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Research Article

In vitro Selective Effect of Imidazole Derivatives as Inhibitors of Human Laryngeal Carcinoma

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Abstract
Laryngeal cancer represents a significant portion of head and neck malignancies, greatly affecting quality of life. Despite advancements in treatment, late-stage mortality remains high. The HEp-2 cell line, derived from human laryngeal carcinoma, serves as an *in vitro* model for studying this cancer. We present *in vitro* results highlighting the activity of imidazole-based ionic liquids and lysosomotropic detergents as selective inhibitors of laryngeal cancer growth using the HEp-2 cell line, alongside non-oncogenic MRC-5 and HEK-293 cell lines. A comparative analysis of the selectivity coefficients reflecting the action of these imidazole derivatives on HEp-2 cancer cells, compared to their effects on MRC-5 fibroblasts and HEK-293 kidney cells, revealed that the compounds 1-dodecyl-3-methylimidazolium chloride, 1-decyloxycarbonylmethylimidazole, and 1-dodecyloxycarbonylmethyl-3-methylimidazolium chloride appear to be promising candidates with selective activity against laryngeal cancer, with anticancer selectivity coefficients ranging from 2.8 to 11.5 and 2.2 to 20.1, respectively. Especially promising is 1-dodecylimidazole, which has an average selectivity coefficient of 47,675 based on IC_{50} values for the two non-oncogenic cell lines. Notably, the selectivity coefficients of these compounds were significantly higher—by two or more times—than those of the drug cisplatin.

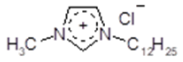
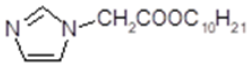
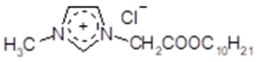
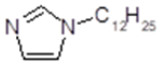
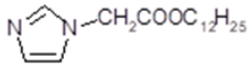
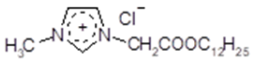
Keywords: anticancer activity, human laryngeal carcinoma, imidazole derivatives

1. INTRODUCTION

In addition to being a universal model in biomedical research and foundational for screening and evaluating new anticancer drugs, the HEp-2 cell line, derived from human laryngeal carcinoma, serves as an important *in vitro* tool for studying the features, nature, and origin of laryngeal cancer [1][2]. From an epidemiological standpoint, laryngeal cancer accounts for a significant portion of head and neck malignancies and continues to affect quality of life adversely. Despite gradual improvements in treatment outcomes, mortality from late-stage laryngeal cancer remains high. Head and neck squamous cell carcinoma ranks as the sixth most common cancer worldwide, causing approximately 500 thousand deaths annually [3][4]. Predictions indicate that the incidence could reach 1.08 million cases annually by 2030. Although advances in treatments such as surgery and combination therapy have been made, overall, 5-year survival rates are still around 40–50% [5][6] due to high recurrence, aggressive growth, and a tendency to metastasize. Additionally, the American Cancer Society highlights a trend toward decreasing five-year survival rates for laryngeal cancer patients, despite advances in treatment options [7]. Squamous cell carcinoma of the larynx is a prevalent cancer type with a poor prognosis in advanced stages. Despite improvements in diagnostics, treatment options remain largely unchanged, typically involving radiation therapy, surgery, chemotherapy, and targeted therapies [8].

A key element in evaluating new anticancer drugs is their ability to selectively target cancer cells based on proliferation patterns, specific genetic mutations, or unique metabolic pathways. Such selectivity is essential in reducing toxicity and improving treatment outcomes by targeting tumour cells. Currently, new derivatives for treating laryngeal cancer include natural compounds such as corilagin and curcumin [9][10], as well as synthetic options like benzimidazole derivatives [11]. Several studies have demonstrated that 7-substituted coumarins can inhibit the proliferation and migration of laryngeal cancer cells *in vitro* [12]. There is also evidence supporting the successful use of 2-aminobenzothiazole on human laryngeal carcinoma cells [13], and investigations into salicin

Table 1. Chemical structures of the studied compounds.

