Department of Pharmacology

Qualifying Examination (Part I)

December 15 & 17, 2009

Please remember that this is a closed-book examination. You must be prepared to answer 4 of the 7 questions. Although not necessary, you may prepare written answers, overhead figures, or any type of materials that you think might be useful in the presentation of your answers. You may bring such preparation materials with you to the examination. The oral examination itself will not extend beyond two hours.

BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

The reinforcing effect of psychostimulants such as cocaine and amphetamine involves the interaction of the drugs with the dopamine (DA) signaling. However, recent data suggest that other neurotransmitter systems may also be involved in mediating aspects of the psychostimulant-induced reward. One line of evidence in support of this notion is that mice lacking DA transporter (DAT) or DA (dopamine-deficient mice, DD; mice have no DA but normal norepinephrine) display behavioral responses associated with psychostimulant reinforcement such as conditioned place preference (CPP). Figure 1 shows that DD mice can form CPP to cocaine to a similar extent as wild type mice (Fig. 1A) and that this action is mimicked by fluoxetine in DD but not wild type mice (Fig. 1B).



Figure 1. Conditioned place preference without dopamine. During the pre-testing session, the mice were tested for the time spent in each of the two (black and white) chambers of the conditioned place preference apparatus without any drugs. During the training period, the mice received a dose of the drug in one chamber and saline in another (pairing). During the post-testing session, the mice did not receive any drugs but were tested for the time spent in the drug- and saline-paired chambers. The difference between the time spent in the drug-paired chamber during pre-testing and post-testing session is indicative of CPP. (A) CPP response to varying doses of cocaine in dopamine-deficient (DD) and wild type (WT) mice. * - p<0.05; ** - p<0.01 as compared to the pre-test. (B) Fluoxetine (5 mg/kg) produces CPP in DD but not WT mice. * - p<0,05 to pre-test.

- 1. What neurotransmitter system(s) and protein(s) are targeted by fluoxetine? What disease(s) fluoxetine is used to treat? What does the absence of CPP to fluoxetine in wild type animals is indicative of in terms of the drug's clinical utility?
- 2. Devise two tests that could confirm that the neurotransmitter system in question 1 above plays the critical role in the fluoxetine-mediated CPP in DD mice.
- 3. Propose a mechanism, including a site of action in the brain, by which cocaine is able to induce CPP in DD mice. Propose experiments to test both molecular and site of action elements of your model.

The disposition of orally administered triazolam was investigated in mice engineered with a knockout of the mouse Cyp3a gene and which also had the human CYP3A gene expressed in the liver only (Cyp3a (-/-)Tg-3A4Hep), in the intestine only (Cyp3a (-/-)Tg-3A4Int), or in both liver and intestine (Cyp3a (-/-)Tg-3A4Hep/Int).

All mice received oral triazolam and subgroups were treated with vehicle (untreated controls), the tyrosine kinase (EGFR) inhibitor gefitinib, or the antifungal ketoconzole. The plasma levels of triazolam and its primary CYP3A metabolite (1'-OH triazolam) were measured by LC-MS and the results presented graphically (Fig. 1) and tabulated as the AUC (Table 1).



Figure 1. Plasma concentration versus time curves of triazolam (A–C) and 1'-OH triazolam (D–F) after oral triazolam administration (0.5 mg/kg) and coadministration of gefitinib (25 mg/kg, B and E) or ketoconazole (35 mg/kg, C and F) are shown for Cyp3a(-/-), Cyp3a(-/-)Tg-3A4_{Hep}, Cyp3a(-/-)Tg-3A4_{Int}, and Cyp3a(-/-)Tg-3A4_{Hep}/Int mice. Note differences in scale for triazolam and 1'-OH triazolam panels. Data are shown as the mean concentrations, and error bars represent the S.D. (n = 4-5 per time point).

TABLE 1

Systemic exposure (AUC) of triazolam and 1'-OH triazolam after oral triazolam administration (0.5 mg/kg) and oral coadministration of gefitinib (25 mg/kg) or ketoconazole (35 mg/kg) to mice

	Vehicle	Gefitinib	Ketoconazole
<u>Triazolam</u>			
Сур3а (-/-)	317 ± 62	294 ± 21	374 ± 50
Сур3а (-/-)Тg-3А4нер	370 ± 49	$248 \pm 14^{*}$ †	588 ± 68**††
Cyp3a (-/-)Tg-3A4Int	$140\pm15^{**}$	132 ± 23**	458 ± 44*†††
Cyp3a (-/-)Tg-3A4Hep/Int	$130\pm19^{**}$	77 ± 15***†	$549 \pm 38^{**} + + +$
Wild-type	$194\pm23^{*}$	N.D.	$467\pm43^*\dagger\dagger\dagger$
<u>1'-OH Triazolam</u>			
Сур3а (-/-)	165 ± 34	145 ± 13	186 ± 19
Сур3а (-/-)Тg-3А4нер	175 ± 21	193 ± 56†	97 ± 13**†††
Cyp3a (-/-)Tg-3A4Int	211 ± 27	$235\pm35^{\star}$	144 ± 23*†
Cyp3a (-/-)Tg-3A4Hep/Int	261 ± 40*	202 ± 45	150 ± 22†
Wild-type	237 ± 29	N.D.	$144 \pm 14^{*}^{++}$

AUC₀₋₃₂₀ min (h . μ g/l), area under plasma concentration-time curve up to 320 min.

