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Neş'e Erdok
Coronavirus, 2020 / Oil on Canvas

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COVER IMAGES

Neş'e Erdok,
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EDITORIAL | Yeditepe Journal of Health Sciences, 13(2):64

From Art as Medicine to Health Sciences Research

Dear Colleagues,

We are pleased to present the August 2025 issue of the *Yeditepe Journal of Health Sciences*, our second issue.

The cover painting of this issue is *Coronavirus* by Neş'e Erdok, a well-known Turkish figurative painter. During the SARS-CoV-2 epidemic, she created two paintings titled *Corona*, and the work featured here is the second. As Shaun McNiff has emphasized in his book *Art as Medicine*:

"Art as medicine does not restrict its interactions to human relationships. Concentration on the 'other' ensouls the world, and paintings are ensouled objects or beings who guide, watch, and accompany their makers and the people who live with them. Their medicine is established by this 'otherness,' which enables them to influence people who open themselves to receiving help from another."

Inspired by this perspective, we intend to continue featuring medicine-related artworks on the cover of our journal. We sincerely thank Neş'e Erdok for allowing us to publish her *Corona* painting on the cover of YJHS.

This issue brings together articles from diverse disciplines, which we hope will contribute to current knowledge and inspire new research directions for our readers. It includes a review article on reference ranges in clinical laboratories as well as research articles ranging from the discovery of a chemical substance to studies on the antibiotic use of adults.

As stated in our inaugural issue, our goal is to be indexed in both national and international databases, and we are working diligently toward this aim. Progress is steady, and each issue brings us closer to achieving it.

Steve Jobs once said, *"Great things are never done by one person; they're done by a team of people."* The same holds true for our journal. We extend our heartfelt thanks to all the authors who contributed their articles, the reviewers who provided valuable feedback—often on very short notice—and our editorial team, as well as .doc, for their meticulous work.

We invite scientists from around the world to submit their work and join us in building this journal into a valuable resource for the global health sciences community.

Warm regards from Istanbul,

Gülderen Yanıkkaya Demirel
Editor-in-Chief

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REVIEW ARTICLE | Yeditepe Journal of Health Sciences, 13(2):65-76

Reference Intervals for Clinical Laboratories

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Abstract

Reference intervals (RIs) are a fundamental tool in many medical disciplines for interpreting patient laboratory test results in clinical laboratories. Ideally, they enable the differentiation of healthy and unhealthy individuals. Clinical laboratories must establish accurate RIs, which is a very important process. Traditionally, RIs have been estimated using the 'direct' approach, which involves collecting laboratory test results from apparently healthy volunteers. An alternative approach is the 'indirect' approach, in which results from specimens collected for routine, screening, diagnostic or monitoring purposes are used to determine the RIs. When a laboratory receives an RI from the literature, manufacturers or another laboratory, the process of confirming its suitability for use is usually referred to as 'verification of RIs'. This raises questions about the transferability of RIs that need to be addressed. Common RIs can be obtained from multicentre studies, providing an opportunity to harmonise RIs within a given population. Clinical decision limits (CDLs) lead to the decision that individuals with values above or below the decision limit should be treated differently. There is still some confusion surrounding the difference between RIs and CDLs. The challenging groups, such as pediatric, geriatric and gestational age groups, as well as for uncommon sample types is a gap in the RIs studies. When individuality is a key factor, personalised RIs are far more effective than population-based RIs for monitoring individuals.

Keywords: Reference intervals, in clinical laboratories, clinical decision limits

INTRODUCTION

Laboratory medicine has long played a key role in diagnosing, treating and monitoring hospitalised patients. Every day, millions of laboratory tests are performed worldwide that need to be interpreted for clinical decision-making purposes. Reliable and accurate reference intervals (RIs) for laboratory analyses are therefore an integral part of correctly interpreting clinical laboratory test results (1).

Studies in this area began around six decades ago. In the mid-20th century, Gräsbeck et al. (2) published the initial paper entitled *Normal Values and Statistics*. In subsequent years, it was realised that the term “normal

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values" was inadequate and even partially incorrect. In 1969, Gräsbeck and Saris (3,4) launched the concept of the reference value(s) in a session devoted to normal values at a Congress of Clinical Laboratory Medicine, and the term "reference values" has since become widely accepted as an alternative to "normal values". From 1987 to 1991, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) published a series of six papers recommending that each laboratory follow defined procedures to produce its own RIs (5-10). Interest in this topic has been renewed as a result of the following regulatory initiatives of the last two decades. According to European Directive 98/79 on *in vitro* diagnostic medical devices, diagnostic kit manufacturers must supply their clients with the appropriate RIs for use with their assay platforms and reagents (11) and the International Organization for Standardization (ISO) 15189 standard for clinical laboratory accreditation states that each laboratory should periodically re-evaluate its own RIs (12).

Despite these requirements, RIs in most clinical laboratories are often out of date and incomplete due to the complex process of establishing them (13). Therefore, rather than developing RIs directly from an apparently healthy population, most laboratories obtain RIs for clinical use from various sources, such as manufacturers' package inserts, publications, textbooks, multicentre studies, published national or international expert panel recommendations and guidelines, local expert groups, or data mining of existing data. The laboratory is required to validate RIs from manufacturers or estimate appropriate RIs from the local population (13). The guideline entitled *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory* (EP28-A3c) provides the necessary steps for selecting reference individuals and considers pre-analytical and analytical factors, as well as analysing reference values for RI establishment studies and transference and verification of RIs (14). However, in the present era of evidence-based medicine, there is still a significant discrepancy between theory and practice with regard to the application of RIs as decision-making tools, despite mandatory requirements.

