REVIEW

dissertation for obtaining an educational and scientific degree 'Doctor',

on *"Synthesis, characterization and toxicity study of bexarotene esters"*

by *Ivelin Rosenov Iliev*

Field of higher education: *7. Health and sport*

Professional field: *7.3. Pharmacy*

Doctoral Program: *Pharmaceutical Chemistry*

Form of doctoral studies: *Full-time*

Scientific leaders:
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Department: "*Pharmaceutical Chemistry" at the Faculty of Pharmacy, Medical University – Varna 'Prof. Dr. Paraskev Stoyanov'*

Reviewer: *Assoc. Prof. Velichka Yordanova Andonova, PhD*

Scientific specialty: Technology of pharmaceutical forms and biopharmacy

Institution: Faculty of Pharmacy, Medical University – Varna 'Prof. Dr. Paraskev Stoyanov'

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General presentation of the procedure

The presented set of materials by the Ph.D. student Ivelin Rosenov Iliev is under the Rules for the Development of the Academic Staff at the Medical University 'Prof. Dr. P. Stoyanov' – Varna and the terms and conditions for acquiring EQD ‚Doctor' at the Medical University – Varna (MU-Varna).

He is enrolled as a full-time Ph.D. student at the Department of Pharmaceutical Chemistry at the Faculty of Pharmacy at MU-Varna with supervisors Assoc. Prof. Svetlana Fotkova Georgieva, PhD and Assoc. Prof. Yana Koleva Koleva, PhD (Order No P-109-53/31.01.2020). During his preparation, Ivelin Iliev strictly followed the procedure regarding the requirements for full-time doctoral studies, which is evident from the submitted documents. He was withheld with the right of defense with Order No R-109-114/09.02.2023.

Brief biographical data about the Ph.D. student

Ivelin Rosenov Iliev was born on 11.11.1994. He graduated from the ‚Sándor Petyofi' Vocational Technical High School in Razgrad with a specialty in Computer Engineering and Technologies and a Computer Systems Technician qualification. In 2019, at the Medical University – Varna, he acquired a Master's degree in Pharmacy. After graduation, he worked in open-type pharmacies as a master pharmacist. Ivelin Iliev is enrolled in 2020 as a full-time Ph.D. student in a Ph.D. Program 'Pharmaceutical Chemistry,' Department of Pharmaceutical Chemistry at the Faculty of Pharmacy at MU-Varna. In 2021, he joined the same department as an Assistant Professor where he currently works. He is fluent in English.

Structure and sections of the dissertation

The dissertation submitted to me for review contains 171 pages and is illustrated with 63 figures (plus 5 in the annex) and 37 tables. In addition, 213 literary sources are cited, two of which are in Cyrillic. The scientific paper includes the following sections: introduction (2 pages), literature review (64 pages), purpose and tasks (1 page), experimental part, which includes materials (2 pages) and methods (12 pages), results and discussion (60 pages), conclusions (1 page), contributions (1 page), literature used (13 pages), list of publications and participations (2 pages), funding (1 page) and annexes (5 pages). The structure of the dissertation is under the procedure for acquiring the Ph.D. at the Medical University – Varna and the Rules of the Medical University.

Topicality of the theme and appropriateness of the set goals and objectives

Retinoid Bexarotene is FDA-approved for treating skin T-cell lymphoma (CTCL); therapeutic effects have been observed in treating breast and lung cancer. In addition, several

studies have described the potential of Bexarotene in treating neurological diseases, such as Alzheimer's disease, Parkinson's disease, and schizophrenia. However, its pronounced hepatotoxicity, teratogenicity in experimental animals, and adverse drug reactions limit its application. These shortcomings can be overcome by implementing a strategy to develop prodrugs of Bexarotene to increase the possibility of its application. This warrants the synthesis of new, not described in the literature, analogs of Bexaroten, their characterization, determination of toxicological profile, and investigation of the possibilities for their therapeutic application as prodrugs. In this sense, the dissertation topic of Master Pharmacist Ivelin Iliev is highly **relevant**.

The purpose of the dissertation and the resulting problems to be solved are precisely and specifically defined.

