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*Rapid Communication*

# An Integrated Method to Determine Epithelial Transport and Bioactivity of Oral Drug Candidates *in Vitro*

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## INTRODUCTION

Development programs to identify orally-active drugs are typically a lengthy process of eliminating numerous potential compounds to identify a few putative leads which have appropriate oral bioavailability characteristics. The physical and metabolic barriers presented by the intestinal mucosa greatly dictate oral bioavailability. The relative impact of these barriers for a given drug molecule depends largely on the route taken by that drug across the mucosa. Drug molecules can cross either through (transcellular) or between adjacent (paracellular) cells or by a combination of both [1, 2]. Each route presents unique permeability and metabolic challenges for a transporting compound.

Recently, *in vitro* systems have been used more and more to assess the potential for drug candidates to overcome these physical and metabolic barriers presented by the intestinal epithelia. At present, confluent monolayers of an adenocarinoma cell line which demonstrates a well-differentiated enterocyte-like phenotype, e.g. Caco-2 cells [3], are frequently being used to assess intestinal drug absorption [4]. Flux measurements for a compound from the mucosal (apical) surface to the serosal (basolateral) chambers of media bathing these cells have been useful in establishing the relative transport properties for several classes of molecules, including peptides and peptide analogs [5]. Metabolism can be assessed by evaluating a drug's stability at both the mucosal and serosal surfaces of these tissues in vitro [6]. Both of these methods commonly involve HPLC methodologies to quantitate drug transport rates and for the separation and characterization of metabolic products.

While these HPLC methods are powerful tools, they do not provide any information about a drug candidate's bioactivity and HPLC sensitivity is typically limited to micromolar drug concentrations. This may be insufficient to assess physiologically significant levels of potent drug candidates. These lower levels of drug concentrations are typically measured by bioassay. Although the combined data from separate studies of epithelial drug transport and metabolism can be evaluated and related favorably to measured values of oral bioavailability [7], the individual assessment of transport and metabolism is too time consuming for a large drug screening effort.

In this manuscript, we describe an integrated method to assess both transport and bioactivity for a potent class of peptides at levels below HPLC sensitivity. This approach uses cells capable of responding to the presence of a biologically active drug. The relative response by these cells, therefore, is a product of the amount transported, metabolic stability and bioactivity properties for each candidate molecule. To illustrate this approach, we have used confluent monolayers of Caco-2 cells set above primary cultures of rat pituitary cells which respond to growth hormone releasing peptide (GHRP) by secreting growth hormone (GH) [8, 9]. The compounds tested in our studies were synthetically derived from the sequential reduction and optimization of GHRP-6 (Figure 1), the most widely studied GHRP [10]. By using a single measurement as a biological endpoint, the release of GH in this case, we were able to rapidly and simultaneously assess relative transport rates and metabolic stability for several GHRP analogs having exceptional bioactivity.

## MATERIALS AND METHODS

*Materials*. The GHRP analogs used were prepared by the Genentech Bioorganic Chemistry Department (Figure 1) and described in detail elsewhere [9]. Dulbecco's modified Eagle's medium (DME), fetal bovine serum (Select) were purchased from JRH Biosciences (Lenexa, Kansas). HEPES, DME low glucose media containing $NaHCO_3$, MEM non-essential amino acids, L-glutamine, Penicillin-Streptomycin solutions, and trypsin-EDTA (0.05% and 0.53 mM, respectively) solutions were from Gibco Laboratories, Life Technologies Inc. (Grand Island, New York). Rat tail collagen was obtained from Collaborative Res. Inc. (Bedford, Massachusetts). Snapwell™ culture dishes (12 mm; 0.4 μm pore size) and T-flasks were obtained from Costar (Cambridge, Massachusetts). The Caco-2 cell line was purchased from the American Type Culture collection (Rockville, Maryland).

*Methods*. GHRP concentrations were analyzed by HPLC using a Vydac $C_{18}$ reversed-phase column. The mobile phases were 0.1% trifluoroacetic acid/water and 0.1% trifluoroacetic acid/acetonitrile (B). The samples were eluted using a gradient of 30-75% B over 9 min at a flow rate of 1 ml/min and with the column temperature maintained at 40 °C. Compounds were detected by UV absorbance at 214 nm. Concentrations were determined using a standard curve. Growth hormone (GH) concentrations were determined using a two-site ELISA [8]. The mean for each group was determined and analyzed by one-way analysis of variance with a post-hoc Student-Newman-Keuls. $EC_{50}$ values were calculated using a 4-parameter curve-fit program from data

