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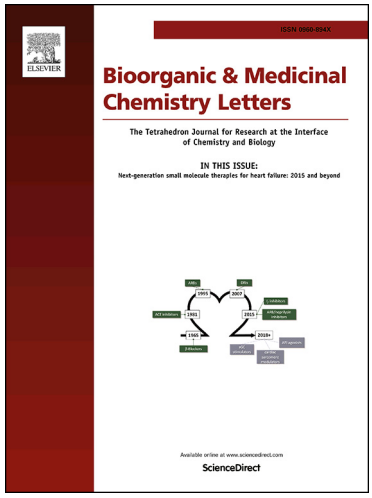

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Analogs of penfluridol as chemotherapeutic agents with reduced central nervous system activity.

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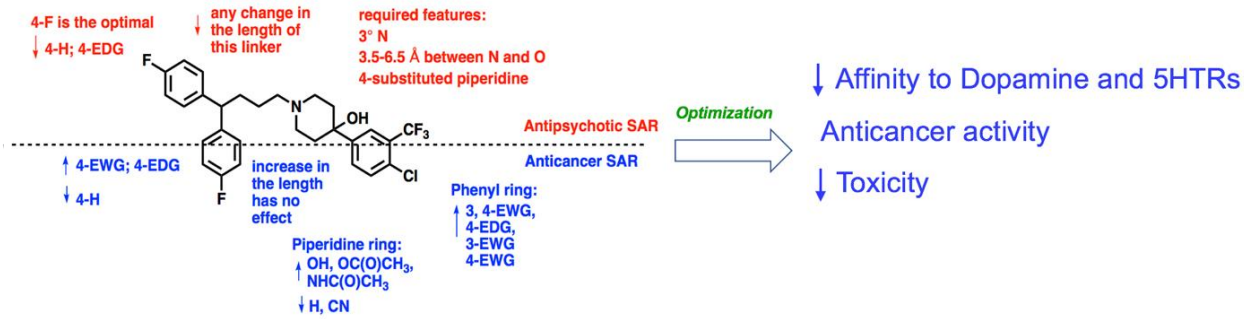
Keywords: penfluridol; anticancer agent; central nervous system; repurposing; toxicity; optimization.

Abstract:

Several recent reports have highlighted the feasibility of the use of penfluridol, a well-known antipsychotic agent, as a chemotherapeutic agent. In vivo experiments have confirmed the cytotoxic activity of penfluridol in triple-negative breast cancer model, lung cancer model, and further studies have been proposed to assess its anticancer activity and viability for the treatment of glioblastomas. However, penfluridol anticancer activity was observed at a dosage significantly higher than that administered in antipsychotic therapy, thus raising the concern for the potential onset of CNS side effects in patients undergoing intensive pharmacological treatment. In this study, we evaluate the potential CNS toxicity of penfluridol side by side with a set of analogs.

Graphical Abstract TOC:



Authors on this paper have filed a provisional patent application (INV 1365, TTU, USA) detailing this work.

Traditionally, de novo drug discovery for cancer treatment is invariably associated with high cost for preclinical and clinical studies which is estimated at approximately 1.042 billion dollars per drug[1]. Therefore, repurposing approved drugs to extend their therapeutic viability to other diseases (such as cancer treatment) is likely to afford the quickest and most cost-effective transition from bench to bedside[2, 3]. At the same time, to avoid potential side toxicity issues, it is crucial to gather all available toxicological information associated with the use of a given drug[4] and prevent scenarios similar to bevacizumab, cetuximab and others[5], the USA Food and Drug Administration (FDA) developed guidance for a systemic approach to collect and report compound-associated toxicities[6]. This is especially important when the proposed dosage and/or frequency of administration of a repurposed drug are substantially different (higher) than those previously associated for its original therapeutic scope.