Knowledge of the problem

The review is set on 64 pages and is based on rich literary material – **213 sources**.

The review shows a broad knowledge of the matter, namely: general characteristics of vitamin A and retinoids, including function, physiological role and administration of vitamin A, structure, ligands, and classification of retinoids; preparation, pharmacokinetics, pharmacodynamics, metabolism, toxicity, drug interactions and administration of Bexarotene; derivatives of Bexarotene (5 methods for their preparation are considered), prodrugs, and esters as prodrugs; and a review of *in silico* and *in vivo* methods for determining the toxicity of newly synthesized drugs. And most of the significant problems existing in the topic discussed are presented. In addition, comprehensiveness with a clear and understandable writing style is demonstrated.

Aim and objectives of the research

Based on the literature review, the Ph.D. student formulates the **purpose of this dissertation**, namely to obtain, structurally characterize, and study a group of new non-described in the literature Bexaroten ester derivatives and to prepare a toxicological profile of retinoid analogs**. Six main tasks** are defined, and sequencing and solving them enables the achievement of the ultimate goal.

Methodology of the study

The chosen methods allow for a logical solution to the tasks set concerning the stated purpose of the dissertation.

The method of synthesis of Bexarotene esters has been described extensively, except that the exact amount of solvent required to dissolve 0.03 g of Bexarotene needs to be specified. This omission would make it difficult to reproduce the synthesis. Furthermore, it needs to be made clear whether preliminary studies have been carried out to determine the optimal reaction conditions of the synthesis process.

The applied instrumental analytical methods are generally known, modern, and adequately used. Unnecessarily, the Ph.D. student has devoted time and space to explain the essence of melting point determination, IR, UV-Vis, and TLC, as they are widely covered in the course of study for the Master's degree in PF 7.3. 'Pharmacy.'

In silico analysis for biological effect prediction and theoretical toxicity assessment of Bexarotene, newly synthesized Bexarotene esters and their metabolites were performed using three software programs.

On p. '2.6. Determination of general toxicity using *in vivo* models' description of the experiment thus made raises some questions:

1. Why do the 5 groups contain different numbers of experimental animals?
2. In point '2.6.2. Preparation of solutions' what necessitates the inclusion of a 'few drops' of Tween 20 as a suspending agent, and how was this surfactant chosen?
3. Have solutions been prepared or suspensions for the treatment of experimental animals?
4. How is a ‚missing effect level' defined?

Analysis of results

The conducted own research is presented on 73 pages and includes 33 tables and 26 figures. The tables and figures are clear. The study has been carried out in the following main directions, which follow the logically set tasks, namely:

1. A simple esterification method of Bexarotene in the presence of oxalyl chloride in the medium of aliphatic alcohols has been applied. There needs to be more information on how the crystal structure of the resulting Bexarotene esters (E1 and E2) and their solubility in the various solvents listed in Table 6 have been determined. It would be correct to consider that solids of white color have been obtained.
2. A TLC analysis was carried out to follow the reaction process with different mobile phases and establish the end of the esterification process. There needs to be an accurate description of all the used relations of the components of the applied two- and multicomponent ones, which would make it difficult to reproduce the experiment. Furthermore, the literature sources based on which these mobile phases are precisely determined need to be indicated. However, according to the doctoral student, the application of mobile phase hexane: ethyl acetate in relation 1 : 1 has given a result.
3. Well-known physical methods have characterized the obtained esters of Bexarotene.

3.1 The melting temperature of the solids E1 and E2 has been determined. Given that E3 and E4 are not in solid form, their presence in Table 10 is excessive. When melting a solid, it passes from a solid to a liquid state and does not solve as specified in the dissertation. Considering the dissertant's conclusion about unreacted Bexarotene and the

incompletely proceeded esterification process, what is the accuracy in determining the experimental yield and the calculated percentage yield?

3.2 To prove the structure of the newly synthesized analogs of Bexarotene, a comparative ATR-FTIR analysis of the spectra of Bexaroten as a parent compound and of its newly obtained esters in the region 4000-500 cm^{-1} was conducted. The results again show that the esterification process only proceeded partially under the experimental conditions indicated.