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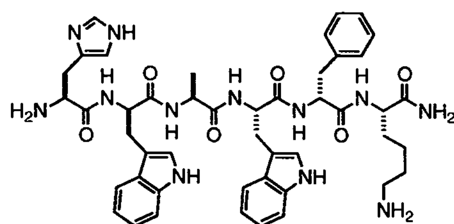
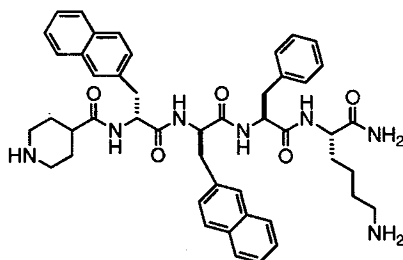


**GHRP-6**

$ED_{50}$ 6.2 ± 1.5

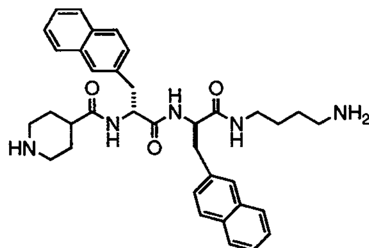
MW = 872



**G-7039**

$ED_{50}$ 0.18 ± 0.04

MW = 797



**G-7083**

$ED_{50}$ 17.1 ± 3.3

MW = 595

**Fig. 1.** Chemical structures of the growth hormone releasing peptides (GHRP) used in these studies. GHRP-6 structure is presented for comparison to G-7039 and G-7083. $EC_{50}$ values obtained from direct pituitary challenges are shown (n = 3-5).

obtained from the direct application of compounds onto pituitary cell cultures. Significance was defined as $p<0.05$ as compared to controls.

*Media.* DME/low: Dulbecco's modified Eagle's low glucose media supplemented with 10% fetal bovine serum (Hyclone A-111-L), 25 mM $NaHCO_3$, 20 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. DME/High: Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. KR: Kreb's Ringer bicarbonate buffer (pH 7.4) supplemented with 40 mM D-glucose.

*In Vitro Cell Culture.* Anterior pituitaries from 200-220g female Sprague-Dawley rats were enzymatically dispersed and cultured in DME/low media as described previously [8]. Briefly, $4 \times 10^5$ pituitary cells were plated in each well of a Corning Costar 6-well plate and incubated in 2 ml of DME/low media for 3 days at 37°C in an atmosphere of 5% $CO_2$ and 90% relative humidity. On day three, the media was discarded and the cells were washed extensively as described previously [8]. Pituitary cells were then ready for use in the integrated transport assay.

Caco-2 cells (passage number 25-35) were grown to confluent monolayers as previously described [11]. Briefly, cells were maintained at 37°C in DME/high media in an atmosphere of 5% $CO_2$ and 90% relative humidity. Cells were passaged every week at a split ratio of 1:3 in 75 $cm^2$ flasks and seeded onto prewetted and collagen coated polycarbonate filters (Snapwells™) at a density of 63,000 cells/$cm^2$. Growth media (DME/high) was replaced every other day and confluent monolayers of Caco-2 cells were used 20-26 days after seeding.

*Integrated Transport Assay.* Exact methods for flux measurements are described elsewhere [11, 12]. Prior to use in transport experiments, Caco-2 monolayers were washed twice with KR media and allowed to recover in KR media added back to the apical or mucosal (0.5 ml) and basolateral or serosal (2 ml) surfaces. Media on the mucosal surface was then exchanged with KR media containing various concentrations of the compound being assessed for transport. For studies where transport rates were determined by HPLC analysis, 1 ml volumes were removed from the serosal surface (with volume replacement and correction for the volume removed in subsequent calculations) at 45 min intervals while the cells were incubated at 37 °C and 90% relative humidity. For integrated transport assays, the basolateral surfaces of the Snapwells™ were rinsed in 2 ml PBS and, with the peptide solution still in the apical chamber, then transferred onto wells containing pituitary cells grown for 3-days as described above. Caco-2 cells and pituitary cells were then co-incubated for 30 - 60 min at 37°C. At the conclusion of an incubation, media was removed from the serosal chamber (bathing the pituitary cells) for subsequent analysis of GH. Pituitary cells were enzymatically removed with 10% trypsin and counted in a Coulter Counter model ZM.

*Assessment of Caco-2 Monolayer Integrity.* Transepithelial resistance (TEER) was determined before and after flux and integrated transport experiments using the Millicell®-ERS (Millipore) as described previously [7]. Monolayer quality was also assessed by direct staining and visualization as described previously [13]. Briefly, monolayers were fixed in 10% formalin-PBS, stained with 12 μg/ml acridine orange for 5 min and washed with PBS. Filters were carefully cut out, placed cell-side up on a slide, mounted with a coverslip and inspected for monolayer integrity using fluorescence microscopy (Leitz Aristoplan, E. Leitz Wetzlar GmbH).

