ORIGINAL ARTICLE

β-arrestin2 recruitment at the β2 adrenergic receptor: A luciferase complementation assay adapted for undergraduate training in pharmacology

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Abstract

In the context of pharmacology teaching, hands-on activities constitute an essential complement to theoretical lectures. Frequently, these activities consist in exposing fresh animal tissues or even living animals to selected drugs and qualitatively or quantitatively evaluating functional responses. However, technological advancements in pharmacological research and the growing concerns for animal experimentation support the need for innovative and flexible in vitro assays adapted for teaching purposes. We herein report the implementation of a luciferase complementation assay (LCA) enabling to dynamically monitor β-arrestin2 recruitment at the β_2 adrenergic receptor in the framework of pharmacological training at the faculty of Pharmacy and Biomedical Sciences. The assay allowed students to quantitatively characterize the competitive antagonism of propranolol, and to calculate pEC_{50}, pK_B, and pA_2 values after a guided data analysis session. Moreover, the newly implemented workshop delivered highly reproducible results and were generally appreciated by students. As such, we report that the luciferase complementation-based assay proved to be a straightforward, robust, and cost-effective alternative to experiments performed on animal tissues, constituting a useful and flexible tool to enhance and update current hands-on training in the context of pharmacological teaching.

KEYWORDS
Arrestin, G protein-coupled receptor, luciferase, pA_2, teaching, undergraduate, β_2 adrenergic receptor

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1 | INTRODUCTION

The teaching of general pharmacology entails an essential focus on the concepts of agonism and antagonism, two notions underpinning the common understanding of drug properties. Parameters such as pEC_{50}, pK_B, and pA_2 quantitatively define the action that biologically active compounds exert on their molecular targets and are thus of utmost importance in the educational curriculum of pharmacology courses. In this context, practical pharmacology classes are instrumental in fostering the learning and comprehension of such parameters among undergraduate students. These workshops typically consist in the characterization of drug-induced effects on fresh animal tissues, notably measuring guinea pig ileum contraction in response to different ligands.[1,2] Students are then required to compute EC_{50}, pK_B, and pA_2 values on the basis of the experimental data that they collected, resulting in an active learning experience, promoting a quality and long-lasting understanding.[3]

Despite their relevant pedagogic value, ex vivo experiments encounter several drawbacks. Their implementation frequently turns out to be cumbersome, poorly reliable, and endowed with low reproducibility, hindering their pedagogical outcomes.[4] Of note, their integration in pharmacology programs has been drastically reduced because of growing concerns related to animal experimentation.[5] Plus, ex vivo experimentation is not aligned with the current drug investigation practices in the pharmaceutical industry, mainly relying on high-throughput screening (HTS) campaigns with cellular bioassays.[6] Given that many students in the health-care sector will be confronted with such technologies in their future workplace, there is an evident unmet need to provide students with an introduction to up-to-date methods in drug research.

In this context, a number of computer-based simulations of ex vivo experiments have been developed in order to provide time- and resource-saving alternative approaches, especially when delivering workshops to large cohorts of students.[2] Such in silico approaches meet most of the educational objectives of pharmacology practical classes, especially concerning data handling and experimental design.[4,7] However, computer-based platforms do not support the practical aspects of laboratory training, which is of particular relevance in Pharmacy and Biomedical Sciences programs.[1,4]

Taken together, these concerns have called for the development of animal-free, screening-compatible experimental alternatives to ex vivo educational pharmacology experiments. We herein report the design of a novel pharmacology workshop based on a split firefly luciferase complementation assay (LCA), inscribed in the undergraduate programs of Pharmacy and Biomedical Sciences. The activity started with the experimental phase, aiming at employing the LCA to characterize the competitive antagonism of propranolol at the beta-2 adrenergic receptor (β2AR), followed by a guided data analysis session leading to the calculation of propranolol pK_B and pA_2 values.

What is already known

- Experimental workshops are an essential complement to theoretical lectures in pharmacology teaching
- Experimental activities based on animal tissues are not aligned with current research practices in pharmacology and with the 3Rs principle.

What this study adds

- The luciferase complementation assay provides a reliable and animal-free alternative for practical activities in pharmacology teaching for undergraduate students
- Combining practical and computer-based activities provides students with a thorough overview of pharmacological research practices, encountering marked appreciation.

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2 | MATERIALS AND METHODS

2.1 | Materials

Isoproterenol, (±)-propranolol, poly-L-lysine, glass coverslips, paraformaldehyde (PFA), and anti-FLAG (RRID:AB_10950495) antibodies were purchased from Sigma-Aldrich (Diegem, Belgium). Primary anti-hemagglutinin antibodies (RRID:AB_390918) were purchased from Roche (Basel, Switzerland). D-Luciferin was purchased from Promega, UK. Secondary antibodies (donkey anti-mouse coupled to AlexaFluor 488, fluorophore (RRID:AB_2556746) and goat anti-rat antibodies coupled to AlexaFluor 555 fluorophore (RRID:AB_2535855)), together with bovine serum albumin, L-glutamine, penicillin/streptomycin, ammonium chloride, trypsin-EDTA, and microscope slides were purchased from Thermo Fisher Scientific (Waltham, MA). Cell culture medium (Dulbecco's Modified Eagle Medium) and Puromycin (50 mg/mL Stock) were purchased from Invitrogen (Merelbeke, Belgium). 96-well plates were purchased form Greiner Bio-One (Wemmel, Belgium). 4′,6-diamidino-2-phenylindole-containing mounting medium was purchased from Biotium (San Francisco, CA). Fetal bovine serum (FBS) was purchased form Biowest (Riverside, MO).

