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Generation of novel radiolabeled opiates through site-selective iodination

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ABSTRACT

Tritiated opioid radioligands have proven valuable in exploring opioid binding sites. However, tritium has many limitations. Its low specific activity and limited counting efficiency makes it difficult to examine low abundant, high affinity sites and its disposal is problematic due to the need to use organic scintillants and its relatively long half-life. To overcome these issues, we have synthesized both unlabeled and carrier-free radioiodinated iodobenzoyl derivatives of 6β-naltrexamine (^{125}I-BNtxA, **18**), 6β-naloxamine (^{125}I-BNalA, **19**) and 6β-oxymorphamine (^{125}I-BOxyA, **20**) with specific activities of 2100 Ci/mmol. To optimize the utility of the radioligand, we designed a synthesis in which the radiolabel is incorporated in the last synthetic step, which required the selective iodination of the benzoyl moiety without incorporation into the phenolic A ring. Competition studies demonstrated high affinity of the unlabelled compounds for opioid receptors in transfected cell lines, as did the direct binding of the ^{125}I-ligands to the opioid receptors. The radioligand displayed very high sensitivity, enabling a marked reduction in tissue, as well as excellent signal/noise characteristics. These new ^{125}I-radioligands should prove valuable in future studies of opioid binding sites.

The opioid receptors are G-protein coupled receptors which mediate pain relief through both the central and peripheral nervous systems.[1,2] The opiate antagonists naltrexone (Ntx, **3**) and naloxone (Nal, **4**) and the agonist oxymorphone (Oxy, **5**) are potent and widely used opiates. Three families of opiate receptors have been identified and cloned: mu (MOR-1), kappa$_1$ (KOR-1) and delta (DOR-1). Radioligands were critical in the initial demonstration of the opioid receptors. Early attempts to detect binding sites using ^{14}C-labeled opioids[3] were not successful due to the low specific activity of the radioligands combined with the high affinity and low abundance of the sites. A number of selective tritiated ligands are currently used to label mu ([D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin; DAMGO), kappa$_1$ (U50,488H) and delta ([D-Pen^2,D-Pen^5]enkephalin; DPDPE) receptors. However, tritium presents a number of limitations. While their specific activity, typically around 50 Ci/mmol, is sufficient to identify the receptors in brain tissue or cell lines expressing them, examination of binding sites with very low abundance and high affinity is limited and may impact the ability to identify proposed subtypes of mu,[4–6] delta,[7,8] and kappa receptors[9–11] proposed from both biochemical and cloning studies. The use of tritium also poses practical issues. These range from detection issues, such as the need to utilize scintillation fluor for counting which destroys the labeled sample and special films and long exposures for autoradiography, to its disposal, which is problematic due to the long half-life and the need to use organic scintillation fluors. To avoid these issues, we developed high affinity ^{125}I-labeled opiate ligands that obviate many of these problems. They have high specific activity (2100 Ci/mmol), which permits the examination of binding at very low concentrations and can detect sites of low abundance. They can be counted without the need for scintillation fluor and their disposal is facilitated by their short half-life.

Traditionally incorporating a radioactive ^{125}I into a molecule requires the direct iodination of a phenolic or an aromatic amine. Problems can arise if a molecule has more than one potential site of incorporation. This is particularly difficult if iodination of one of the sites adversely impacts binding affinity or functional activity, as is the case with the A ring of opiates. Our goal to develop ^{125}I-labeled opioids suitable for general use required an approach to incorporate the iodine as the final synthetic step into a specific location of the molecule. Prior studies suggested benzoic acid substitutions at the 6-position of the opiate scaffold can maintain activity.[12,13] We now report the design and synthesis of three novel, high affinity ^{125}I-labeled opiates based upon 6-substituted amines of naltrexone (**6**), naloxone (**7**), and oxymorphone (**8**).

Abbreviations: NHS, *N*-hydroxy succinimide; THF, Tetrahydrofuran; DCC, Dicyclohexyl carbodiimide; NH_4OAc, Ammonium acetate; $NaBH_3CN$, Sodium cyanoborohydride; MeOH, Methanol; rt, room temperature; DCM, Methylene chloride; DIEA, *N*,*N*-Diisopropyl ethyl amine; *n*-Bu_3SnCl, Tributyl tin chloride; BuLi, Butyl lithium; $Na_2S_2O_5$, Sodium metabisulfite; AcOH, Acetic acid.

