In vitro potential virucidal effect evaluation of Xibornol on *Human Adenovirus type 5, Human Rhinovirus type 13, Human Coronavirus 229E, Human Parainfluenza Virus type 1* and *Human Respiratory Syncytial Virus*

Abstract: The availability of virucidal compounds to reduce the impact of respiratory viruses is a relevant topic for public health, especially during the recent Coronavirus Disease (COVID-19) pandemic. Antimicrobial properties of Xibornol are known since the 70s, but its activity on viruses is currently little explored. In this study, Xibornol activity at a fixed concentration of 0.03 mg/100 ml has been evaluated on five respiratory viruses (*Human Adenovirus 5*, *Human Rhinovirus type 13*, *Human Coronavirus 229E*, *Human Parainfluenza Virus type 1* and *Human Respiratory Syncytial Virus*) through *in vitro* experiments based on adapted European standard UNI EN 14476-20019. The experiments were carried out under two different environmental conditions, one with the addition of foetal bovine serum to simulate an *in vivo* condition (dirty condition) and the other without the addition of any organic substances (clean condition). The viral abatement of Xibornol (expressed as Log_{10} reduction – LR) was statistically significant under both clean and dirty environmental conditions. Namely, in clean condition, LR ranged from 2.67 to 3.84, while in the dirty one the abatement was slightly lower (from 1.75 to 3.03). *Parainfluenza Virus* and *Human Adenovirus* were most resistant compared to the other viruses. The obtained data confirmed Xibornol activity and its use as topic substance for viral inactivation to prevent upper respiratory tract disease.

Keywords: Virucidal activity – Respiratory Viruses – Upper respiratory tract diseases – Xibornol – cell culture tests

1. Introduction

Respiratory viruses have a public health relevance, since majority of respiratory diseases are caused by viruses compared to other pathogens, increasing morbidity and mortality in worldwide population (Roth et al., 2018). From the end of 90's, several re-emerging and new respiratory

viruses (i.e., various subtypes of *Avian Influenza* and *coronavirus*) continue to challenge medical and public health systems (Liu et al., 2016). Such biological agents are a heterogeneous group that infect all aged people, carrying from acute-mild illnesses limited to the upper airways, to severe disease with interstitial pneumonia and serious systemic problems (Hodinka, 2016; Elrobaa and New, 2021). The recent pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) highlighted the importance of face masks, social distancing, hand washing, environmental disinfection and vaccination in reducing the virus spreading among the susceptible hosts (WHO, 2020; Leung, 2021). Moreover, various therapeutic approaches have been also widely investigated, such as the evaluation of virucidal compounds for the inactivation of viral particles in the upper respiratory tract, before cells infection (Rabie, 2021; Rabie, 2022). The Xibornol (6-isobornyl-3-4-xylenol) is a chemical compound, whose antimicrobic properties are well known since the 1970s (Capponi, 1969; Morandini et al., 1985; Fabbri et al., 1988). Xibornol appears as white crystalline powder, slightly soluble in water and freely soluble in methanol and other organic solvents as acetone and Toluene. It is stable after light exposure as well as after thermal and humidity treatment while it seems slightly sensitive to acid and basic stress. It is produced by the reaction of 3,4-dimethylphenol and camphene which are condensed in presence of tin tetrachloride: Xibornol is obtained through the rearrangement of camphene into isoborneol, followed by attack on 3,4-dimethylphenol. Xibornol is marketed as spray formulation at concentration of 3% for the topical treatment of upper respiratory tract inflammations, infections and, in general, for mouth hygiene (AIFA, 2016).

However, there is a paucity of information on virucidal properties of Xibornol owing to the lack of international standard procedures to test lipophilic drugs insoluble in water (Damery et al., 1989), and comparisons of Xibornol effectiveness among structurally and taxonomically different respiratory viral are missing.

To overcome such technical constrain, standards on other antiseptics were adopted for the *in vitro* study of Xibornol. In particular, the European standard UNI EN 14476-2019 (*Virucidal activity of chemical disinfectants or anti-septic products for instruments, surfaces or hands, which form a physically stable homogeneous preparation when diluted with hard water or with water*) has been adapted by diluting Xibornol at different concentrations in a non-ionic oil-in-water surfactant called Labrasol (Caprylocaproyl Polyoxyl-8 glycerides) (Cirri et al., 2007; Verani et al. 2017). The obtained data on a limited number of viruses (*Human Adenovirus, Human Coronavirus and Human Rhinovirus*) was also already published in the patent of 2019 (Bigini, 2019).