2.2 | Molecular cloning

The human $β_2AR$ coding sequence was cloned and amplified from Human Embryonic Kidney 293 (HEK 293) cells (RRID:CVCL_0045) cDNA, while the rat β-arrestin2 coding sequence was amplified from β-arrestin2 green fluorescent protein (GFP; 35411; Addgene, Cambridge, MA). Both sequences were engineered as described by Dupuis et al.[8] The N-terminus of the $β_2AR$ sequence was fused to a signal sequence (KTIIALSYIFCLVFA)[9] and a FLAG epitope (DYKDDDDK), whereas its C-terminus was attached to a flexible linker (GSSGGG)

followed by the C-terminal fragment of the firefly luciferase enzyme (Fc-Luc, amino acids 413–549, as described by Takakura et al [10]). The β-arrestin2 protein was flanked with a HA epitope (YPYDVPDYA) and the N-terminal moiety of the luciferase enzyme (FnLuc, amino acids 1–415), also followed by a flexible linker (GGGS). The modified β_2AR and β-arrestin2 sequences were, respectively, cloned into pIRESpuro and pIREShygro3 plasmids (TakaraBio, Kusatsu, Japan) yielding constructs pIRESpuro-β_2AR-FcLuc and pIREShygro3-β-arr2-FnLuc.

2.3 | Cell culture and transfection

HEK293 cells were cultivated at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% FBS. After the transfection and selection steps described by Dupuis et al.[8] a clonal population of cells stably expressing the β-arrestin2-FnLuc protein (HEK293-β-arr2-FnLuc cells) was obtained. HEK293-β-arr2-FnLuc cells were then transfected with the pIREShygro3-β_2AR-FcLuc vector using the calcium phosphate precipitation method.[11] Three days after transfection, the cells were selected for puromycin resistance (1 μg/mL) in order to obtain stable transfectants, herein referred to as HEK-LCA cells. Cells were then tested for the expression of β-arr2-FnLuc and β_2AR-FcLuc proteins by immunofluorescence. After selection of clones, cell lines were routinely cultured with medium containing hygromycin and puromycin to maintain good expression levels.

2.4 | Immunofluorescence

HEK-LCA cells plated on poly-L-lysine-coated glass coverslips were fixed with a 4% PFA solution for 30 minutes. Background fluorescence was reduced by exposing the coverslips for 15 minutes to a 50 mM solution of ammonium chloride. Non-specific binding of antibodies to the samples was prevented with a blocking solution composed of 2% BSA diluted in PBS. Cell membranes were permeabilized through the addition of 0.3% Triton X-100 detergent to the blocking solution. Immunodetection of the FLAG and HA epitopes was performed by incubating coverslips overnight at 4°C with mouse anti-FLAG and rat anti-HA primary antibodies (1/1000 dilution) followed by a 1-h exposure (at room temperature) to anti-mouse antibodies coupled to Alexa Fluor 488 (1/1000 dilution), and anti-rat antibodies coupled to Alexa Fluor 555 (1/1000 dilution), respectively. After a washing step, nuclei were stained with 4′,6-diamidino-2-phenylindole, mounted on microscope slides, and analyzed with an EVOS fluorescence microscope.

2.5 | Luciferase complementation assay

The day of the experiment, students were provided with confluent T175 flasks with HEK-LCA cells and with 100 mM stock solutions

of isoproterenol and propranolol. They were instructed to harvest cells from one confluent flask with a trypsin-EDTA 0.05% solution at room temperature, to resuspend pelleted cells in 5 mL HBSS buffer and distribute them in 96-well plates (50 μl per well, with approximately 2×10^5 cells per well). Students were also instructed to dilute the drugs in HBSS buffer (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, and 10 mM HEPES, pH 7.4) in microtubes. Isoproterenol was to be tested at different concentrations (logarithmic dilutions) combined or not with defined concentrations of propranolol. Luciferase activity was tested after the addition of 50 μL of a 500 μM D-luciferin (Promega, UK) solution to each well, and, after an incubation period of 20–30 minutes, emitted luminescence was measured using a Victor X3 Plate Reader (Perkin Elmer, Waltham, MA).

2.6 | Participants and organization of the laboratory class

The laboratory activity was integrated in the pharmacology course given during the second and third years of the Pharmacy and Biomedical Sciences programs, respectively. During the academic year 2019–2020, a total of 139 students participated in the activity (103 were registered in the bachelor programs in Pharmacy and 36 in Biomedical Sciences). The practical pharmacology session was scheduled after completion of a series of theoretical lectures. These covered the concepts necessary to the understanding of the experimental design, granting the necessary background for the insightful interpretation of results. The attendance together with the completion of a laboratory report were compulsory requirements to pass the final exam. The objectives and the experimental protocols were presented in an introductory lecture and in a series of online videos. Each workshop session involved 20–24 students, divided into groups of three on a voluntary basis. The day of the experiment, before the start of the activity, student knowledge about the key theoretical concepts required to accomplish the laboratory activity was evaluated by the means of a short written, closed-book test.