Reference intervals are derived from the reference population value distribution, usually the central 95% interval, and describe a specific population using a minimum sample size of 120, as recommended by the Clinical Laboratory Standards Institute (CLSI) guideline EP28-A3c (14). The traditional method for establishing RIs, known as the direct approach, is based on collecting samples from members of a preselected reference population, making the measurements and then determining the intervals. An alternative approach is to perform analysis of results generated as part of routine pathology testing and using appropriate statistical techniques to determine RIs. This is known as the indirect approach (15). The methods and processes for determination of reference RIs using indirect methods have been in development for over 50 years. This approach is not only a useful adjunct to traditional direct methods but also has a number of significant benefits and advantages (15).

In practice, this is very challenging because it is difficult to recruit a sufficient number of reference individuals, control pre-analytical variables and apply statistical methods appropriately (14). Therefore, the Committee for Reference Intervals and Decision Limits (C-RIDL) of the IFCC has emphasised the importance of common Rls and has conducted multicentre RI studies since 2009 (16). Where there are no apparent regional differences in reference values for any of the analytes and the assays are standardised nationwide, the reported RIs can be used throughout the country (17).

Clinical decision limits (CDLs) should be distinguished from RIs. While RIs describe the typical distribution of results seen in an apparently healthy reference population, CDLs are based on the diagnostic question and are obtained from specific clinical studies to define the probability of of a certain disease or another outcome (18).

This review describes the methodologies for establishing and verifying RIs, and provides a detailed evaluation of common RIs, CDLs, personalized RIs. The differences between these types of RI are explained (e.g., direct versus indirect RIs, RIs versus CDLs), to help readers avoid confusion. The review also discusses the importance of RIs for specific age groups, such as paediatric and geriatric patients.

REFERENCE INTERVALS

The concept of RIs is now well established and is based on including a fixed percentage of a reference population within the interval described withupper and lower reference limits (RLs). The reference population is generally made up of a statistically significant number of predefined condition-free subjects, but the concept can be applied to any defined population. Generally, it is the responsibility of laboratories to either validate a RI derived elsewhere or determine their own interval for use with their population and analytical methods. The pre-analytical, analytical, and post-analytical factors affect RIs (19).

Reference intervals are divided into two main subgroups: direct RIs and indirect RIs.

Direct Reference Intervals

Direct approach to RIs is the recommended process by

the EP28-A3c guideline, where subjects representing the reference population are selected and sampled and the specimen analyzed for this purpose (14). In this process, individuals from a population (the reference population) are selected for sampling based on defined criteria. Specimens are then collected from these individuals and analyzed for the selected measurands. This approach has been subdivided into a priori and a posteriori selection process. The a priori approach is to select individuals for specimen collection and analysis if they meet defined inclusion criteria and it is the more appropriate approach when the biology of an analyte is known. In the a posteriori approach, specimens collected from a population will be included in the analysis based on other factors such as clinical details or other measurement results, which were not used to define the collection. Thus, in the posteriori approach, not all specimens that were collected would be included in the reference population for further analysis. Ideally, a direct approach would use randomly selected members of the reference population; however, this is rarely achieved as the tested population is usually influenced by convenience and cost factors (20).

Pre-analytical and analytical aspects must be taken into consideration in the implementation of a RI study. Generally, the pre-analytical considerations involve biological (i.e. sampling time in relation to biological rhythms, fasting or non-fasting and physical activity) and methodological factors (i.e. sample collection techniques, type of additives, with or without tourniquet and sampling equipment, specimen handling, transportation, time and speed of centrifugation, and storage conditions) (14). For reproducibility and standardization, it is essential that the pre-analytical aspects are accurately defined and described as the preanalytical phase is known to have the highest error rate in the total test process (21).

Analytical aspects include the analytical variability of the method used for the measurement, equipment/instrumentation, reagents, calibration standards, and calculation methods. Different commercial methods may be used in a trueness-based approach to the reference measurement system, providing results traceable to the system and thus, comparable results can be produced in clinical laboratories. When performing a RI study, the reference measurement systems and standard reference materials are of great importance to ensure the traceability of the test results in comparisons (22).

Establishing of RIs involvels parametric and nonparametric calculation methods, detection of outliers, partitioning, and confidence intervals (CIs). In the parametric calculation method, the most suitable transformation method must be selected (e.g., logarithmic, Box-Cox power or some other function) and testing is then applied to establish whether the transformed reference values conform to Gaussian distribution (23). Box-Cox power transformation often has been used to transform data to a Gaussian distribution for parametric computation of RIs (23). In addition to the calculation of the RIs, detection, and exclusion of the outliers are very important to obtain reliable RIs. A simple but effective method for the detection of outliers is a visual inspection of the data. Although the method proposed by Dixon (24) is presented in the guideline, EP28-A3c (14), it is not very sensitive when there is more than one outlier. The Tukey method is a more sophisticated method, which includes Box-Cox transformation of the data to obtain Gaussian distribution followed by identification of the outliers in interquartile ranges (25). The latent abnormal value exclusion (LAVE) method proposed by Ichihara and Boyd (26) is a secondary exclusion method to exclude possibly abnormal results hidden within the reference values.

