Determination of an appropriate dose ratio of a synergistic combination of long-acting bronchodilators for the maintenance treatment of asthma and COPD

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Summary

Introduction. The combination of inhaled drugs used in maintenance therapy of non-communicable respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) has shown significant improvement in symptoms and pulmonary function. As the co-administration of a long-acting β2-agonist (LABA) with a long-acting muscarinic antagonist (LAMA) could provide synergistic benefit on airway smooth muscle relaxation, pharmacological interactions between formoterol fumarate dihydrate (FOR) and tiotropium bromide monohydrate (TIO), and indacaterol maleate (IND) and TIO were studied using an ex vivo technique. Methods. An experimental model of isolated perfused guinea pig tracheal rings was used to highlight the dose ratios generating strong synergistic interactions that could be used in the formulated combinations. Recorded data were analysed with two widely used pharmacological models, the Bliss independence (BI) theory and the unified theory. These enable the synergy to be identified by accurate statistical analysis and the magnitude of interaction to be quantified, respectively[1]. Results. Synergistic interactions were found among the dose ratios tested, with the maximum interaction magnitudes for FOR:TIO 2:1 (w/w) and IND:TIO 10:1 (w/w). Conclusion. The dose ratio showing the strongest synergy and leading to the best dose reduction when compared with the same drugs as monotherapy will be chosen to be formulated. However, this synergy at the selected dose ratio requires to be confirmed on a preclinical model of asthma or COPD by assessing lung function.

Key Message

It was proposed that dose ratios of LABA/LAMA (w/w) combinations resulting in synergistic airway relaxation were highlighted using an appropriate ex vivo model of isolated perfused guinea pig tracheal rings. This was done to optimise the benefits of the co-administration of these drugs in the treatment of asthma and COPD.

Introduction

The control of non-communicable respiratory diseases such as asthma and COPD remains a challenge for many patients[2][3]. Simplifying their treatment regimen by developing formulations containing combinations of drugs in a single device that can be administered at a lower daytime frequency could improve the control of these diseases, especially for patients with problems related to adherence or inhalation technique[4][5]. In addition, significant improvement in symptoms and lung function has been shown with combinations of drugs used in maintenance therapy[6][7]. Similarly, a recent synergistic effect has been demonstrated for the combination of LABA and LAMA for the treatment of COPD[1]. Therefore, the identification and quantification of a synergistic interaction can be included as a step before formulation in the development of an inhaled medicine comprising a combination of long-acting bronchodilators for asthma and/or COPD patients. In this context, this research is carried out by studying the impact of LABA/LAMA combinations on the contractile tone of airway smooth muscle, with a specific focus on the LABA/LAMA (w/w) dose ratio promoting an optimisation of the airway smooth muscle relaxation.

In this work, FOR and IND were each associated with TIO, the only LAMA approved for both COPD and asthma, to assess their synergistic potential. The selection of these two LABAs was based on their difference in duration of action (i.e., acting for 12 hours and over 24 hours, respectively). In addition to their long duration of action, these drugs were chosen for their promising combined effects on airway relaxation in both COPD and asthma. Isolated and perfused tracheal rings from guinea pigs were used as an experimental model. This was because they allow the assessment of the relaxation effect of the drugs on airway smooth muscle on an appropriately sized organ from a small animal model with a high
degree of similarity to the human airway in terms of pharmacological receptors\cite{8}. There is currently no ideal model for the analysis of pharmacological interactions\cite{9}. Therefore, two of the most widely used pharmacological models, the BI theory and the unified theory, were applied to the resulting data to highlight the dose ratios of LABA/LAMA (w/w) providing strong synergistic interactions. Indeed, the complementary approaches of these models, and their applicability to various experimental systems (i.e. in vitro, ex vivo, and in vivo)\cite{10} seem to be relevant for the determination of dose ratios of new and existing inhaled LABA/LAMA combinations\cite{1}.

**Materials and methods**

**Materials**

FOR, IND, and TIO were obtained from CHEMO Industriale Chimica s.r.l. (Saronno (VA), Italy). Methacholine (MCH) was purchased from Sigma-Aldrich (St.Louis, USA) and atropine sulfate from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). All components used for the preparation of the Krebs-Henseleit (K-H) solution (i.e., KCl, NaCl, NaHCO$_3$, MgSO$_4$, KH$_2$PO$_4$, CaCl$_2$ and glucose) were purchased from VWR (Pennsylvania, USA). Ultrapure water was obtained from a Purelab-Ultra system (Elga LabWater, Lane End, UK).

Solutions of bronchodilators were prepared daily using DMSO for the stock solution and were diluted in ultrapure water. Solutions of MCH and atropine sulfate were prepared in ultrapure water.