2.7 | Survey

One hundred and six students (76% of all participants) anonymously completed an online survey (in French language), consisting of seven, closed-ended, unipolar Likert scale items, answerable with 5-point agreement options ("Strongly Disagree", "Rather Disagree", "Neutral", "Rather Agree", and "Strongly Agree"). These items were designed to assess the overall opinion of students in regard to the main objectives of the workshop, notably improving the understanding of relevant pharmacological parameters, introduce students to research practices and data handling, together with reducing teaching-related animal experimentation. Completion of the questionnaire necessitated approximately
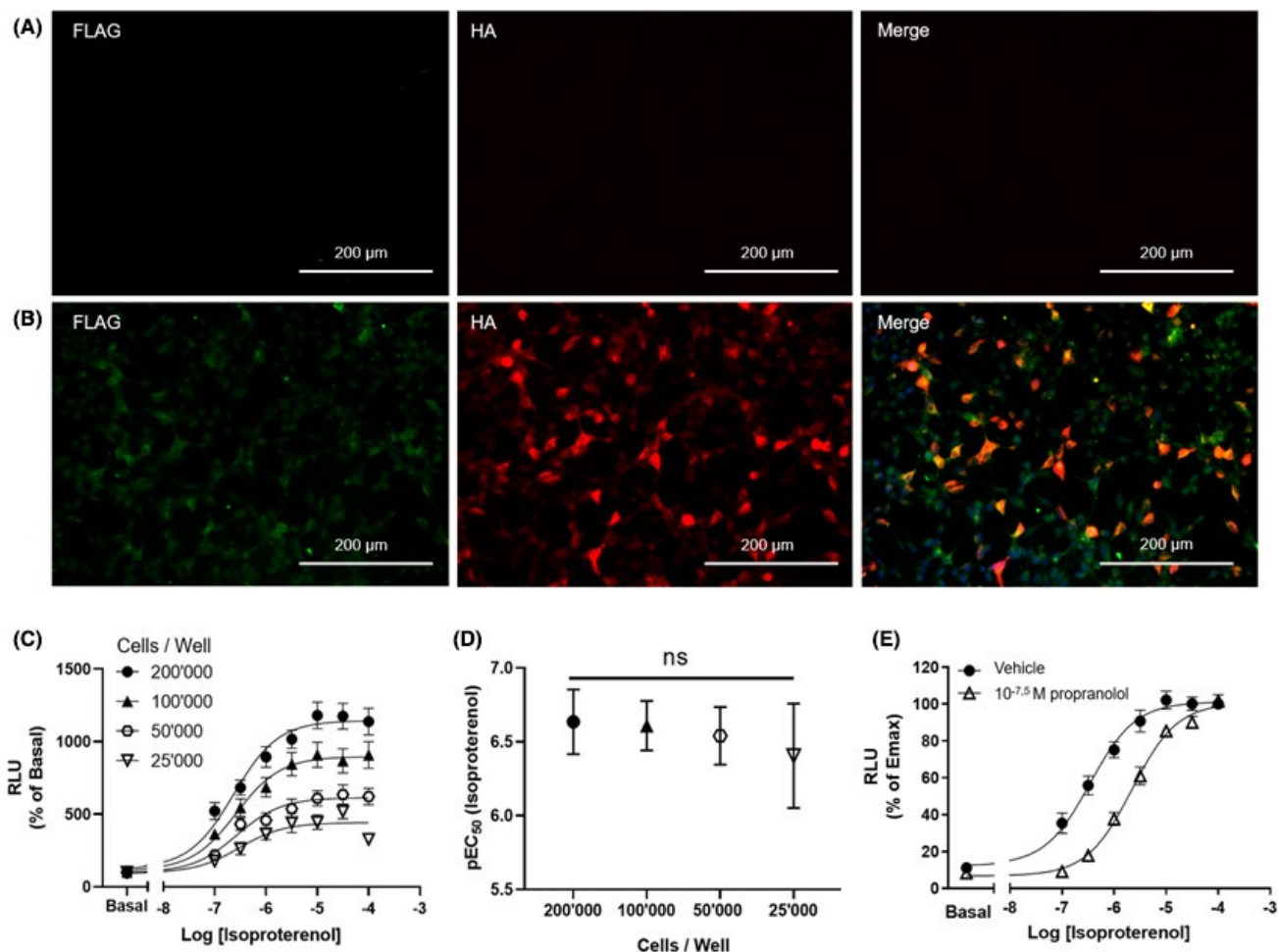
FIGURE 1 Validation of the LCA. Immunodetection of β_2AR-FcLuc and βarrestin2-FnLuc proteins in non-transfected (A) and HEK-LCA cells (B). β_2AR-FcLuc (green) and βarrestin2-FnLuc (red) were, respectively, immunodetected using anti-FLAG and anti-HA primary antibodies. (C) Isoproterenol-induced β-arrestin2 recruitment at the β_2AR at increasing cellular densities (25.000, 50.000, 100.000, and 200.000 cells per well). Data represent the mean ±SEM of six independent experiments performed in triplicate, normalized to the signal obtained from non-stimulated cells. D) pEC$_{50}$ values of isoproterenol obtained at the different tested cellular densities, revealing no significant difference (one-way ANOVA (ns, $p > 0.05$)). Data represent the mean ±SEM of six independent experiments performed in triplicate. E) Effect of $10^{-7.5}$ M propranolol on the cellular response induced by isoproterenol. Data represent the mean ±SEM of six independent experiments performed in triplicate, normalized to the maximal response of each condition

5 minutes, and was not mandatory to the successful completion of the pharmacology class.

2.8 | Data and statistical analysis

LCA luminescence readouts were expressed in relative light units (RLUs) as means ±SEM. Results were normalized to the maximal response obtained with isoproterenol (considered 100%) or to the signal obtained from non-stimulated cells (considered 100%) to control for receptor expression variation. During the validation steps, linear regression and statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, CA). During the teaching activities, students performed data analysis using an in-house developed Microsoft Excel spreadsheet including the Solver.xlam add-in macro. Survey answers were first summarized by descriptive statistics and then compared using a two-sided Fisher's test. In order to facilitate comparisons, we merged together the categories "Strongly Disagree", "Rather Disagree", and "Neutral". The same procedure was performed for the "Rather Agree" and "Strongly Agree" categories. Answer quantification was performed by transforming the aforementioned categories into quantitative variables. The score of 1, 2, 3, 4, and 5 was, respectively, assigned to the "Strongly Disagree", "Rather Disagree", "Neutral", "Rather Agree", and "Strongly Agree" categories.