Stratification of RIs by age and gender is the minimum pre-requisite and other means include race, ethnicity, body mass index (BMI) or nutritional habits. The most widely-used partitioning method is that of Harris and Boyd (27), in which the means and standard deviations (SDs) of the subgroups are considered as a separate different SD that may produce different limits. A similar method was proposed by Lahti et al. (28) allowing the estimation specifically of the percentage of subjects in a subclass outside the RIs of the entire population in any situation. More recently, Klee et al. recommended a partitioning method on the basis of the magnitude of the SDs of test results named SD ratio (SDR). An SDR greater than 0.3 can be regarded as a guide for the consideration of partitioning reference values (29).

The CI is a range of values including the true percentile (e.g., the 2.5th percentile of the population) with a specified probability, usually of 90% or 95%, as the "confidence level" of the interval. It was recommended that RLs should always be presented together with their 90% CIs in the C28-A3 guideline. In the C28-A3 guideline, non-parametric CIs are given from the observed values corresponding to certain rank numbers from Reed et al. (30). Although one can theoretically determine 95% RIs with a lower number (as few as 39 samples), it is clearly recommended that at least 120 subjects are required to calculate the CIs of the lower and upper RIs in the guideline). Horn et al. (31) proposed a "robust method" method based on transformation of the original data according to Box and Cox followed by a "robust" algorithm giving different weights to the data, depending upon their distance from the mean (32).

Indirect Reference Intervals

An alternative approach is called the indirect approach where results from specimens are collected for routine purposes, which have been collected for screening, diag-

nostic or monitoring purposes and are used to determine the RIs. Data mining, or "big data", is the process of using previously generated data to identify new information. Routine pathology databases often contain many thousands or millions of results from many 100s or 1000s of patients, which can be used in this manner (15).

A key difficulty of standard statistical techniques is the high likelihood (or indeed expectation) of values from diseased individuals in the data set, which has been extracted from the pathology database, to influence RI results. As standard statistical techniques are strongly influenced by the extremes of the data set, and these extremes are those most likely to be from affected subjects, great attention needs to be given to outlier removal (15). There have been a number of examples of approaches to attempt to minimize the presence of results from diseased subjects in database extracts. For example, Ozarda et al. (33) applied data exclusion criteria to reduce contamination of the database by results from subjects with the disease. In this IFCC study, a two-step data cleaning process was applied as follows: 1) After excluding the test results of inpatients, only the results of outpatients were included, except for those ordered from outpatient clinics specialising in emergency care, oncology, anaesthesia and resuscitation, gastroenterology, and nephrology. 2) If a patient had multiple records in a given year, all records from that year were excluded except the first result, based on the assumption that the necessity for multiple testing implies a higher likelihood of an unhealthy status (33).

Standard parametric (mean and SD) or non-parametric statistics (percentiles), such as those used in direct RI studies, can also be used for indirect studies. This will involve outlier removal, either before or after transformation, followed by calculation of the mean and SD or median and relevant percentiles (33). The indirect RIs are usually determined by statistical methods based on identifying a distribution in the midst of the data such as Bhattacharya (34) and Hoffmann (35), rather than requiring assessment of all individual results in the database as belonging to the reference population or otherwise. Standard parametric or non-parametric processes have been used for indirect RI studies. This will involve outlier removal, either before or after transformation, followed by calculation of the mean and SD or median and relevant percentiles formation of the source data by use of Box-Cox formula. The truncated maximum likelihood (TML) method (36) and the truncated minimum chi-squared (TMC) method (37) are two indirect methods of estimating RIs. These methods use a software programme consisting of an Excel spreadsheet for the front end and an R script for the calculations. Both methods use an iterative algorithm to determine the optimal truncation segment of the reference value distribution and estimate the parameters of the corresponding distribution. The TML method is similar, except that it provides a more accurate estimation of λ and more reliable normality testing for the central truncated segment.

Direct & Indirect Reference Intervals

Direct sampling techniques require a series of structured steps that together require significant resources (6,7). These steps include the following: definition of the reference population; locating/recruiting members of the reference population; obtaining informed consent; sample collection, processing and storage; sample analysis; statistical evaluation (including outlier exclusion); and development of RIs for routine use. The processes of identifying subjects, collecting specimens and performing analysis are, at the very least, expensive and time consuming. By contrast, the indirect approach is based on data that have already been generated as part of routine care, thus excluding the resource-intensive components, i.e. patient identification, recruiting, specimen collection and measurement, of the direct approach (7).

Important benefits of the indirect approach, relative to the direct approach, include that it is faster and cheaper. It is also based on the actual preanalytical and analytical conditions used in routine practice. Additionally, the reference population is the one from which a patient is actually being distinguished from, i.e., a person presenting to a health care service who does not have the condition under consideration is compared with the person attending for medical care of that condition (6).

There are however risks and difficulties associated with indirect approaches. The most important risk is the question as to whether the presence of diseased individuals influences the RIs. This will depend on the nature of the disease state, i.e. clearly separated or overlapping with the nondisease population, and the relative prevalence in the population. Data sets can be "biochemically filtered" to reduce the frequency of results from subjects where there is a higher likelihood of disease affecting the result. An additional recommended approach is to limit results to a single result per patient. As a diseased patient is more likely to be retested than a non-diseased patient, failure to do this is likely to lead to overrepresentation of results from unwell subjects. The removal of probable outliers from a data set can be a useful tool, even if more robust statistical processes are used. However, there is no consensus on the best statistical model to calculate the indirect RIs (33).