## RESULTS AND DISCUSSION

Components of the method described in this report are depicted in Figure 2. This method, established here for the

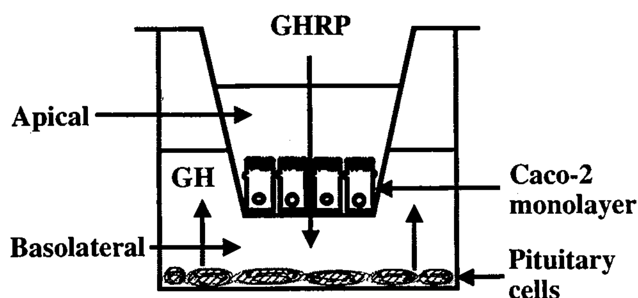



Fig. 2. Cartoon depicting cell culture system used in the integrated assay system. The upper reservoir is the site of GHRP application to the apical (mucosal) surface of the Caco-2 monolayer. The lower reservoir baths both the basolateral (serosal) surface of the Caco-2 monolayer and the pituitary cells adhering to the bottom of the culture well. A polycarbonate membrane physically separates the basolateral surface of the Caco-2 and pituitary cells. The transported GHRPs elicit GH release from the pituitary cells.

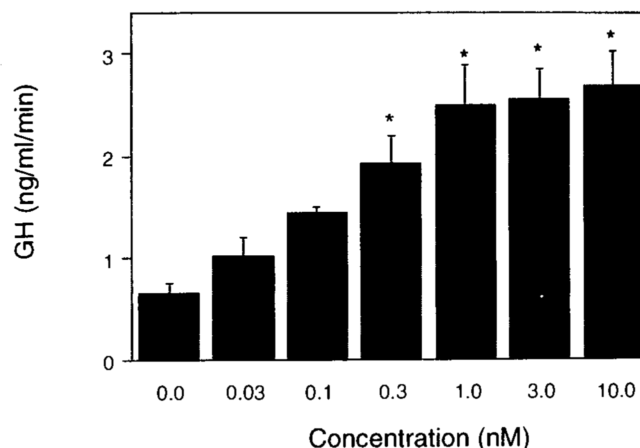



Fig. 3. Sensitivity and linearity of a response for a GHRP analog. Levels of released growth hormone following direct application of G-7039 to the pituitary (bioassay) cell layer are presented (mean ± SEM). Note linearity at concentrations from 0.03 - 1.0 nM and a plateau of response above 1.0 nM. Statistical significance ($p<0.05$) vs. control is denoted by an asterisk.

analysis of GHRP analogs, consists of two fairly generic components: an intestinal cell barrier and a bioassay readout or endpoint. The intestinal cell barrier selected was that of Caco-2 cell monolayers, a cell line which emulates many of the biochemical and physiological properties of the absorptive apical villus region of the small intestine and a well-established cell model system for intestinal drug transport [5, 14]. In the present studies, Caco-2 monolayer TEER values were determined prior to ($195 \pm 17\ \Omega \cdot cm^2$) and at the conclusion ($158 \pm 31\ \Omega \cdot cm^2$) of flux experiments to verify maintenance of barrier function. These values are consistent with previously reported values for intact monolayers of Caco-2 cells [7, 12, 14]. Visual inspection of monolayers also suggested the maintenance of intact Caco-2 monolayers during the course of these transport studies (data not shown).

For most of the GHRP analogs studied, no transport across Caco-2 monolayers could be detected by HPLC methodologies unless initial donor compartment concentrations were at least 500 μM (data not shown). Unfortunately, such high concentrations of some compounds resulted in significant disruption of the Caco-2 monolayers. This lack of measurable compound transport (using an HPLC method with a level of sensitivity of ~ 0.125 nM) at lower donor concentrations was consistent with an intact barrier function by these Caco-2 monolayers. G-7083 was the only compound of a limited series of analogs tested which did not produce monolayer disruption at higher (500 μM) concentrations and its permeability across Caco-2 monolayers was determined by HPLC measurements to be $2.0 \pm 0.1 \times 10^{-6}$ cm/sec. This permeability value is similar to that previously observed for peptides of similar molecular mass [15].

The bioassay in this report involved cultures of isolated rat anterior pituitary cells and the readout was an ELISA for released GH. The ability for this system to respond to physiological levels of a GHRP (i.e. G-7039) analog is demonstrated in Figure 3. At higher concentrations a saturation of the response was observed, consistent with a receptor-mediated stimulation of pituitary cells by the GHRP analog.