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The synthesis of the unlabeled compounds is presented in Scheme 1 (Fig. 1). The ketone at the 6-position of the three opiates (**3**–**5**) was transformed to an amine (**6**–**8**) by reductive amination using $NaBH_3CN$ and NH_4OAc to yield a mixture of beta and alpha isomers.[14] The beta isomer was purified by column chromatography. In a parallel synthesis, 3-iodobenzoic acid (**1**) was converted to its *N*-succinimidyl ester (**2**) by reacting it with *N*-hydroxysuccinimide in presence of DCC and THF. The corresponding activated ester (**2**) was then reacted with the beta isomer of the opiate (**6**–**8**) in presence of DIEA and DCM. The 3-iodo-benzoyl amido derivatives of opiates (**9**–**11**) were then purified by column chromatography.

The synthesis of the radiolabelled derivatives of the opiates is presented in Scheme 2 (Fig. 2). 3-Bromobenzoic acid (**12**) was converted to 3-tributylstannanyl-benzoic acid (**13**) using BuLi and tributylstannayl chloride.[15] 3-Tributylstannnanyl-benzoic acid (**13**) was converted to the corresponding *N*-succinimidyl derivative (**14**) by reacting it with *N*-hydroxysuccinimide, DCC and THF. The β-isomers of naltrexamine (**6**), naloxamine (**7**) and oxymorphamine (**8**) were then reacted with the N-hydroxysuccinimidyl ester of 3-tributylstanannayl-benzoic acid (**14**) in presence of DIEA and DCM. The purified opiate-stannyl analog (**15**–**17**) was then used as a precursor for incorporating radioactive iodine. The opiate-stannyl analog precursor (**15**–**17**) is stable at 4 °C for extended times. Incorporation of the radioiodine was performed in the final step using $Na^{125}I$ and chloramine-T in methanol by incubating the contents at room temperature for 2 min, after which the reaction was terminated by adding sodium metabisulfate. The radioactive derivative (**18**–**20**) was purified by RP–HPLC using a gradient of 0.1% TFA/water (A) and 0.1% TFA/ACN (B) as the solvents, with the product eluting at 50% 0.1% TFA/ACN (B).

To determine whether the compounds (**9**–**11**) retained high affinity for the opioid receptors, we assessed their affinity (K_i) in CHO cell lines stably transfected with MOR-1, DOR-1 or KOR-1[16,17] in competition studies which utilized ^{3}H-radioligands specific to the transfected receptor (Table 1). All three compounds (**9**–**11**) retained high affinity for mu (MOR-1) receptors and for kappa$_1$ (KOR-1) receptors. Although both IBNtxA (**9**) and IBNalA (**10**) displayed high affinity for delta (DOR-1) receptors, IBOxyA (**11**) did not.

In view of their high affinity for the opioid receptors, we proceeded to synthesize and examine the binding of ^{125}I-labeled versions (**18**–**20**) of the compounds directly. Association at 25 °C in MOR-1/CHO cells was rapid for all three radioiodinated ligands, reaching steady-state within 1 h and remaining stable for at least 90 min (data not shown). The ratio of total binding to non specific binding typically was 6:1 or greater, providing excellent signal/noise differentiation. With the far higher specific activity and counting efficiency of ^{125}I-ligands, we were able to use only 1/10th the protein in the binding assay that was required with the ^{3}H-labeled ligands and still have more than five-fold more counts of specific binding.

We next carried out saturation studies with ^{125}IBNtxA (**18**), ^{125}IBNalA (**19**) and ^{125}IBOxyA (**20**) in stably transfected CHO cell

