REVIEW

of the dissertation by Prof. Iva Ugrinova, PhD, entitled “Common Target, Different Agents: Fundamental Vulnerabilities of the Tumor Cell as a Basis for Selective Antitumor Action”, submitted for the award of the scientific degree “Doctor of Sciences”

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GENERAL INFORMATION ABOUT THE DISSERTATION

The dissertation of Prof. Iva Ugrinova, PhD, is devoted to a topical and scientifically significant problem: the possibility that structurally diverse synthetic and natural agents may exert selective antitumor effects through convergence on a limited number of fundamental vulnerabilities of the tumor cell. These include disturbances of redox homeostasis, dependence on the maintenance of genomic stability, and weakened control of cell survival and death. The topic is formulated ambitiously and offers a conceptual framework linking studies on ferrocene-containing camphor sulfonamides, heterocycle-annulated naphthalimides, natural products of marine, plant, and fungal origin, and polymeric nanosystems for drug delivery.

The dissertation comprises 281 pages, with the main authorial text ending on numbered page 279. It includes an introduction, literature review, aim and objectives, a results section, an integrated discussion, conclusion, scientific contributions, 15 conclusions, perspectives, a list of abbreviations, a list of 10 publications, and 158 references. The dissertation contains 96 figures, 23 tables, and 9 schemes, including those from the publications reproduced in the “Results” section, a substantial part of which consists of full-text reproduction of the publications on which the dissertation is based. The studies cover the period 2021–2026 and were supported by four projects of the Bulgarian National Science Fund and by the National Scientific Programme “BioActiveMed”. As a critical comment on the overall presentation of the dissertation, I would point out that its Table of Contents does not include page numbers, which makes it difficult for the reader to locate the individual sections.

The literature review is comprehensive, up to date, and logically structured. It covers p53 and NF-κB signaling, apoptosis, cell-cycle control, DNA damage and repair, replication stress, autophagy, the main classes of antitumor agents studied, and nanoplatforms. A positive feature is the inclusion, at the end of the thematic subsections, of passages entitled “Own Contribution”, which help distinguish general scientific knowledge from the results of the author’s research team. The review is not merely descriptive: it establishes the biological rationale for the subsequent synthesis and leads logically to the aim of the dissertation. In some places, however, the boundary between the literature review, the presentation of the authors’ own results, and their discussion becomes blurred, leading to repetition and making it more difficult to distinguish the primary data from independent literature sources.

The stated aim is clear and corresponds to the title. It is stated that six scientific objectives were formulated, but only five are listed in the text. This is a formal inconsistency that does not detract from the value of the dissertation. The first four objectives cover separate experimental directions of the dissertation, whereas the fifth is more integrative in nature. As presented, the fifth objective concerning the common vulnerabilities of the tumor cell can be regarded as a meta-analysis and conceptual synthesis of the results obtained in carrying out the other four objectives.

METHODOLOGICAL ASSESSMENT

The format of the dissertation makes a unified assessment of the methodology difficult. Because individual articles are reproduced, there is no common “Materials and Methods” section systematizing the cell lines, the number of independent biological replicates, exclusion criteria, statistical tests, corrections for multiple comparisons, and the method used to calculate

the selectivity index (SI). Overall, the methodological toolkit is broad and appropriate to the objectives. A comprehensive experimental approach was used to assess the biological and antitumor activity of synthetic compounds, natural products, and nanocarriers. The studies were conducted mainly *in vitro* using panels of human tumor cell lines, with control non-tumor cells also used to assess selectivity.

The synthetic compounds were obtained by multistep organic synthesis and characterized by nuclear magnetic resonance ($^{1}H/^{13}C$ NMR), high-resolution mass spectrometry (HRMS), elemental analysis, and chromatographic techniques. To improve water solubility and enable targeted release of active substances, micellar and pH-sensitive niosomal systems were developed and characterized in terms of size, surface charge, morphology, encapsulation efficiency, and release profile. Natural products were obtained using various extraction procedures—aqueous and ethanolic extraction, Soxhlet extraction, and modern extraction approaches. The chemical composition of the extracts was investigated by chromatographic and mass-spectrometric methods, with specific metabolites and biologically active components being determined. For plant extracts, total phenolic/flavonoid content and antioxidant activity were analyzed.

