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A preliminary study of the metabolic stability of a series of benzoxazinone derivatives as potent neuropeptide Y5 antagonists

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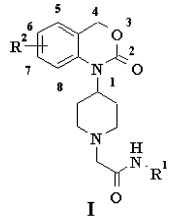
Abstract—The metabolic stability of benzoxazinone derivatives, a potent series of NPY Y5 antagonists, has been investigated. This study resulted in the identification of the structural moieties prone to metabolic transformations and which strongly influenced the in vitro half-life. This provides opportunities to optimize the structure of this new class of NPY Y5 antagonists.

Obesity is a disease that has reached epidemic proportions in many countries in the developed world. In the clinic, the body mass index (BMI) is used as the standard measure and any person with a BMI >30 kg/m^2 is termed as obese. It is now well accepted that even those people who are mildly obese have an enhanced risk of premature death, hypertension, atherosclerosis, coronary heart disease, diabetes mellitus, arthritis, sleep apnea, as well as certain forms of cancer. It is currently estimated that there are over 35 million obese people in the United States alone.

Neuropeptide Y (NPY) is a 36 amino acid peptide isolated in 1982 from porcine brain and is named for the presence of an N-terminal tyrosine and C-terminal tyrosine amide. NPY is the most abundant peptide observed in the mammalian brain, inducing a variety of behavioural effects, including stimulation of food intake, anxiety and depression, as well as the regulation of the cardiovascular and neuroendocrine systems. Currently, six different G protein coupled receptors (GPCR), designated NPY1–NPY6, are know to bind NPY with high affinity.[1] Through utilizing these receptor pathways, NPY induces orexigenic signals thereby increasing food intake and decreasing energy expenditure. It is believed that two of these subtypes, NPY1 and NPY5, appear to mediate the effects of NPY on food uptake.[2] There is some pharmacological evidence on the feeding of rodents that the NPY Y5 receptor most influences feeding.[3] This is also supported by the inhibitory effects of NPY Y5 receptor antisense oligonucleotides[4] as well as by using NPY Y5 antagonists. Although it is accepted that NPY is involved in regulation of food intake, its exact role has still to be determined.

The first NPY Y5 selective antagonists were reported in 1997.[5] Since then many NPY Y5 antagonists of diverse structural types have been reported in the literature as potential drugs for the treatment of obesity.[6]

In our previous letter, we reported the synthesis and the initial structure–activity relationship (SAR) study of benzoxazinone derivatives **I** as potent NPY Y5 antagonists.[7]



I

A range of compounds with modifications to the substituents on the northern benzoxazinone ring (R^2) and to the southern amide (R^1) were prepared and tested. Most of the compounds were found to be potent and selective

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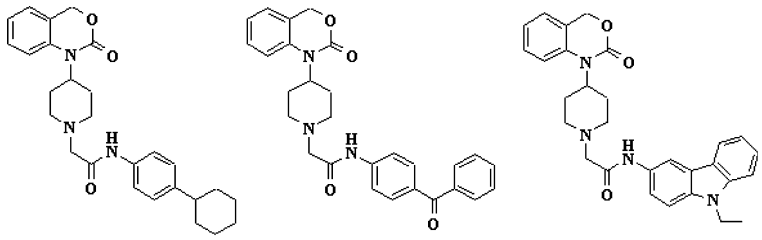


1 2 3

NPY Y5 antagonists having nanomolar binding affinities and showing functional antagonism in the forskolin-induced cyclic AMP test.

The in vitro metabolic stability was investigated[8] (percent of parent drug remaining at 1 h and half-life) using human and rat liver microsomes in independent experiments. Compounds **1**–**3** were incubated at 37 °C up to 1 h at 10 μM concentration, incubation mixtures were sampled at selected times (0, 15, 30, 45 and 60 min), stopped with acetonitrile and analyzed using HPLC (reverse phase XTerra MS C18 column; Waters, 50 × 3.0 mm, particle size 2.5 μm) with PDA detection (Waters). The results showed that the parent compounds were significantly metabolized during this period by liver microsomal enzymes from both species (Table 1).