Among the many drugs being tested for their off-label activities, penfluridol, an oral antipsychotic agent[7], was investigated for its anticancer properties[8]. Several groups have reported that this compound reduces cancer growth *in vitro* and *in vivo*[9-15]. Encouraging tumor-killing data and ability of this drug to cross the blood-brain barrier (BBB) prompt the idea of using this agent in the treatment of glioblastoma[12, 13] and cancers that have high rates of metastasis in the brain, including metastatic triple-negative breast cancer[10] and lung cancers[14]. However, *in vivo* data also indicated that anticancer dosage should be considerably higher (estimated at 50 mg of daily dosing in human)[10-12, 15, 16] than those used for the treatment of chronic schizophrenia and similar psychotic disorders (weekly oral dosing of 20 mg to 100 mg, 160 mg for resistant cases)[17]. Earlier, clinical data report neurological side effects attributed to the antipsychotic use of penfluridol, including epilepsy, fatigue, dyskinesias, Parkinsonism, akathisia, dystonia and depression, although the numbers are at the lower range[18-20]. Because penfluridol is capable of penetrating the BBB we estimated that the dosage required to produce chemotherapeutic activities[10-12] would substantially increase the incidence of neurological side effects already associated with its use, thus impacting the sustainability and overall benefits associated with the treatment.

To assess these potential side-effects, we have evaluated the ability of penfluridol to inhibit major groups of G-coupled protein receptors (GPCRs) that are expressed in the brain[21]. Our data have confirmed that this compound has a multitarget CNS profile with the majority of Ki values being in the nanomolar range.

Table 1. *Inhibitory activity of penfluridol at selected CNS receptors.*

	5HT1A	***5HT1D***	***5HT2A***	***5HT2B***	***5HT2C***	***5HT5a***	***5HT6***	***5HT7***	***D1***	***D2***	***D3***	***D4***	***D5***
Inhibition, %	100	91.3	95.2	97.3	95.3	95.0	96.0	98.4	96.5	71.5	98.2	77.6	82.9
Ki, nM	356	3560	361	184	881	10^3	10^3	280	147	159	136	10^3	125

	KOR	***MOR***	***DOR***	***H1***	***H2***	***NET***	***SERT***	***DAT***	***Alpha 1D***	***Alpha 2B***	***Alpha 2C***	***Beta 3***
Inhibition, %	94.6	87.0	89.1	84.0	98.4	86.2	92.7	89.1	95.7	95.6	100	94.0
Ki, nM	10^3	867	1714	10^3	10^3	588	10^3	1714	602	401	445	515

An initial literature analysis has shown that the available pharmacophore for CNS activity of penfluridol[22, 23] somewhat diverges from the one reported for its anticancer property[24, 25](Figure 1). Therefore, we have hypothesized that by leveraging the pharmacophores activities to promote its anticancer properties while reducing its GPCRs activities we can develop a more effective and safer chemotherapeutic penfluridol analog. Multiple modifications of the right - side motif containing the 4-substituted piperidine ring highlighted the importance of spatial orientation of the piperidine residue and hydroxy group for anticancer and antipsychotic activity[22-25]. It appears that this part of the molecule interacts with both targets via hydroxyl bonding and stabilizes the active conformation required for both types of biological activity. Hence, we have prepared analogs with the modifications in the linker motif and the left part of the original penfluridol structure. During the initial stage of our project, we have screened the obtained derivatives for their ability to inhibit selected GPCRs and to inhibit the growth of cancer cells *in vitro*, followed by evaluation of the top compound for its ability to cross the BBB *in vivo*. In addition, we have identified the dosing regimen that is required to achieve a therapeutic dose of selected compounds in mice.