3.3 A rapid UV-Vis method for quantifying Bexarotene has been developed and validated. The spectra of the newly synthesized E1, E2, E3, and E4 in different solvents and their mixtures have been taken. It has been found that the developed UV-Vis method for quantifying Bexarotene is unsuitable for determining its mixtures with E1, E2, E3, and E4.

3.4 A modified HPLC analysis was applied to determine the purity of the esters E1, E2, E3, and E4. The results show that impurities in E2, E3, and E4 are present that have not been identified.

4. *In silico* methods have been applied as a theoretical approach to predict the biological activity of E1, E2, E3, and E4.

4.1. The QSAR Toolbox used to simulate hepatic *in vivo* metabolism and hepatic S9 metabolism in rats, as well as skin metabolism, presents data on the theoretical possibility of using Bexarotene's esters as prodrugs. However, the first two highlight the need to study their genotoxicity and cell dysfunction

4.2. thoroughly.

4.3. Molinspiration software found that Bexarotene has the most significant biological activity of all the compounds studied. However, the analysis shows that esters also have activity relative to the receptors and enzymes considered, which means that the esters still need to meet the definition of prodrugs.

4.4. In addition, the theoretical prediction of absorption, distribution, metabolism, excretion, and toxicity of Bexarotene and newly synthesized Bexarotene esters was carried out using PreADME/Tox software. Intestinal absorption after oral administration for esters was found to be excellent (100%), and all analyzed compounds had a high affinity for plasma proteins (100%). Bexarotene has the lowest absorption rate across the blood-brain barrier compared to newly synthesized Bexaroten's esters (which have a value above 10.00). Of the compounds analyzed, all were inhibitors of CYP2C9, and only Bexarotene did not inhibit CYP3A4. Of the esters analyzed, only E1 showed carcinogenic activity in rats. The compounds studied had an average risk of inhibiting the hER gene and were not potentially cardiotoxic derivatives according to *in silico* tests. E2, E3, and E4 had the most promising ADME/T *in silico* profile showing high intestinal absorption and satisfactory distribution profile. The three compounds have negligible renal excretion. The compound E1 showed high toxicity due to mutagenic and carcinogenic action, probably due to the hydrolysis of E1 to Bexarotene and methanol, described in section 4.1. Compounds E2,

E3, and E4 showed lower toxicity. According to ADME/T, the profile of compounds E2, E3, and E4 makes them potential antineoplastic agents with action in the CNS.

5. On p. 5 of 'Results and discussion' are presented the results of an *in vivo* study with experimental animals – male Wistar rats. After a single treatment of the animals with Bexarotene and ethyl ester of Bexarotene in two doses, psycho-somatic changes in the behavior of part of the animals were detected with Bexarotene's ethyl ester. It could only be assumed that this effect is due to the separated ethanol due to the hydrolysis of the ester. The observed effect on the body weight of experimental animals and their behavior for me is not sufficient to conclude that there are no symptoms of toxicity when considering body weight after a single oral administration of Bexarotene and its ethyl ester since any swelling of an organ resulting from an inflammatory process or intoxication would lead to an increase in body weight. The Ph.D. student further conducted a biochemical study for the liver enzymes ASAT and ALAT and C-reactive protein (CRP) levels. Minor changes in ASAT and CRP concentrations were observed in the exposed groups relative to the control. It is apparent from the data presented that the ALAT concentration in the animals treated with E2 at a dose of 500 mg/m^2 body surface area was lower than all other groups. The results have not been discussed. The macroscopic examination of the internal organs after a single administration of Bexarotene and Bexarotene ethyl ester could not be considered a reliable diagnostic method because of the possibility that the observed changes in lung and GIT may be due to several other factors not related to the compounds studied. At the same time, the condition of the liver was not commented on, provided that ASAT and ALAT levels were examined. The Ph.D. student concluded that Bexarotene and its newly synthesized ethyl ester did not significantly affect body weight and did not possess organ toxicity. A definite conclusion about the latter can be drawn only after a histological examination, which has not been conducted.