The principal method used to determine cytotoxicity and cell viability was the MTT assay, by which IC_{50} values and other effective concentrations were determined after treatment with different concentrations. The long-term antiproliferative effect was also assessed by clonogenic assay. Mechanisms of cell death were investigated by flow cytometry, including Annexin V/propidium iodide staining to distinguish viable, early- and late-apoptotic, and necrotic cells, as well as cell-cycle analysis. Apoptosis was additionally monitored through caspase-3/7 activity. Immunofluorescence microscopy and immunoblotting were used to elucidate molecular mechanisms, allowing analysis of DNA replication stress and damage, expression and cellular localization of regulatory proteins (e.g., p53, NF-κB), and markers of autophagy. The formation of reactive oxygen species (ROS) and changes in mitochondrial membrane potential were also investigated using fluorescent probes. The quantitative data obtained were processed using standard statistical methods, mainly ANOVA followed by multiple comparisons.

MAIN SCIENTIFIC RESULTS

Research Direction 1: Ferrocene-Containing Camphor Sulfonamides

The two publications in this research direction constitute a logically connected and progressively developed line of research on the antitumor potential of ferrocene-containing camphor sulfonamides. The first study compares DK-164 with its more water-soluble structural analogue CC-78, while the second addresses the principal pharmaceutical drawback of the more selective DK-164—its low solubility—by incorporating it into polymeric micelles.

A strength of the first study is its comprehensive experimental approach: cytotoxicity was investigated in two non-small-cell lung carcinoma lines (A549 and H1299) and control non-cancerous MRC5 cells. The inclusion of cisplatin and tamoxifen as comparator drugs further increases the informativeness of the study. The result for DK-164 is particularly convincing: the IC_{50} is 18.7–22 μM in tumor cells versus 130.7 μM in MRC5 cells. In contrast, CC-78 is more cytotoxic (IC_{50} 10–19 μM) but practically non-selective, which substantially limits the therapeutic value of its increased solubility. The observed ROS production in MRC5 cells, reaching levels comparable to the positive control after 48 h, is also an important indication of potential toxicity.

The second study offers a rational solution through nanoformulation of DK164. The micelles are well characterized physicochemically: a size of approximately 45 nm, low dispersity, and 98±2% encapsulation efficiency; approximately 42% of the substance is released over 24 h without an initial burst release. A substantial advantage is that the formulated DK164-

NP retains the selectivity and biological properties of free DK164, although its cytotoxicity is slightly lower.

It may be concluded that the studies in the first research direction are methodologically diverse, conceptually consistent, and provide convincing preliminary evidence that DK-164 is the more promising candidate, while its micellar formulation successfully resolves an important technological problem. The data presented link the effect of DK-164 to oxidative stress, p53-dependent responses, modulation of NF-κB, cell-cycle arrest, and apoptosis. A strength of this research direction is the comparison of closely related structural analogues, which makes it possible to explore structure–activity relationships. At this stage, the results primarily reveal the preclinical *in vitro* potential of the compound. An important next step in these promising studies is an *in vivo* evaluation of the pharmacokinetics, antitumor efficacy, and safety of DK164-NP.

Research Direction 2: Heterocycle-Annulated Naphthalimides

The two studies in this research direction focus on the development of novel heterocycle-annulated 1,8-naphthalimide derivatives as potential antitumor agents. The first study investigates benzodioxin- annulated naphthalimides and identifies **5a** as the lead compound, while the second extends the concept to benzofuran- annulated analogues, among which **5d** shows the highest activity and selectivity. In both cases, nanomolar cytotoxicity was demonstrated and a mechanism involving inhibition of DNA synthesis, accumulation of DNA damage, cell-cycle arrest, and apoptosis was proposed.

A major strength of these two studies is the comprehensive experimental approach (chemical synthesis and analysis (NMR), cytotoxicity testing (MTT) and clonogenic assay, flow cytometry, incorporation of 5-ethynyl-2′-deoxyuridine (EdU), fluorescence-microscopy-based quantitative measurement of p53, γH2AX, Annexin V/PI, caspase-3/7, and, for the benzofuran series, LC3), which allows the observed cytotoxic effect to be followed at several biological levels. The methodological reliability of the experiments is supported by at least three independent replicates, a large number of events in flow-cytometric analyses, and appropriate statistical analysis. Particularly valuable is the comparison between p53-positive A549 and p53-deficient H1299 lung cancer cells: the difference between G1 and G2/M cell-cycle arrest provides a convincing functional indication that the cellular response depends on p53 status.