Table 1 involves the comparison of direct and indirect methods for RI determination showing mostly benefits of indirect methods. However, it should be born in mind that EP28-A3c still recommends the direct methods to establish RIs.

Table 1. Comparison of direct and indirect methods for reference interval determination.

Direct	Indirect
Ethical issues with sample collection and responses to new information identified on patient, obtaining informed consent may be difficult	No ethical issues with sample collection and no new information identified on patient
Costs of performing the study are high	Costs of performing the study are very low
Difficult and expensive to get statistically significant numbers	Significant numbers readily available
Difficult to define "healthy" status	Defining "health" is not required. Exclusion criteia would be heplful to exclude "unhealthy population"
Preanalytical and analytical conditions may not match routine conditions	Preanalytical conditions match routine conditions
Hard to perform direct RI studies	Easy to repeat indirect RIs
Recommended method by the guideline, EP28-A3c	Recommended especially for uncommon sample types and challenging groups (i.e., pediatric and geriatric patients)

VERIFICATION OF REFERENCE INTERVALS

Under optimal conditions, a laboratory should perform its own RI study to establish RIs specific for its method and local population. However, the process of developing RIs is often beyond the capabilities of an individual laboratory due to the complex, expensive and time-consuming nature of the process to develop them. Often, clinical laboratories lack the necessary resources to determine RIs adapted to their local patient population and therefore refer to manufacturers of laboratory devices and test kits.

Clinical laboratories may transfer adequate RIs from external sources. Assuming the original RI study was performed using robust methodology and statistical procedures, transferring an RI requires certain conditions to be fulfilled before it can be verified and accepted. There are two main scenarios in which RIs are transferred. First, reference values may originate from a different population or laboratory method than the receiving laboratory, and second, reference values may originate from a laboratory that shares the same laboratory method/population as the receiving laboratory (38).

In the first instance, comparing the laboratory methods serves as an instructive early screening tool to assess the suitability of the reference values for the receiving laboratory (13). Laboratory methods can be compared by a method comparison study between the method used during the development of the RI and the method used by the receiving laboratory to determine the statistical validity of an RI transfer (39). For a method comparison study, samples must be collected with an appropriate distribution of values spanning the RI, as aninsufficient range may underestimate and a range too large may overestimate the strength of the correlation. The correlation between the two methods is subsequently analyzed and, if appropriate, linear regression analysis is performed to determine the slope and y-intercept values of the best-fit regression line (40). These values are subsequently used to transfer the RI. According to the CLSI EP28-A3c guideline, the best-fit regression line should have a slope bias close to 1, a y-intercept close to 0 and a correlation coefficient (r^2) close to 1 (14). Furthermore, according to CLSI EP09-A3 guidelines, the scatter and bias plots should be examined for constant scatter to ensure there are no dramatic differences between the variation at the upper and lower ends of the range of values (41). To sufficiently assess the acceptability of the method bias, it is also important that the magnitude of the y-intercept is small compared to the range of the data and the RI. If the y-intercept is large compared to the RI, it is recommended to reject transference and establish an RI directly from a healthy reference population. If the preanalytical processes (e.g., preparation of reference individuals, specimen collection, transportation, and handling), the laboratory methods and the populations (e.g., a relatively homogenous population within the same geographical region) are very similar to those of the laboratory where the RIs originated, the method comparison study is still recommended to confirm the comparability, although the bias between the laboratory methods is expected to be very small (42).

Following transference, the CLSI EP28-A3c guideline recommends subsequently verifying the transferred RI.

It is important that laboratories verify their RIs before applying them for routine clinical care. This requirement applies to RIs derived using the indirect approach. This can be achieved by the conventional approach where the laboratory analyses samples from 20 subjects without the predefined condition in the reference population. The RIs is considered verified if two or less results out of 20 fall outside of the RIs that would correspond to a 95% probability (14). However, this procedure is not practical for clinical laboratories and is not often used for routine verification (38).

Alternatively, laboratories can assess if the given RI is appropriate for their testing patient population and analytical method by monitoring the percentage of abnormal results (that would be typically flagged by the laboratory information system) and comparing it with the expected percentage that may be easily derived from the original indirect study calculations. When a change in the flagging rate in any direction (increased or decreased) does not exceed a predefined expected value, the RI under evaluation is acceptable for use. This method does not require additional patient testing and may be programmed in the laboratory information system as a continuous quality control monitoring measure (15).

COMMON REFERENCE INTERVALS

Establishment of well-controlled, reliable RIs is an important mission for all clinical laboratories (43). Although direct RIs are most established using a well-defined and representative reference population, with sample analysis performed by a single laboratory, RIs can also be determined with the intention of serving a much broader population demographic and/or geographic location with sample analysis performed by a single platform or multiple platforms; these are termed common RIs. There are two types of common RIs. The first is objective RIs, which require many prerequisites (44) and defined by well-conducted multicenter studies (45). The second is subjective RIs, which are defined by the survey(s) and guidance from a group of experts using the harmonization approach (46).

The derivation of RIs on a national level by conducting a multicenter study that follows a common protocol, comprehensive standard operating procedures (SOPs), and secondary integration of the results on a global scale is probably the most effective way to establish globally applicable, or common RIs (47). The C-RIDL has published papers including a protocol and SOPs for multicenter RI studies (51), with indication of the utility of a panel of sera for the alignment of test results among laboratories in the multicenter studies (48).