The aim of this work was to improve the knowledge on viral abatement of Xibornol by testing and elaborating all the available data related to the *in vitro* experiments and considering a fixed

xibornol concentration (3%). The tested viruses were chosen on the basis of high circulation in humans (Shi et al., 2022) and their clinical relevance for respiratory diseases (Table 1), namely:

- *Human Adenovirus type 5* (HAdV-5) that has high stability to physical and chemical agents, thus being able to survive long time outside the host;
- *Human Rhinovirus type 13* (RhV13) is transmitted through close contact and respiratory secretions;
- *Human Coronavirus 229E* (HCoV-229E), commonly considered as surrogates of pandemic coronaviruses;
- *Human Parainfluenza Virus type 1* (PIV), which circulation is common throughout the year, but mainly in autumn;
- *Human Respiratory Syncytial Virus strain 18537* (RSV) that infects children under the age of 5 and the elderly.

2. Materials and Methods

Experimental phases were performed according to European Standard UNI EN 14476-2019 adapted as cited above (Bigini, 2019).

2.1 Xibornol solution

Xibornol, kindly provided by Abiogen Pharma spa (Pisa, Italy), was supplied dissolved in Labrasol solution at concentrations 3% (g/100 ml). The obtained Xibornol is a racemic mixture of two enantiomers (1R, 2S, 4S) and (1S, 2R, 4R). Indeed, the molecule of Xibornol contains three asymmetric centers (positions 1, 2 and 4 of the bicycloheptane ring) but only the formation of the two isomers occurs owing to the mechanisms of involved reactions in the synthetic process. The final active substance has a crystalline form which is the stable from a thermodynamic point of view. The formation of the right polymorph is controlled routinely during process, together with the chiral purity, the assay (≥ 99.0%), and the impurity profile which are monitored for the release and stability of the active pharmaceutical ingredient (API).

2.2 Cell cultures viral replication

Tested viruses were obtained by American Type Culture Collection (ATCC) and propagated in susceptible cell lines. In particular, HAdV-5 (ATCC VR-5) and RhV13 (ATCC- VR-286) were propagated and assayed on HeLa cell line (ATCC CCL-2); HCoV-229E (ATCC- VR-740) on

MRC-5 (ATCC CCL-171) cell line; PIV (ATCC VR-94) on LLC MK-2 cell line (supplied by Pisa Hospital Virological Unit), and RSV (ATCC VR-1580) was seeded on Hep-2 cell line (ATCC CCL-23). For each virus a small volume with 0.1 multiplicity of infection (MOI) was absorbed on 25-cm^2 flasks for 1 hour at 37°C in 5% CO_2 atmosphere. After adsorption, Minimum Essential Medium (MEM) with 2% Fetal Bovine Serum (FBS), 10% L-glutamine and 0.125% gentamycin, was added and the flasks were incubated for 2 - 3 days. The typical viral specific cytopathic effect (CPE) was revealed by observation on microscope (Carducci et al., 2009).

2.3 Infectivity quantification

Each viral suspension was tenfold diluted (10^{-1} to 10^{-4}) in MEM supplemented with 1% L-glutamine and 0.125% gentamycin. Then, each dilution was seed into five wells, each of them received 75 µl of sample, 75 µl of MEM supplemented with 0.125% gentamycin and 0.1% HEPES buffer to stabilize pH, and 50 µl of each susceptible virus cell suspension (see 2.1) (approximately 10^6 cells/ml). Plates were covered and incubated at 37°C under 5% CO_2 for 5 days. Examination for cytopathic effects was performed with inverted light microscopy. The highest dilution producing a cytopathic effect in 50% of the inoculated cells was determined using the Spearman-Karber formula (Hamilton et al., 1977; Verani et al., 2020, Ramakrishnan, 2016), and the results were expressed in 50% tissue culture infective dose per millilitre ($TCID_{50}$/ml). The minimum detectable limit for this procedure was $10^{1.12}$ $TCID_{50}$/ml.