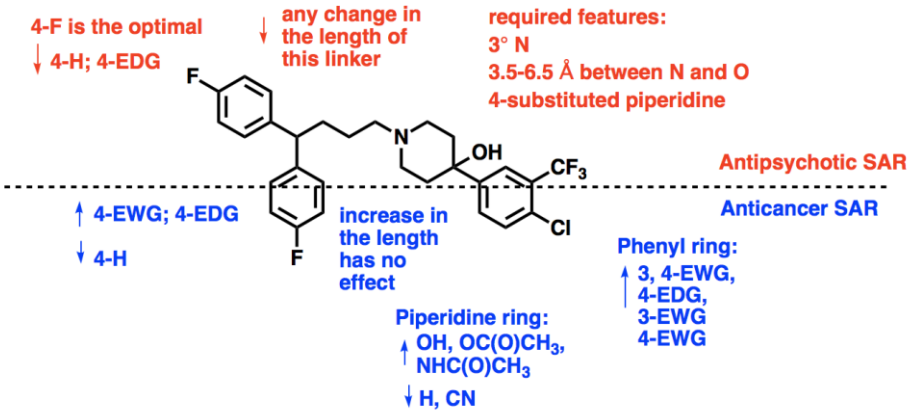
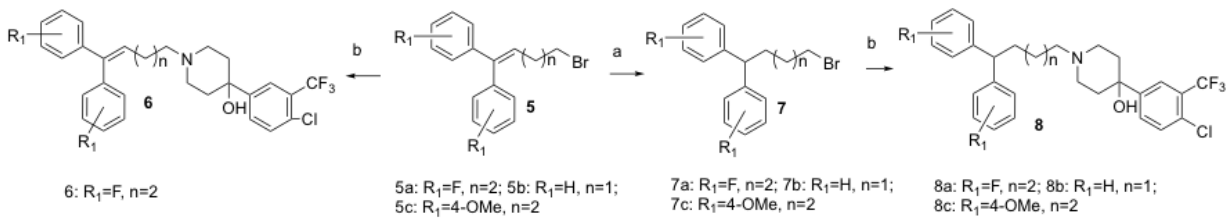
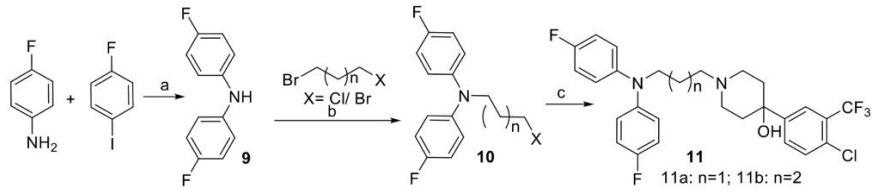


Figure 1. *Structural requirements for antipsychotic and anticancer activities of penfluridol and its analogs*[23, 24].

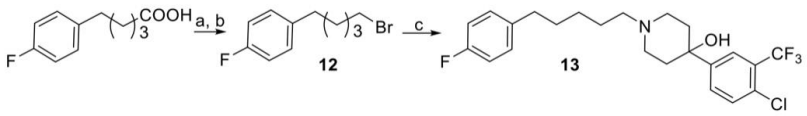
The proposed derivatives were prepared in good yields using a previously published procedure[24, 26-28] as depicted in Schemes 1 through 3 and in schemes S1-S2 (supplemental material). In particular, the right moiety of all analogs was prepared by reacting a Grignard reagent with the N-BOC piperidone, followed by deprotection step in the presence of hydrochloric acid in ethanol[24] (Scheme S1). As shown in Scheme S2 (supplemental material), the left part of compounds was constructed using selected benzene derivatives and the corresponding lactones to produce the intermediate **5** that was further utilized in preparing a key intermediate **7** (Scheme 1). This compound was coupled with a moiety **2** (scheme S1, supplemental material) to obtain the final diphenyl butyl- or diphenyl pentylpipreridines **8** and **6**. In scheme 2, synthesis of analogs **11a** and **11b** started with the amination of aryl chloride using 1,1'-bis(diphenylphosphino)ferrocene (DPPH) as a catalyst.[27] Next, the coupling of **9** with dibromo- or bromo-chloroalkane afforded intermediate **10** which was further reacted with **2** to afford compounds **11a** and **11b** in 10% and 24% overall yield respectively. Finally, in Scheme 3 monophenyl analog **13** was prepared in three steps following the published procedure[26] to afford the desired product in 8% overall yield. All products were purified by flash chromatography and characterized using ^{1}H and ^{13}C NMR. The purity of all compounds was at or above 95% (supplemental material).