2.9 | Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[12] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[13]

3 | RESULTS

3.1 | Cell model and protocol optimization

3.1.1 | Immunodetection of β_2AR-FcLuc and β-arr2-FnLuc proteins in HEK-LCA cells

HEK-LCA cells were obtained after stably transfecting HEK293 cells with pIRESpuro-β_2AR-FcLuc and pIREShygro3-β-arr2-FnLuc constructs. Immunofluorescence experiments were performed to verify the co-expression of β_2AR-FcLuc receptor and β-arrestin2-FnLuc protein, respectively, tagged with FLAG and HA epitopes. We analyzed the fluorescent microscopy images from non-trans-fected control cells (Figure 1A) and transfected cells selected with hygromycin and puromycin (Figure 1B). In these cells, we observed the superposition of green and red fluorescent signals, absent in images obtained from non-transfected cells, confirming the expression of the β_2AR-FcLuc and β-arr2-FnLuc proteins in HEK-LCA cells.

3.1.2 | Determination of the optimal cellular density for the design of the workshop experimental protocol

Next, we aimed at establishing the best working protocol enabling students to successfully perform LCAs. Cells were plated onto 96-well plates at different densities (200.000, 100.000, 50.000, and 25.000 cells per well) and exposed to increasing concentrations of isoproterenol. RLUs were normalized to the signal obtained from unstimulated cells plated at the same density ("basal"). The signal amplitude was maximal after approximately 15 min and remained stable for up to 45 min (data not shown). We could observe a con-centration-dependent increase in luciferase activity at all the tested cellular densities, yielding isoproterenol pEC_{50} values consistent with those from the literature [13] (Figure 1C). Plus, variations in cell densities did not result in significant alterations in the potency of isoproterenol ($p > 0.05$; one-way ANOVA) (Figure 1D). The analy-sis of concentration-response curves indicated a cell density-de-pendent nature of the luminescent signal, with the highest cellular density (200.000 cells/well) delivering the largest signal amplitude. Hence, we designed an experimental protocol requiring students to seed HEK-LCA cells at a 200.000 cells/well density, increasing the chances of success of the laboratory activity and facilitating its setup, since a single confluent T175 flask typically yields the 20 mil-lion cells needed for a single 96-well plate.

3.1.3 | The LCA allows the detection of propranolol competitive antagonism at the β_2AR.

Prior to organizing the workshop, we tested the effect of inhibit-ing isoproterenol-induced β-arrestin2 recruitment at the β_2AR using the beta-blocker propranolol. RLUs were normalized to the maximal value obtained for every condition and plotted in Figure 1E. When analyzing the concentration-response curves, we observed that propranolol ($10^{-7.5}$ M) induced a rightward shift in bioluminescent signal, indicating its expected competitive antagonism at the β_2AR (Figure 1E). Using the Gaddum equation,[14] we calculated the pK_B of propranolol at 8.23, in line with the available literature on this compound.[13]

3.2 | Laboratory activity

3.2.1 | Workshop setup and results

The pharmacology workshop was delivered to students enrolled in the second year of the bachelor's degree in Pharmacy and in the third year of the bachelor's degree in Biomedical Sciences, respec-tively, referred to as "Pharm" and "BioMed" students. The labora-tory activity consisted of the quantification of the competitive antagonism of propranolol for β-arrestin2 recruitment at the β_2AR. Students were required to harvest cells and seed them in 96-well plates at a density of 200.000 cells/well. Then, they treated cells with increasing concentrations of isoproterenol in combination with propranolol (at a concentration of 10^{-7}, $10^{-7.5}$, or 10^{-8} M) or a vehi-cle solution. After 20–30 min, luciferase activity readouts were col-lected and analyzed during a supervised data analysis session. An in-house developed Microsoft Excel® sheet enabled students to plot concentration-response curves and derive pEC_{50} and pK_B values from the experimental results they gathered, together with perform-ing Schild linear regression analysis leading to the obtention of the pA_2 value for propranolol.

In total, 46 groups of students participated in the activity. Forty-two groups obtained a detectable response in the described experi-mental conditions, setting the success rate of the LCA-based activity at 91%. Pooling together the experimental results of these 42 groups resulted in the concentration-response curves shown in Figure 2B, revealing the concentration-dependent inhibition of isoprotere-nol-mediated β-arrestin2 recruitment at the β_2AR in response to in-creasing concentrations of propranolol. The Schild linear regression analysis of the same data set, plotted in Figure 2C, determined a pA_2 value of 8.45 ± 0.04.