In the two publications, the evidence for “replication stress” is predominantly indirect. Reduced EdU incorporation and accumulation of γH2AX convincingly demonstrate suppressed DNA synthesis and DNA damage, but do not by themselves establish the primary molecular target. For **5a**, the authors explicitly note the lack of direct data on DNA binding, topoisomerase inhibition, and replication-fork dynamics. The direct target of **5d** has likewise not been identified. The benzofuran study adds interesting data on LC3, but accumulation of LC3-II and LC3-positive structures does not in itself demonstrate increased autophagic flux; in this regard, the authors correctly note the need for flux analysis with lysosomal inhibitors or tandem-LC3 reporters. Both publications in this research direction examined p53-positive (A549) and p53-deficient (H1299) cells. Because of the genetic differences between A549 and H1299, the observed experimental differences cannot be attributed solely to p53, as the authors correctly acknowledge in the publication. Claims regarding therapeutic potential and tumor selectivity should therefore be regarded as promising but preliminary. Their validation requires additional studies using isogenic p53+/p53− cell lines, a broader panel of tumor cells, 3D and *in vivo* experimental models, as well as pharmacokinetic and toxicological evaluation of the compounds.

In conclusion, the second research direction may be considered the most mechanistically focused block of the dissertation. The studies are methodologically well supported and mutually complementary. They thus provide convincing evidence of strong *in vitro* antitumor activity and a cellular phenotype associated with replication stress. The benzodioxin- and benzofuran-

annulated derivatives show activity in the low nanomolar range and high selectivity, reaching an SI of 32 for the lead benzofuran compound. The data on suppressed DNA synthesis, replication stress, activation of DDR signaling, phase-specific cell-cycle arrest, and apoptosis form a coherent mechanistic model. Comparison of p53-competent and p53-deficient lines demonstrates different types of cellular response and the presence of p53-independent pathways. The scientific novelty and potential for further optimization of this research direction are convincing.

Research Direction 3: Natural Products

The six publications presented in this research direction examine various natural sources of biologically active substances—higher plants (*Croton lechleri*, *Cannabis sativa*), fungi (*Amanita muscaria* and other species), a marine organism (*Rapana venosa*), and the natural polyphenol curcumin incorporated into a nanocarrier system. Their common scientific logic is consistent: identification/extraction of natural compounds/fractions, chemical or physicochemical characterization, and assessment of biological, predominantly antitumor, activity *in vitro*. A strength of these studies is their interdisciplinary nature. They combine analytical chemistry, extraction technologies, cell biology, proteomics, and nanotechnology.

The study on twigs of the tropical tree *Croton lechleri* (dragon's blood) is one of the most methodologically complex. It compares plant extracts obtained by three methods (conventional Soxhlet extraction, supercritical-fluid extraction (CO_2), and pressurized ethanol extraction), determines total phenolic content and antioxidant activity, and investigates the cytotoxicity of the extracts toward A375 melanoma cells and non-tumor HaCaT cells. Particularly valuable are the demonstrated higher selectivity of the ethanolic Soxhlet extract toward A375 and the LC-MS finding that it is rich in polyphenols, especially flavonols. The biological activity of the extracts is demonstrated, while identification of some of the components remains only preliminary because there are no data establishing which specific component or combination of components is responsible for the effect.

The work on pH-sensitive curcumin-loaded niosomes (K-niosomes) is conceptually somewhat different: the principal contribution is not the discovery of a new natural substance, but improvement of the delivery of an already known natural polyphenol. The system was characterized in terms of size, polydispersity, ζ-potential, cryo-transmission electron microscopy (cryo-TEM), loading capacity, pH-dependent release, and stability. An optimal K-niosome with a size of approximately 302 nm and 83% curcumin loading was obtained. The biological part includes several tumor cell lines, MTT assays, and analysis of apoptosis/inflammatory markers, allowing a sound functional assessment. pH-dependent release and higher antitumor activity of some K-niosomes compared with free curcumin were observed. Targeted delivery of the curcumin-loaded niosomes was demonstrated using simulated pH conditions and cellular models, and the resulting data support the pH sensitivity and enhanced cellular activity of the K-niosomes.

The publication on *Amanita muscaria* (fly agaric) makes a distinctive scientific contribution because it combines a toxicologically significant question with the search for antitumor activity. Commercial preparations of ibotenic acid (IBO), muscimol (MUS), and ergosterol (ERG) were investigated by HPLC-HILIC, capillary electrophoresis, and UHPLC-MS/MS, while a standardized mushroom extract was tested on two cancerous and one non-cancerous lung cell lines. The authors show that the extract is cytotoxic, but IBO, MUS, and ERG—the compounds of interest—are not present in quantities that could convincingly explain this effect. This is a scientifically important "negative" result because it points to other, as yet unidentified compounds. Interpretation of the data is also complicated by variability among geographical populations of *A. muscaria*, which limits the reproducibility and standardization of the analyses.