In control experiments, compounds **1**–**3** were shown to be hydrolytically stable (data not shown). This leads to the conclusion that the metabolic profiles observed are the result of transformations under the rat and human microsomal conditions used. Of the three, compound **3** showed a better stability profile, although still at an unsatisfactory level. The metabolic stabilities observed for compound **1** (Table 1, entry a) under the experimental conditions employed were similar, which may suggest related metabolic pathways.

Prompted by the poor metabolic stability displayed in the series, further studies on the initial compounds **1**–**3** were carried out to identify the major sites of metabolism. In separate experiments, the compounds were exposed to human and rat microsomes and the resulting mixtures analyzed by LC–MS using electro spray ionization (ESI) technique[9]. This technique allowed a number of metabolites and transformations to be identified (Fig. 1).

Analysis of the data, led us to realize that the northern oxazinone moiety is prone to ring opening leading to several products **a**, **b** and **c** as summarized in Figure 2. It is likely that this transformation is promoted by the oxidation of the benzylic position. As for the southern amide section, typical phase 1 metabolism was observed depending upon substrate, such as hydroxylation, reduction and dealkylation.

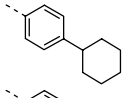
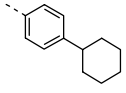
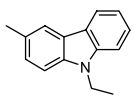
To improve the overall metabolic profile of the series, synthetic analogues of the core structure were prepared. Based on the initial metabolism data, work was undertaken in two key areas: first, the effect of substitution on the northern benzoxazinone ring system, and second, modifications to the southern amide substituent.

A range of compounds were prepared as outlined in Tables 2 and 3 and tested for in vitro metabolic stability and half-life using both rat and human liver microsomes.

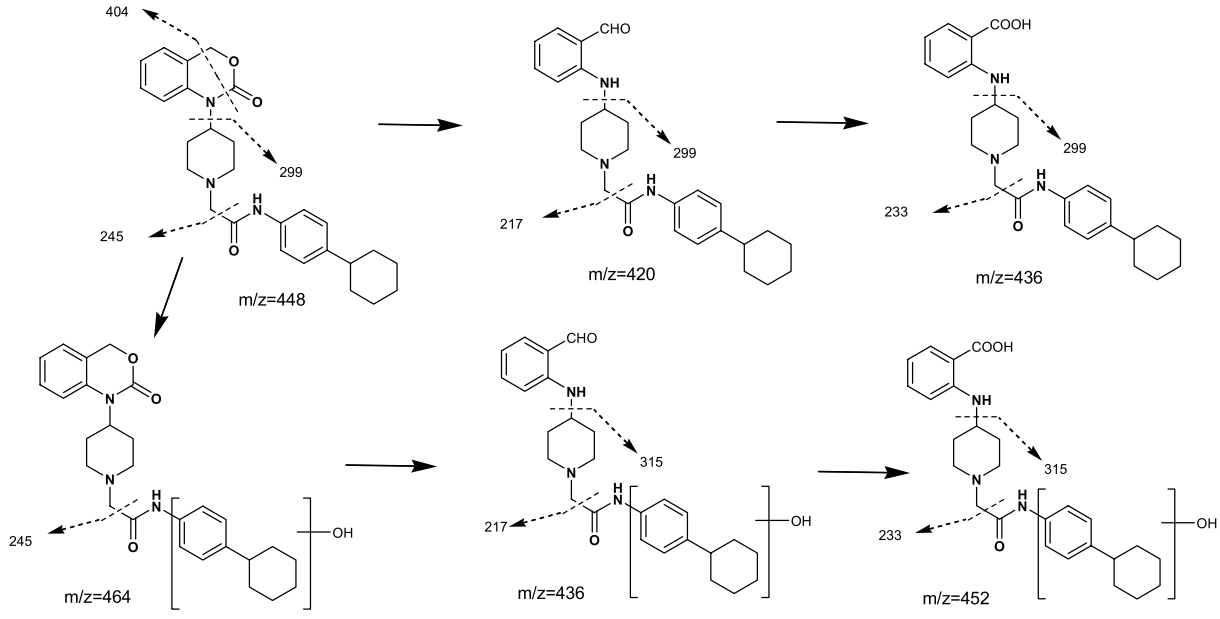
We initially examined the benzoxazinone ring; thus a small number of derivatives were synthesized to examine the effect of the nature and the position of the substituents on the half-life (Table 2). The majority of the compounds tested showed a better metabolic profile than those displayed by the parent compounds. The 6-methoxy derivative was of particular interest as it displayed a good balance of affinity and increased half-life.

