding site such as the alcohol functions of serine, threonine and tyrosine or the thiol of a cysteine. Docking analysis of **15n** suggests that Ser_{336} and Tyr_{373} surround the electrophilic Michael acceptor in a range of 3.9 and 4.6 Å. However, the actual possibility of covalent bond formation to FXR has to be further investigated.

For exploration of the SAR of the acidic head group the 2-naphthoyl residue of **15j** was selected as aromatic acyl substituent. With the 2-naphthoyl residue as acyl substituent the aliphatic C_4-side chain of the head group was elongated or replaced by various aromatic carboxylic acids. Elongation of the aliphatic carboxylic acid of **15j** by one (**17**) or two (**18**) carbon atoms improved the EC_{50} value but decreased relative FXR activation. Improvement of the EC_{50} value was also achieved by introduction of an additional aromatic moiety (compounds **19**–**22**). 4-aminobenzoic acid derivative **19** yielded the lowest EC_{50} value with 1.0 ± 0.2 μM, which is an approximately 10-fold improvement over the butyric acid side chain (**15j**). However, at the same time maximal relative FXR activation was diminished to 23 ± 1%. More flexible aromatic head groups with an additional methylene spacer between amide and aromatic ring (**21**) or between aromatic ring and carboxylic acid (**22**) only slightly changed the EC_{50} value (1.3 ± 0.1 μM for **21** and 3.1 ± 0.3 μM for **22**) but further diminished maximal relative FXR activation to ~10%. 3-Aminobenzoic acid derivative **20** showed the best overall characteristics with an EC_{50} of 1.6 ± 0.2 μM and 37 ± 1% maximal relative FXR activation.

We investigated the role of the amides in the anthranilic acid core by selective methylation, which eliminates the possibility to act as hydrogen bridge donors. In addition, the acyl anilide moiety was replaced by a sulfonic amide, which allows polar interactions but features a different geometry and bears strong N–H acidity. Both methylated derivatives **23** and **24** were inactive suggesting that both amide nitrogen functions might contribute to binding to the nuclear receptor via hydrogen bridges. The docking pose of **15j** shows an intramolecular hydrogen bond that includes the hydrogen of the acyl anilide and cannot be formed in compound **24**. Potentially, this interaction is crucial to maintain an appropriate geometry for receptor binding.

4. Conclusion

Our SAR study of anthranilic acid derivatives as FXR agonists revealed that the anthranilic acid core constitutes a promising scaffold for the development of novel FXR ligands. All functional groups of the anthranilic acid moiety seem to be crucial for FXR activation. By replacement or blockade of any of these groups activity was lost. By enlargement of the lipophilic tail as exemplified in compounds **15c**, **d**, **j**, we were able to improve both the EC_{50} value and maximal relative FXR activation. By introduction of an aromatic moiety within the acidic head group the potency was further enhanced. The resulting compound **20** constitutes a potent partial FXR agonist with an EC_{50} value of 1.5 ± 0.2 μM and 37 ± 1% maximal relative FXR activation. Further structural optimization and SAR studies should also tend to improve solubility and metabolic stability of this class of FXR ligands. Compound **20** provides a promising structure for further optimization.

5. Experimental section

General All chemicals and solvents were of reagent grade and used without further purification, unless otherwise specified. ^1H NMR and ^{13}C NMR spectra were measured in DMSO-d_6, on a Bruker AV 500 spectrometer. Chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on a Fisons Instruments VG PlattformII measuring in the positive- or negative-ion mode (ESI-MS system). The purity of the final compounds was determined by combustion analysis, which was performed by the Microanalytical Laboratory of the Institute of Organic Chemistry and Chemical Biology, Goethe-University Frankfurt, on an Elementar Vario Micro Cube. All tested compounds described here have a purity ⩾95%. Intermediates were not analyzed.

Docking simulations were performed using the Molecular Operating Environment (MOE) (Version 2012.10; The Chemical Computing Group, Montreal, Canada). The crystal structure of FXR (PDB ID: 3OLF[23]) was downloaded from the Protein Data Bank (PDB).[23] Prior to ligand docking one monomer of the dimer crystal structure was isolated and the crystallized ligand was removed. Subsequently, the structure was prepared with Protonate 3D and the active site was isolated using MOE Site Finder. The structures were placed in the site with the Triangle Matcher method, and then ranked with the London dG scoring function. For the energy minimization in the pocket MOE Forcefield Refinement was used and ranked with the GBVI/WSA dG scoring function.

5.1. Synthesis

5.1.1. General Procedures

(a) Ortho-aminobenzoylation with isatoic anhydride: Isatoic anhydride derivative (**4a**/**b**, 1.0 equiv) and 4-*N*,*N*-dimethylaminopyridine (**7**, 0.1 equiv) were dissolved in a mixture of pyridine (2 mL/mmol **4a**/**b**) and DMF (0.5 mL/mmol **4a**/**b**) and heated to 80 °C. After a clear brown solution had formed, the respective amine (**5a**–**i**, 1.1 equiv) was added in one portion. With addition of NEt_3 (0.5 mL/mmol **4a**/**b**) the formation of carbon dioxide started. The reaction mixture was kept at 80 °C for 16 h. Then the solvents were evaporated in vacuum and the crude product dissolved in ethyl acetate. The organic phase was washed twice with 10% hydrochloric acid and brine and dried over Na_2SO_4. Further purification was performed by recrystallization or column chromatography on silica.

(b) Acylation of anthranilic acid derivatives: Anthranilic acid derivative (**6a**–**j**, 1.0 equiv) was dissolved in THF (3 mL/mmol **6a**–**j**) and pyridine (3.0 equiv) was added. After a clear solution had formed, the respective acyl chloride (**8a**–**p**, 1.3 equiv) was added in THF (2 mL/mmol **6a**–**j**). The reaction mixture was kept at room temperature for 4–8 h and the reaction progress was monitored by TLC. When anthranilic acid derivative (**6a**–**j**) was consumed, the reaction mixture was diluted with ethyl acetate, washed three times with 10% hydrochloric acid and dried over Na_2SO_4. Further purification was performed by column chromatography on silica.

5.1.1.1. 4-(2-(3,5-Dinitrobenzamido)benzamido)butanoic acid (15a). Preparation according to general procedure b using **6a** and 3,5-dinitrobenzoyl chloride (**8a**). Yield 44.6%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.44. ^1H NMR (500 MHz, DMSO) δ 12.66 (s, 1H), 12.03 (s, 1H), 9.04 (d, J = 2.0 Hz, 2H), 9.03–9.01 (m, 1H), 8.88 (t, J = 5.6 Hz, 1H), 8.40 (dd, J = 8.3, 1.0 Hz, 1H), 7.84 (dd, J = 7.9, 1.4 Hz, 1H), 7.64–7.58 (m, 1H), 7.30 (td, J = 7.7, 1.1 Hz, 1H), 3.32 (s, 2H), 2.29 (t, J = 7.4 Hz, 2H), 1.78 (p,

J = 7.1 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 206.28, 173.77, 167.77, 160.19, 147.80, 137.18, 136.70, 131.29, 127.66, 126.57, 123.42, 120.85, 120.54, 29.79, 29.37, 22.76. $C_{18}H_{16}N_4O_8$. MS (ESI−): m/z 415.2 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 51.64 (51.93); H 3.84 (3.87); N 13.25 (13.46).

5.1.1.2. 4-(2-(4-Methoxybenzamido)benzamido)butanoic acid (15b). Preparation according to general procedure b using **6a** and 4-methoxybenzoyl chloride (**8b**). Yield 62.5%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.45. ^{1}H NMR (500 MHz, DMSO) δ 12.50 (s, 1H), 8.93 (s, 1H), 8.65 (d, J = 8.2 Hz, 1H), 7.90 (d, J = 8.7 Hz, 2H), 7.83 (d, J = 7.7 Hz, 1H), 7.55 (t, J = 7.6 Hz, 1H), 7.17 (t, J = 7.6 Hz, 1H), 7.13 (d, J = 8.7 Hz, 2H), 3.85 (s, 3H), 3.32–3.30 (m, 2H), 2.30 (t, J = 7.2 Hz, 2H), 1.78 (p, J = 7.0 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.85, 168.25, 163.40, 161.69, 139.00, 131.48, 128.12, 127.46, 125.92, 121.71, 119.28, 119.22, 113.40, 54.30, 29.85, 29.37, 22.83. $C_{19}H_{20}N_2O_5$. MS (ESI+): m/z 379.60 ((M+Na)$^+$, 100). Combustion analysis: measured (calculated): C 63.96 (64.04); H 5.63 (5.66); N 7.63 (7.86).