Using the integrated Caco-2/pituitary cell assay, the evaluation of two GHRP analogs are described for illustration (Figure 4). Three to four independent experiments were run for each compound with the donor GHRP concentration starting at ~ 5 nM and going up to ~ 500 nM. The absolute concentrations for each compound varied slightly due to differences in molecular mass. These donor compartment concentrations are well below those which elicited damage to the Caco-2 monolayers in the direct transport studies described above. HPLC analysis of the serosal samples from these flux experiment failed to show any detectable concentrations of GHRP analogs (data not shown) while the elicitation of GH release readily demonstrated bioactive peptide transport rates across the Caco-2 monolayer. G-7083 applied to the mucosal surface of Caco-2 monolayers over a concentration range of 8-800 nM resulted in a linear response of GH release. A similar escalating dose (5-500 nM) of a more potent analog, G-7039, demonstrated linearity at the lower

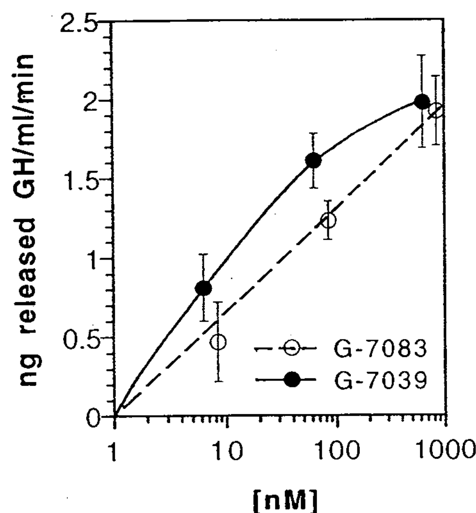



Fig. 4. Integrated assay evaluation of GHRP analogs. Values represent GH release (mean ± SEM) in response to increasing concentrations of biologically active GHRP analogs following transport through Caco-2 monolayers. Background GH release values have been subtracted to normalize the data.

doses. The apparent lack of linearity at higher doses may reflect a saturation of GHRP receptors on the pituitary cells.

An estimate of GHRP analog biological activity in this static transport system can be made using values obtained in the linear assay range. For example, the estimated $EC_{50}$ of G-7039 in the integrated transport assay is approximately 40 nM, based on initial concentrations in the apical chamber prior to flux (Figure 4). The corresponding $EC_{50}$ from pituitary cells challenged directly with G-7039 is $0.18 \pm 0.04$ nM [9]. This value was essentially reproduced in our challenge system (Figure 3) and corresponds roughly to a 200-fold lower amount of bioactive GHRP, suggesting that less than 0.5 % of bioactive GHRP analog in the donor chamber successfully crossed the Caco-2 monolayer. This value is in agreement with transport measurements of other peptides of similar molecular mass [1, 2].

The present study describes an *in vitro* method to simultaneously assess aspects of epithelial drug transport and bioactivity. Since transport and metabolism, along with relative biological activity, for an individual molecule are interwoven aspects of lead compound selection, this assay can potentially provide a streamlined approach to evaluate several classes of compounds. In our hands the time required to perform the studies described presently was half that required if transport and bioassay studies were performed sequentially rather than in an integrated format. This time savings, however, would be variable and should be more significant if a number of molecule classes were being assessed since the development of new analytical methods for each class of molecule to be tested can drastically slow a large drug screening effort. Fast through-put and the possibility for system miniaturization and automation for such an assay might make it even more appealing for large drug screens. In our illustration of this approach we have demonstrated the translocation of two bioactive GHRP compounds across Caco-2 monolayers based on GH release from pituitary cells. It is likely that such *in vitro* approaches will become even more crucial in the future as a means of increasing the screening rate for the numerous compounds being generated as a result of breakthroughs in combinatorial chemistry approaches. Further, the use of a sensitive detection system may be crucial where non-physiological drug concentrations may be harmful to the integrity of the barrier but are required to accommodate an HPLC detection limit.

Although this integrated assay approach offers the advantages described above, it requires the maintenance and coordination of two different cell types and does not provide direct information on drug permeabiity or metabolic events. Rather, it focuses on bioactivity information. One must consider that, because of differences in transport properties, the apparent rank order of net potency for drug candidates may be different from rankings obtained directly with a bioassay. It is also important to note that Caco-2 monolayers may or may not represent a realistic metabolic and transport barrier for certain classes of molecules. Caco-2 cells do appear useful in modeling human colonic drug transport [12], but little information is currently available as to such a correlation with human small intestinal tissues. In summary, we have assessed the bioactivity and transport of GHRP analogs using an integrated assay with a single endpoint. The utility of such an assay approach may be useful in accelerating oral drug discovery.

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