Turning our attention to the southern substituent, 10 amide substituents **A**–**J** were chosen based on the SAR from the original investigation[7] (Fig. 3). A selection of compounds was tested in the in vitro metabolism studies and the results are shown in Table 3. When the 6-methoxy substituent was coupled with the carbazole moiety

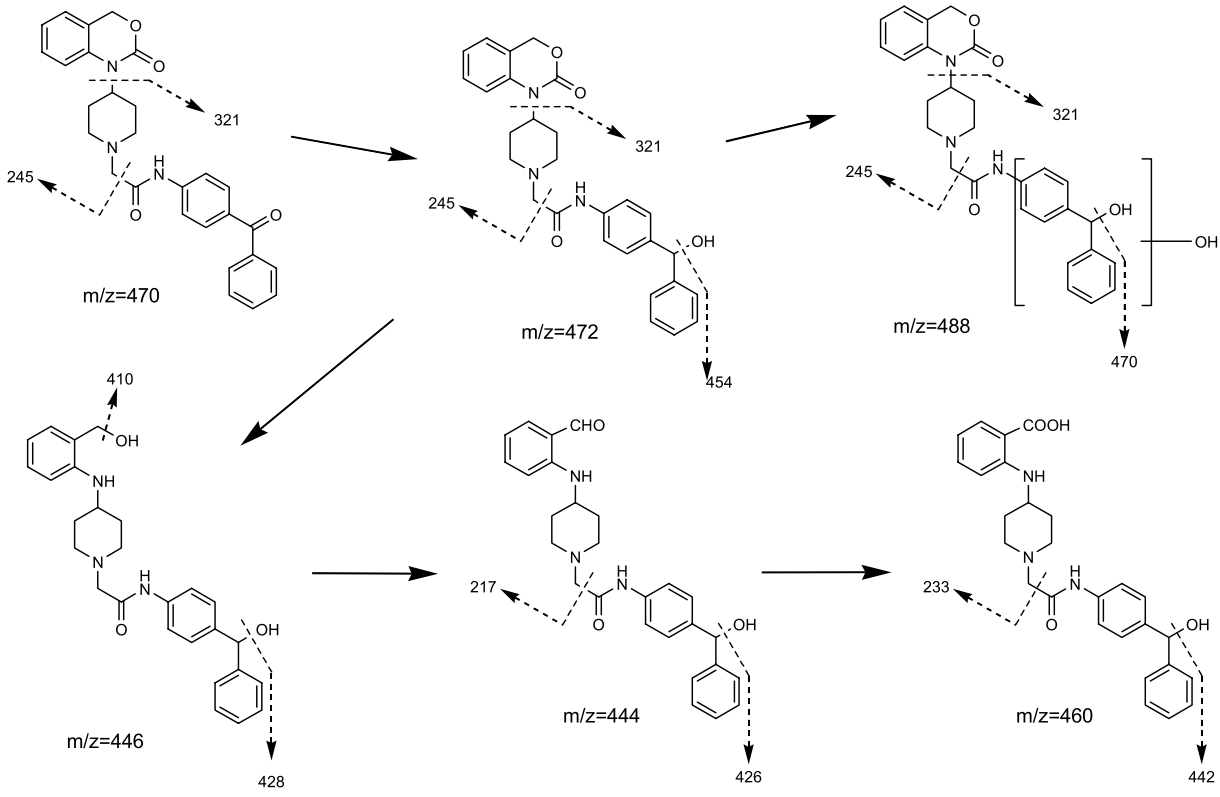
Table 1. Metabolic stability of compounds **1–3**

Entry	Compound	I: R^1	R^2	Parent drug remaining after 1 h (%)[a]			In vitro half life (min)	
				IC_{50}, nM	Rat	Human	Rat	Human
a	**1**		H	>1000	0	0	15	16
b	**2**		H	7.6	7	6	4	15
c	**3**		H	3.4	10	25	18	29

[a] Incubation concentration: 10 μM.