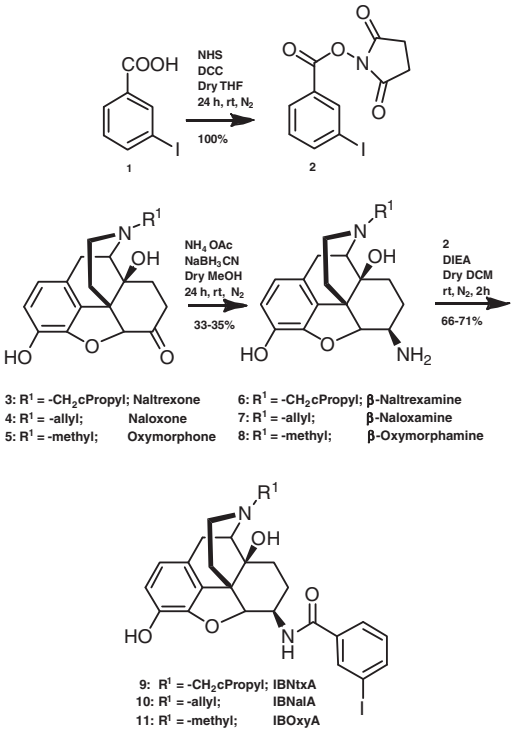

Figure 1. Synthesis of 3-iodobenzoyl derivatives of β-naltrexamine, β-naloxamine and β-oxymorphamine.

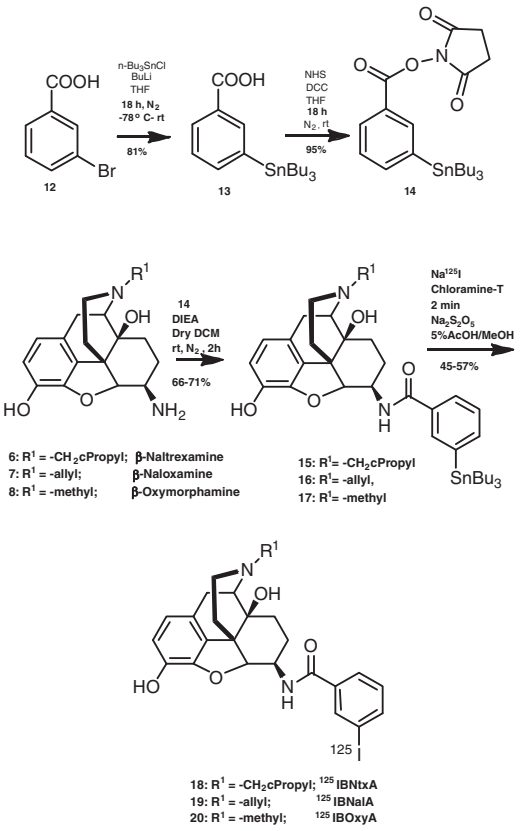

Figure 2. Synthesis of radiolabelled compounds.

Table 1
Competition by IBNtxA, IBNalA and IBOxyA against ^{3}H-opioids in transfected cell lines

Drug	K_i (nM)		
	MOR-1	KOR-1	DOR-1
IBNtxA (**9**)	2.50 ± 0.82	0.23 ± 0.16	0.58 ± 0.16
IBNalA (**10**)	0.70 ± 0.18	0.08 ± 0.006	2.55 ± 0.74
IBOxyA (**11**)	1.75 ± 0.33	9.0 ± 4.0	70.76 ± 15.40

Competition studies in membranes from CHO cells stably transfected with the indicated opioid receptors were performed with ^{3}H-DAMGO (MOR-1), ^{3}H-U69,593 (KOR-1) ^{3}H-DPDPE (DOR-1) (1 nM) and K_i values determined from the IC_{50} values obtained by nonlinear regression analysis (Prism, Carlsbad, CA).[20] Results are the means ± SEM of at least three independent replications.

lines (Fig. 3; Table 2). As anticipated, all three radioligands displayed very high affinity in the MOR-1/CHO cells (Fig. 3A). Both ^{125}IBNtxA (**18**) and ^{125}IBNalA (**19**) labeled the KOR-1/CHO cells even more potently (Fig. 3B). No specific ^{125}I-IBOxyA (**20**) binding could be detected in either the KOR-1/CHO or the DOR-1/CHO cells at the highest concentrations of radioligands used, presumably due to its very poor affinity. ^{125}IBNtxA (**18**) displayed high affinity for delta sites in the DOR-1/CHO cells, consistent with its affinity in competition studies while ^{125}I-BNalA (**19**) labeled sites with a 10-fold lower affinity (Fig. 3C). When assessing the affinity of a drug, measuring its binding directly with the ^{125}I-radioligand is preferable and gives a better estimate of the K_D values as compared to competition studies.