**Animals**

Male Dunkin Hartley guinea pigs (body weight 250-500g) were obtained from Charles River Laboratories and housed in groups of four or five in the facilities of the Belgian research institute of public health Sciensano. They received dry food (Carfil Quality, Oud-Turnhout, Belgium) and water *ab libitum*. All animal procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments.

**Preparation of isolated tracheal rings from Dunkin Hartley guinea pig**

Guinea pigs were euthanised using increasing concentrations of carbon dioxide. The trachea was removed and immediately placed in a K-H solution with the following composition (in mM): NaCl 118.1, KCl 4.7, MgSO$_4$.7H$_2$O 1.2, KH$_2$PO$_4$ 1.2, CaCl$_2$ 2.5, NaHCO$_3$ 25, and glucose 5. After removing surrounding tissues, the trachea was cut into 3-5 mm long rings. The rings were mounted on metal rods under a 1.5 g resting tension in organ baths containing 20 mL of K-H solution gassed gently with 95% O$_2$ / 5% CO$_2$ and maintained at 37°C. Changes in isometric forces were measured using force displacement transducers and continuously recorded with a transducer amplifier and IOX computer software, version 1.8.3.25 (EMKA Technologies, Paris, France).

Equilibration of the bath conditions was carried out for at least 1 hour before the experimental stimulations with refreshment of the K-H solution every 20 minutes. Following equilibration, the tracheal rings were exposed to 60 mM KCl to ensure their viability. Organ baths were rinsed several times to restore the resting tension before stimulating the tracheal rings with the tested drugs.

**Experimental protocol**

A previously defined concentration of MCH (10 μM) was added to the organ baths to obtain a stable submaximal contraction size between 50% and 70% of the maximum MCH contraction. The assessment of the tracheal relaxation was carried out on precontracted tracheal rings by multiple additions of the combined drugs in a cumulative dose fashion to generate semi-logarithmic concentration-relaxation curves. A time interval of 30 minutes between concentrations was applied. At the end of the experiment, 1 μM of atropine sulfate was added to the organ baths as full agonist to achieve the maximal relaxant response ($E_{\text{max}}$) for each tracheal ring, allowing calculation of a normalized relaxation percentage. The contractile relaxation of tracheal rings was expressed as a percentage of $E_{\text{max}}$ induced by atropine sulfate (1 μM) on the submaximal contractile tone induced by MCH.

For both studied combinations, two dose ratios were selected based on a preliminary screening study (data not shown). Concentration-relaxation curves were generated for these combinations at a constant dose ratio by varying only the amounts of drugs that are cumulatively added to the organ baths. Recorded data were analysed using the BI theory and the unified theory, respectively. The interaction between drugs is synergistic if the delta effect ($\Delta E$) value (i.e., the difference between the observed
effect and the expected calculated effect) is positive with a positive 95% confidence interval, according to the BI theory, and if the calculated combination index (CI) value is lower than 1, according the unified theory. CompuSyn® software (Paramus, NJ, USA) was used to calculate the CI value.

**Statistical analysis**

For each studied combination, values are represented as mean ± SEM of n=5-6 different animals. The statistical significance at each dose level was assessed by the one-way analysis of variance (ANOVA) with a Bonferroni correction. The level of statistical significance was defined as p < 0.05. Statistical analysis was performed using GraphPad Prism 5 computer software (San Diego, CA, USA).

**Results and discussion**

Amounts of drugs in the combinations were defined based on TIO, which has a higher relaxing potency than the LABAs in guinea pig tracheal rings (experimental pEC50 values: TIO 8.8 ± 0.3, FOR 5.4 ± 0.3, IND 5 ± 1). Consequently, this drug was added in the same way for all combinations tested, generating curves showing similar profiles (Fig.1). Synergistic interactions were identified for all combinations, but only from a certain concentration that depended on the combination and dose ratio. A combination index plot describes the magnitude of interaction due to the CI value in relation to the fraction affected (Fa), which is the relaxant effect obtained for each studied combination (Fig.1B). This plot confirms that pharmacological interactions are synergistic (CI below 1) beyond a defined dose that is cumulatively added. The FOR/TIO combination with the dose ratio 2:1 (w/w) showed earlier synergistic results, although only after three cumulative additions (Fig.1).

![Figure 1](image)

**Figure 1** – Pharmacological interaction analysis in guinea pig tracheal rings induced by FOR plus TIO (green and blue curves), and by IND plus TIO (orange and purple curves) for the range of concentrations tested in the study. (A) ΔE value between observed and expected relaxant response predicted by the BI theory. (B) Combination index plot obtained according the unified theory with 7 data points that are outside the axis limits. Data are expressed as mean ± SEM from experiments performed using samples from n=5-6 different subjects. BI: Bliss independence; CI: combination index; Fa: fraction affected.