Scheme 1: *Reagents and conditions: (a) Pd/C, H_2, EtOH, r.t. (b)* ***2****, Na_2CO_3, KI, CH_3CN, reflux.*



Scheme 2: *Reagents and conditions: (a) 1,1'-Ferrocenediyl-bis(diphenylphosphine), $Pd(dppf)Cl_2.CH_2Cl_2$, KtBuO, anhydrous THF, 100 ºC; (b) NaH, THF, 80 ºC; (c)* ***2****, Na_2CO_3, KI, CH_3CN, reflux.*



Scheme 3: *Reagents and conditions: (a) $LiAlH_4$, THF, 0 °C to r.t.; (b) CBr_4, Ph_3P, DCM, 0 °C to r.t.; (c)* ***2****, Na_2CO_3, KI, CH_3CN, reflux.*

To confirm that the proposed changes retain similar cytotoxic as penfluridol, analogs were evaluated *in vitro* using two cancer cell lines of both mouse and human origin: mouse-derived luciferin-expressing Lewis Lung Carcinoma (LLC luc) cell line and human triple-negative breast cancer cell line (MDA MB-231) using the 3-[4, 5-dimethylthiazol-

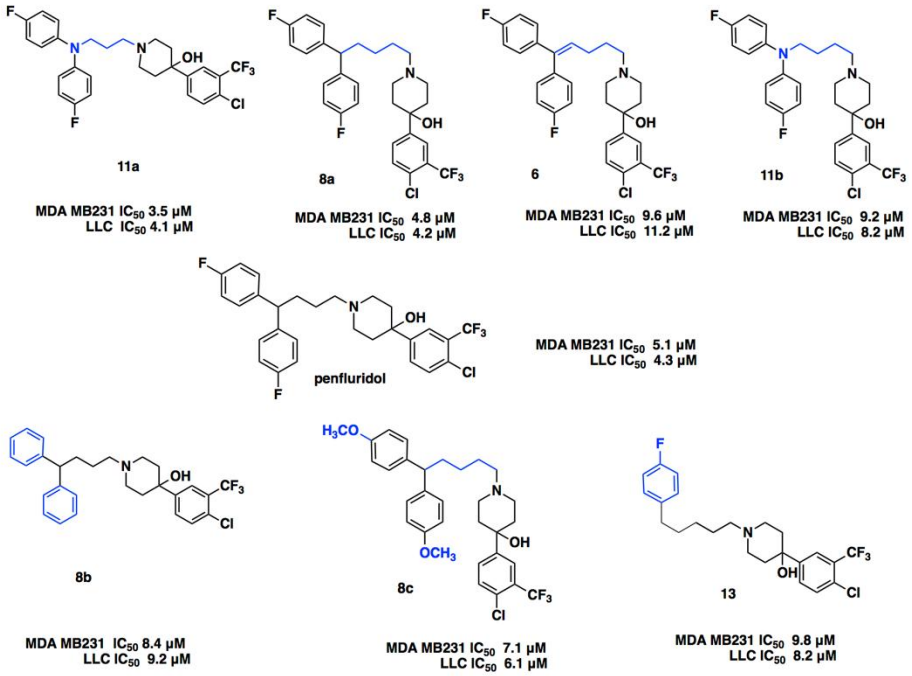


Figure 2. *Synthesized analogs of penfluridol with the corresponding activity against MDA MB231 and LLC cell lines.*

2-yl]-2, 5-dimethyltetrazolium bromide (MTT) colorimetric assay[29]. All experiments were performed in triplicates to ensure good reproducibility and penfluridol was used as a positive control. Data were obtained at 24h, 48h, and 72 hours and IC_{50} values (μM) were calculated (supplementary materials). Among all derivatives tested, compounds **8a**, **8c** and **11a** showed the best activity (IC_{50} 3.5 - 7.1 μM), with little to no reduction when compared to penfluridol (IC_{50} 4.3 - 5.1 μM). All three compounds have modifications in the spacer linker including elongation of the chain by one carbon (**8a** and **8c**) or introduction of nitrogen atom (**11a**). An unsaturated linker with the elongated chain **6** (IC_{50} 9.6 - 11.2 μM) caused a two-fold reduced cytotoxicity when compared to penfluridol. Interestingly, when two favorable features, such as elongated linker and N-containing linker, were combined, the activity has decreased (**11b**, IC_{50} 8.2 - 9.2μM). Next, modifications of the left motif, including unsubstituted phenyl moieties (**8b**) and monophenyl analog (**13**) were marked by the reduced activity, whereas introduction of methoxy group was tolerated (**8c**,

IC_{50} 6-7 μM). Overall, *in vitro* cytotoxicity data have shown that all designed analogs were active against selected cancer cell lines, supporting our hypothesis that these modifications will not alter significantly the anticancer activity of the original penfluridol molecule.