2.4 Xibornol effect on cell lines

To choose the right concentration for the subsequent tests, cytotoxicity assays were preliminarily performed to evaluate the effect of Xibornol on the different cell lines, using the methodological approach of European Standard UNI EN 14476-2019. Serial dilution of each supplied Xibornol 3% solution were prepared using Labrasol and seeded into 25-cm^2 flasks with a confluent cell monolayer. To verify a possible toxic effect of the diluent, Labrasol was seeded without Xibornol. Moreover, a negative control for each cell line was also prepared. After 24-h incubation period, the cell morphology was observed under inverted microscope to verify the integrity of the monolayer. All tests were analysed in triplicate. The obtained data allowed to define the cytotoxic concentrations (CC) of Xibornol by considering cells death or morphological modifications and the highest dilution that revealed no effect.

2.5 Xibornol effect on chosen viruses

The experiments were performed by considering the concentration coming from Sect. 2.4 and two different mixture virus-Xibornol, to simulate different environmental conditions (Figure 1): the absence of interference substances (hereafter "clean conditions") allowed to evaluate the direct effect of Xibornol on virus infectivity, while the addiction of FBS at 3% was applied to simulate the *in vivo* condition of the presence of various compounds with possible virucidal action, as suggested by the European Standard UNI EN 14476-2019 (hereafter "dirty conditions").

Briefly, mixtures of 10 ml were prepared with 1 ml of virus, 8 ml of Xibornol solution from Sect. 2.4 (non-toxic concentration for cell lines) and 1 ml of Labrasol for clean condition, while the dirty one was simulated by replacing Labrasol with 1 ml of FBS. Moreover, for each test, a positive control was prepared with 10 ml of virus and Labrasol instead of Xibornol. After a contact time of 15 minutes at temperature of 25°C, viral concentration was estimated by endpoint dilution assay as described above. A total of 10 combinations (type of virus and environmental condition), each one carried out in triplicate, were analysed. The starting titers of each virus were the following: 1.2×10^5 $TCID_{50}$/ml for HAdV-5, 7.5×10^5 $TCID_{50}$/ml for HCoV-229E, 1.5×10^4 $TCID_{50}$/ml for RhV13, 2.37×10^3 $TCID_{50}$/ml for PIV, and 3.16×10^5 $TCID_{50}$/ml for RSV.

2.6 Data analysis

The infectivity reduction was estimated comparing viral titers obtained from untreated and Xibornol-treated samples. The abatement was expressed as logarithmic (Log_{10}) reduction (LR) calculated as $LR = Log_{10}(Nt/Nn)$, where Nt is the viral titer estimated after Xibornol treatment and Nn is viral titer obtained from untreated samples.

Unpaired Student's t-test was used to compare the effect of Xibornol on viruses' infectivity under the clean and dirty conditions. The resistance to the Xibornol was compared between the five viruses (HAdV-5, HCoV-229E, RhV13, PIV, RSV) and the two environmental conditions ("clean" and "dirty") using two-way analysis of variance (ANOVA), then post hoc comparisons using Bonferroni test were conducted to locate the differences. Values of $p \leq 0.05$ were considered as statistically significant. All statistical analyses were performed using *GraphPad Prism 5* (GraphPad, San Diego, CA, USA).

3. Results and discussion

The role of respiratory viruses is well known and their high rate in mutations can lead to the escape from immune system and inefficacy to protect from a novel variant.

The research of effective measures to reduce the risk of infections and diseases is one of the main objectives for public health protection. Vaccination is a milestone for the viral infection prevention, but the use of antivirals is also needed to achieve this aim also by improving the knowledges of already existing and used products.

Xibornol effect is known and its efficacy on respiratory bacteria is recently studied and confirmed (Celandroni et al., 2021). Few data are available regarding the effect on viruses for its strong lipophilic nature that doesn't allow to strictly follow the European Standard on cell culture assays. To overcome such technical problem, we used a non-ionic oil-in-water surfactant, as reported by existing publication and patent (Cirri et al., 2007; Bigini, 2019), to explore the possible antiviral effect of Xibornol. In the present study, the cytotoxicity assays revealed a clear toxic effect using Xibornol 3% on the three cell lines (HeLa, MRC-5, and LLC MK-2) with concentrations ranging from 3 to 0.3 mg/100 mL responsible for a loss of viability and alteration of morphology in at least 30% on observed flasks. The cell cultures remained alive for the other tested dilutions (Table 2); therefore, the concentrations of 0.03 mg/100 mL represented the highest dilution that did not influence the replication of cell line and it was chosen for the viral survival experiments.