The study of bioactive substances from the hemolymph of *Rapana venosa* is among the strongest in terms of the biological model: six different molecular subtypes of breast cancer and the non-tumor MCF-10A line were used. A protein fraction with a molecular mass of 50–100 kDa demonstrated the strongest cytotoxic effect. A particularly positive aspect is that the presumed synergism with cisplatin and tamoxifen was not assessed merely by visual inspection of dose–response curves, but was analyzed using the Chou–Talalay combination index and an additional synergy model. The inclusion of a non-tumor control allows selectivity to be assessed, although the authors themselves describe it as "weak". A critical point is that the active fraction is a mixture of macromolecules. Tandem mass spectrometry and bioinformatic homology analyses suggest candidates but do not prove which protein causes the effect. The data on autophagy and p53 are interesting and are more associative than definitive evidence of the mechanism. A logical continuation of the research reported in this article would be subfractionation of the active fraction into individual components and their *in vitro*/*in vivo* validation.

The study of cannabidiol (CBD) in lung epithelial cancer cells (A549 and H1299) clearly formulates a mechanistic question—the significance of p53 and apoptosis. CBD induces a dose-dependent increase in apoptosis in p53-positive A549 cells, whereas in p53-deficient H1299 cells apoptosis is weaker and not dose-dependent despite similar overall viability. A strength of this study is the comparison of two genetically distinct lines and the analysis of Annexin V and caspase-3/7 alongside the assessment of viability. Because only two tumor cell lines were examined, without a non-tumor lung control, no conclusion can be drawn regarding selectivity toward cancer cells. The authors themselves characterize the data as preliminary and non-quantitative. Therefore, the claim regarding the antitumor potential of CBD can only be regarded as a basis for future research.

The studies on Bulgarian wild-growing fungi are screening in nature. Aqueous and ethanolic extracts of *Trametes versicolor*, *Lenzites betulina*, *Fomes fomentarius*, *Fomitopsis betulina*, and *Amanita muscaria* were tested by MTT, and different IC_{50} values were established depending on the species, solvent, and cell line. Among the wood-decay fungi, extracts of *F. betulina* showed the highest overall cytotoxicity, with the authors suggesting a role for betulinic acid. The word "suggesting" captures the key limitation: the chemical composition of the crude extracts is not sufficiently characterized, and a causal relationship with specific compounds has not been demonstrated. IC_{50} is expressed as a volume percentage of the extract rather than as the concentration of a defined active substance, which hampers direct comparison and reproducibility. A positive aspect of this study is the inclusion of non-tumor MRC-5 cells in the lung panel and the clear characterization of the work as primary screening, which logically led to the subsequent, more in-depth investigation of *A. muscaria*.

The publications in the third research direction provide convincing evidence that certain extracts, fractions, and compounds from natural sources can affect the viability, apoptosis, cell cycle, and autophagy of tumor cells *in vitro*. These publications generate compelling hypotheses regarding new bioactive substances and systems for their delivery. In this way, they establish a fundamental preclinical basis for future studies aimed at molecular identification and standardization of the active components, assessment of their stability, pharmacokinetic/toxicological evaluation, and *in vivo* validation.

OVERALL ASSESSMENT OF THE DISSERTATION

The individual sections of the dissertation provide a logical and coherent account of the literature data, the authors' own experimental results, and their in-depth analysis. A major strength of the dissertation is the successful attempt to integrate the experimental results around the concept that structurally diverse synthetic and natural antitumor agents act on a limited set of fundamental vulnerabilities of the tumor cell—disturbances in redox homeostasis, genomic stability, and cell-survival mechanisms. This gives the necessary conceptual coherence to what is otherwise a substantial body of experimental material.

Particularly convincing is the use of tumor selectivity as a unifying criterion of biological significance, as well as the inclusion of polymeric nanosystems as a logical transition from establishing biological activity to potential pharmaceutical applicability. The scientific contributions are clearly distinguished as fundamental, experimental, and applied and, overall, correspond to the results presented. Among the most significant are the characterization of new antitumor agents and their mechanisms of action, the identification of p53-dependent and p53-independent effects, and the development of approaches to improve their pharmaceutical characteristics.