Data are presented as means \pm S.D., n = 4-5.

* P <0.05, ** P <0.01, and *** P <0.001 compared with Cyp3a(-/-) mice;

+ P < 0.05, + P < 0.01, and + + P < 0.001 compared with untreated strain.

N.D., not determined.

Questions

- 1. Present an evaluation of the pharmacokinetic data with preliminary conclusions on the principal means of elimination of orally administered triazolam and the effects of gefitinib and ketoconazole.
- 2. Outline experiments to establish the mechanisms of action of gefitinib and ketoconazole.
- 3. How would you explore the involvement of factors besides CYP3A4 in the disposition of triazolam?
- 4. What additional information would be required to judge the utility of these humanized mice in predicting human DMPK?

A young man is seen by an endocrinologist for weight loss and hypoglycemic (low blood sugar) episodes.

1) You test his blood insulin level, and it is appropriately low given his low blood sugar. What other endocrine system (other than the pancreas) may be dysfunctional? What laboratory finding(s) would confirm dysfunction in the system you suspect is involved?

2) How could you differentiate between a problem in the central nervous system and a problem outside of the CNS to explain his clinical findings? In particular, describe the regulation of the level of the hormone you suspect is involved by the CNS and the measurements you could make to determine the cause. How would you determine if the endocrine gland is responding appropriately?

3) This person is also found to have hypotension (low blood pressure). You are concerned that a second hormone system could be dysfunctional. Name a hormone that regulates blood pressure. What laboratory test(s) could confirm a deficiency in this hormone system? Can a single CNS problem explain deficiencies in the hormone discussed in #1 as well as this one?

You receive samples from a cancer cell biopsy that shows unusually aggressive growth, proliferation, and metastasis properties. After successfully generating a stable cell line that retains the same properties in culture, you determine that the mTOR pathway is constitutively activated. S6-kinase activity is chronically elevated and other hallmarks of cellular events downstream of mTOR are similarly upregulated. The cancer cells appear to be unresponsive to rapamycin. Preliminary analysis of the signaling pathways indicates that the PIP₃ levels are similar to those in control cells, but phosphatidic acid (PA) production is abnormally high.

Task- Outline in detail three distinct pathways for generating PA.

(a)The distinct substrates/starting materials should be identified for each of these three pathways.

(b) Design an experiment to determine where the abnormally high levels of PA are coming from. Discuss how you determine flux through these pathways? Describe your working hypothesis and different outcomes you would expect to find if each of these pathways contained the transforming mutation.

(c) Discuss some of the practical constraints associated with interrogating each of the three pathways.

A novel, cytoplasmic tyrosine kinase (Bmx) was identified that is expressed only in cardiac myocytes. Mice with targeted deletion of Bmx were viable and appeared normal. Placing a band on the thoracic aorta, a procedure that greatly increases afterload, results in pressure-induced cardiac hypertrophy in wild type animals. The phenotype of the Bmx null animals is described below.



Figure 1. Loss of Bmx prevents pressure overload–induced hypertrophy: functional and anatomic indices. A, Mice were euthanized 8 weeks after Thoracic Aortic Constriction (TAC) or sham surgery, and heart weight to body weight (HW/BW) ratio was determined. *P=0.004 vs WT sham for HW/BW, #P=NS vs Bmx KO sham. Bars are SEM. B, Images of hearts from mice euthanized 8 weeks after surgery. C, Kaplan–Meier survival curve (starting n values: WT TAC, 23; WT sham, 10; Bmx KO TAC, 9; Bmx KO sham, 7). D, M-mode echocardiographic images from WT and Bmx KO mice. E, Ejection fraction and left ventricular mass data obtained by echocardiography at 8 weeks after sham or TAC surgery. *P<0.01. Bars are SEM (n values for all groups: WT TAC, 6; WT sham, 9; Bmx KO TAC, 9; Bmx KO sham, 7).

Summarize your interpretation of the data above and the role of Bmx in cardiac hypertrophy. Outline an *in vitro* and *in vivo* strategy to identify small molecules that interact with Bmx. Assuming that you will identify several compounds with activity at Bmx, outline a strategy to compare the relative activities of these compounds using both *in vitro* and *in vivo* approaches.