5.1.1.3. 4-(2-(4-Ethylbenzamido)benzamido)butanoic acid (15c). Preparation according to general procedure b using **6a** and 4-ethylbenzoyl chloride (**8c**). Yield 64.8%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.44. ^{1}H NMR (500 MHz, DMSO) δ 12.53 (s, 1H), 12.10 (s, 1H), 8.89 (t, J = 5.4 Hz, 1H), 8.66 (dd, J = 8.4, 0.9 Hz, 1H), 7.88–7.81 (m, 3H), 7.56 (td, J = 1.3, 7.9 Hz, 1H), 7.42 (d, J = 8.3 Hz, 2H), 7.19 (td, J = 1.2, 7.5 Hz, 1H), 3.33–3.30 (m, 2H), 2.69 (q, J = 7.5 Hz, 2H), 2.31 (t, J = 7.3 Hz, 2H), 1.79 (p, J = 7.1 Hz, 2H), 1.22 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO) δ 173.78, 168.20, 163.83, 147.67, 138.85, 131.49, 131.35, 127.63, 127.47, 126.48, 126.30, 121.88, 119.37, 37.42, 29.75, 26.72, 22.81, 13.85. $C_{20}H_{22}N_2O_4$. MS (ESI−): m/z 353.22 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 67.47 (67.78); H 6.04 (6.26); N 7.85 (7.90).

5.1.1.4. 4-(2-(4-(*tert*-Butyl)benzamido)benzamido)butanoic acid (15d). Preparation according to general procedure b using **6a** and 4-*tert*-butylbenzoyl chloride (**8d**). Yield 69.3%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.46. ^{1}H NMR (500 MHz, DMSO) δ 12.55 (s, 1H), 12.09 (s, 1H), 8.92 (t, J = 5.1 Hz, 1H), 8.66 (dd, J = 8.4, 1.0 Hz, 1H), 7.89–7.85 (m, 2H), 7.83 (dd, J = 8.0, 1.4 Hz, 1H), 7.64–7.59 (m, 2H), 7.58–7.53 (m, 1H), 7.19 (td, J = 7.8, 1.2 Hz, 1H), 3.32–3.30 (m, 2H), 2.31 (t, J = 7.3 Hz, 2H), 1.78 (p, J = 7.1 Hz, 2H), 1.32 (s, 9H). ^{13}C NMR (126 MHz, DMSO) δ 174.72, 169.15, 164.82, 155.48, 140.01, 132.68, 132.33, 128.69, 127.28, 126.30, 123.14, 120.55, 39.18, 35.24, 31.58, 31.37, 24.66. $C_{22}H_{26}N_2O_4$. MS (ESI−): m/z 381.24 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 68.96 (69.09); H 6.65 (6.85); N 7.12 (7.32).

5.1.1. 5. 4-(2-([1,1′-Biphenyl]-4-carboxamido)benzamido)butanoic acid (15e). Preparation according to general procedure b using **6a** and [1,1′-biphenyl]-4-carbonyl chloride (**8e**). Yield 66.0%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.42. ^{1}H NMR (500 MHz, DMSO) δ 12.65 (s, 1H), 12.11 (s, 1H), 8.93 (t, J = 5.5 Hz, 1H), 8.68 (dd, J = 8.3, 1.1 Hz, 1H), 8.05–8.00 (m, 2H), 7.93–7.88 (m, 2H), 7.85 (ddd, J = 8.0, 3.4, 1.5 Hz, 1H), 7.79–7.76 (m, 2H), 7.61–7.55 (m, 1H), 7.55–7.49 (m, 2H), 7.46–7.41 (m, 1H), 7.25–7.19 (m, 1H), 3.34–3.31 (m, 2H), 2.41 (t, J = 7.4 Hz, 2H), 1.83 (dt, J = 14.2, 7.1 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 172.67, 168.23, 163.52, 142.93, 138.78, 138.26, 132.58, 131.54, 128.37, 127.53, 127.50, 126.88, 126.44, 126.22, 122.05, 119.51, 119.44, 37.31, 29.40, 22.73. $C_{24}H_{22}N_2O_4$. MS (ESI−): m/z 401.2 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 71.82 (71.63); H 5.83 (5.51); N 6.56 (6.96).

5.1.1.6. 4-(2-([1,1′-Biphenyl]-3-carboxamido)benzamido)butanoic acid (15f). Preparation according to general procedure b using **6a** and [1,1′-biphenyl]-3-carbonyl chloride (**8f**). Yield 61.4%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.42. ^{1}H NMR (500 MHz, DMSO) δ 12.71 (s, 1H), 9.15 (s, 1H), 8.66 (dd, J = 8.4, 1.0 Hz, 1H), 8.21 (t, J = 1.7 Hz, 1H), 7.92 (dd, J = 10.7, 4.6 Hz, 2H), 7.86 (dd, J = 7.9, 1.3 Hz, 1H), 7.76 (dd, J = 8.2, 1.1 Hz, 2H), 7.69 (t, J = 7.7 Hz, 1H), 7.60–7.55 (m, 1H), 7.53 (dd, J = 10.5, 4.8 Hz, 2H), 7.43 (t, J = 7.4 Hz, 1H), 7.24–7.18 (m, 1H), 3.32 (dd, J = 12.1, 6.7 Hz, 2H), 2.29 (t, J = 7.2 Hz, 2H), 1.79 (p, J = 7.0 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 174.15, 168.13, 163.84, 140.13, 138.69, 138.64, 134.72, 131.46, 129.51, 128.98, 128.42, 127.52, 127.26, 126.09, 125.08, 124.63, 122.13, 119.72, 119.47, 30.55, 29.37, 22.85. $C_{24}H_{22}N_2O_4$. MS (ESI−): m/z 401.2 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 71.76 (71.63); H 5.58 (5.51); N 6.93 (6.96).

5.1.1.7. 4-(2-([1,1′-Biphenyl]-2-carboxamido)benzamido)butanoic acid (15g). Preparation according to general procedure b using **6a** and [1,1′-biphenyl]-2-carbonyl chloride (**8g**). Yield 62.0%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.43. ^{1}H NMR (500 MHz, DMSO) δ 12.09 (s, 1H), 11.47 (s, 1H), 8.68 (d, J = 5.1 Hz, 1H), 8.39 (d, J = 8.3 Hz, 1H), 7.66 (dd, J = 7.9, 1.2 Hz, 1H), 7.64 (dd, J = 7.6, 1.2 Hz, 1H), 7.60 (td, J = 7.6, 1.4 Hz, 1H), 7.52 (td, J = 7.5, 1.3 Hz, 1H), 7.47 (dd, J = 11.8, 4.4 Hz, 2H), 7.41–7.37 (m, 2H), 7.37–7.32 (m, 2H), 7.30–7.26 (m, 1H), 7.13 (td, J = 7.7, 1.2 Hz, 1H), 3.18 (q, J = 6.9 Hz, 2H), 2.26 (t, J = 7.4 Hz, 2H), 1.70 (p, J = 7.2 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.78, 167.42, 166.85, 139.08, 138.62, 138.07, 136.03, 131.12, 129.64, 127.58, 127.55, 127.29, 127.00, 126.89, 126.59, 122.05, 119.98, 119.29, 37.30, 29.76, 22.81. $C_{24}H_{22}N_2O_4$. MS (ESI−): m/z 401.1 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 71.50 (71.63); H 5.54 (5.51); N 7.03 (6.96).