Comp. No.	Molecular Weight	Chemical Structure	Name
1	287.9		1-dodecyl-3-methylimidazolium chloride
2	266.4		1-decyloxycarbonyl-methylimidazole
3	317.8		1-decyloxy-carbonylmethyl-3-methylimidazolium chloride
4	236.4		1-dodecylimidazole
5	294.4		1-dodecyloxy-carbonylmethyl-imidazole
6	345.9		1-dodecyloxy-carbonylmethyl-3-methylimidazolium chloride

dimethyl ether as an inhibitor of laryngeal cancer growth also deserve attention [14]. Despite the availability of numerous anticancer drugs, significant challenges persist in their clinical effectiveness. Issues such as multi-drug resistance, which hampers treatment success, and limitations in therapeutic efficacy can undermine patient outcomes [15]. Additionally, many existing drugs face problems with solubility that hinder their absorption and bioavailability, ultimately impacting their therapeutic potential. Moreover, adverse side effects often lead to treatment discontinuation, further complicating patient management. These challenges underscore the urgent need for the development of innovative anticancer agents that can overcome these barriers and provide more effective, safer treatment options for patients battling cancer [16].

In our previous research, we presented *in vitro* findings demonstrating the effectiveness of both common and ester-functionalized long-chain imidazole derivatives, including lysosomotropic detergents and cationic biocides, also known as ionic liquids, as selective agents for cancer treatment (see Table 1) [17]. Additionally, our earlier publications provide detailed *in vitro* and *in silico* analyses of the cytotoxic and anticancer properties of imidazole derivatives **3** and **6**, specifically using the HEp-2 human laryngeal carcinoma cell line (as shown in Table 1) [18]. To confirm the selectivity of test compounds **1**–**6** as inhibitors of human larynx carcinoma, experiments were conducted, including studies of two conditionally non-oncogenic cell lines - human fibroblast cell lines MRC-5 and human embryonic kidney cell lines HEK-293. A comparative analysis of the calculated selectivity coefficients of the studied imidazoles was used as a unit of selectivity measure against non-malignant cell lines. The ecotoxicology of anticancer drugs has recently attracted considerable attention [19]. Because they were designed to disrupt or prevent cell proliferation, usually by interfering with DNA synthesis, they have been shown to have potent cytotoxic, genotoxic, mutagenic, carcinogenic, endocrine-disrupting, and/or teratogenic effects on several organisms. Therefore, there is serious concern that cytostatic drugs, which alter the structure and function of DNA, may affect not only tumor cells but also normal cells. Consequently, all living organisms may be susceptible to their genotoxic effects [20].

Although anticancer drugs are present at lower concentrations in the environment than other classes of pharmaceuticals, they can potentially affect all living organisms due to their specific molecular mechanism of action. It is believed that they can have an effect even at very low concentrations. Furthermore, the excretion of cytostatic drug metabolites into the aquatic environment, where

they can cause toxicity in aquatic organisms, is a serious issue. Therefore, this study also examined the acute toxicity of various imidazole derivatives against Daphnia magna, the most sensitive aquatic organism.

2. MATERIALS AND METHODS

2.1. In vitro Cytotoxicity Testing

Human larynx carcinoma HEp-2 cell lines (ATCC CCL-23) were used as cancer cell lines. Human fibroblast cell lines MRC-5 (ANCC CCL-171) and human embryonic kidney cell lines HEK-293 (ATCC CRL-1573) were used as conditionally non-oncogenic cell lines. The HEp-2 cell line was grown at 37 °C for 72 h in 96-well plates in RPMI-1640 medium (Sigma, USA) supplemented with 5% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 200 µg L-glutamine. HEK293 and MRC-5 cell lines were cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F12) medium. DMEM/F12 is a 1:1 mixture of DMEM and F-12. The combination of DMEM's high concentration of amino acids, vitamins, and glucose, enriched with Ham's Nutrient Mixture F-12's additional components, was found to support the growth of a wide range of mammalian cells.