Next, we have investigated the effect of the proposed changes on the inhibition of selected G-protein coupled receptors (GPCRs). Receptor binding profile and Ki determinations were provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, where primary binding assay has identified compounds with significant inhibition effect of 50% and more[21]. These derivatives were further

evaluated using a radioligand binding assay, and Ki values were calculated accordingly.

As shown in Tables 2–4, the greatest reduction in the inhibitory activity for spacer modified analogs was observed for compound **11b**. For some serotonin receptors, this N-butyl analog has shown decreased inhibition (50% - 80%) when compared to penfluridol (95% - 100%) (Figure 2), and for other serotonin receptor subtypes it retained inhibition levels at >90% but decreased the Ki valued by 3-16-fold (Table 3). Comparable results were obtained for dopamine receptors, opioid receptors, NET, SERT and DAT (Figure 3, Tables 4). Moreover, **11b** has shown abolished activity at H1 and H2 receptors, significantly improving CNS-toxicity profile of the original penfluridol molecule. Similar effect was observed for

compound **8a**, a homologated analog of penfluridol. However, it appears that elongation of the chain doesn't reduce the ability of this compound to inhibit dopamine receptors (Table 4). On a contrary, **8a** produced significantly lower Ki values for D4 receptor subtype (Ki 25 nM) when compared to the original penfluridol molecule (Ki 10000nM). An introduction of N-propyl linker, **11a,** resulted in higher affinity of a molecule to histamine receptors and in substantially increased binding to the D4 receptor subtype, whereas the rest of the receptor's groups were not affected. Available results for unsaturated analog **6** suggest that this compound has slight improvement in the CNS binding profile, although increased affinity to D4 receptor is observed here as well.

In the second group of analogs with the modifications on the left side of the diphenylbutylpiperidine structure, the most favorable profile is associated with the compound **8c**. The data suggest a significant reduction in the Ki values for the majority of the serotonin and dopamine receptor subtypes, opioid receptors, and histamine receptors (Figure 3, Tables 2-4). The monophenyl analog **13** has shown no substantial changes in the binding profile, whereas compound **8b** lacking the 4-F substituent displays increased ability to inhibit all dopamine receptor subtypes, MOR, H2, and DAT. These data were not in line with the previously reported structure-activity relationship trends[22, 23], where the importance of the electron withdrawing groups at para-position was highlighted for the antipsychotic activity.

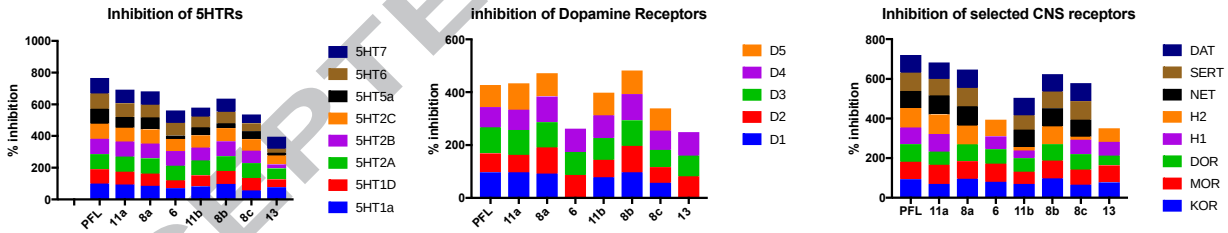


Figure 3. *Inhibition of selected CNS receptors by penfluridol and its analogs. 5HT– serotonin receptors, D – dopamine receptors, KOR – kappa opioid receptor, MOR – mu opioid receptor, DOR – delta opioid receptor, H1-histamine receptor 1, H2 – histamine receptor 2, NET – norepinephrine transporter, SERT – serotonin transporter, DAT – dopamine transporter.*