3.2.2 | The LCA-based activity showed marked reproducibility features

In order to assess the reproducibility of the LCA, the combined results of student groups having participated in the workshop on different days were compared by one-way ANOVA analysis fol-lowed by a Tukey post hoc test, revealing no statistically signifi-cant difference among the average pK_B or pA_2 values obtained by the different groups (Figure 3A). As mentioned above, students who took part in this workshop were enrolled in two distinct curricula: the bachelor degree in Pharmacy and the bachelor de-gree in Biomedical Sciences. Considering their distinct practical
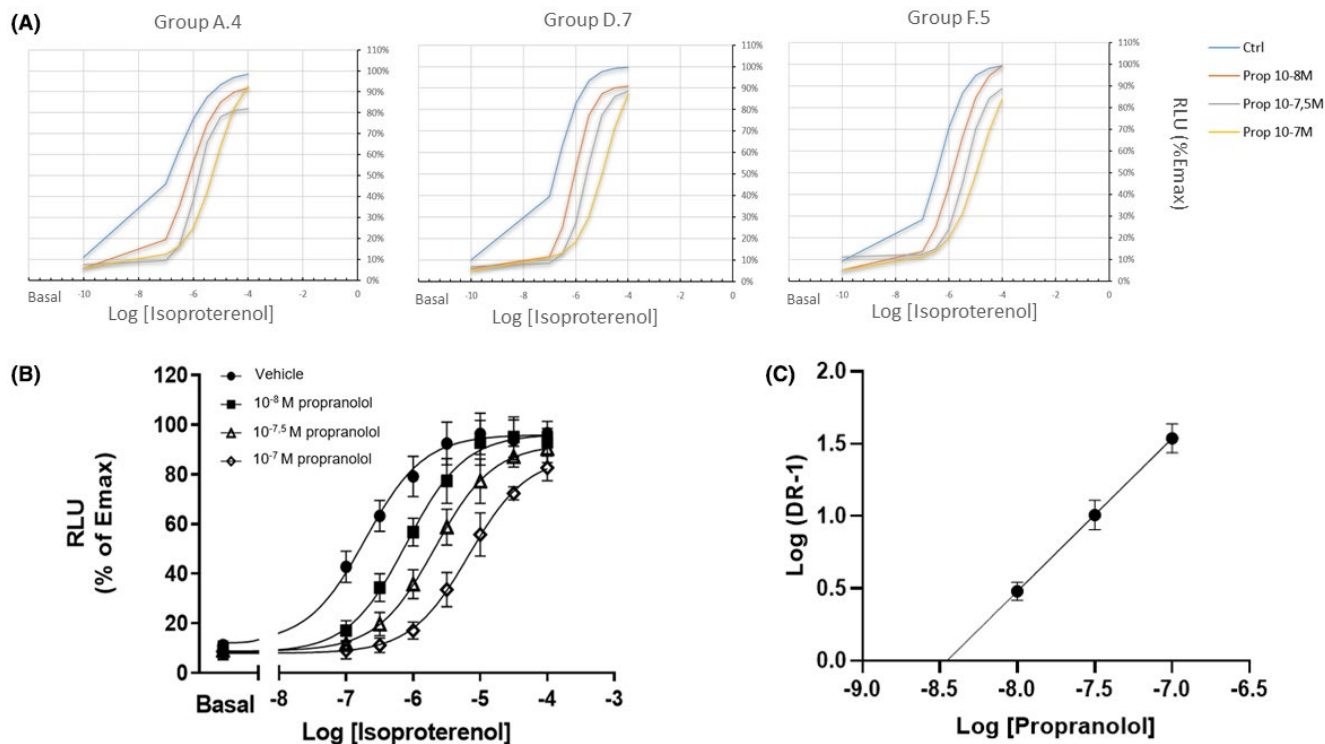
FIGURE 2 Results of the workshop. (A) Experimental results obtained by three different student groups at different days of the pharmacology workshop. Data represent the mean ±SEM of one experiment performed in triplicate and the non-linear fitting obtained using Microsoft Excel. (B) Concentration-response curves for isoproterenol-induced β-arrestin2 recruitment at the β_2AR in the presence of vehicle or increasing concentrations of the competitive antagonist propranolol. Data represent the mean ±SEM of 42 independent experiments performed in triplicate and the sigmoidal curve obtained using GraphPad Prism. (C) Schild Plot obtained after the analysis of data represented in A. Linear regression analysis yielded an X-axis intercept of 8.45 ± 0.04, corresponding to the pA_2 for propranolol. Data represent the mean ±SEM of 42 independent experiments performed in triplicate

laboratory experience, we hypothesized that their experimental results may substantially differ. Comparison of the mean pK_B or pA_2 values obtained by the two different student cohorts using a two-sided Welch t-test did not reveal any statistically significant difference (Figure 3B).

3.3 | Survey

Students having participated in the workshop were invited to complete an online survey on voluntary basis. In detail, we evaluated to what extent students agreed to the seven statements concerning (1) the perceived value of the workshop in their curriculum, (2) its contribution to their understanding of pEC_{50}, pK_B, and pA_2 pharmacological parameters, (3) the theoretical knowledge required for the understanding of the workshop, (4) the utility of this workshop in providing useful insights in pharmacological research practices, (5) the utility of this workshop in the development of practical laboratory skills, (6) to what extent the practical, hands-on approach was a useful complement to the computer-based feature of the workshop, and (7) whether reducing animal experimentation was a priority. Students answered expressing their agreement to the presented items employing a Likert scale with five modalities: "Strongly Agree", "Agree", "Neutral", "Disagree", and "Strongly

Disagree". Answers to the survey are summarized in Figure 4. Data analysis was performed by pooling together "Strongly Agree" and "Agree" answers under the category "Agree", whereas "Neutral", "Disagree", and "Strongly Disagree" answers were grouped under the category "Disagree". A total number of 106 students answered the questionnaire, implying a total response rate of 76%. Seventy-four of 103 Pharm and 32 of 36 BioMed students took part in the online evaluation, yielding a participation rate of 72% and 89%, respectively. In a defined scale from 0 to 5, Pharm and BioMed students responded with an average score of 4.27 ± 0.18 and 4.42 ± 0.18, respectively, placing the average answer in the "Agree" category. A two-tailed Student t-test did not reveal any statistical difference between the average response of the two cohorts. Nevertheless, differences emerged when comparing the answers using the two-sided Fisher's test. In detail, students enrolled in the Biomedical Sciences curriculum agreed significantly more than students enrolled in the Pharmacy curriculum with the statements 1, 2, 3, and 7 (Figure 5).