5.1.1.8. 4-(2-(Isonicotinamido)benzamido)butanoic acid (15h). Preparation according to general procedure b using **6a** and isonicotinoyl chloride (**8h**). Yield 48.7%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.19. ^{1}H NMR (500 MHz, DMSO) δ 12.76 (s, 1H), 12.09 (s, 1H), 8.94 (t, J = 5.3 Hz, 1H), 8.85 (dd, J = 4.4, 1.7 Hz, 2H), 8.60 (dd, J = 8.3, 1.0 Hz, 1H), 7.87 (dd, J = 8.0, 1.4 Hz, 1H), 7.82 (dd, J = 4.4, 1.7 Hz, 2H), 7.63–7.57 (m, 1H), 7.25 (td, J = 7.7, 1.1 Hz, 1H), 3.33–3.31 (m, 2H), 2.31 (t, J = 7.3 Hz, 2H), 1.78 (p, J = 7.1 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.79, 168.03, 162.26, 150.26, 140.88, 138.12, 131.59, 127.55, 122.71, 120.00, 119.92, 119.71, 37.44, 29.73, 22.79. $C_{17}H_{17}N_3O_4$. MS (ESI+): m/z 328.18 ((M+H)$^+$, 100). Combustion analysis: measured (calculated): C 62.25 (62.38); H 5.33 (5.23); N 12.76 (12.84).

5.1.1.9. 4-(2-(Thiophene-2-carboxamido)benzamido)butanoic acid (15i). Preparation according to general procedure b using **6a** and thiophene-2-carbonyl chloride (**8i**). Yield 51.9%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.37. ^{1}H NMR (500 MHz, DMSO) δ 12.62 (s, 1H), 12.09 (s, 1H), 8.92 (t, J = 5.5 Hz, 1H), 8.53 (dd, J = 8.4, 1.0 Hz, 1H), 7.91 (dt, J = 4.5, 2.2 Hz, 1H), 7.84 (dd, J = 8.0, 1.4 Hz, 1H), 7.72 (dd, J = 3.8, 1.2 Hz, 1H), 7.59–7.52 (m, 1H), 7.28–7.25 (m, 1H), 7.22–7.16 (m, 1H), 3.32 (d, J = 6.9 Hz, 2H), 2.33–2.27 (m, 2H), 1.84–1.73 (m, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.78, 168.14, 158.70, 139.21, 138.51, 131.59, 131.56, 127.75, 127.74, 127.51, 122.02, 119.29, 119.16, 29.74, 29.37, 22.80. $C_{16}H_{16}N_2O_4S$. MS (MALDI): m/z 332.7 ((M+1), 100), 333.7 (34), 334.7 (20). Combustion analysis: measured (calculated): C 57.73 (57.82); H 4.89 (4.85); N 8.01 (8.43); S 9.63 (9.65).

5.1.1.10. 4-(2-(2-Naphthamido)benzamido)butanoic acid (15j). Preparation according to general procedure b using **6a** and 2-naphthoyl chloride (**8j**). Yield 55.0%. Rf (pentane/ethyl

acetate 1:1 + 2% acetic acid) = 0.43. ^{1}H NMR (500 MHz, DMSO) δ 12.70 (s, 1H), 8.98 (s, 1H), 8.68 (dd, J = 8.3, 0.8 Hz, 1H), 8.55 (d, J = 0.9 Hz, 1H), 8.12 (d, J = 8.3 Hz, 2H), 8.03 (t, J = 6.0 Hz, 1H), 7.99 (dd, J = 8.6, 1.8 Hz, 1H), 7.86 (dd, J = 7.9, 1.2 Hz, 1H), 7.70–7.56 (m, 4H), 7.25–7.20 (m, 1H), 3.29–3.23 (m, 2H), 2.32 (t, J = 7.3 Hz, 2H), 1.80 (p, J = 7.1 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.80, 169.15, 164.96, 139.87, 134.90, 132.73, 132.66, 132.43, 129.63, 129.19, 128.73, 128.62, 128.31, 128.19, 127.57, 123.71, 123.37, 120.99, 120.86, 39.30, 31.76, 24.69. $C_{22}H_{20}N_2O_4$. MS (ESI−): m/z 375.21 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 69.95 (70.20); H 5.44 (5.36); N 7.12 (7.44).

5.1.1.11. 4-(2-(1-Naphthamido)benzamido)butanoic acid (15k). Preparation according to general procedure b using **6a** and 1-naphthoyl chloride (**8k**). Yield 58.7%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.43. ^{1}H NMR (500 MHz, DMSO) δ 12.09 (s, 1H), 8.85 (t, J = 5.5 Hz, 1H), 8.66 (d, J = 8.2 Hz, 1H), 8.38–8.34 (m, 1H), 8.12 (d, J = 8.3 Hz, 1H), 8.06–8.02 (m, 1H), 7.85 (dd, J = 7.1, 1.1 Hz, 1H), 7.82 (dd, J = 7.9, 1.4 Hz, 1H), 7.66–7.59 (m, 4H), 7.24 (td, J = 7.7, 1.2 Hz, 1H), 3.23 (dd, J = 12.6, 6.9 Hz, 2H), 2.26 (t, J = 7.3 Hz, 2H), 1.73 (p, J = 7.2 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.77, 167.83, 166.18, 138.37, 133.57, 132.67, 131.32, 130.23, 128.87, 127.71, 127.48, 126.46, 125.81, 124.55, 124.45, 124.37, 122.40, 120.44, 119.83, 29.77, 29.37, 22.78. $C_{22}H_{20}N_2O_4$. MS (ESI−): m/z 375.20 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 70.11 (70.20); H 5.59 (5.36); N 7.09 (7.44).

5.1.1.12. 4-(2-(2,2-Diphenylacetamido)benzamido)butanoic acid (15l). Preparation according to general procedure b using **6a** and 2,2-diphenylacetyl chloride (8 l). Yield 64.8%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.42. ^{1}H NMR (500 MHz, DMSO) δ 12.69 (s, 1H), 11.39 (s, 1H), 8.70 (s, 1H), 8.36 (d, J = 8.0 Hz, 1H), 7.66 (dd, J = 7.9, 1.4 Hz, 1H), 7.50–7.45 (m, 1H), 7.40–7.31 (m, 8H), 7.28–7.23 (m, 2H), 7.14 (t, J = 7.5 Hz, 1H), 5.19 (s, 1H), 3.20 (dd, J = 12.6, 6.8 Hz, 2H), 2.37–2.30 (m, 2H), 1.72 (p, J = 7.2 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 172.65, 169.58, 167.56, 138.89, 137.71, 130.95, 127.93, 127.71, 127.26, 126.19, 122.19, 121.05, 119.97, 50.07, 37.14, 29.40, 29.36, 22.75. $C_{25}H_{24}N_2O_4$. MS (ESI−): m/z 415.26 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 71.97 (72.10); H 6.08 (5.81); N 6.92 (6.73).

5.1.1.13. 4-(2-(3,3-Diphenylpropanamido)benzamido)butanoic acid (15m). Preparation according to general procedure b using **6a** and 3,3-diphenylpropanoyl chloride (**8m**). Yield 68.3%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.44. ^{1}H NMR (500 MHz, DMSO) δ 12.13 (s, 1H), 11.28 (s, 1H), 8.71 (t, J = 5.4 Hz, 1H), 8.24 (d, J = 8.3 Hz, 1H), 7.66 (d, J = 7.9 Hz, 1H), 7.44–7.38 (m, 1H), 7.35 (d, J = 7.4 Hz, 4H), 7.26 (dd, J = 10.5, 4.9 Hz, 4H), 7.18–7.12 (m, 2H), 7.09 (td, J = 7.7, 1.1 Hz, 1H), 4.53 (t, J = 7.9 Hz, 1H), 3.32–3.28 (m, 2H), 3.15 (d, J = 8.0 Hz, 2H), 2.31 (t, J = 7.2 Hz, 2H), 1.78 (p, J = 7.0 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.81, 168.57, 167.70, 143.40, 137.88, 130.92, 127.68, 127.24, 126.81, 125.48, 121.77, 120.35, 119.68, 45.65, 41.78, 37.32, 29.77, 22.91. $C_{26}H_{26}N_2O_4$. MS (ESI−): m/z 429.26 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 72.72 (72.54); H 5.98 (6.09); N 6.22 (6.51).