Eight years ago, the C-RIDL performed a global multicentre study to evaluate the importance of age, BMI and levels of alcohol consumption and smoking as major sources of variations of reference values in various countries (ethnic groups). Multiple regression analysis was used to confirm differences related to ethnicity in BMI-related changes in reference values. This was done to confirm ethnicity-related differences in BMI-related changes in reference values. The aim was also to make a BMI-adjusted comparison of reference values among the countries and to delineate gender- and age-related profiles of reference values from a large number of datasets compiled from the 12 countries (49,50). This was a direct multicenter RI study with total recruitment of 13,386 healthy adults to determine global RIs of 25 analytes were measured chemically and 25 immunologically and an example of a well-conducted multicenter RI study, in which each laboratory acts as a central laboratory and sample analysis is performed using multiple platforms. In this type of multicenter study, it is essential to perform rigorous quality control monitoring to detect analytical deviations and use internationally accepted reference materials for standardized analytes to ensure traceability in each center. In addition to internationally accepted reference materials, the global IFCC, C-RIDL study is based on a common protocol (47) and the use of a panel of sera (48) to harmonize measurement results. This approach resulted in a method comparison and successful transference of the data obtained from the global study. As part of the global study, a multicenter RIs study was also performed in Türkiye, including seven geographical regions, using traceable materials and panel of sera from 40 reference individuals from the global study in the central laboratory, using a single platform, as an example of studies where the measurements were performed in one center acting as the central laboratory (51). With the lack of regional differences and the well standardized status of test results, common RIs for Türkiye have been derived from this nationwide study. Additionally, “cross-check testing” using at least 20 samples has been performed to compare results among the participating laboratories in Türkiye as recommended in the protocol for multicenter studies (47). Thus, common RIs were transferred from the multicenter study to each participating laboratory in Türkiye using the linear regression slope and intercept (45).

REFERENCE INTERVALS & CLINICAL DECISION LIMITS

Every laboratory request has a purpose, with specific questions. The question “Is the patient healthy or not healthy?” relates to RIs that describe the typical distribution of results seen in an apparently healthy refer-

ence population. However, the questions ("Is the patient at risk of a developing a disease, or is the patient diseased, or worsening?") are related to CDLs, where values above or below the threshold are associated with a significantly higher risk of adverse clinical outcomes or are defined as diagnostic for the presence of a specific disease (52).

Clinical decision limits are thresholds above or below which a specific medical decision is recommended and are derived from receiver operating characteristic (ROC) curves and predictive values (53). Reference intervals are focused on optimizing specificity (typically to 95%) while CDLs are also focused on optimizing sensitivity for the disease. The approaches to identifying CDLs can be categorized (52): 1) The Bayesian approach is probably the most evidence-based approach to modifying the management of the patient. Following these criteria, a value resulting from a diagnostic test that serves to distinguish between two clinical subgroups is based on stated assumptions regarding: (i) the clinical sensitivity of the diagnostic test; (ii) the clinical specificity of the diagnostic test; (iii) the relative distribution of individuals between the two subgroups; and (iv) the clinical costs of misclassification (54). 2) The epidemiological approach for defining CDLs is based on clinical outcome derived from population-based studies and is typically applied to lipid parameters (low density lipoprotein cholesterol, high density lipoprotein cholesterol, etc.) (55). 3) The physiopathological approach involves the use of "critical values" that represent a pathophysiological state with such variance from normal as to be life-threatening unless prompt action is taken. While many clinical endpoints can be difficult to define, the endpoint of mortality is clear and, because it defines the risk of dying or of major patient harm, it defines a particular set of high risk CDLs often called "critical values" (56).

There are two limits (upper and lower) of the RIs, conraversly there is only one CDL, which is usually an upper limit. However, according to the likelihood of various clinical situations or different clinical questions, multiple low and high CDL may be used. RIs are defined by laboratory experts using different methods (direct, indirect). Clinical decision limits sare defined by clinicians and laboratory experts. Consensus standards of RIs are well-defined (14) while than those of CDL's still to be developed.

As there are key differences between RIs and CDLs (see Table 2), it is important to note that RIs and CDLs should not be viewed as the same in clinical laboratories. Rls are generally considered as a distribution of test values in the predefined population, whereas CDLs are mostly determined by assessing the patients' outcomes or response to management change. Clinical decision limits are based on the diagnostic question and are obtained from specific clinical studies to define the probability of the presence of a certain disease or a different outcome (57). Reference intervals relate to studies based on apparently healthy individuals while CDLs are based mainly on clinical outcome studies (e.g., prospective cohort studies, meta-analysis), guidelines and consensus values. These limits lead to the decision that individuals with values above or below the decision limit should be treated differently. To avoid confusion, the EP28-A3c recommended reporting decision limits or RIs but not both, with a clear indication of which has been used (14).

Analytical quality affects the reliability of both RIs and CDLs. The biological variability theory suggests that the

Table 2. Comparison of reference intervals and clinical decision limits.