As a critical point, a certain degree of overlap and repetition can be noted among the "Integrated Discussion", "Conclusion", "Scientific Contributions", and the final summary conclusions. Some formulations are more categorical than is warranted by the predominantly *in vitro* results, particularly with regard to therapeutic applicability. In places, it would be more precise to distinguish demonstrated molecular effects from hypotheses and prospects for subsequent preclinical studies. Nevertheless, the concluding sections of the dissertation convincingly demonstrate its scientific value, originality, and internal conceptual unity.

Another strength of the dissertation is that it opens broad prospects for future development, including more in-depth studies of the leading antitumor agents and improvement of their applicability. Quite logically and justifiably, emphasis is placed on elucidating the mechanisms of action and structure–activity relationships of the naphthalimide derivatives, improving polymeric nanosystems for targeted delivery, expanding studies of the bioactive components from *Rapana venosa,* and developing green biotechnologies as a source of new antitumor agents.

Compliance with the Minimum National Requirements for the Scientific Degree "Doctor of Sciences"

The dissertation submitted by Prof. Iva Ugrinova, PhD, fully complies with the Minimum National Requirements (MNR) for the acquisition of the scientific degree "Doctor of Sciences" in professional field 4.3 "Biological Sciences". According to the Development of Academic Staff in the Republic of Bulgaria Act (DASRBA), the Regulations for its Implementation, and the Regulations of IMB-BAS, the candidate must meet the relevant indicators: 100 points in Group B (dissertation), 100 points in Group G (publications), and 100 points in Group D (citations). Prof. Ugrinova presents 100 points in Group B, 227 points in Group G, and 174 points in Group D. Thus, the requirement for Group B is met, while the minimum thresholds for Groups G and D are exceeded by 127% and 74%, respectively. More importantly, Prof. Ugrinova's dissertation complies with Article 12(4) of DASRBA, according to which a dissertation must contain theoretical generalizations and solutions to major scientific or applied-scientific problems. The 10 publications presented by Prof. Ugrinova are not a mechanical collection of co-authored works; rather, they are united by the original idea of "tumor vulnerabilities as organizing axes" and address an exceptionally important applied-scientific problem—the fight against cancer. It is particularly important that Prof. Ugrinova's position within the author teams and her contribution to the publications demonstrate a leading rather than peripheral role. In eight of the ten publications she is a lead author, and in several she is also the corresponding author. The studies on naphthalimides are especially indicative, as Prof. Ugrinova participates in the conceptualization, data analysis and interpretation, supervision, administration, and funding of the research. Thus, the combination of the quantitative indicators, her consistent position as a lead author, and Prof. Ugrinova's substantial contribution to conceptualization, scientific supervision, and interpretation of the results provides grounds to conclude that her personal contribution is sufficiently clearly delineated and meets the regulatory requirement that the dissertation represent a significant and original contribution to science.

CONCLUSION

The dissertation of Prof. Iva Ugrinova, PhD, represents a large-scale, interdisciplinary, and conceptually integrated scientific study of a topical fundamental and applied-scientific problem of high societal significance—the search for new approaches to selectively targeting tumor cells. The results presented are characterized by scientific novelty and originality and are both fundamental and applied in nature. I particularly value the successful conceptual integration of the substantial and diverse experimental material around the idea that structurally diverse synthetic and natural antitumor agents can converge on a limited set of fundamental vulnerabilities of the tumor cell. The critical comments made in this review concern primarily the structure and presentation of the dissertation, as well as the need for some of the predominantly *in vitro* results to be validated in more complex experimental models. They delineate the natural limitations of the present studies and the prospects for their further development, but do not diminish the scientific value, originality, or significance of the results obtained.

Prof. Ugrinova's scientometric indicators significantly exceed the Minimum National Requirements for the award of the scientific degree "Doctor of Sciences". Her substantial personal contribution is also of major importance to my positive assessment. Her position within the author teams of the publications, as well as the declared specific author contributions, demonstrate her leading role in the conceptualization of the studies, scientific supervision, analysis, and interpretation of the results. On the basis of the overall assessment, I consider that Prof. Ugrinova's dissertation fully meets the requirement of DASRBA to contain theoretical generalizations and solutions to significant scientific and applied-scientific problems corresponding to contemporary achievements and representing a significant and original scientific contribution. **All of the above gives me convincing grounds to give a positive assessment of the dissertation and to vote "IN FAVOR" of awarding Prof. Iva Ugrinova, PhD, the scientific degree "Doctor of Sciences" in professional field 4.3 "Biological Sciences".**

September 14, 2026

/Prof. Roumyana Mironova/