Finally, we determined whether the binding of the ^{125}I-radioligands to the various transfected cells showed the same sensitivity towards traditional ligands as determined previously in traditional ^{3}H-radioligand binding assays. Overall, the various selective drugs all competed ^{125}I-radioligand binding to various receptors with high affinity and K_i values similar to those seen with ^{3}H-ligands (Table 3).

Our initial goal in these studies was to develop a ^{125}I-labeled opioid radioligand for routine use in receptor binding assays. ^{125}I-labeled compounds have a number of important advantages over analogous ^{3}H-labeled radioligands. ^{125}I-ligand binding is far more sensitive due to its far greater specific activity (2100 Ci/mmol) compared to ^{3}H-drugs (~50 Ci/mmol). This enables the use of far less tissue in the assays, a major help when dealing with limited sources, such as with tissue culture samples. Equally important, counting ^{125}I-drugs can be done without the need for scintillation fluors, eliminating a major problem with disposal seen with the ^{3}H-agents. Its far shorter half-life also helps with the eventual disposal of the materials. Finally, since scintillation fluor is not necessary, samples can be counted without sacrificing them, an important consideration when having to follow multi-step processes. Thus, the use of ^{125}I-ligands has scientific, environmental and financial incentives.

Table 2
Saturation studies of the ^{125}I-labeled compounds in cell lines

Radioligand	K_D (nM)		
	MOR-1	KOR-1	DOR-1
^{125}IBNtxA (**18**)	0.11 ± 0.02	0.027 ± 0.001	0.24 ± 0.05
^{125}IBNalA (**19**)	0.22 ± 0.03	0.05 ± 0.01	2.5 ± 0.22
^{125}IBOxyA (**20**)	0.08 ± 0.03		

Saturation studies were performed with the indicated radioligand in CHO cells stably transfected with the designated opioid receptor. The saturation curves were fit a single site using nonlinear regression analysis (Prism, Carlsberg, CA). K_D values are the means ± SEM of at least three independent determinations. Bmax values were dependent upon the tissue used and, therefore, varied among the studies. No specific binding could be detected for ^{125}I-BOxyA in either the DOR-1/CHO or KOR-1/CHO cells at the highest concentrations. B_{max} values for MOR-1 were 3.81 ± 0.42, 1.18 ± 0.2 and 1.49 ± 0.5 pmol/mg protein for ^{125}I-labeled IBNtxA (**18**), IBNalA (**19**) and IBOxyA (**20**), respectively. B_{max} values for KOR-1 were 0.57 ± 0.02 and 1.15 ± 0.01 pmol/mg protein for ^{125}I-labeled IBNtxA (**18**) and IBNalA (**19**), respectively, and for DOR-1 it was 0.96 ± 0.2 and 2.7 ± 1.04 fmol/mg protein for IBNtxA (**18**) and IBNalA (**19**), respectively.

Direct iodination of opiates presents a problem since incorporation of iodine into the phenolic A ring of the opiate scaffold markedly decreases activity. We addressed this by introducing a second iodination site in the molecule. However, the presence of two potential iodination sites required a method to selectively iodinate only the site of interest, in this case the site on the benzoylamide. To achieve this, we generated the tributyl stannate precursor. This route permits the iodination of the molecule at the last step in the synthesis, minimizing manipulation of radioactive species. Iodine