Overall, through the analysis of the receptor binding profiles, we were able to identify three compounds, **8a, 11b** and **8c**, with the reduced affinity to selected CNS receptors. Compounds **11b** and **8c** showed a significant reduction in the inhibition of dopamine receptors, a group of receptors strongly associated with the neurological side-effects produced by penfluridol. Similarly, **8a** had pronounced effect on the inhibition of serotonin receptors, while moderate changes in the affinity to dopamine receptors. Therefore, we

expect **8a, 11b** and **8c** to have diminished CNS toxicity if used as chemotherapeutic agents. Due to the minimal changes in the penfluridol structure, we assumed that our analogs yielded minimal alterations in the BBB permeability and no additional organ toxicity. To confirm this hypothesis, we have selected compounds **8a** (IC_{50} 4.8 μM, MDA MB231; IC_{50} 4.2μM, LLC) and **8c** (IC_{50} 6.1 μM, MDA MB231; IC_{50} 7.1 μM, LLC) for

***Table 2**. Binding activity (Ki, nm) of penfluridol and its analogs at serotonin receptor subtypes. Data represent mean inhibition (n=4). NA – not active; ND – not determined.*

	5HT1A	***5HT1D***	***5HT2A***	***5HT2B***	***5HT2C***	***5HT5a***	***5HT6***	***5HT7***
PFL	356	3560	361	184	881	10000	10000	280
11a	1262	752	164	177	402	ND	1680	1574
8a	1395	844	1000	423	1712	894	1671	969
6	1363	*NA*	1105	*2199*	1603	*ND*	*ND*	*10000*
11b	1094	1074	6059	509	1126	4152	1670	812
8b	481	628	1000	374	2131	NA	1695	326
8c	10000	10000	900	729	740	4973	1400	949
13	901	*4431*	889	*NA*	2548	*ND*	*ND*	*1120*

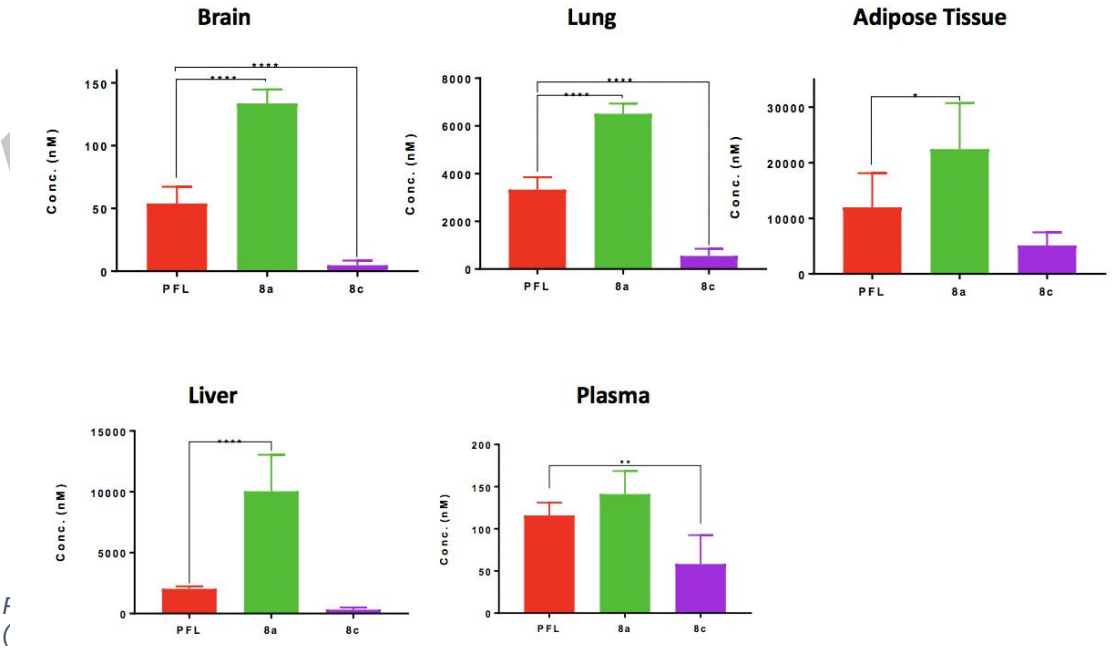
***Table 3.** Binding activity (Ki, nm) of penfluridol and its analogs at dopamine receptor subtypes. Data represent mean inhibition (n=4). NA – not active; ND – not determined.*