The soluble mediator(s) of tubuloglomerular feedback (TGF) remain uncertain. Recently, two separate lines of knock-out mice were investigated to determine the involvement of two enzymes capable of dephosphorylating adenine nucleotides in the TGF response. One enzyme, ecto-5'-nucleotidase (e-5'NT), catalyzes the dephosphorylation of adenosine monophosphate (AMP), and the second enzyme, nucleoside triphosphate diphosphohydrolase 1 (NTPDase1), dephosphorylates ATP and ADP (final product is AMP). Both enzymes are expressed on glomerular and mesangial cells near the macula densa. Mice with homozygous deletion alleles (i.e., complete knockouts, denoted by -/-) are healthy and have normal renal function at baseline. Specifically, urine osmolality, baseline GFR, blood pressure, body weight, renal blood flow, and plasma renin concentration were not statistically different between knockout and wildtype (WT) littermates. However, there were significant differences in TGF responses as illustrated in Figures 1 and 2.



Questions:

- 1. Explain the observations made with both sets of genetically engineered mice, then propose one unifying hypothesis that incorporates these findings.
- 2. What other approaches could have been used to investigate the involvement of these two enzymes in TGF?
- 3. Predict the effect of NTPDase1 knockout on the mechanism of renin release.

Recent findings indicate that in regulating the signaling by Gq-coupled GPCRs, GRK2 has two modes of action: a) GRK2 phosphorylates the receptor, preparing it for arrestin binding; b) GRK2 (via its N-terminal RGS-like domain) also directly binds the GTP-liganded α -subunit of Gq and prevents it from activating effectors. The following mutant forms of GRK2 were created in which one or both of these functions were disrupted: Gq-binding deficient (R106A and D110A), kinase dead (K220R), or the Gq-binding deficient/kinase-dead (R106A/K220R) GRK2.

A group of investigators is studying the regulation of endogenously expressed M3 muscarinic acetylcholine receptor (M3) using RNA interference and over-expression, focusing on GRK2 and arrestins. In these cells M3 is the only receptor responsive to carbachol. In each case below they demonstrated that only the intended protein (indicated in each figure) is knocked down. Their results are shown in Figs 1-3.



Fig. 1. GRK2-mediated regulation of calcium mobilization after M3 mAChR activation. **A**, effect on calcium mobilization. Seventy-two hours after siRNA transfection, HEK-293 cells were loaded with Fura-2/AM and stimulated with 10 μM M3 agonist carbachol.

Mean <u>+</u> SEM increase in the peak calcium transient after stimulation with 10 μ M carbachol from five individual experiments (***, p < 0.001 using two-tailed *t* test). **B**, calcium mobilization in HEK-293 cells stably expressing indicated forms of GRK2 at comparable levels. Mean <u>+</u> SEM increase in peak calcium mobilization in cells expressing vector (pcDNA3), wild-type, Gq- binding deficient (R106A, D110A), kinase dead (K220R), or the Gq-binding deficient/kinase- dead (R106A/K220R) GRK2 (*, p < 0.05 for GRK2-K220R; ***, p < 0.001 for wild-type GRK2, as compared to pcDNA3 control).



Fig. 2. Effect of arrestin knockdown on calcium mobilization after M3 activation. A. Cells were harvested 72 h after transfection and processed as in Fig.1. Shown is a representative calcium trace from five independent experiments. B. Mean \pm SEM increase in the peak calcium transient after stimulation with 10 µM carbachol from five individual experiments (***, p < 0.001).



Fig. 3. Effect of GRK and arrestin knockdown on ERK activation by M3. A. effect of GRK2 knockdown on ERK1/2 activation. Serum-starved cells were treated with 100 µM carbachol for the indicated times. B, mean fold increase in ERK2 activation. Blots were incubated simultaneously with primary antibodies

specific for phospho-ERK1/2 and total ERK2 overnight. Phospho-ERK1/2 fluorescence was normalized to total ERK2 fluorescence. The data are presented as the fold increase in ERK2 activation over basal (*, p < 0.05; **, p < 0.01). C, effect of arrestin knockdown on ERK1/2 activation. D, mean fold increase in ERK2 activation. Phospho-ERK1/2 was normalized to total ERK2. The data are presented as the fold increase in ERK2 activation over basal (**, p < 0.01).

Questions:

- 1. Compare the effects of the knockdown of GRK2 and arrestins 2 and 3 on M3-induced calcium mobilization and ERK activation. Compare the effects of over-expression of WT GRK2 and its mutants with specific functions disrupted by mutagenesis to identify their relative importance in reducing calcium mobilization.
- 2. Propose a model of signaling which accounts for the effects on calcium and ERK activation.
- 3. Propose experiments to test your model.
- 4. Describe at least one alternative mechanism of GPCR-induced ERK activation and compare it to your model.
- 5. Describe at least one GPCR-independent mechanism of ERK activation.