5.1.1.14. 4-(2-Cinnamamidobenzamido)butanoic acid (15n). Preparation according to general procedure b using **6a** and cinnamoyl chloride (**8n**). Yield 50.2%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.42. ^{1}H NMR (500 MHz, DMSO) δ 12.10 (s, 1H), 11.51 (s, 1H), 8.79 (t, J = 5.6 Hz, 1H), 8.59 (d, J = 7.5 Hz, 2H), 8.50 (t, J = 8.8 Hz, 1H), 8.01 (dd, J = 7.9, 1.6 Hz, 2H), 7.57–7.50 (m, 2H), 7.20–7.15 (m, 1H), 7.13 (t, J = 7.4 Hz, 1H), 6.86 (d, J = 1.7 Hz, 1H), 6.82 (d, J = 1.6 Hz, 1H), 3.28–3.24 (m, 2H), 2.30 (t, J = 7.3 Hz, 2H), 1.78 (p, J = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.72, 168.74, 164.11, 134.97, 132.22, 131.64, 130.43, 129.41, 128.64, 128.58, 123.24, 123.04, 122.95, 70.26, 68.35, 31.57, 31.17, 24.73. $C_{20}H_{20}N_2O_4$. MS (MALDI): m/z 352.9 ((M+1), 100). Combustion analysis: measured (calculated): C 67.90 (68.17); H 5.88 (5.72); N 8.14 (7.95).

5.1.1.15. 4-(2-(3-Phenylpropanamido)benzamido)butanoic acid (15o). Preparation according to general procedure b using **6a** and 3-phenylpropanoyl chloride (**8o**). Yield 71.2%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.41. ^{1}H NMR (500 MHz, DMSO) δ 12.06 (s, 1H), 11.25 (s, 1H), 8.73 (t, J = 5.4 Hz, 1H), 8.36 (d, J = 8.2 Hz, 1H), 7.69 (dd, J = 7.9, 1.3 Hz, 1H), 7.48–7.44 (m, 1H), 7.29–7.22 (m, 3H), 7.17 (ddd, J = 8.5, 6.4, 4.0 Hz, 2H), 7.12 (td, J = 7.8, 1.1 Hz, 1H), 3.27 (dd, J = 12.7, 6.8 Hz, 2H), 2.92 (t, J = 7.6 Hz, 2H), 2.66 (t, J = 7.7 Hz, 2H), 2.29 (t, J = 7.3 Hz, 2H), 1.75 (p, J = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.71, 170.61, 168.71, 141.23, 139.18, 135.58, 132.19, 130.90, 128.78, 128.72, 126.47, 122.99, 46.07, 39.07, 33.17, 31.60, 31.08, 24.71. $C_{20}H_{22}N_2O_4$. MS (ESI−): m/z 353.4 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 68.12 (67.78); H 6.33 (6.26); N 7.50 (7.90).

5.1.1.16. 4-(2-(2-(Naphthalen-2-yl)acetamido)benzamido)butanoic acid (15p). Preparation according to general procedure b using **6a** and 2-(naphthalen-2-yl)acetyl chloride (**8p**). Yield 65.0%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.48. ^{1}H NMR (500 MHz, DMSO) δ 11.30 (s, 1H), 8.71 (s, 1H), 8.34 (d, J = 8.3 Hz, 1H), 7.94–7.83 (m, 4H), 7.66 (d, J = 7.6 Hz, 1H), 7.55–7.42 (m, 4H), 7.12 (t, J = 7.5 Hz, 1H), 3.86 (s, 2H), 3.20 (q, J = 6.3 Hz, 2H), 2.25 (t, J = 7.2 Hz, 2H), 1.70 (p, J = 6.9 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.74, 169.58, 168.56, 139.01, 133.55, 133.37, 132.43, 132.10, 128.45, 128.42, 128.32, 128.14, 127.97, 126.63, 126.19, 123.21, 122.09, 120.96, 45.03, 39.06, 31.61, 24.67. $C_{23}H_{22}N_2O_4$. MS (ESI−): m/z 389.21 ((M−H)$^-$, 100). Combustion analysis: measured (calculated): C 70.51 (70.75); H 5.63 (5.68); N 6.92 (7.17).

5.1.1.17. Methyl 4-(2-(2-naphthamido)benzamido)butanoate (16). Preparation according to general procedure b using **6b** and 2-naphthoyl chloride (**8j**). Yield 74.0%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.62. ^{1}H NMR (500 MHz, DMSO) δ 12.66 (s, 1H), 8.93 (t, J = 5.4 Hz, 1H), 8.67 (d, J = 7.7 Hz, 1H), 8.55 (s, 1H), 8.12 (t, J = 8.4 Hz, 2H), 8.04 (d, J = 7.8 Hz, 1H), 7.99 (dd, J = 8.6, 1.6 Hz, 1H), 7.88–7.83 (m, 1H), 7.66 (qd, J = 6.9, 3.4 Hz, 2H), 7.63–7.58 (m, 1H), 7.27–7.20 (m, 1H), 3.55 (s, 3H), 3.39–3.35 (t, J = 7.0 Hz, 2H), 2.42 (t, J = 7.3 Hz, 2H), 1.84 (p, J = 7.1 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 173.60, 169.17, 164.97, 139.82, 134.90, 132.74, 132.69, 132.43, 129.60, 129.17, 128.71, 128.61, 128.31, 128.19, 127.57, 123.70, 123.39, 121.07, 120.96, 51.71, 39.06, 31.22, 24.62. $C_{23}H_{22}N_2O_4$. MS (ESI+): m/z 391.24 ((M+H)$^+$, 100). Combustion analysis: measured (calculated): C 70.51 (70.75); H 5.68 (5.68); N 7.08 (7.17).

5.1.1.18. 5-(2-(2-Naphthamido)benzamido)pentanoic acid (17). Preparation according to general procedure b using **6c** and 2-naphthoyl chloride (**8j**). Yield 58.2%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.46. ^{1}H NMR (500 MHz, DMSO) δ 12.72 (s, 1H), 8.92 (t, J = 5.5 Hz, 1H), 8.69 (dd, J = 8.4, 1.0 Hz, 1H), 8.55 (d, J = 1.1 Hz, 1H), 8.13 (d, J = 8.2 Hz, 2H), 8.04 (d, J = 7.7 Hz, 1H), 7.99 (dd, J = 8.6, 1.8 Hz, 1H), 7.86 (dd, J = 8.0, 1.3 Hz, 1H), 7.70–7.62 (m, 2H), 7.62–7.58 (m, 1H), 7.23 (td, J = 7.9, 1.2 Hz, 1H), 3.32 (d, J = 3.6 Hz, 2H), 2.26 (t, J = 6.9 Hz, 2H), 1.58 (dt, J = 6.9, 3.6 Hz, 4H). ^{13}C NMR (125 MHz, DMSO) δ 173.95, 168.06, 163.97, 138.74, 133.73, 131.55, 131.48, 131.25, 128.42, 128.00, 127.47, 127.41, 127.10, 126.98, 126.36, 122.45, 122.13, 119.73, 119.62, 37.63, 31.95, 26.97, 20.60. $C_{23}H_{22}N_2O_4$. MS (ESI−): m/z

389.22 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 70.41 (70.75); H 5.55 (5.68); N 6.91 (7.17).

5.1.1.19. 6-(2-(2-Naphthamido)benzamido)hexanoic acid (18). Preparation according to general procedure b using **6d** and 2-naphthoyl chloride (**8j**). Yield 59.0%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.47. ^{1}H NMR (500 MHz, DMSO) δ 12.70 (s, 1H), 11.99 (s, 1H), 8.89 (t, J = 5.4 Hz, 1H), 8.67 (dd, J = 8.3, 0.7 Hz, 1H), 8.54 (s, 1H), 8.12 (d, J = 8.5 Hz, 2H), 8.02 (t, J = 9.6 Hz, 1H), 7.98 (dd, J = 8.6, 1.7 Hz, 1H), 7.85 (dd, J = 7.9, 1.2 Hz, 1H), 7.70–7.62 (m, 2H), 7.62–7.57 (m, 1H), 7.27–7.19 (m, 1H), 3.31–3.30 (m, 2H), 2.20 (t, J = 7.4 Hz, 2H), 1.61–1.48 (m, 4H), 1.39–1.29 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.93, 168.99, 164.94, 139.83, 134.90, 132.73, 132.63, 132.42, 129.62, 129.20, 128.67, 128.62, 128.31, 128.19, 127.59, 123.68, 123.39, 121.11, 120.91, 34.02, 31.18, 29.00, 26.45, 24.69. $C_{24}H_{24}N_2O_4$. MS (ESI−): m/z 403.21 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 71.67 (71.27); H 5.98 (5.98); N 6.89 (6.93).