Metabolic pathway compound 1



Metabolic pathway compound 2

Figure 1. Metabolic profile of compounds **1–3**.

15, improved metabolic stability and a longer half-life (human) was observed whilst affinity was maintained.

From the data it is clear that the southern substituent plays an important role in the overall metabolism profile of this series. The benzophenone (B), fluorenone (D), and the reduced version (C) all correspond to low in vitro half-life in both rat and human microsomes. As the ring system becomes more electron rich, an improvement in half-life is noted as with the carbazole derivatives (G and H) (Figs. 3 and 4).

The combined data from Tables 2 and 3 when represented graphically, revealed several trends. In general, substitution on the benzoxazinone ring led to an increase in the in vivo half-life with substitution at the 5-position

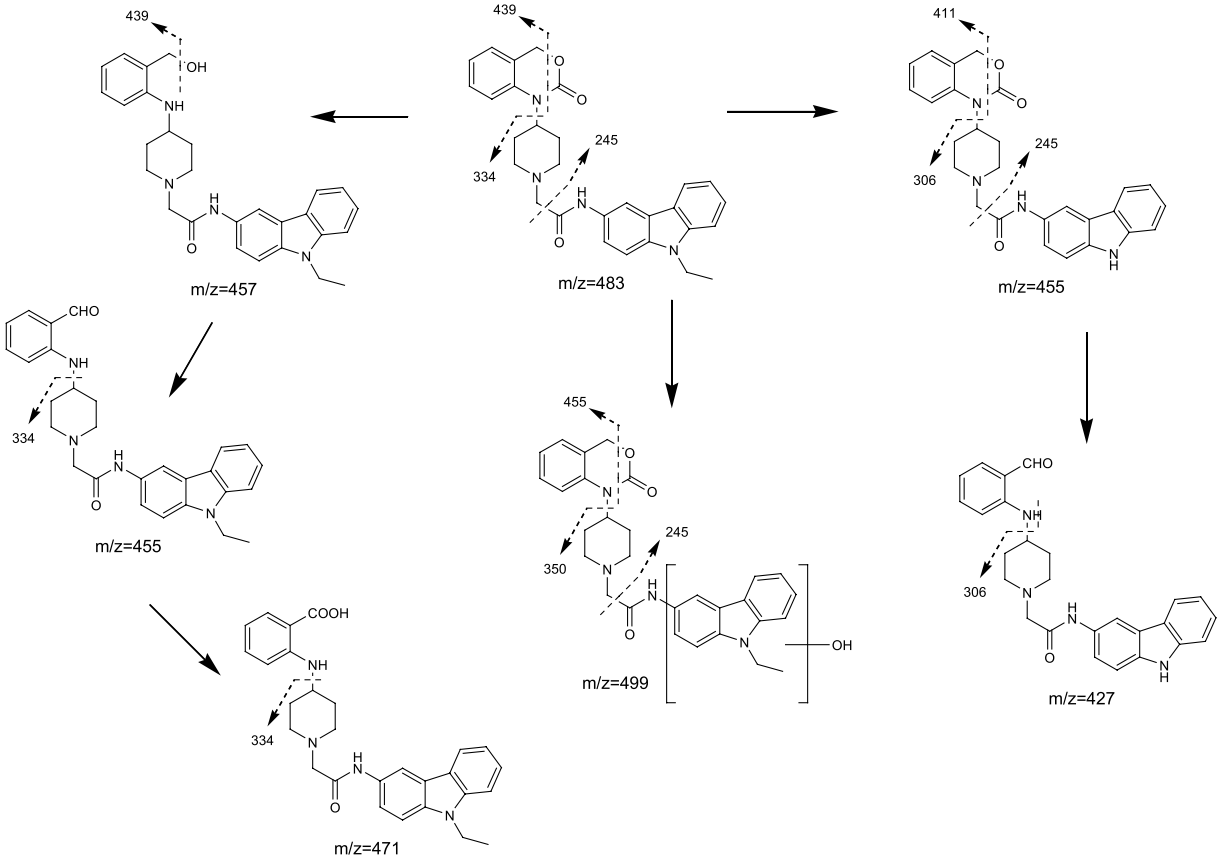


Figure 1. (*continued*)