Cells were maintained in logarithmic growth phase at 37°C in a humidified atmosphere with 5% CO_2. The cells were counted using a Goryaev chamber after being stained with trypan blue. The cells were plated in sterile 384-well, flat-bottom plates at a volume of 30 µL per well, at the following seeding densities: HEK293: 3,000 cells/well; MRC-5: 3,000 cells/well; HEp-2: 5,000 cells/well. After plating, the plates were sealed with transparent, sterile, non-breathable film, centrifuged for 30 s at 350 rpm, and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, the test or reference compound was added to the appropriate wells of the plate at a volume of 15 µL per well.

The reference compound, cisplatin, was tested at concentrations ranging from 0.015 to 300 µM (ten points at a threefold dilution; each concentration tested in n = 4). The tested compounds were examined at concentrations ranging from 0.02 to 408 µM (10 points at a 3-fold dilution; each concentration tested in n = 4). The final concentration of dimethyl sulfoxide (DMSO) was 0.5%. The plates were then incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, 30 µL of the CelltitreGlo development agent was added to each well. The plates were then shaken for 1 min. The results were recorded using a PheraStar FSX spectrophotometer.

To determine the metabolic activity of the cells after 48 h of incubation with the tested compounds, resazurin was added to the HEK293 and HEp-2 cell lines at a final concentration of 50 µM. The cells were then incubated for three hours in a humidified atmosphere at 37 °C and 5% CO_2. The presence of resorufin, the fluorescent product of resazurin, was quantified by measuring fluorescence under excitation at 555 nm and emission at 585 nm. The MRC-5 cell line was analysed for viability using the CellTiter-Glo® test kit, which determines the number of viable cells in culture by quantifying adenosine triphosphate (ATP), an indicator of metabolically active cells. The luminescence index directly determined the number of viable cells in culture. Before seeding, the cells were concentrated by centrifugation at 1,200 rpm for 5 min at room temperature, after which they were mixed with an appropriate volume of RPMI-1640 or DMEM/F12 medium. The effect of the test compounds on cell viability was normalised against the blank control, which comprised untreated wells containing 0.5% DMSO and a 300 µM concentration of cisplatin as a positive control. A four-parameter equation with a non-linear sigmoidal fit was used in GraphPad Prism 9.0 to generate dose-response curves and calculate the IC_{50} value, which is an indicator of the compound's potency. Four parallel measurements were performed, and the results are reported as the mean ± SD.

2.2. In vivo Acute Toxicity Evaluation

The acute toxicity of the compounds was evaluated using *Daphnia magna*, with the lethal concentration (LC_{50}) defined as the concentration that causes 50% mortality in the tested organisms. Tests with *D. magna* followed the procedures outlined in the Organization for Economic Cooperation and Development (OECD) Guideline 202 [21], using a light incubator maintained at 22±1°C with a 16-hour light and 8-hour dark

Table 2. Comparative analysis of the compounds' selectivity and acute toxicity.

Comp. No.	HEp-2, IC_{50} (μM)	MRC-5, IC_{50} (μM)	MRC-5/ HEp-2, SC	HEK-293 IC_{50} (μM)	HEK-293/ HEp-2, SC	Acute Toxicity, LC_{50} (mg/mL)
1	0.78	8.98±3.7	11.5	15.7±8.9	20.1	<0.01
2	39.4	400.3±3.2	10.2	782.2±0.0	19.9	0.18 ± 0.06
3	67.9	192.3±2.7	2.8	149.7±11.5	2.2	0.83 ± 0.02
4	0.0004	19.2±4.7	48000.0	18.9±5.4	47350.0	<0.01
5	126.9	189±2.5	1.5	363.7±5.8	2.9	0.17 ± 0.04
6	18.9	280±2.5	15.5	40.5±10.35	2.2	<0.01
CP[a]	39.8	265.5±14.8	6.7	855.4±6.2	21.5	34.88 ± 3.67