	Reference intervals	Clinical decision limits
Population based on	General population	Clinical population
Method for derivation	95% central interval of the reference distribution	Clinical outcome studies, guidelines and consensus values, ROC curves, predictive values
Based on	Healthy population	Diagnostic question
Focused on	Optimizing specificity (typically to 95%)	Optimizing specificity and sensitivity for the disease
Data number	Two (lower and upper limits)	One or more, according to the likelihood of clinical situation or different clinical questions
Dependence	Type of population, age range, gender	Clinical problem, patient's category
Consensus standard	Well defined	Still to be developed
Defined by experts	Laboratory experts	Clinicians and laboratory experts

desirable bias for RI classification takes into account between-subject /intraindividual (CVI) and within-subject /interindividual variability (CVG) and that it will prevent an unacceptable increase in the proportion of healthy individuals flagged as outside RIs. Analytical quality will similarly affect the application of CDLs, although the impact is defined not by the statistics of the reference population distribution but by the clinical risk definitions as well as the prevalence of disease (58). Increasing measurement uncertainty generally causes greater clinical uncertainty; similarly, the impact of uncorrected measurement bias will lead to clinical bias. The traceability of method calibration is vitally important for both RIs and CDLs. Neither universal CDLs (e.g., for lipids and HbA1c) nor common CDLs (e.g., for routine analytes) can be clinically reliable without traceability and analytical quality standards (63).

CHALLENGING GROUPS FOR THE DETERMINATION OF REFERENCE INTERVALS

As the concentrations of many routinely measured analytes vary significantly with growth and development, the use of inappropriate pediatric RIs can result in misdiagnosis and misclassification of disease. It is well known that the determination of pediatric RIs is an extremely difficult task, primarily because of ethical limitations related to blood drawing in very young children and neonates. The most significant step in this area has been taken by Adeli et al. (45) in the Canadian Laboratory Initiative in Pediatric Reference Intervals (CALIPER) Project, which is a collaboration between multiple pediatric centers across Canada, that aims to address the current gaps in pediatric RIs and has established a database of age- and gender-specific pediatric RIs. The German Health Interview and Examination Survey for Children and Adolescents (Kinder- und Jugendgesundheitssurvey, KiGGS) is an another excellent example in this area (60). As these direct studies were well conducted and of large sample size, the current problems in pediatric RIs could be resolved through evaluation and application of the findings. However, as an alternative, indirect methods can be used for the pediatric group as recommended in the EP28-A3c (14).

The major difficulty in obtaining geriatric RIs is the selection of healthy individuals, as most elderly subjects do not meet the CLSI EP28-A3c guideline for inclusion in a healthy reference population (14). The width of the RI is altered by factors such as the regular use of medications or unrecognized subclinical diseases. Therefore, it becomes very difficult to differentiate the effects of age, aging, or a pathological condition. Although there has been increasing interest and studies in this subject (61,62), this issue remains inadequately addressed (63). It would be of great benefit to conduct a large, multicenter study with pediatric, adult, and geriatric reference individuals to develop common RIs, subsequently transfer them to local laboratories. They can be then verify them with respect to these specific age-groups using a limited number of healthy subjects and/or existing laboratory data (63).

Laboratory RIs during pregnancy, delivery, and the early postpartum period are another specific group as physiological changes during pregnancy may affect laboratory parameters and there is a need to establish reference values during pregnancy to recognize pathological conditions (64). Reporting the correct gestational age-specific reference values can also improve the sensitivity of the RIs.

The RIs for uncommon sample types (e.g., cerebrospinal fluids [CSFs], amniotic fluids) are usually interpreted on the basis of values reported in reference texts or handbooks; however, current reference texts either present normal CSF parameters without citation or cite studies with significant limitations. Recent developments to determine accurate, age-specific reference values for glucose tein concentrations and white blood cell counts in CSF, amniotic fluids and aspirations in a large population of neonates and young infants will bring literature up to date at a time when molecular tools are commonly used in clinical practice (65,66).

Integrating genetic and laboratory information would increase the accuracy of RIs by eliminating extreme results related to genetic variation. It has been reported that the use of genetic information to partition Rls could reduce the between-person variation and therefore with the reduced variance obtained from partitioning based on genetic differences, there could be potentially less misidentification of unusual test results caused by non-disease associated genetic variations. It has been reported that serum folate and homocysteine status are impaired by subgroup stratification of the rate of methylenetetrahydrofolate reductase (MTHFR) 677C > T i 1298A > C (67). However, there is often a lack of knowledge of the genetic status of the reference individuals. Integrating genetic information with RI values would improve the sensitivity of the RIs (20).

PERSONALIZED REFERENCE INTERVALS

Knowledge of major sources of variation inbiological quantities is a part of the concept of reference values.

There are many analytes that are affected by biological characteristics, such as age, gender, or pregnancy, or by factors, such as season or geographic location. Certain quantities have predictable cyclical biological variation (BV) (daily, monthly, seasonal) and the knowledge of the expected values throughout the cycle is vital for clinical interpretation of laboratory data (68).

When individuality is still a key factor, subject-based RIs are far more effective than population-based RIs for monitoring individuals (69). For clinicians, the main concern is whether the actual test result from a specific patient is indicative of disease or not. To answer this question, a personalized RI; i.e., an RI for that specific individual would be useful (70). The within-subject BV (CVI) describes the fluctuation of a measurand around its homeostatic set point in steady-state conditions in an individual, whereas the variation between the set point of different individuals is defined as the between-subject BV (CVG) (76). Many investigators have previously produced estimates for CVI and group CVG variation. However, there is now a better understanding of the need to produce and promulgate accurate estimates generated from significant sample sizes using the best statistical tools available (72). Important statistical considerations include determining the BV parameters, outlier removal, and their CIs (73).