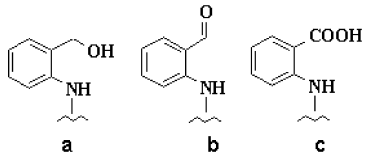
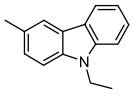
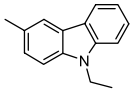
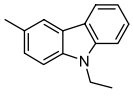
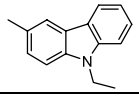


Figure 2. Meatabolic transformations of the benzoxazinone ring system.

being preferred over the alternative positions (Fig. 5A).

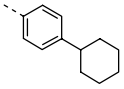
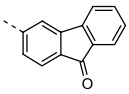
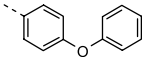
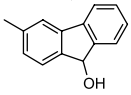
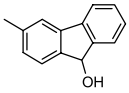
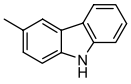
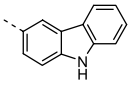
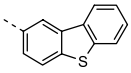
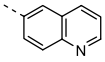
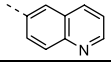
No examples of disubstituted compounds were prepared in this study. Although the selected substituents do not cover a wide property space, they do provide an indication of the effect of electronic and steric factors on the overall microsomal stability profiles. In general, there is

Table 2. Study of the benzoxazinone substitution on metabolic stability

Entry	Compound	I: R^1	R^2	Parent drug remaining after 1 h (%)[a]			In vitro half life (min)	
				IC_{50}, nM	Rat	Human	Rat	Human
d	**4**		5-F	27.5	42.29	38.3	53.3	48.0
e	**5**		5-Cl	54.6	43.3	75.2	63.6	106.4
f	**6**		6-MeO	9.2	41.6	48.1	50.5	69.4
g	**7**		7-Me	28.9	16.2	76.9	25.8	86.1

[a] Incubation concentration: 10 μM.

Table 3. Study of the variation of the amide moiety on metabolic stability

Entry	Compound	I: R^1	R^2	Parent drug remaining after 1 h (%)[a]			In vitro half life (min)	
				IC_{50}, nM	Rat	Human	Rat	Human
h	**8**		7-F	48.4	24.3	31.1	30.1	39.1
i[b]	**9**		6-MeO	7.5	0	2.2	3.5	2.6
j	**10**		5-F	16.5	14.4	44.0	21.5	64.8
k	**11**		7-Me	>1000	0	63.0	3.5	90.1
l	**12**		7-F	39.7	0	2.9	7.1	12.6
m	**13**		8-MeO	222	0	8.4	6.8	17.2
n	**14**		5-F	461	11.3	62.0	19.3	95.0
o	**15**		6-MeO	17	31.0	63.5	36.2	106.9
p	**16**		7-Cl	134.9	24.0	19.6	32.7	28.4
q[c]	**17**		6-F	7.7	0.9	5.4	2.5	14.4
r	**18**		6-MeO	41.8	23.2	43.5	28.9	43.4

[a] Incubation concentration: 10 μM.
[b] Incubation concentration: 5 μM.
[c] Incubation concentration: 1 μM.

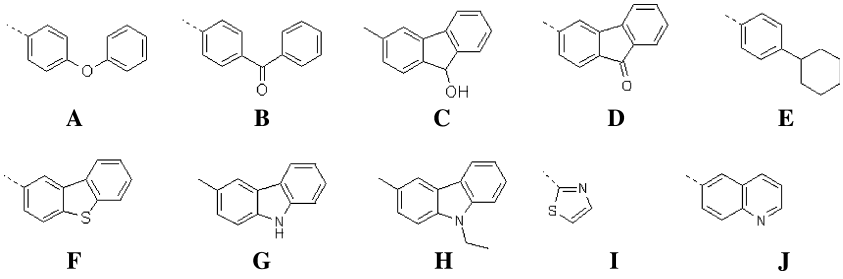


Figure 3. Amide substients.