Conclusions

In the resume, 7 conclusions are presented, stemming from the results of each task separately. Instead, their formulation shows the results of the experimental work performed on Ivelin Iliev's dissertation.

Contributions and relevance of development to science and practice

My detailed acquaintance with the dissertation work of Ivelin Rosenov Iliev showed that there are original scientific, theoretical, and applied scientific contributions that are actually protected in the presented work.

Judgment of dissertation publications

The Ph.D. student has a total of 4 (four) publications on the dissertation. The published articles reflect the experimental research included in the dissertation. In addition, Ivelin Rosenov Iliev has participated in 14 (fourteen) scientific forums in Bulgaria and abroad. The presented materials are related to the dissertation.

Personal participation of the doctoral student in the dissertation study

The individual participation of the doctoral student in the review of the problem, formulation of the goal and tasks, the conduct of the experimental research, analysis of the results, and the derived contributions are available. The support of learning leaders is also evident.

Abstract

The abstract is prepared according to the requirements. It includes the purpose and tasks and the materials and methods used. The research and discussion presented fully reflect the main results achieved in the dissertation. The results obtained are illustrated with a sufficient number of figures and tables. The conclusions coincide with those in the dissertation. Scientific contributions, publications list, and participation in scientific forums in connection with the dissertation are included. Familiarization with the abstract makes it possible to fully understand the problem being developed, the research carried out, and the interpretation of the results.

Critical remarks and recommendations

My recommendations are in the following directions:

1. To pay attention to the terms and their basic meaning using scientifically accepted ones. For example, in the literature review, some terms such as 'ointments,' 'application instead of 'administration or 'route of administration,' 'pharmaceutical properties of medicines', 'chemotherapy index,' 'chemoprophylactic,' etc. are incorrectly used; in 'Methods' – 'clear, homogeneous solution,' 'after tempering the solution remains clear and the precipitate is released into the flask,' 'solution is dried' (p. 76), 'risk assessment of chemical compounds' (p. 83), it is not clear what is meant by the term 'pharmaceutical profiles' (page 142).
2. To follow the rule 'from the general to the particular' by following the logic of processes/research. Notably, Bexarotene's pharmacodynamics was considered before its pharmacokinetics in the literature review. Also, since the methods of synthesis of Bexarotene derivatives precede the prodrug point, it needs to be clarified from the statement whether its analogs and derivatives are prodrugs and whether there are other studies of prodrugs of Bexarotene.
3. Are the concepts used in the dissertation 'analog,' 'derivative,' and 'prodrug' of Bexarotene unambiguous?
4. To reproduce the experiments, it is necessary to describe them adequately and to avoid expressions such as 'sufficient quantity' and 'a few drops' such as those in the described methodologies for dissolving Bexarotene in 'General methodology for the preparation of Bexarotene esters' and 'Preparation of solutions' in 'Determination of general toxicity utilizing *in vivo* models.'
5. The selected reaction conditions, particularly the reaction time for obtaining the esters of Bexarotene, are probably the result of preliminary studies not mentioned anywhere in the

dissertation. Therefore, a thorough analysis of the preliminary studies is necessary to achieve the desired result.

The recommendations made are within the value of the work done.

CONCLUSION

The dissertation works ***contain original scientific results that contribute to science*** and **meet all** the requirements of the Law on the Development of Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for the Implementation of the LDASRB, and the relevant Regulations of MU-Varna.

The dissertation shows that the Ph.D. student **Ivelin Rosenov Iliev has** theoretical knowledge and professional skills in the scientific specialty ‚Pharmaceutical Chemistry' by **demonstrating** qualities and skills for the independent conduct of scientific research.

Due to the above, I give my ***positive assessment*** of the conducted research, presented by the above-reviewed dissertation, the abstract, the achieved results, and the contributions. Therefore, ***I propose to the honorable scientific jury*** to ***award the educational and scientific degree ‚Doctor' to Ivelin Rosenov Iliev*** in the doctoral program ***'Pharmaceutical chemistry' for the dissertation paper on 'Synthesis, characterization and toxicity study of Bexarotene esters.'***

12.04. 2023

Varna

Reviewer: .....

(Assoc. Prof. Velichka Andonova, PhD)