Xibornol showed a marked virucidal effect under the experimental setup (Figure 2) and, when data from all viruses were combined, the differences in virus survival between untreated and Xibornol-treated samples were statistically significant (unpaired t-test, $p < 0.0001$), under both clean and dirty conditions (Figure 3). Overall, Xibornol treatment determined at least 2 $\log_{10}$ abatement of virus infectivity, but with some differences according to the type of virus and the experimental condition (Table 3).

Regarding the effect of experimental condition on LR, the mean viral Log_{10} reduction was around 3 in clean condition and approx. 0.6 Log_{10} lower in the dirty ones and the differences of viral abatement between clean and dirty conditions was statistically significant for HAdV-5 and HCoV-229E (two-way ANOVA, $p < 0.001$). For such viruses, the abatement in dirty condition was approx. 1 Log10 higher compared to the clean condition, namely 1.05 Log_{10} for HAdV and 1.43 Log_{10} for HCoV-229E (Table 3, Figure 4). Such results suggested the possible protective role of organic material against chemical compounds: aggregation phenomena can protect viruses when they are released in the environmental matrices, as reported in the water treatment plants where the absorption to the organic particles, not eliminated during physical treatments, protects viruses from chemical disinfection (Templeton et al., 2008).

Moreover, the comparison of LR among viruses showed statistically significant differences (two-way ANOVA, $p < 0.05$), with HAdV-5 and PIV the most resistant viruses, in both environmental conditions (Figure 4). Such results can be supported by the biochemical and structural properties

of such viruses, since non-enveloped viruses (i.e., HAdV-5) are more resistant to chemical inactivation compared to some enveloped viruses (Firquet et al., 2015), while PIV has a virion structure that provide a higher environmental stability compared to other enveloped viruses (Henrickson, 2003).

Limitation of the study. In this study, Xibornol effect has been evaluated *in vitro* and not directly on respiratory tract mucosa. Although, *in vitro* experiments are commonly used to simulate real conditions, *in vivo* tests allow a better understanding of Xibornol action on viruses (Combe et al., 1988; Fabbri et al., 1988; Scaglione et al., 1988). Moreover, we compared the effect of Xibornol on various viruses, but through the evaluation of only one concentration of Xibornol (0.03 mg/100 ml).

4. Conclusion

The data presented in this study give a wide view on antiviral activity of Xibornol, focusing on several representative respiratory viruses. The Xibornol effect on HCoV229E (a surrogate of SARS-CoV-2) give some insights on the susceptibility of coronaviruses to such chemical treatment, that could be useful in the perspective of using Xibornol as topical agent.

Although the experiments have been carried out using protocols for testing disinfectant on the environment, the obtained results support the validity of Xibornol as topical antiviral agent for human usage, also considering that its effectiveness against viruses was maintained in the "dirty" condition tested in this paper. Clinical trials for assessing antiviral properties on oral mucous membranes are worth.

Funding: This research received a grant from Abiogen Pharma S.p.A.

Acknowledgments: Specific thanks go to Abiogen Pharma S.p.A. and Pisa Hospital Virological Unit for experimental materials supply.

References

1. Bigini C (2019) Patent US20190336456 - Use of xibornol as active agent in the treatment of viral infections
2. Capponi M. (1969) Action de l'isobornyl-6-dimethyl-3-4-phenol sur le rickettsies pathogenes. Bull Ste Path exotique 62: 658-661.