[a]Cisplatin - as a reference drug; SC - selectivity coefficient.

photoperiod. Neonates of *D. magna* (aged 6–24 h) served as both controls and test subjects across a geometric series of concentrations for each compound, adhering to the 48-hour acute toxicity protocol without the addition of food or organic extracts. Five neonates were randomly selected and placed in 50 mL glass beakers containing 30 mL of the test solution - compounds of different concentrations (0.000001%, 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1, 1%), and a control with culture water. Mortality was assessed after 48 h, which served as the endpoint for determining the effect. Organisms unable to swim within 15 seconds after gentle agitation were classified as immobilized. Additionally, the sensitivity of *D. magna* to the reference toxicant potassium dichromate ($K_2Cr_2O_7$) was tested. Acute toxicity measurements were performed in triplicate. LC_{50} values were statistically analyzed using the Statistica 7 program. The degree of toxicity of the compounds was defined according to the classification of D.R. Passino and S.B. Smith [22].

3. RESULTS AND DISCUSSION

In this experimental study, we present novel findings that validate the selectivity of the anticancer effects of imidazole derivatives. The research involved the examination of human fibroblast cell lines MRC-5 and human embryonic kidney cell lines HEK-293, which were included as conditionally non-oncogenic cell lines in our investigations. Cytostatic cisplatin was used as one of the common chemotherapeutic drugs to treat heavy or recurrent laryngeal cancer [23][24]. The degree of the action selectivity of the studied derivatives was expressed through the selectivity coefficient (SC). This coefficient is derived from the ratio of the half-maximal inhibitory concentration (IC_{50}) values for non-oncogenic cell lines compared to the HEp-2 cell line, a representative of laryngeal cancer. The findings of this evaluation are detailed in Table 2, showing the varying degrees of action selectivity exhibited by the compounds examined.

The data presented in Table 2 indicate that the 1-dodecylimidazole (compound **4)** has the lowest IC_{50} value against the HEp-2 cell line. This compound also demonstrates the highest level of selectivity in

cytotoxic action against the HEp-2 cell line, with selectivity coefficients of 48000.0 and 47350.0 when compared to the non-oncogenic cell lines MRC-5 and HEK-293, respectively. Ester-functionalized imidazole-based compounds (compounds **2** and **5**) showed much less cytotoxic effect on HEp-2 than compound **4**. Compound 1-dodecylimidazole is known to exhibit extracellular pH-dependent cytotoxicity, since it acquires detergent properties under acidic conditions inside lysosomes. Once protonated, 1-dodecylimidazole disrupts lysosomal and endosomal membranes, releasing their contents into the cytoplasm and ultimately killing the cell [17]. The lower cytotoxicity of compounds **2** and **5** against HEp-2 is due to their lower basicity compared to 1-dodecylimidazole and, therefore, lower ability to be protonated inside lysosomes. These compounds also had a low cytotoxicity in non-malignant MRC-5 and HEK-293 cells. However, the high difference in the activity between compounds **2** and **5** indicates that the mechanism of cell death induced by compounds may be more complex.

Excluding 1-dodecylimidazole as the most active compound, all other studied imidazole derivatives can be ranked according to the calculated SC as **6** > **1** > **2** > **3** > **5** relative to non-oncogenic human fibroblast cell lines MRC-5, and as **2** > **1** > **6** > **5** > **3** SC relative to non-oncogenic human embryonic kidney cell lines HEK-293. Among the long-chain imidazolium salts (compounds **1**, **3,** and **6**), 1-dodecyl-3-methylimidazolium chloride (**1**) demonstrated the highest *in vitro* cytotoxic effect against the HEp-2 cell line, as well as high selectivity to the non-oncogenic cell lines MRC-5 and HEK-293. Ester-functionalized imidazolium salt, 1-dodecyloxycarbonylmethyl-3-methylimidazolium chloride also showed higher activity against HEp-2 cells than cisplatin, and high selectivity to MRC-5 cell line. As expected, the compounds **3** and **6** are less toxic to the normal cells than compound **1**. The introduction of polar functional groups into the hydrocarbon chains of long-chain onium salts is known to decrease their penetration through the cell membrane due to reduced lipophilicity [17]. Figure 1 illustrates the selectivity coefficient for test compounds in comparison to the reference control, cisplatin, within the MRC-5 and HEK293 cell lines. The data are presented as a percentage relative to cisplatin.