4 | DISCUSSION

Understanding and interpreting pharmacological parameters is of pivotal importance for health-care professionals. For this reason,
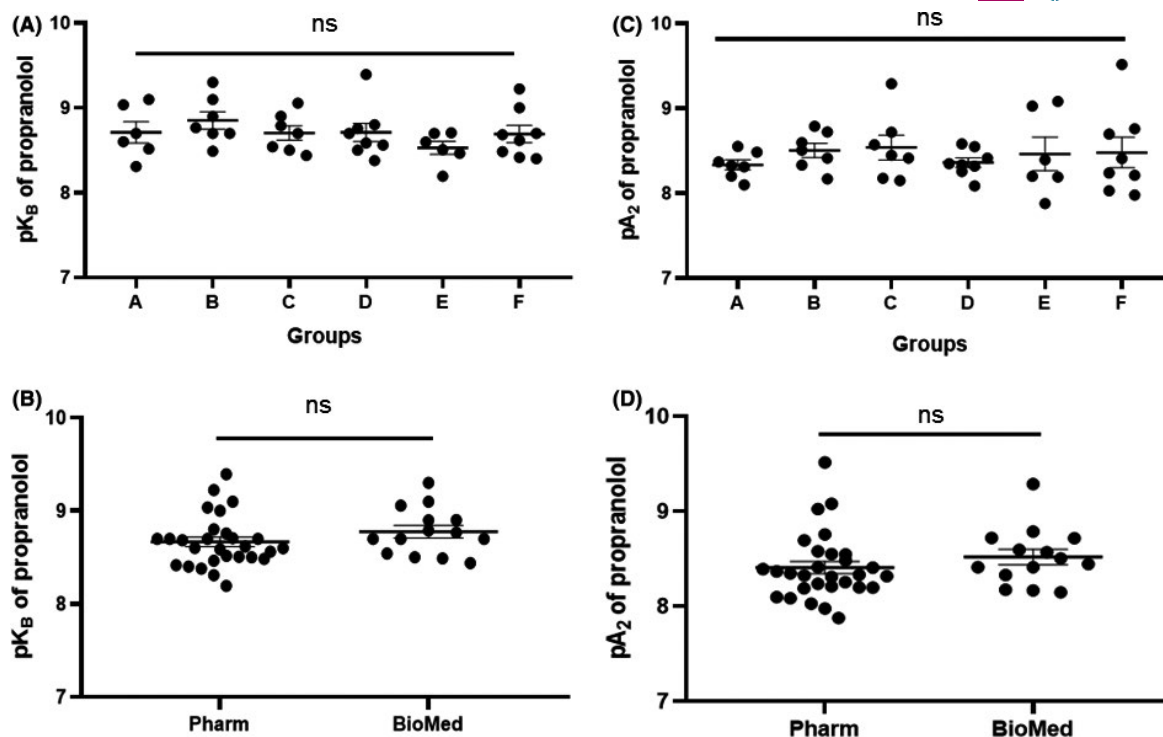
FIGURE 3 LCA reproducibility. (A) Mean pK_B and pA_2 values obtained for propranolol obtained by eight different student groups on eight different days of the workshop were compared using a one-way ANOVA analysis followed by a Tukey post hoc test, revealing no statistical difference (ns, $p > 0.05$). Data for each group represent the mean ±SEM of at least six independent experiments performed in triplicate. (B) Mean pK_B and pA_2 values for propranolol calculated by students enrolled in the Pharmacy (Pharm) or Biomedical Sciences (BioMed) programs were compared using a two-sided Welch t-test, revealing no statistical difference (ns, $p > 0.05$). Data for each group represent the mean ±SEM of at least 14 independent experiments

pharmacology undergraduate training frequently includes practical, laboratory-based activities aiming at manipulating pEC_{50}, pA_2, and pK_B values. Commonly, these activities involve experiments on fresh tissues obtained from laboratory animals. Albeit its physiological relevance, such experimental model implies a complex setup, resulting in scarce robustness and reproducibility. We herein report the development and implementation of an original LCA-based practical class, aiming at providing a reliable, straightforward, and animal-free option for pharmacology teaching. The assay has been applied to the human β_2AR, a G protein-coupled receptor (GPCR) of major interest in an educational context due to its importance in human physiology as well as pharmacotherapy. The LCA allowed the monitoring of agonist-evoked recruitment of β-arrestin2 at the β_2AR, a molecular event triggering not only receptor desensitization and internalization, but also contributing to downstream signaling.[15]

The LCA consists in the detection of protein–protein interactions in intact living cells by means of two complementary fragments of the firefly luciferase enzyme, FcLuc and FnLuc, respectively, fused to the proteins of interest. As these proteins come into close contact in response to defined triggers, the recombined enzyme is able to catalyze the oxidation of its substrate, which is accompanied by light emission.[10] After validating the expression of β_2AR and β-arrestin2 carrying the luciferase fragments in transfected HEK293 cells by immunofluorescence, we optimized

the experimental protocol to be communicated to student groups. Seeding cells at a high cellular density (200.000 cells/well) yielded the strongest bioluminescent signal amplitude and, considering that students had limited laboratory experience at the time of the workshop, opting for an elevated signal to noise ratio appeared as ideal to improve their chances to successfully accomplish the experimental tasks. Hence, the estimated potency of isoproterenol was found to be independent from cellular density. We also verified that propranolol, a β_2AR competitive antagonist, could inhibit isoproterenol-induced signal with a pK_B value comparable to literature data obtained from diverse in vitro approaches,[13] including LCA[10,16] and BRET.[17] As such, the LCA proved to be a valid tool for the pharmacological characterization of β_2AR ligands and convenient for didactic purpose in the context of a pharmacology workshop.