Variations in the concentration of the analyte still within the RI can be significantly outside the subject's usual values, in which case it is useful to calculate if the reference change value has been surpassed or to calculate the statistical significance of a trend. The reference change value, which can be defined by absolute (±delta) or relative (±delta%) means, can help in the interpretation of the results of serial measurements (74). The progress of a disease or recovery from it is often reflected by the dynamics of test results (delta values/delta change). The example of absolute and relative kinetic changes of cTn in patients with acute coronary syndrome shows that serial measurements may assist in diagnosis and may be used to rule out non-ST elevation myocardial infarction (75).

The prerequisites to calculate delta changes from serial measurements are a well-accepted clinical algorithm with defined time points (e.g., baseline, 3 h, 6 h for cTn) and the knowledge of the intra-individual BV of the measurand CVI.

Although the source of BV data is typically from a healthy reference population, its application to disease assumes that BV is the same in chronic disease as in health (76), and this has been adopted as a surrogate for clinically significant changes.

CONCLUDING REMARKS

Interpreting the results of a clinical laboratory test requires comparison with a RI, a clinical decision point or previous results. Clinicians and laboratory experts should clearly distinguish between these concepts. Direct methods are still the gold standard for establishing RIs. However, this method is time-consuming and expensive for laboratories, and in many cases, laboratories prefer to use recommended RIs provided by manufacturers or modified RIs obtained from other sources. Indirect methods of deriving RIs are inexpensive, easy and fast. Although very important progress has been made over the last decade, there is still no consensus on the most effective model for establishing reliable RIs.

It should be borne in mind that RI is only an estimation. They involve uncertainties and assumptions that may or may not be true. Once a second sample has been collected, comparing it with the previous result may be more important than comparing it with the RI. Each patient should be assessed individually using all available clinical and laboratory data. Clinicians should realize that test result is not an absolute number but rather a range that is determined by a combination of analytical and BVs.

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RESEARCH ARTICLE | Yeditepe Journal of Health Sciences, 13(2):77-85

Synthesis and Biological Evaluation of Novel N-Substituted 3-Methylindole Derivatives

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Abstract

Objective: Ten novel compounds with the general structure of 1-[(substituted-1-piperidinyl)methyl)]-3-methyl-1H-indole and 1-[(4-(substituted-1-piperazinyl)methyl)]-3-methyl-1H-indole, were synthesized using one-pot reaction method and investigated for their cytotoxic activity against MCF-7 and noncancerous human umbilical vein endothelial cells (HUVECs).

Materials and Methods: The synthesis of the target compounds was carried out using a one-pot reaction method, in a typical procedure, 3-methylindole (2.2 mmol, 300 mg) was dissolved in 20 mL of ethanol in a round bottom flask, then formaldehyde 37% (3 mmol) and substituted piperidine or piperazine derivatives (2.2 mmol) were added. The reaction mixture was then refluxed for 4–6 hours.

Results: *In vitro* cytotoxic activity screening of the compounds was performed against breast cancer (MCF-7) and noncancerous HUVEC lines. Compounds **1, 2, 3, 9** and **10** exhibited selective inhibitory effect on MCF-7 cells with IC_{50} values of 27, 53, 35, 32 and 31 μM respectively.

Conclusion: The anticancer activity of the target compounds was examined against breast cancer cell line MCF-7 and noncancerous HUVEC cell line using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay to investigate their selective cytotoxicity effect, tamoxifen was used as reference drug. Compounds **1, 2, 3, 9** and **10** exhibited moderate cytotoxic activity comparing to the standard and showed a selective inhibitory effect on MCF-7 cells with selectivity index (SI) values of 3.21, 1.08, 2.90, 1.48 and 2.29 respectively. The IC_{50} values of these compounds on MCF-7 cell line were determined as follows: 27, 53, 35, 32 and 31 μM. These compounds' IC_{50} values on HUVECs were obtained as 85, 57, 100, 48 and 71 μM.

Keywords: Indole, piperidine, piperazine, 3-Methyl Indole, anticancer.

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INTRODUCTION

Cancer is a serious and critical life-threatening illness that was responsible for 9.7 million deaths in 2022, and it is the main cause of death universally after ischaemic heart diseases (1).

Cancer Cancer can be treated and managed by various methods like chemotherapy, radiation therapy, immunotherapy and local treatments like surgery (2). Although studies are continuously updating, chemotherapy remains the most effective and commonly applied treatment against various cancers. However, this effectiveness is impacted by its side effects on patient's physical and psychological health which might limit its clinical potential (3). Therefore, the development of new and safer anticancer agents with minimum toxicity and high potency should be continuously pursued (4).

Due to the rising academic interest in the indole ring as a key scaffold in pharmaceutical chemistry (5), numerous studies over the past decade have been investigating the anticancer properties of various indole-based compounds (6). This increasing attention is supported by the fact that the indole ring is one of the most versatile medicinally important heterocyclic scaffolds, recognized as a privileged moiety with a wide range of pharmacological activities (7). It has become a fundamental nucleus in many synthesized anticancer candidates, as reflected in numerous published studies (8).

Recently, the indole ring has been used as a main scaffold for several U.S. Food and Drug Administration (FDA) approved anticancer agents such as vincristine, vinblastine etc. (Figure 1). Due to the indole ring's aromaticity and weak acidity on N-H bond, it can have different substitutions which allows variety of derivatives. As already mentioned, vinblastine and vincristine that bear an indole ring in their structure, have been widely used in clinic for the treatment of several different cancer types including breast cancer, through tubulin polymerization inhibition (9-11).