A comparative analysis of the compounds' selectivity **1**–**3**, **5**, and **6** relative to the non-oncogenic cell lines indicates that the selectivity of the studied imidazole derivatives on human laryngeal cancer cells HEp-2 in relation to non-oncogenic human fibroblast cell lines MRC-5 is more pronounced than to the non-oncogenic HEK-293 cell lines. Moreover, test compounds **1**, **2**, and **6**, which act most selectively on cells in relation to the most sensitive non-oncogenic human fibroblast cell lines MRC-5, demonstrated SC values of 175%, 148% and 230% respectively, compared to the SC of cisplatin (100% as a control). Compounds **3** and **5** exhibited the least selective effect in comparison to cisplatin and compounds **1**, **2**, and **6**.

The acute toxicity evaluation results, as detailed in Table 2, indicate that all imidazole derivatives, irrespective of their cytotoxic or anticancer efficacy, are classified as super-, extremely-, and highly toxic according to the D.R. Passino classification system [22]. It is well-known that cancer drugs have a high acute toxicity profile [25] [26]. Any drug can cause side effects/toxicity. The severity of toxic effects depends on the dose, method, and duration of the treatment process [27]. The results of the acute toxicity assessment are critical to the development of potential chemotherapy protocols. These findings must be considered when formulating guidelines for the administration of new drug candidates, to ensure that side effects, including toxicity, are evaluated realistically. It is important to emphasize that the most critical element in studying the anticancer effects of potential drug candidates is their ability to selectively inhibit oncogenic cells. The main limitation of many traditional chemotherapeutic agents, such as taxanes or doxorubicin, is their low selectivity, which results in systemic toxicity and numerous side effects [28]. In the search for new anti-cancer drugs, evaluating their selectivity and safety profile for healthy cells is essential. The presented study, which continues our previous research on imidazole derivatives as anticancer agents, demonstrates experimental results of the *in vitro* anticancer activity of several long-chain imidazole derivatives as selective inhibitors of laryngeal cancer growth. Research involving the

HEp-2 cancer cell line, along with the non-oncogenic human fibroblast cell line MRC-5 and the embryonic kidney cell line HEK-293, suggests that at least four compounds—specifically **1**, **2**, **6**, and notably **4** —exhibit promising selective activity against laryngeal cancer.

Non-oncogenic (normal) cell lines are often used to assess the action of anticancer drugs, allowing comparisons of toxicity between cancerous and healthy cells. This helps determine the selectivity and therapeutic window of the drug. Non-oncogenic cell lines are crucial for calculating the selectivity coefficient of anticancer effects, serving as models for studying cancer biology and validating drug targets. They are essential for evaluating the effectiveness and resistance of drugs, as they enable researchers to observe the impact of various compounds on cancer cell lines without considering the tumor's complex microenvironment.

For example, human lung fibroblasts (embryonic) MRC-5, mouse fibroblasts 3T3 (NIH 3T3), and normal human dermal fibroblasts NHDF are commonly used as models of healthy connective tissue. To assess toxicity on normal epithelium, human embryonic kidney HEK-293 cells and human retinal epithelial cells (hTERT - immortalized RPE-1) are employed. Human microvascular endothelial cells HMEC-1 effectively model the vascular wall and are vital in studying angiotoxicity [29]-[31]. Currently, cancer research faces significant biological, technological, and systemic challenges that hinder the development of effective treatments. Preclinical models often fail to replicate the architecture, microenvironment, and complex immune interactions in human carcinogenesis. While positive laboratory results are achievable, clinical success remains elusive. Nevertheless, ongoing scientific advances in oncology continue, and new experimental and *in vitro* strategies are increasingly important [32]-[34].