The goal of such experimental activity, organized in the bachelor programs in Pharmacy or Biomedical Sciences, was to exploit the LCA to assess and quantify the competitive antagonism of propranolol when detecting isoproterenol-induced β-arrestin2 recruitment at β_2AR. Students performed LCAs with a success ratio of 91%. The reported experimental failures were mostly associated with incomplete cell harvesting and inaccurate pipetting, leading to large variations or even to totally odd data. After data collection, students were able to observe that the concentration-response of isoproterenol underwent a rightward shift depending on the
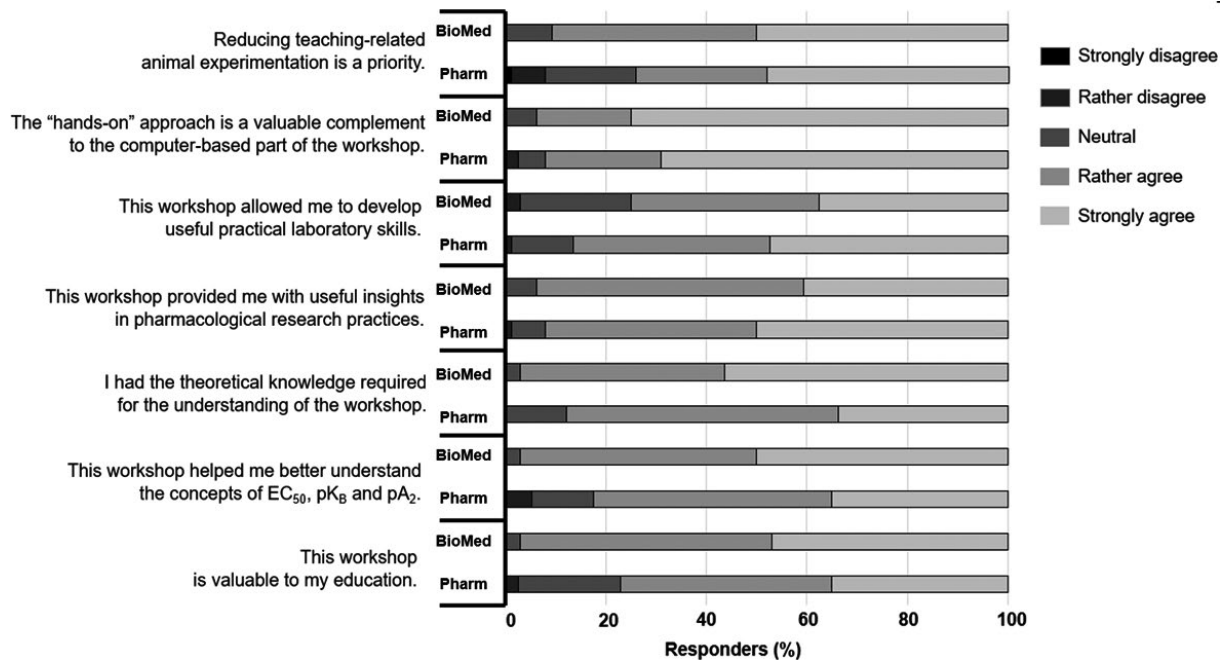
FIGURE 4 Evaluation of students' opinion on the pharmacology workshop. Students voluntarily and anonymously reported their agreement to seven items concerning the main features and objectives of the workshop. They answered using a 5-point Likert scale ("Strongly Disagree", "Rather Disagree", "Neutral", "Rather Agree", and "Strongly Agree"). Answers are hereby reported as a percentage of responders for each student cohort
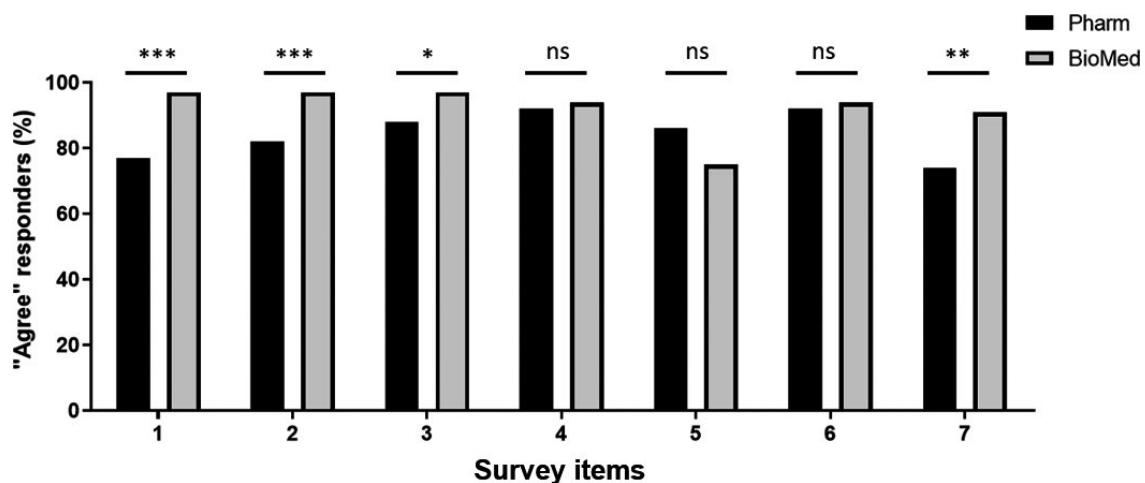