5.1.1.20. 4-(2-(2-Naphthamido)benzamido)benzoic acid (19). Preparation according to general procedure b using **6e** and 2-naphthoyl chloride (**8j**). Yield 54.6%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.49. ^{1}H NMR (500 MHz, DMSO) δ 12.78 (s, 1H), 11.48 (s, 1H), 10.81 (s, 1H), 8.54 (s, 1H), 8.35 (dt, J = 8.3, 4.1 Hz, 1H), 8.12–8.05 (m, 2H), 8.02 (d, J = 8.0 Hz, 1H), 7.98–7.86 (m, 6H), 7.69–7.60 (m, 3H), 7.34 (td, J = 7.7, 1.1 Hz, 1H). ^{13}C NMR (125 MHz, DMSO) δ 167.03, 166.41, 164.42, 142.32, 137.48, 133.70, 131.51, 131.46, 131.15, 129.50, 128.42, 128.33, 127.79, 127.33, 126.97, 126.30, 125.05, 123.66, 122.92, 122.82, 121.45, 119.13, 119.03. $C_{25}H_{18}N_2O_4$. MS (ESI−): m/z 409.22 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 73.10 (73.16); H 4.56 (4.42); N 6.63 (6.83).

5.1.1.21. 3-(2-(2-Naphthamido)benzamido)benzoic acid (20). Preparation according to general procedure b using **6f** and 2-naphthoyl chloride (**8j**). Yield 56.8%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.50. ^{1}H NMR (500 MHz, DMSO) δ 13.02 (s, 1H), 11.63 (s, 1H), 10.72 (s, 1H), 8.54 (s, 1H), 8.42 (dd, J = 18.2, 9.5 Hz, 2H), 8.09 (t, J = 7.0 Hz, 2H), 8.04–7.93 (m, 4H), 7.71 (d, J = 7.6 Hz, 1H), 7.64 (dd, J = 15.9, 7.6 Hz, 3H), 7.49 (t, J = 7.9 Hz, 1H), 7.33 (t, J = 7.5 Hz, 1H). ^{13}C NMR (125 MHz, DMSO) δ 166.95, 166.62, 164.36, 138.27, 137.67, 133.71, 131.50, 131.48, 131.19, 130.52, 128.31, 128.21, 127.83, 127.34, 127.16, 126.97, 126.32, 124.28, 124.14, 123.08, 122.80, 122.77, 121.17, 120.90, 120.80. $C_{25}H_{18}N_2O_4$. MS (ESI−): m/z 409.4 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 72.94 (73.16); H 4.50 (4.42); N 6.54 (6.83).

5.1.1.22. 4-((2-(2-Naphthamido)benzamido)methyl)benzoic acid (21). Preparation according to general procedure b using **6g** and 2-naphthoyl chloride (**8j**). Yield 57.9%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.50. ^{1}H NMR (500 MHz, DMSO) δ 12.32 (s, 1H), 11.83 (s, 1H), 10.56 (s, 1H), 8.54 (s, 1H), 8.50 (d, J = 8.0 Hz, 1H), 8.10 (t, J = 7.6 Hz, 2H), 8.02 (d, J = 7.8 Hz, 1H), 7.96 (dd, J = 14.0, 5.2 Hz, 2H), 7.65 (dt, J = 14.9, 7.4 Hz, 5H), 7.32 (t, J = 7.1 Hz, 1H), 7.25 (d, J = 8.4 Hz, 2H), 3.54 (s, 2H). ^{13}C NMR (125 MHz, DMSO) δ 172.32, 166.80, 164.20, 137.94, 136.38, 133.71, 131.51, 131.14, 130.39, 130.34, 128.88, 128.38, 128.27, 127.91, 127.36, 127.17, 126.96, 126.33, 122.62, 122.43, 120.80, 120.21, 120.08, 38.97. $C_{26}H_{20}N_2O_4$. MS (ESI−): m/z 423.19 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 73.51 (73.57); H 4.89 (4.75); N 6.82 (6.60).

5.1.1.23. 2-(4-(2-(2-Naphthamido)benzamido)phenyl)acetic acid (22). Preparation according to general procedure b using **6h** and 2-naphthoyl chloride (**8j**). Yield 58.0%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.47. ^{1}H NMR (500 MHz, DMSO) δ 12.90 (s, 1H), 12.56 (s, 1H), 9.55 (t, J = 5.9 Hz, 1H), 8.68 (dt, J = 8.3, 2.1 Hz, 1H), 8.50 (d, J = 1.2 Hz, 1H), 8.09 (dd, J = 8.7, 3.4 Hz, 1H), 8.06 (dd, J = 7.8, 0.6 Hz, 1H), 8.01 (t, J = 6.2 Hz, 1H), 7.95 (dd, J = 8.5, 1.7 Hz, 2H), 7.92 (d, J = 8.3 Hz, 2H), 7.68–7.60 (m, 3H), 7.49 (d, J = 8.4 Hz, 2H), 7.26 (tt, J = 4.0, 2.0 Hz, 1H), 4.62 (d, J = 5.8 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 168.33, 166.66, 164.06, 143.49, 138.77, 138.61, 133.72, 131.77, 131.51, 131.21, 128.74, 128.36, 127.96, 127.56, 127.40, 127.07, 126.97, 126.92, 126.47, 126.42, 126.34, 122.49, 122.30, 119.87, 119.66, 41.27. $C_{26}H_{20}N_2O_4$. MS (ESI−): m/z 423.22 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 73.43 (73.57); H 4.78 (4.75); N 6.81 (6.60).

5.1.1.24. 4-(2-(2-Naphthamido)-*N*-methylbenzamido)butanoic acid (23). Preparation according to general procedure b using **6i** and 2-naphthoyl chloride (**8j**). Yield 51.3%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.50. ^{1}H NMR (500 MHz, DMSO) δ 12.13 (s, 1H), 10.45 (s, 1H), 8.16–7.96 (m, 4H), 7.82–7.64 (m, 3H), 7.59–7.50 (m, 1H), 7.42 (dd, J = 23.5, 11.6 Hz, 1H), 7.34 (t, J = 7.5 Hz, 1H), 3.46 (dd, J = 13.6, 6.5 Hz, 2H), 2.99 (s, 3H), 2.32 (t, J = 7.3 Hz, 2H), 1.85 (dt, J = 14.4, 7.1 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.69, 169.36, 165.72, 135.81, 134.79, 132.58, 132.06, 131.42, 130.01, 129.47, 128.59, 128.48, 128.36, 128.13, 128.06, 127.35, 125.80, 125.35, 124.57, 46.55, 37.51, 31.59, 22.41. $C_{23}H_{22}N_2O_4$. MS (ESI−): m/z 389.22 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 70.56 (70.75); H 5.59 (5.68); N 6.87 (7.17).

5.1.1.25. 4-(2-(*N*-Methyl-2-naphthamido)benzamido)butanoic acid (24). Preparation according to general procedure b using **6j** and 2-naphthoyl chloride (**8j**). Yield 44.7%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.54. ^{1}H NMR (500 MHz, DMSO) δ 12.07 (s, 1H), 8.28 (s, 1H), 7.95 (d, J = 6.0 Hz, 1H), 7.79 (dd, J = 12.5, 4.8 Hz, 1H), 7.76 (t, J = 8.3 Hz, 1H), 7.66 (d, J = 8.5 Hz, 1H), 7.54–7.43 (m, 2H), 7.41–7.35 (m, 1H), 7.27 (t, J = 7.2 Hz, 1H), 7.24–7.18 (m, 2H), 3.31 (s, 3H), 3.30–3.25 (m, 2H), 2.30 (t, J = 7.4 Hz, 2H), 1.80–1.68 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.73, 169.74, 167.31, 142.86, 134.47, 134.29, 133.34, 132.26, 131.07, 130.26, 128.98, 128.78, 128.65, 127.93, 127.61, 127.48, 127.28, 126.81, 125.72, 38.98, 38.23, 31.61, 21.53. $C_{23}H_{22}N_2O_4$. MS (ESI−): m/z 389.22 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 70.39 (70.75); H 5.62 (5.68); N 7.00 (7.17).