	D1	***D2***	***D3***	***D4***	***D5***
PFL	147	159	136	10000	125
11a	300	148	474	25	437
8a	798	194	209	385	529
6	*ND*	317	552	1643	*ND*
11b	10000	550	782	10000	744
8b	135	38	62	58	121
8c	10000	417	437	459	623
13	*ND*	460	*ND*	132	*ND*

Table 4. *Binding activity (Ki, nm) of penfluridol and its analogs at selected CNS receptors. Data represent mean inhibition (n=4). NA – not active; ND – not determined.*

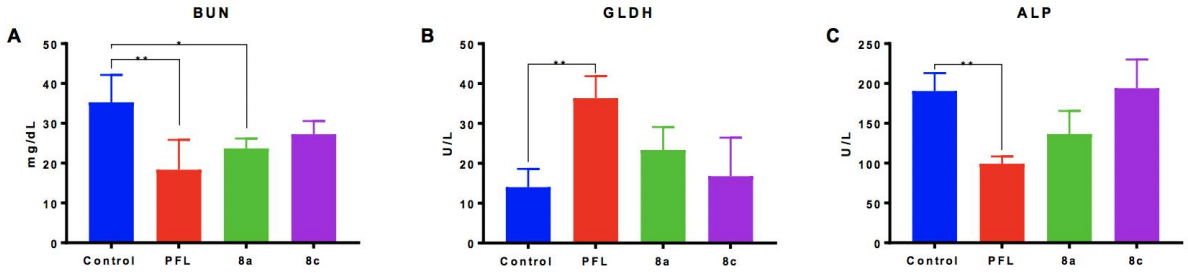
	KOR	***MOR***	***DOR***	***H1***	***H2***	***NET***	***SERT***	***DAT***
PFL	10000	867	1714	10000	10000	588	10000	1714
11a	667	902	1558	814	252	902	903	1191
8a	2669	356	1413	*ND*	546	77	428	529
6	1021	328	*ND*	718	526	*ND*	*ND*	*ND*
11b	10000	10000	10000	*NA*	*NA*	458	10000	10000
8b	1328	70	1526	*ND*	278	518	1180	121
8c	10000	8253	10000	7031	*NA*	1438	1314	622
13	1705	536	*NA*	718	401	*ND*	*ND*	*ND*

further evaluation *in vivo*. The LLC cell line is a syngeneic cell line for mice of the C57BL6 background that have functional immune system[30]. Therefore, we have selected the C57BL6 mice (females) for our *in vivo* experiments. In the initial study, three groups of animals were treated with penfluridol, **8a** or **8c** compounds (5 mg/ kg) by intraperitoneal (i.p.) injection for seven consecutive days. This dosing regimen was chosen to match the published *in vivo* data, where seven days were reported as the minimum length of treatment for therapeutic effect in an *in vivo* lung cancer model[31]. Twenty-four hours after the last injection, all mice were sacrificed, and organs were collected and stored at -80 ºC. LC-MS/MS analysis of plasma, brain, lungs and adipose tissue was performed to identify concentration levels of the compounds (Figure 4). The observed distribution pattern for analog **8a** was very similar to the one displayed by



penfluridol, where an accumulation of the drug occurred predominantly in the adipose tissue and lungs. At the same time, compound **8a** has higher level of the drug in the brain relative to penfluridol. Analog **8c**, on the other hand, displayed lower concentration of the drug in all tested samples, although the overall distribution pattern was similar to penfluridol and **8a**. Our preliminary analysis of the pharmacokinetic profile of **8c** has shown that the maximum concentration of drug in the brain (Figure S5, supplemental material) is achieved at a 6-hour time point following the i.p. injection of 10 mg/kg dose. Currently, we are investigating if lower levels of **8c** in animal samples are associated with the faster metabolic degradation of this compound when compared to penfluridol. In particular, we are looking at the stability of the 4-methoxyphenyl moiety under experimental conditions.