a good correlation between rat and human half-life data for the methoxy derivatives although the carbazole derivative **15** has a significantly longer half-life in human than rat. This trend also holds true for the fluoro derivative **14**. The methyl substituent in particular confers better stability in the human microsomes (Fig. 5B). It is interesting to note the lack of correlation between rat and human half-life data with the 7-methyl derivative **11** which is much more stable to human microsomes than to rat

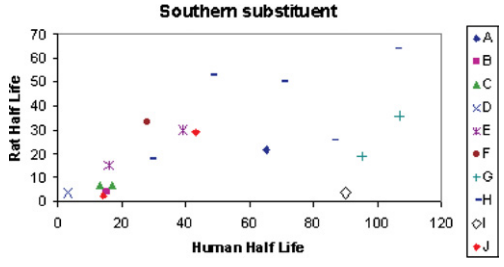


Figure 4. Analysis of the amide substituents.

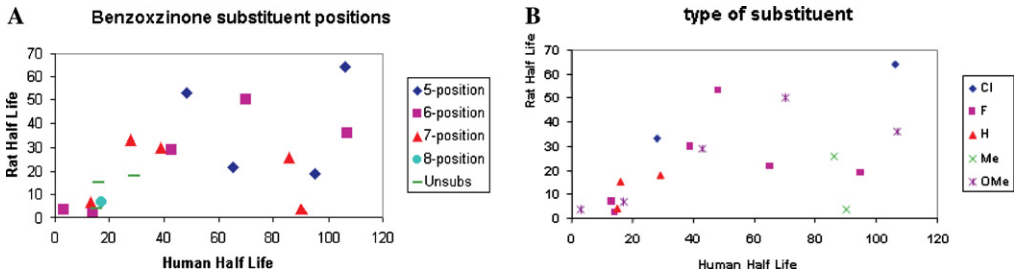


Figure 5. Analysis of the benzoxazinone substituents.

($t_{1/2}$ 90 min in human versus $t_{1/2}$ 3.5 min in rat). To a lesser extent this is also true for compounds **7** and **14**, which may suggest species differences with regards to metabolism. Fortunately, this increase in metabolic stability did not come at the expense of activity and these compounds retained potency in the low nanomolar range. Based on the current results, it is reasonable to suggest that microsomal stability can be achieved through substitution of the benzoxazinone ring without loss of activity. Additional studies would be required to see the effect of polysubstitution of the benzoxazinone ring system on microsomal stability and activity.

In summary, the metabolic profile of a series of potent benzoxazinone NPY5 antagonists was investigated with the aim to relate their metabolic stability with the structural features. From the results, the two sections of the molecule, the northern benzoxazinone ring and the southern substituent are both prone to metabolism. The data set shows that by altering the substituent pattern on the benzoxazinone moiety, rather than the amide section, compounds with better microsomal stability and with equal potency to the original lead compounds (**1**–**3**) were obtained. This leads to the conclusion that the metabolism observed is not a factor of each part of the molecule independently but rather is a factor related to the molecule as a whole, with the different sections acting in combination. Hence, it seems that the metabolic pathway leading to opening of the benzoxazinone ring does not favour the presence of an electron rich moiety in the southern amide section of the molecule resulting in compounds with increased stability to microsomal degradation. The results obtained from this study will allow further optimization of this new structural class of NPY Y5 antagonists.

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8. For the in vitro metabolic study male rat liver microsomes from TEBU-Xenotech, code R3000 and human liver microsomes (mixed gender) from TEBU-Xenotech, code H0610 have been used. Concentration of the studied compounds was 10 μM. The in vitro elimination half-life ($t_{1/2}$) was obtained using the expression $t_{1/2} = 0.693/b$, b being the slope found in the linear fit of the natural logarithm of the parent drug remaining percentage versus incubation time.
9. All metabolites were detected by LC/MS run on a quadrupole Ion Trap instrument (DECAXP, ThermoFinnigan-Corporation, San Jose, CA) operating in positive electrospray. The chosen mobile phase was (A) formic acid pH 2.5 (B) acetonitrile in a gradient elution performed from 15% to 45% in (B) in 30 min, at a flow rate of 0.20 ml/min. The column used was a X-Terra MS 2.1 × 150 mm (Waters).