Furthermore, the mean of the maximum ΔE calculated according to the BI theory for the FOR/TIO combination and the IND/TIO combination for each studied dose ratio is reported in Table 1. Considering the FOR/TIO combination, the maximum ΔE was obtained with the 2:1 (w/w) dose ratio, while the dose ratio showing a higher ΔE value with IND/TIO was 10:1 (w/w). There are no significant differences between the maximum ΔE for the constant dose ratios studied (p > 0.05). However, the slight differences for the same LABA/LAMA combination show that promoting a combination with lower concentrations could lead to stronger synergistic interactions, as also reported by certain authors. As the aim is to determine an appropriate dose ratio of LABA/LAMA combinations resulting in synergistic airway relaxation, the dose ratio showing strong synergy and leading to the best dose reduction when compared with the same drugs as monotherapy was chosen to be formulated.

However, the data collected must be set against the limitations of the experimental model as relaxant responses given by the tracheal rings are concentration- and time-dependent. Furthermore, while the CI values are closely related to the amounts of drugs added to the organ baths, the ΔE value assessment is limited by the maximum relaxation value that can be achieved (i.e., 100% relaxation). Therefore, each pharmacological model has its advantages and limitations, hence the interest in highlighting synergy through several pharmacological models.
Table 1 – Most relevant AE value and related pharmacological interaction values for each fixed dose ratio of FOR plus TIO, and IND plus TIO combinations, calculated according to BI theory and unified theory analysis in guinea pig tracheal rings. AE data are expressed as mean ± SD from experiments performed using samples from n=5-6 different subjects.

<table>
<thead>
<tr>
<th>FOR</th>
<th>TIO</th>
<th>Dose ratio FOR:TIO (w/w)</th>
<th>Relaxant effect (ΔE)</th>
<th>Mean ΔE (%)</th>
<th>CI</th>
<th>Interaction magnitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.42 nM</td>
<td>2.08 nM</td>
<td>2:1</td>
<td>94.60</td>
<td>27.8 ± 5.8</td>
<td>0.155</td>
<td>++++</td>
</tr>
<tr>
<td>4.56 nM</td>
<td>2.60 nM</td>
<td>3:1</td>
<td>96.28</td>
<td>21.5 ± 4.8</td>
<td>0.137</td>
<td>++++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IND</th>
<th>TIO</th>
<th>Dose ratio IND:TIO (w/w)</th>
<th>Relaxant effect (ΔE)</th>
<th>Mean ΔE (%)</th>
<th>CI</th>
<th>Interaction magnitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.04 nM</td>
<td>2.08 nM</td>
<td>10:1</td>
<td>92.04</td>
<td>21.4 ± 11.7</td>
<td>0.231</td>
<td>++++</td>
</tr>
<tr>
<td>40.07 nM</td>
<td>2.08 nM</td>
<td>20:1</td>
<td>90.15</td>
<td>19.1 ± 18.1</td>
<td>0.314</td>
<td>+++</td>
</tr>
</tbody>
</table>

The smaller the CI value, the greater the magnitude of the interaction. - - - - - Very strong antagonism; - - - - moderate antagonism; - - - - - slight antagonism; - - - - nearly additive; + slight synergism; + + moderate synergism; + + + + strong synergism; + + + + + very strong synergism; CI: Combination index.

Finally, complementary kinetic studies with the selected amounts of drugs will be performed to confirm the synergy and to refine the selection of the appropriate dose ratio without the influence of previous cumulative additions of drugs on the resulting relaxation, while assessing the airway relaxant effect over time.

Conclusion

The use of an ex vivo model such as guinea pig tracheal rings to highlight appropriate synergistic dose ratios of LABA/LAMA combinations represents a promising strategy before the formulation step in the development of optimised synergistic LABA/LAMA (w/w) combinations in the maintenance therapy of asthma and COPD. Synergy was found for all dose ratios previously selected and assessed in this study, but only for specific concentration ranges, and with different interaction magnitudes. These experiments show that airway relaxation can be improved by combining lower concentrations of drugs than with monotherapies, which also reduces related side effects[1]. The dose ratio showing synergy and leading to the best dose reduction when compared with the same drugs as monotherapy was chosen to be formulated (i.e., FOR:TIO 2:1 (w/w) or IND:TIO 10:1 (w/w)). However, due to the limitations of the experimental model (e.g. isolated perfused organ), the synergy at the selected dose ratio first requires confirmation on a preclinical model of asthma or COPD by assessing bronchodilation through measurement of lung function before proceeding to clinical trials[12].

References