5.1.1.26. 4-(2-(Naphthalene-2-sulfonamido)benzamido)butanoic acid (25). Compound **6a** (670 mg, 3 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (15 mL, abs.) triethylamine (1.3 mL, 9 mmol, 3.0 equiv) was added and the solution was cooled to 0 °C. Naphthalene-2-sulfonyl chloride (**9**, mg, mmol, 1.3 equiv) was dissolved in CH_2Cl_2 and drop wise added to the solution of **6a**. The reaction mixture was kept at 0 °C and the reaction was monitored by TLC. After 4 h the mixture was diluted with 50 mL ethyl acetate, washed three times with 10% hydrochloric acid (50 mL) and dried over Na_2SO_4. Yield 68.9%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.31. ^{1}H NMR (500 MHz, DMSO) δ 12.13 (s, 1H), 11.77 (s, 1H), 8.71 (t, J = 5.4 Hz, 1H), 8.46 (d, J = 1.7 Hz, 1H), 8.13 (d, J = 8.0 Hz, 1H), 8.05 (d, J = 8.8 Hz, 1H), 7.98 (d, J = 8.1 Hz, 1H), 7.71–7.61 (m, 4H), 7.58 (dd, J = 8.3, 1.0 Hz, 1H), 7.48–7.42 (m, 1H), 7.12–7.06 (m, 1H), 3.19 (dd, J = 12.6, 6.9 Hz, 2H), 2.22 (t, J = = 7.3 Hz, 2H), 1.65 (p, J = 7.2 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.70, 167.51, 137.41, 134.93, 133.65, 131.72, 130.81, 128.86, 128.58, 128.48, 127.60, 127.57, 127.07, 127.05, 122.83, 120.98, 120.22, 119.27, 37.29, 29.63, 22.65. $C_{21}H_{20}N_2O_5S$. MS (ESI−): m/z 411.4 ((M−H)⁻, 100). Combustion analysis: measured (calculated): C 61.35 (61.15); H 4.95 (4.89); N 6.56 (6.79).

5.1.1.27. 4-(2-((Naphthalen-2-ylmethyl)amino)benzamido)butanoic acid (26). Compound **6a** (670 mg, 3 mmol, 1.0 equiv) and 2-naphthaldehyde (10, 470 mg, 3 mmol, 1.0 equiv) were dissolved in acetic acid (9 mL) and stirred for 2 h at room temperature. CH_2Cl_2 (15 mL, abs.) was added and the mixture was cooled to 0 °C. $NaBH_4$ (450 mg, 12 mmol, 4.0 equiv) was added in small portions over 1 h. The mixture was stirred for an additional hour at 0 °C and overnight at room temperature. Saturated NH_4Cl solution (5 mL) was added and the mixture was stirred at room temperature for additional 15 min. The mixture was then partitioned between ethyl acetate (50 mL) and water (50 mL) and the aqueous layer was extracted twice with ethyl acetate (2*50 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuum. The crude product was purified by column chromatography on silica. Yield 52.3%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.31. ^{1}H NMR (500 MHz, DMSO) δ 12.08 (s, 1H), 7.94–7.84 (m, 3H), 7.74 (s, 1H), 7.67 (d, J = 7.4 Hz, 1H), 7.56–7.44 (m, 4H), 7.19 (dd, J = 11.1, 4.2 Hz, 1H), 6.65 (dd, J = 12.0, 7.8 Hz, 2H), 6.02 (d, J = 2.2 Hz, 1H), 4.05–3.96 (m, 1H), 2.84 (dt, J = 7.6, 6.8 Hz, 1H), 2.31–2.18 (m, 2H), 1.89–1.69 (m, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.60, 161.85, 145.58, 137.78, 132.52, 132.05, 131.65, 127.81, 127.23, 126.79, 126.71, 125.83, 125.64, 124.04, 123.37, 116.36, 114.13, 113.46, 69.10, 42.61, 29.69, 21.76. $C_{22}H_{22}N_2O_3$. MS (ESI−): m/z 361.6 ($(M-H)^-$, 100). Combustion analysis: measured (calculated): C 72.95 (72.91); H 5.80 (6.12); N 7.65 (7.73).

5.1.1.28. 4-((2-(2-Naphthamido)benzyl)amino)butanoic acid (27). Compound **14** (1.4 g, 5 mmol, 1.0 equiv) and 4-aminobutyric acid (**5a**, 520 mg, 5 mmol, 1.0 equiv) were dissolved in acetic acid (15 mL) and stirred for 2 h at room temperature. CH_2Cl_2 (25 mL, abs.) was added and the mixture was cooled to 0 °C. $NaBH_4$ (750 mg, 20 mmol, 4.0 equiv) was added in small portions over 1 h. The mixture was stirred for an additional hour at 0 °C and overnight at room temperature. Saturated NH_4Cl solution (8 mL) was added and the mixture was stirred at room temperature for additional 15 min. The mixture was then partitioned between ethyl acetate (50 mL) and water (50 mL) and the aqueous layer was extracted twice with ethyl acetate (2*50 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuum. The crude product was purified by column chromatography on silica. Yield 54.6%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.18. ^{1}H NMR (500 MHz, DMSO) δ 10.49 (s, 1H), 8.67 (s, 1H), 8.15–7.98 (m, 4H), 7.75–7.56 (m, 3H), 7.41–7.35 (m, 1H), 7.34–7.31 (m, 1H), 7.26 (td, J = 7.5, 1.0 Hz, 1H), 5.76 (s, 2H), 3.39–3.36 (m, 2H), 2.29 (t, J = = 8.1 Hz, 2H), 1.98–1.90 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 175.26, 166.05, 136.75, 134.81, 132.60, 132.20, 131.10, 129.70, 129.50, 128.69, 128.45, 128.32, 128.13, 127.28, 126.58, 126.03, 124.86, 42.99, 31.18, 30.65, 17.93. $C_{22}H_{22}N_2O_3$. MS (ESI−): m/z 361.6 ($(M-H)^-$, 100). Combustion analysis: measured (calculated): C 72.57 (72.91); H 5.86 (6.12); N 8.05 (7.73).

5.1.2. Intermediates

5.1.2.1. 4-(2-Aminobenzamido)butanoic acid (6a). Preparation according to general procedure a using isatoic anhydride (**4a**) and 4-aminobutyric acid (**5a**). The crude product was purified by recrystallization from 2-propanol and 10% hydrochloric acid. Yield 71.6%. ^{1}H NMR (500 MHz, DMSO) δ 8.71 (s, 1H), 7.72 (d, J = 7.8 Hz, 1H), 7.44 (t, J = 7.7 Hz, 1H), 7.25–7.17 (m, 1H), 7.13 (t, J = 6.8 Hz, 1H), 3.27 (q, J = 6.3 Hz, 2H), 2.29 (t, J = = 7.4 Hz, 2H), 1.76 (p, J = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.72, 167.57, 138.69, 132.40, 129.01, 123.45, 123.11, 122.06, 38.97, 31.65, 24.82. $C_{11}H_{14}N_2O_3$. MS (ESI−): m/z 221.5 ($(M-H)^-$, 100).

5.1.2.2. Methyl 4-(2-aminobenzamido)butanoate (6b). Preparation according to general procedure a using isatoic anhydride (**4a**) and methyl 4-aminobutanoate (**5b**). The crude product was purified by column chromatography on silica. Yield 69.8%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.65. ^{1}H NMR (500 MHz, DMSO) δ 8.21 (t, J = 5.3 Hz, 1H), 7.46 (d, J = 7.9 Hz, 1H), 7.14–7.09 (m, 1H), 6.68 (d, J = 8.2 Hz, 1H), 6.54–6.46 (m, 1H), 6.37 (s, 2H), 3.57 (s, 3H), 3.22 (q, J = 6.8 Hz, 2H), 2.36 (t, J = 7.2 Hz, 2H), 1.76 (p, J = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 173.66, 169.41, 162.78, 150.03, 132.03, 128.48, 116.74, 115.00, 38.54, 36.24, 31.30, 24.97. $C_{12}H_{16}N_2O_3$. MS (ESI+): m/z 237.6 ($(M+H)^+$, 100).