Research involving various imidazole derivatives using the human HEp-2 cell line model has empirically demonstrated their selective mechanism of action as inhibitors of laryngeal cancer. Experiments with the non-oncogenic human fibroblast cell line MRC-5 showed selectivity coefficients for the imidazole derivatives **1**–**6** as 11.5, 10.2, 2.8, 48000.0, 1.5, and 15.5, respectively. Likewise, using the non-oncogenic human embryonic kidney cell line HEK-293, the coefficients for the same compounds were 20.1, 19.9, 2.2, 47350.0, 2.9, and 2.2. Analyzing these coefficients indicates that both non-oncogenic cell lines are effective models for evaluating the selective effects of new potential anticancer therapies. Notably, 1-dodecylimidazole (compound **4**) stands out as the most promising candidate for selective anti-laryngeal cancer activity.

4. CONCLUSIONS

Non-oncogenic (normal) cell lines play a crucial role in anticancer drug evaluation by enabling the assessment of drug toxicity in healthy cells

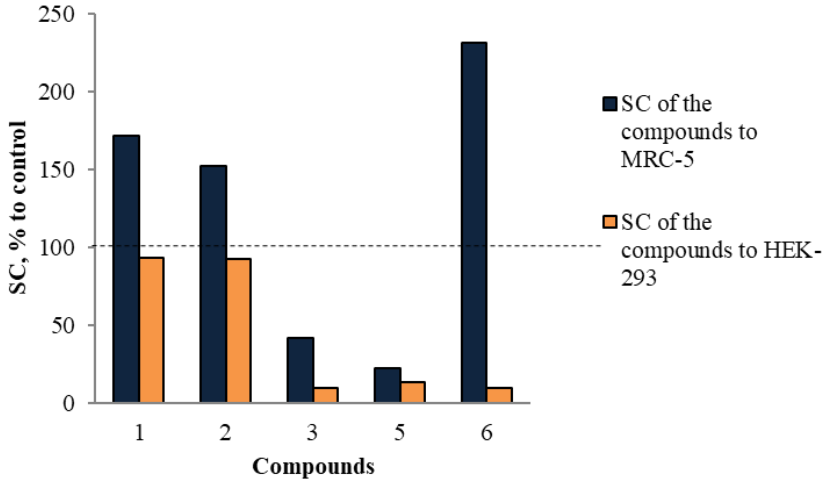


Figure 1. SC expressiveness of the studied imidazole derivatives **1**–**3**, **5**, and **6** in relation to non-oncogenic human cell lines.

alongside cancerous ones, thereby supporting the determination of selectivity and therapeutic potential. Commonly used normal cell models, including fibroblast, epithelial, and endothelial cell lines, provide essential benchmarks for calculating selectivity coefficients and validating the targeted action of candidate compounds in controlled *in vitro* conditions. Despite persistent biological and technological limitations in cancer research—particularly the inability of preclinical models to fully reproduce the complexity of human tumor microenvironments—such in vitro systems remain indispensable for early-stage drug screening. Studies using imidazole derivatives on the human HEp-2 laryngeal cancer cell line, in comparison with non-oncogenic MRC-5 and HEK-293 cell lines, demonstrated marked differences in selectivity coefficients among tested compounds, indicating differential anticancer specificity. The consistently high selectivity values observed for 1-dodecylimidazole highlight its strong preferential activity against laryngeal cancer cells, underscoring the effectiveness of non-oncogenic cell lines as reliable models for identifying and prioritizing promising selective anticancer agents.

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Conflicts of Interest

The authors declare no conflict of interest.

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