3. Carducci A, Battistini R, Rovini E, et al. (2009) Viral removal by wastewater treatment: monitoring of indicators and pathogens. Food Env Virol 1: 85-91
4. Celandroni F., Mazzantini D., Calvigioni M., et al (2021) Antimicrobial Activity of Xibornol and a Xibornol-Based Formulation Against Gram-Positive Pathogens of the Respiratory Tract. Adv Exp Med Biol. https://doi.org/10.1007/5584_2021_664
5. Cirri M, Mura P, Mora PC (2007) Liquid spray formulations of xibornol by using self-microemulsifying drug delivery systems. Int J Pharm 340: 84-91
6. Combe J, Simonnet F, Simonnet G. (1988) Action of xibornol on cell division and macromolecular synthesis of gram-positive bacterias. Ann Pharm Fr 46(1):19-26
7. Damery B., Cremieux A (1989) Virucidal activity against Herpes and Vaccina virus of 8 antiseptic formulations, Int. J. Pharmac., 49: 205-208
8. Elrobaa IH, New KJ (2021) COVID-19: Pulmonary and Extra Pulmonary Manifestations. Front Public Health 9:711616.
9. Fabbri A, Tacchella A, Belli ML (1988) Activity of xibornol against *Staphylococcus aureus*. Int J Med Soc Chem 7: 86-88
10. Firquet S, Beaujard S, Lobert PE, et al. (2015) Survival of Enveloped and Non-Enveloped Viruses on Inanimate Surfaces. Microbes Environ 30(2):140-144
11. Hamilton MA, Russo RC, Thurston RV (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11: 714–719
12. Henrickson KJ (2003) Parainfluenza Viruses. Clinical Microbiology Reviews, 16(2):242–264.
13. Hodinka RL (2016) Respiratory RNA Viruses. Microbiol Spectr. 4(4).
14. Italian Agency for Pharmaceuticals 2016. Description of Xibornol for commercial use. Agenzia Nazionale del Farmaco. Available at: https://farmaci.agenziafarmaco.gov.it/aifa/servlet/PdfDownloadServlet?pdfFileName=footer_000972_026642_RCP.pdf&sys=m0b1l3 (Italian language)
15. Knipe D.M., Howley P.M. Fields Virology. (2013). Editor Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia
16. Leung HL (2021) Transmissibility and transmission of respiratory viruses Nature Rev Microbiol 19: 528–545.
17. Liu Q, Zhou YH, Ye F, et al. (2016) Antivirals for Respiratory Viral Infections: Problems and Prospects. Semin Respir Crit Care Med. 2016;37(4):640-646. doi:10.1055/s-0036-1584803

18. Morandini G, Finiguerra M, Magno M (1985) Lo xibornolo nelle bronchiti Clin Ter 112: 233-239.
19. Rabie AM (2021) CoViTris2020 and ChloViD2020: a striking new hope in COVID-19 therapy Mol Divers 25(3):1839-1854.
20. Rabie AM. (2022) Potent Inhibitory Activities of the Adenosine Analogue Cordycepin on SARS-CoV-2 Replication. ACS Omega. 11;7(3): 2960-2969.
21. Ramakrishnan MA (2016) Determination of 50% endpoint titer using a simple formula World J Virol. 5: 85–86
22. Roth GA, Abate D, Abate KH, et al. (2018) Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017 Lancet 10:1736-1788
23. Scaglione F, Trazzi R, Odero A, et al. (1988) Xibornol: multiple dose pharmacokinetics and diffusion in lung, tonsillar tissue and laryngeal mucosa. Int. J. Clin. Pharmacol. Res. 8, 457–461.
24. Shi M, Zhao S, Yu B, et al. (2022) Total infectome characterization of respiratory infections in pre-COVID-19 Wuhan, China. PLoS Pathog. 18(2): e1010259.doi:10.1371/journal.ppat.1010259
25. Templeton MR, Andrews RC, Hofmann R (2008), Particle-Associated viruses in water: impacts on disinfection processes, Crit.Rev. Env. Sc. Technol, 38: 137–164
26. UNI EN 14476 (2019) European Committee for Standardization. Chemical disinfectants and antiseptics – Virucidal quantitative suspen-sion test for chemical disinfectants and antiseptics used in human medicine – Test method and requirements (phase 2 step 1).
27. Verani M, Di Giuseppe G, Federigi I, et al (2020) Preliminary data related to the effect of climacostol produced by the freshwater ciliate Climacostomum virens on human adenovirus, Viruses 12: 658.
28. Verani M, Federigi I, Bigini C, et al. (2017) Evaluation of the virucidal effect by contact with water-insoluble substances: the case of Xibornol. Int J Pharm SciRev Res 44:159–164.
29. WHO (2020) Water, sanitation, hygiene, and waste management for SARS-CoV-2, the virus that causes COVID-19: interim guidance, 29 July 2020

Figure Legends

Figure 1. Schematic flowchart of the experimental setup

Figure 2. Variation of viral titers for samples treated with Xibornol and untreated ones, in clean (a) and dirty (b) experimental conditions.

Figure 3. Effect of Xibornol treatment under clean (a) and dirty (b) conditions (data from all viruses were combined, separately for untreated and Xibornol-treated samples). Statistical significance was determined by Student's t test.

Figure 4. Viral abatement after Xibornol treatment in clean (dotted line) and dirty (longdash line) conditions, for each virus separately.