5.1.2.3. 5-(2-Aminobenzamido)pentanoic acid (6c). Preparation according to general procedure a using isatoic anhydride (**4a**) and 5-aminopentanoic acid (**5c**). The crude product was purified by recrystallization from 2-propanol and 10% hydrochloric acid. Yield 76.7%. ^{1}H NMR (500 MHz, DMSO) δ 8.82 (s, 1H), 7.75 (dd, J = 18.6, 4.7 Hz, 1H), 7.48 (t, J = 7.7 Hz, 1H), 7.29 (t, J = 9.0 Hz, 1H), 7.21 (t, J = 7.0 Hz, 1H), 3.25 (d, J = 5.3 Hz, 2H), 2.25 (t, J = 6.4 Hz, 2H), 1.58–1.50 (m, 4H). ^{13}C NMR (126 MHz, DMSO) δ 174.85, 167.19, 142.04, 132.39, 129.05, 127.84, 124.67, 122.92, 39.18, 34.44, 28.83, 22.47. $C_{12}H_{16}N_2O_3$. MS (ESI−): m/z 235.1 ($(M-H)^-$, 100).

5.1.2.4. 6-(2-Aminobenzamido)hexanoic acid (6d). Preparation according to general procedure a using isatoic anhydride (**4a**) and 6-aminohexanoic acid (**5d**). The crude product was purified by recrystallization from 2-propanol and 10% hydrochloric acid. Yield 71.8%. ^{1}H NMR (500 MHz, DMSO) δ 8.74 (s, 1H), 7.78–7.71 (m, 1H), 7.52–7.40 (m, 1H), 7.34–7.12 (m, 2H), 3.24 (q, J = 6.7 Hz, 2H), 2.21 (t, J = 7.4 Hz, 2H), 1.57–1.48 (m, 4H), 1.35–1.28 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.92, 167.19, 132.37, 128.99, 127.80, 123.91, 122.38, 107.42, 39.37, 34.09, 29.09, 26.48, 24.71. $C_{13}H_{18}N_2O_3$. MS (ESI−): m/z 248.8 ($(M-H)^-$, 100).

5.1.2.5. 4-(2-Aminobenzamido)benzoic acid (6e). Preparation according to general procedure a using isatoic anhydride (**4a**) and 4-aminobenzoic acid (**5e**). The crude product was purified by column chromatography on silica. Yield 62.4%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.57. ^{1}H NMR (500 MHz, DMSO) δ 10.62 (s, 1H), 7.95–7.91 (m, 2H), 7.91–7.86 (m, 2H), 7.80 (d, J = 7.6 Hz, 1H), 7.42 (t, J = 7.3 Hz, 1H), 7.14 (d, J = 7.2 Hz, 1H), 7.03 (d, J = 5.8 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 167.42, 167.30, 143.70, 132.94, 132.46, 130.63, 129.73, 129.64, 126.24, 125.96, 120.35, 120.10. $C_{14}H_{12}N_2O_3$. MS (ESI−): m/z 255.0 ($(M-H)^-$, 100).

5.1.2.6. 3-(2-Aminobenzamido)benzoic acid (6f). Preparation according to general procedure a using isatoic anhydride (**4a**) and 3-aminobenzoic acid (**5f**). The crude product was purified by column chromatography on silica. Yield 64.0%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.58. ^{1}H NMR (500 MHz, DMSO) δ 10.54 (s, 1H), 8.44 (d, J = 1.2 Hz, 1H), 7.96 (t, J = 8.8 Hz, 1H), 7.82 (d, J = 7.8 Hz, 1H), 7.71–7.66 (m, 1H), 7.48 (t, J = 7.9 Hz, 1H), 7.46–7.41 (m, 1H), 7.26–7.15 (m, 1H), 7.07 (d, J = 6.6 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 167.64, 167.02, 139.72, 133.08, 132.81, 131.64, 130.58, 129.63, 129.28, 128.29, 125.09, 125.01, 121.78, 120.83. $C_{14}H_{12}N_2O_3$. MS (ESI−): m/z 254.9 ($(M-H)^-$, 100).

5.1.2.7. 4-((2-Aminobenzamido)methyl)benzoic acid (6g). Preparation according to general procedure a using isatoic anhydride (**4a**) and 4-(aminomethyl)benzoic acid (**5g**). The crude product was purified by column chromatography on silica. Yield 70.7%. $\boldsymbol{R_f}$ (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.58. $^{\mathbf{1}}$H NMR (500 MHz, DMSO) $\boldsymbol{\delta}$ 9.97 (s, 1H), 7.69–7.56 (m, 3H), 7.25–7.15 (m, 3H), 6.74 (dd, $\boldsymbol{J}$ = 8.2, 0.9 Hz, 1H), 6.63–6.54 (m, 1H), 6.32 (s, 2H), 3.52 (s, 2H). $^{\mathbf{13}}$C NMR (126 MHz, DMSO) $\boldsymbol{\delta}$ 173.31, 168.23, 150.20, 138.25, 132.54, 130.44, 129.87, 129.14, 120.93, 116.82, 115.69, 115.15, 40.62. $\mathbf{C_{15}H_{14}N_2O_3}$. MS (ESI−): $\boldsymbol{m/z}$ 269.0 ($(M-H)^-$, 100).

5.1.2.8. 2-(4-(2-Aminobenzamido)phenyl)acetic acid (6h). Preparation according to general procedure a using isatoic anhydride (**4a**) and 4-(aminophenyl)acetic acid (**5h**). The crude product was purified by column chromatography on silica. Yield 62.8%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.59. ^{1}H NMR (500 MHz, DMSO) δ 9.39 (s, 1H), 7.91 (d, J = 8.3 Hz, 2H), 7.85 (t, J = 9.2 Hz, 1H), 7.46 (t, J = 8.0 Hz, 3H), 7.26–7.22 (m, 1H), 7.14 (t, J = 7.7 Hz, 1H), 4.53 (d, J = 5.7 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 167.73, 167.64, 144.97, 139.35, 132.67, 129.86, 129.78, 129.66, 129.09, 127.76, 123.33, 122.11, 42.76. $C_{15}H_{14}N_2O_3$. MS (ESI−): m/z 269.0 ((M−H)$^-$, 100).

5.1.2.9. 4-(2-Amino-*N*-methylbenzamido)butanoic acid (6i). Preparation according to general procedure a using isatoic anhydride (**4a**) and 4-(methylamino)butanoic acid (**5i**). The crude product was purified by recrystallization from acetone. Yield 61.5%. ^{1}H NMR (500 MHz, DMSO) δ 7.09–7.04 (m, 1H), 6.96 (d, J = 7.3 Hz, 1H), 6.69 (d, J = 8.0 Hz, 1H), 6.55 (td, J = 7.5, 1.0 Hz, 1H), 3.32–3.28 (m, 2H), 2.69 (s, 3H), 2.20–2.14 (m, 2H), 1.93–1.85 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.28, 170.38, 145.86, 130.15, 127.82, 120.75, 115.97, 115.92, 39.31, 30.58, 29.47, 17.68. $C_{12}H_{16}N_2O_3$. MS (ESI−): m/z 234.8 ((M−H)$^-$, 100).

5.1.2.10. 4-(2-(Methylamino)benzamido)butanoic acid (6j). Preparation according to general procedure a using ***N***-methylisatoic anhydride (**4b**) and 4-aminobutanoic acid (**5a**). The crude product was purified by column chromatography on silica. Yield 50.4%. R_f (pentane/ethyl acetate 1:1 + 2% acetic acid) = 0.41. ^{1}H NMR (500 MHz, DMSO) δ 12.07 (s, 1H), 8.29 (t, J = 5.5 Hz, 1H), 7.62 (d, J = 4.2 Hz, 1H), 7.51 (dd, J = 7.8, 1.4 Hz, 1H), 7.27 (ddd, J = 8.5, 7.3, 1.5 Hz, 1H), 6.61 (d, J = 8.1 Hz, 1H), 6.58–6.51 (m, 1H), 3.22 (dd, J = 12.7, 6.8 Hz, 2H), 2.76 (d, J = 4.3 Hz, 3H), 2.26 (t, J = 7.4 Hz, 2H), 1.73 (p, J = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 174.77, 169.62, 150.49, 132.72, 128.62, 115.64, 114.36, 110.89, 38.72, 31.65, 29.73, 24.98. $C_{12}H_{16}N_2O_3$. MS (ESI−): m/z 235.16 ((M−H)$^-$, 100).