None of the tested compounds had a significant effect on the organ weight (Figure S6, supplemental material) supporting previously published data for penfluridol[10]. In addition, we performed clinical chemistry analysis of blood samples of the animals treated with penfluridol, compound **8a,** and compound **8c** (Figure 5, Figure S7). Specifically we measured the levels of electrolytes, minerals, protein metabolism



(total protein, albumin, globulin, A/G ratio), kidney function (blood urea nitrogen - BUN), liver injury

*Figure 5 Effect of drug treatment (5 mg/kg, daily i.p. dosing for 7 days) on A. Kidney function: blood urea nitrogen (BUN); B. Liver injury: glutamate dehydrogenase (GLDH) and C. Liver injury: alkaline phosphatase (ALP), n=5, *= p<0.05, ** = p< 0.01.*

(including alanine aminotransferase - ALT, aspartate aminotransferase - AST, and glutamate dehydrogenase - GLDH), cholestasis, alkaline phosphatase (ALP), bilirubin, pancreatic function (amylase), and muscle injury (creatine kinase (CK), AST, ALT) [32]. In penfluridol-treated animals our results were consistent with the onset of hepatic stress or injury and inflammation of the GI tract. In fact, only penfluridol - treated animals have shown decreased BUN levels and increased GLDH corresponding to hepatic stress or injury, whereas decreased ALP levels may result from low zinc level or can be caused

by inflammation of GI tract. None of these changes was observed in mice treated with compound **8c**, thus suggesting that this analog have a better toxicity profile when compared to penfluridol.

To summarize, our study started with the evaluation of the potential CNS-related toxicity of penfluridol at the doses proposed for the anticancer therapy. As we have shown, this compound inhibits a majority of CNS-related GPCRs at the nanomolar level, raising concerns about a potential burden on a patient under treatment. In our study we have identified 2 compounds (**8a** and **8c)** with anticancer activity but lesser CNS affinity (hence reduced CNS-related side effects) t when compared to penfluridol. In addition, these compounds have shown no toxicity in mice. The metabolic stability of these compounds and their utilization in metastatic triple-negative breast cancer model (**8a**) and lung cancer model (**8c**) is currently under investigation and data will be presented in due course. Furthermore, comparative side by side studies will be performed in the near future to evaluate the anticancer activity of these penfluridol analogs against commercially available treatments.

Abbreviations:

ALP; alkaline phosphatase, creatine kinase; ALT; alanine aminotransferase, AST; aspartate aminotransferase, BBB; blood brain barrier, BOC; *tert*-butoxycarbonyl, BUN; blood urea nitrogen test, CK; creatine kinase, CNS; central nervous system, D1-5; dopamine receptor subtypes, DAT; dopamine transporter, DCM; dichloromethane, DOR; delta opioid receptor, DPPH; 1,1'-bis(diphenylphosphino)ferrocene, ESI electrospray ionization, EtOH; ethanol, FDA; Food and Drug Administration, GI; gastrointestinal, GLDH; glutamine dehydrogenase, H1-2; histamine receptor subtypes, 5HT; serotonin receptors, IC_{50}; half-maximum inhibitory concentration, i.p.; intraperitoneal injection, Ki; inhibition constant, KOR; kappa opioid receptor; LC; liquid chromatography, LC-MS; liquid chromatography-mass spectrometry, LLC luc; mouse-derived luciferin expressing Lewis lung carcinoma cell line, MDA MB231; human triple-negative breast cancer cell line, MOR; mu opioid receptor, MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide, NET; norepinephrine transporter, NMR; nuclear magnetic resonance, r.t.; room temperature, SERT; serotonin transporter, THF; tetrahydrofuran, TLC; thin-layer chromatography.

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Highlights

- Anticancer doses of penfluridol inhibit a majority of G-protein coupled receptors.
- Analysis of antipsychotic pharmacophore allowed to design less toxic analogs.
- Confirmed in vitro cytotoxicity of analogs, in vivo permeability of the BBB.
- Clinical chemistry analysis confirmed optimized toxicity profile.