FIGURE 5 Differences in agreement with the survey items among student cohorts. "Rather Agree" and "Strongly Agree" answers were grouped under the "Agree" category. The percentage of agreeing responders was compared between the Pharmacy (Pharm) and Biomedical Sciences (BioMed) student cohorts through a Fisher's test, revealing a statistical difference for items 1, 2, 3, and 7 (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), while no statistical difference (ns $p > 0.05$) could be observed for statements 4, 5, and 6

concentration of propranolol. Thanks to a supervised data analysis session using an in-house developed Microsoft Excel sheet, students calculated pEC_{50} and pK_B values, together with performing Schild linear regression analyses yielding the pA_2 value for propranolol. The comprehensive analysis of the LCA experiments (N = 42) set the pA_2 value of propranolol at 8.45 ± 0.04, closely comparable to the estimated potency of propranolol reported in animal tissues.[13,18,19] This result supports the validity of LCA as a reliable alternative to

pharmacological experiments performed on animal-derived fresh tissue in the context of undergraduate training. Importantly, when analyzing the experimental results collected on different days of the workshop, we observed the robustness provided by the LCA. Mean pK_B and pA_2 values obtained for propranolol at different days were not statistically different.

BioMed students participated in the workshop after having acquired more laboratory experience than Pharm students, raising the

possibility of a cross-cohort variation in experimental results. Yet, statistical analysis of mean pK_B and pA_2 values obtained by Pharm and BioMed students did not reveal any statistically significant difference. As of rather straightforward application, LCAs may likely be exploited in any health care-related programs, without requiring any particular practical skill. However, the successful outcome of the workshop requires a series of activities aiming at priming students to the practical class. In the hereby reported case, students gained theoretical knowledge through pharmacology lectures, together with attending a specific session describing the rationale and the timeline of the experiment. Moreover, the preparation to the practical activity was supported by a series of short videos, available on the online platform of the pharmacology course. Student groups of up to 24 were then expected to run the experiment autonomously, under the supervision of two designed postgraduate teaching assistants, capable of providing guidance throughout the workshop, including the data analysis session.

Of importance was the opinion of students in regard to the newly implemented activity. Their appreciation of the laboratory activity was evaluated through a survey exploring their agreement with seven statements over the main goals and features of the LCA-based pharmacology workshop. Seventy-six percent of the students participated in the evaluation activity. Overall, the assay received a high degree of satisfaction, as the vast majority of students agreed with the presented statements. Supported by the survey results, we can conclude that the LCA-based workshop provided students with a laboratory activity considered as valuable for their education (indistinctively to their curriculum), helping them to familiarize with pEC_{50}, pK_B, and pA_2 parameters and providing them with useful insights in pharmacological research practices. The preparatory activities were also regarded as useful in the understanding of the workshop. Of note, the practical orientation of the activity was regarded as an asset, providing the occasion to develop useful technical skills and as a valuable complement to the computer-based data analysis session. Importantly, the majority of students showed to be particularly concerned by the reduction of animal experimentation, supporting the introduction of in vitro experiments in a teaching-related context.

Interestingly, the opinion of BioMed and Pharm students slightly differed. Students enrolled in the Biomedical Sciences curriculum considered the workshop as being more useful in the context of their education, together with showing more concern for animal experimentation. These results may be explained by the strong focus that the Biomedical Sciences program brings on fundamental research. Indeed, the practical aspects of bench-related activities, together with animal experimentation, may appear as more compelling to future professionals in the field of biomedical research rather than for future pharmacists.

In conclusion, we hereby report the successful implementation of an LCA in the context of undergraduate pharmacological teaching. Requiring currently available material, and delivering reproducible results, this assay offers a valuable and cost-effective in vitro alternative to educational experiments performed on living animals or on freshly extracted animal tissues, providing a suitable option for the reduction of teaching-related animal experimentation. In this context, computer-based simulations of pharmacological experiments have found widespread application in pharmacology teaching. Nevertheless, these approaches fail to initiate undergraduate students to practical laboratory activities and to the acquisition of practical skills that are necessary in the context of pharmacological research.[1] The pharmacology workshop developed at our university included both practical, on-the-bench experimentation with computer-based data handling and interpretation. Its implementation resulted in a fruitful blend that encountered strongly positive appreciation by students, together with facilitating their understanding of crucial pharmacological concepts such as pEC_{50}, pK_B, and pA_2. Moreover, the assay presented a relevant degree of reproducibility and robustness, together with a straightforward implementation, providing academics with a flexible methodological tool to design and run pharmacological experiments with an educational purpose. Its screening-compatible format aligns it with current practices in pharmacological research and the technological advances in the field. Of note, the assay was applied to the β_2AR after being initially designed to investigate the molecular interaction between β-arrestin2 and the GPR27 orphan receptor.[8] Similarly, the LCA might easily be adapted for a wide array of receptors and signaling pathways, providing teachers with relevant implementation flexibility. Of note, selecting drugs that are relatively stable in solution at room temperature, such as isoproterenol and propranolol, constitutes an advantage for student-oriented laboratory activities. In conclusion, we propose that likewise in vitro assays blending practical and computer-based activities—in combination with theoretical lectures and digital supports—may contribute in enhancing pharmacology teaching for students enrolled in health care-related university programs.

5 | DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research as stated in the BJP guidelines for Design & Analysis, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

N.D. and J.H. developed the LCA. M.F. and N.M. performed the preliminary experiments. E.H conceived and designed the didactic application of the LCA, together with supervising the workshop and the whole pharmacology course. N.M. developed the Microsoft Excel spreadsheet employed during the computer-based part of the workshop. N.M., M.R., and L.R. developed the teaching material and the protocol for the students. M.F. and E.H. designed and delivered the survey. M.F and P.B. performed data analysis. M.F. and E.H. wrote the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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