5.1.2.11. 2-Aminobenzaldehyde (12). 2-(Aminophenyl)ethanol (11, 2.4 g, 20 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (40 mL, abs.) and cooled to 0 °C. Pyridiniumchlorochromate (PCC, 13, 6.5 g, 30 mmol, 1.5 equiv) was added in small portions. The mixture was kept at 0 °C for 1 h and then stirred for 4 h at room temperature. Silica was added to the mixture and the solvent was evaporated. Purification was performed by distillation. Yield 71.9%. ^{1}H NMR (500 MHz, DMSO) δ 6.60 (s, 2H), 6.66 (d, J = 8.1 Hz, 1H), 6.73–6.77 (m, 1H), 7.27–7.32 (m, 1H), 7.45 (d, J = 8.2, 1H), 9.89 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 115.5, 116.2, 118.4, 134.9, 135.5, 149.3, 193.1. C_7H_7NO. MS (ESI+): m/z 122.6 (4.5, (M+H)$^+$), 144.6 (100, (M+Na)$^+$). (agrees with[28])

5.1.2.12. *N*-(2-Formylphenyl)-2-naphthamide (14). Preparation according to general procedure b using **12** and 2-naphthoyl chloride (**8j**). Purification was performed by recrystallization from pentane/acetone. Yield 80.1%. ^{1}H NMR (500 MHz, DMSO) δ 11.87 (s, 1H), 10.09 (s, 1H), 8.61 (s, 1H), 8.53 (d, J = 8.2 Hz, 1H), 8.14 (d, J = 8.3 Hz, 2H), 8.09–8.02 (m, 2H), 7.99 (dd, J = 7.7, 1.5 Hz, 1H), 7.83–7.74 (m, 1H), 7.74–7.59 (m, 2H), 7.45–7.37 (m, 1H). ^{13}C NMR (126 MHz, DMSO) δ 196.16, 166.02, 140.53, 136.22, 135.17, 135.03, 132.67, 131.76, 129.62, 129.21, 128.80, 128.61, 128.22, 127.66, 124.49, 124.39, 123.95, 121.13. $C_{18}H_{13}NO_2$. MS (ESI−): m/z 274.13 ((M−H)$^-$, 100).

5.2. Reporter gene assay

5.2.1. Cell culture

HeLa cells were grown in DMEM high glucose, supplemented with 10% fetal calf serum (FCS), 1% sodium pyruvate (SP) and 1% penicillin/streptomycin (PS) at 37 °C and 5% CO_2.

5.2.2. Plasmids for full length FXR transactivation assay

pcDNA3-hFXR contains the sequence of human FXR and was already published elsewhere,[29] pGL3basic (Promega, Mannheim, Germany) was used as a reporter plasmid, with a shortened construct of the promotor of the bile salt export pump (BSEP, sequence of construct from[30]) cloned into the SacI/NheI cleavage site in front of the luciferase gene. pRL-SV40 (Promega) was transfected as a control for normalization of transfection efficacy and cell growth. pSG5-hRXR was already published elsewhere as well.[31]

5.2.4. Full length FXR transactivation assay

24 h before transfection, HeLa cells were seeded in 96-well plates with a density of 8000 cells per well. 3, 5 h before transfection, medium was changed to DMEM high glucose, supplemented with 1% SP, 1% PS and 0.5% charcoal-stripped FCS. Transient transfection of HeLa cells with BSEP-pGL3, pRL-SV40 and the expression plasmids pcDNA3-hFXR and pSG5-hRXR was carried out using calcium phosphate transfection method. 16 h after transfection, medium was changed to DMEM high glucose, supplemented with 1% SP, 1% PS and 0.5% charcoal-stripped FCS. 24 h after transfection, medium was changed to DMEM without phenol red, supplemented with 1% SP, 1% PS, 1% L-glutamate and 0.5% charcoal-stripped FCS, now additionally containing 0.1% DMSO and the respective test compound or 0.1% DMSO alone as untreated control. Each concentration was tested in triplicate wells and each experiment was repeated independently at least three times. Following 24 h incubation with the test compounds, cells were assayed for luciferase activity using Dual-Glo™ Luciferase Assay System (Promega) according to the manufacturer's protocol. Luminescence was measured with a Tecan Infinite M200 luminometer (Tecan Deutschland GmbH, Crailsheim, Germany). Normalization of transfection efficacy and cell growth was done by division of firefly luciferase data by renilla luciferase data resulting in relative light units (RLU). Fold activation was obtained by dividing the mean RLU of the tested compound at a respective concentration by the mean RLU of untreated control. Relative activation was obtained by dividing the fold activation of the tested compound at a respective concentration by the fold activation of FXR full agonist GW4064 (**2**) at 3 μM. EC_{50} and standard error of the mean values were calculated with the mean relative activation values of at least three independent experiments by SigmaPlot 10.0 (Systat Software GmbH, Erkrath, Germany) using a four parameter logistic regression.

6. Metabolism assay

The solubilized test compounds (5 μL, final concentration 10 μM in DMSO) were preincubated at 37 °C in 432 μL of phosphate buffer (0.1 M, pH 7.4) together with a 50 μL NADPH regenerating system (30 mM glucose-6-phosphate, 4 U/mL glucose-6-phosphate dehydrogenase, 10 mM NADP, 30 mM $MgCl_2$). After 5 min, the reaction was started by the addition of 13 μL of microsome mix from the liver of Sprague–Dawley rats (Invitrogen, Darmstadt, Germany; 20 mg protein/mL in 0.1 M phosphate buffer) in a shaking water bath at 37 °C. The reaction was stopped by addition of 250 μL of ice-cold methanol at 0, 15, 30, and 60 min. The samples were diluted with 250 μL of DMSO and centrifuged at 10,000*g* for 5 min at 4 °C. The supernatants were analyzed, and test compounds were quantified by HPLC: mobile phase,

MeOH 83%/H_2O 17%/formic acid 0.1%; flow-rate, 1 mL/min; stationary phase, MultoHigh Phenyl phase, 5 μm, 250 × 4 precolumn, phenyl, 5 μm, 20 × 4; detection wavelength, 330 and 254 nm; injection volume, 50 μL. Control samples were performed to check the stability of the compounds in the reaction mixture: first control was without NADPH, which is needed for the enzymatic activity of the microsomes, second control was with inactivated microsomes (incubated for 20 min at 90 °C), third control was without test compounds (to determine the baseline). The amounts of the test compounds were quantified by an external calibration curve, where data are expressed as means ± SEM of single determinations obtained in three independent experiments. The metabolism experiments showed the following curves: **15j** (n = 4): 0 min–96 ± 2%; 15 min–93 ± 2%; 30 min–93 ± 2%; 60 min–92 ± 2%; 20 (n = 3): 0 min–100 ± 1%; 15 min–86 ± 2%; 30 min–76 ± 4%; 60 min–61 ± 2%.

7. WST-1 assay in HepG2 cells

The WST-1 assay from Roche was performed according to manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). In brief, HepG2 cells were seeded in 96-well plates (30,000 cells per well) in DMEM containing 1% PS and 1% FCS. After 24 h cells were incubated with compounds **15j**, **20** (final concentrations 10 μM, 30 μM, 60 μM 100 μM), Revlotron (100 μM, Sigma Aldrich) as positive control, and Zileuton (100 μM, Sigma Aldrich) and DMEM + 1% DMSO as negative controls. After 48 h WST reagent (Roche) was added to each well according to manufacturer's instructions. After 45 min incubation absorption (450 nm/reference: 620 nm) was determined with a TEACAN Infinite M200 luminometer. Each experiment was repeated three times in triplicates. Results (expressed as percent of untreated control): **15j**: 10 μM = 92 ± 8%; 30 μM = 90 ± 7%; 60 μM = 81 ± 1%; 100 μM = 84 ± 4%. **20**: 10 μM = 93 ± 5%; 30 μM = 65 ± 1%; 60 μM = 50 ± 7%; 100 μM = 46 ± 9%. Values are expressed as mean ± SEM; n = 3.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.02.053.

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