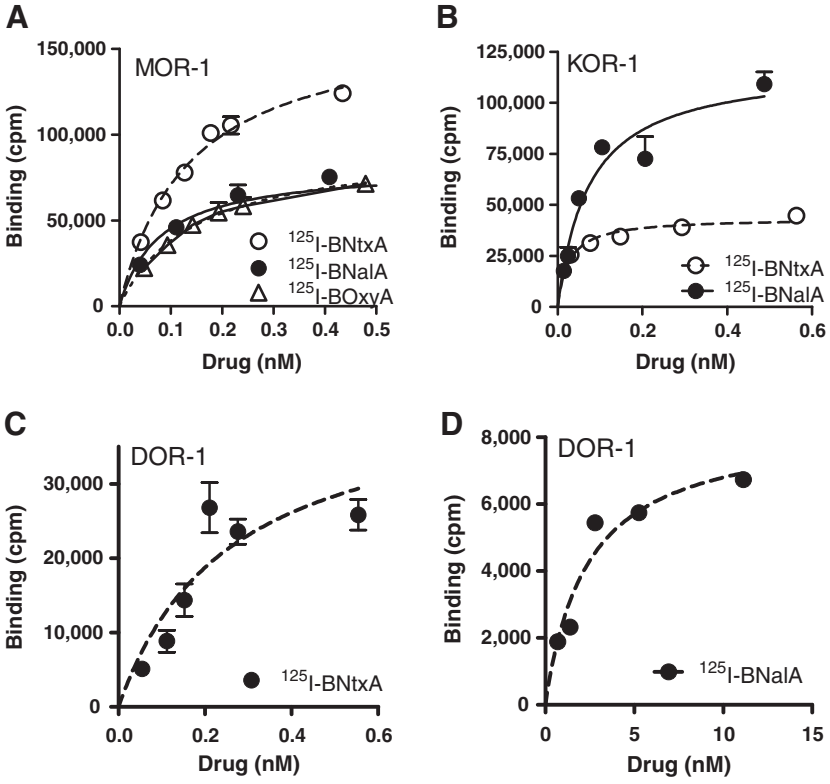

Figure 3. ^{125}I-opioid saturation studies in stably transfected CHO cells. Saturation studies were performed with indicated radioligand in CHO cells stably transfected with the designated opioid receptor. Each figure is a representative experiment that has been independently replicated at least three times. Only specific binding is presented. Differences in B_{max} values reflect the use of different tissue preparations with varying levels of expression and do not impact the measure of affinity (K_D). Error bars represent the SEM of triplicate samples. Error bars that cannot be seen are smaller than the size of the symbol. All radioligands were used carrier-free, with the exception of the ^{125}I-BNalA saturation in the DOR-1 transfected cells where the specific activity was diluted 10-fold due to the need to examine higher ligand concentrations.

Table 3
Competitions of radioiodinated ligand binding in cell lines

Drug	K_i (nM)		
	^{125}I-IBNtxA (**18**)	^{125}I-IBNalA (**19**)	^{125}I-IBOxyA (**20**)
MOR-1			
CTAP	2.33 ± 0.48	2.96 ± 1.07	1.06 ± 0.56
Naloxone	4.23 ± 0.42	2.27 ± 0.60	0.57 ± 0.12
Levallorphan	1.29 ± 0.14	1.69 ± 0.60	0.48 ± 0.11
Naltrexone	1.15 ± 0.10	2.71 ± 1.0	0.07 ± 0.00
DAMGO	3.34 ± 0.43	0.45 ± 0.12	1.55 ± 0.84
Morphine	4.60 ± 1.81	2.52 ± 0.28	1.92 ± 0.86
DOR-1			
DPDPE	1.39 ± 0.67	1.77 ± 0.80	—
Naltrindole	0.46 ±0.32	0.50 ± 0.20	—
KOR-1			
norBNI	0.23 ± 0.03	0.19 ± 0.08	—
5′GNTI	0.15 ± 0.07	0.19 ± 0.05	—
(-)U50,488H	0.73 ± 0.32	0.95 ± 0.37	—

Competition studies were performed with the indicated compounds against the indicated ^{125}I-ligands (0.1 nM) in membranes from CHO cells stably expressing opioid receptors. K_i values were calculated from the IC_{50}.values[20] and represent the means ± SEM of at least three independent replications.

substitutes for the aromatic tributyl stannate far more rapidly than it incorporates into phenol rings.[18,19] By using an excess of the opioid relative to Na^{125}I, we were able to ensure that we selectively placed the ^{125}I into the desired benzyolamide.

In conclusion, we have synthesized a series of ^{125}I-labeled opioids of high affinity that should be of value in the study of opioid receptors. The approach required the development of a method to selectively radiolabel a specific site within the molecule which was achieved using tributyl tin. These ^{125}I-radioligands have a number of advantages over ^{3}H-radiolabels. They are more sensitive, enabling the exploration of very high affinity sites and sites of very low abundance. The also offer practical and environmental advantages, including the need for far less tissue, the ability to detect binding without organic scintillation fluors and a less problematic disposal.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.008.

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