Other versatile pharmacologically important heterocyclic moieties especially in the rational drug design field, are piperidine and piperazine, which both exhibit various pharmacological activities (12,13). For instance, the piperidine structure has been reported to target cancer progression by either inhibiting farnesyl transferase activity or modulating cell signalling and cell mobility through Ras protein family modifications (14).

MATERIALS AND METHODS

Materials

3-Methylindole, 4-phenylpiperidine, 4-hydroxy-4-phenylpiperidine, 3,5-dimethylpiperidine, 4-methylpiperidine, bis(4-fluorophenyl) methylpiperazine, trifluoromethylphenylpiperazine, 4-bromophenylpiperazine, 4-methoxyphenylpiperazine, 2,3-dichlorophenylpiperazine, 4-*tert*-butylpiperazine-1-carboxylate, ethanol,

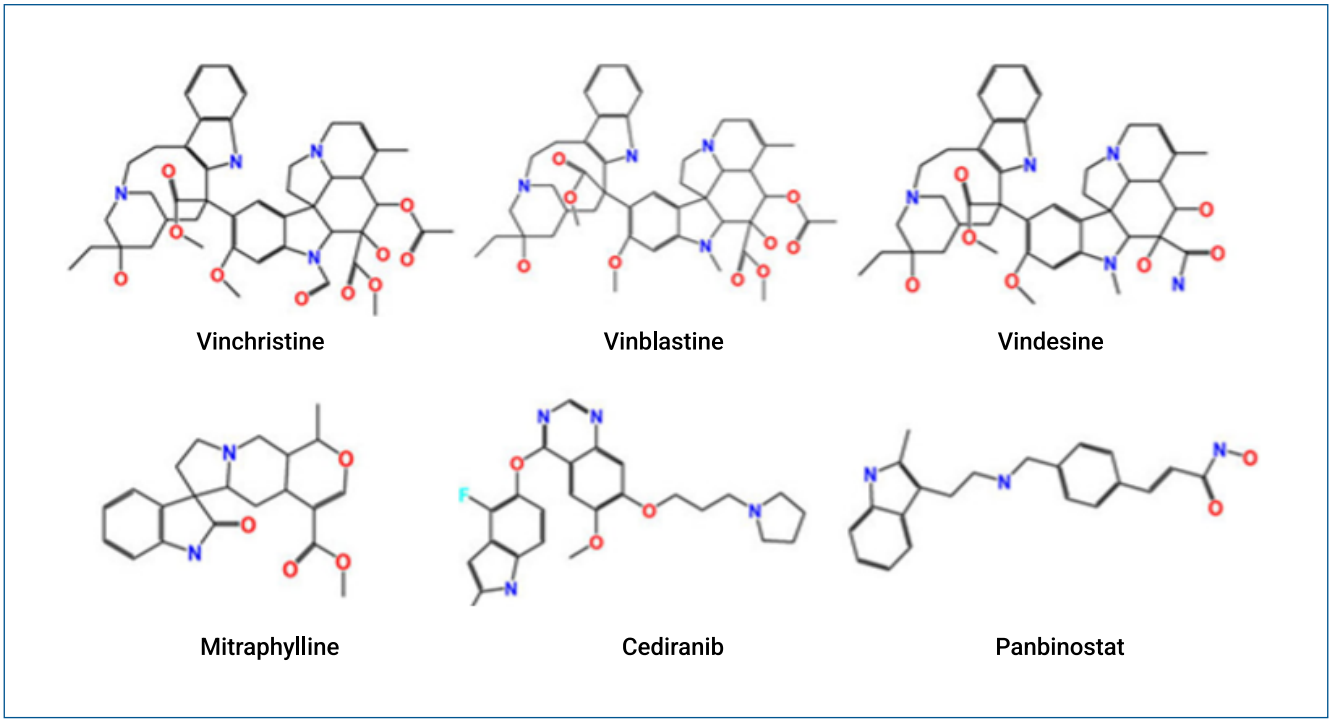


Figure 1. FDA approved anticancer drugs containing indole scaffold.

methanol, formaldehyde, toluene, ethyl acetate, and chloroform were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis Methods

General Synthesis Method of Compounds 1-4

3-Methylindole (2.2 mmol, 300 mg) was dissolved in 20 mL of ethanol in a round bottom flask, then formaldehyde 37% (3 mmol) and substituted piperidine (2.2 mmol) were added. The mixture was refluxed for 4–6 hours, and the reaction was controlled by thin-layer chromatography (TLC) with silica gel plate as a stationary phase and toluene:ethyl acetate (10:1) as a mobile phase. After the reaction was completed, the mixture was placed in the freezer for 24 hours, and the resulting precipitate was filtrated and dried. Purification of the synthesized compounds was carried out using a combination of crystallization with either ethanol or methanol and column chromatography, using toluene: ethyl acetate (10:1) solvent system.

General Synthesis Method of Compounds 5-10

3-Methylindole (2.2 mmol, 300 mg) was dissolved in 20 mL of ethanol in a round bottom flask, then formaldehyde 37% (3 mmol) and substituted piperazine (2.2 mmol) were added. The mixture was refluxed for 4–6 hours, and the reaction was controlled by TLC with silica gel plate as a stationary phase and toluene: ethyl acetate (10:1) as a mobile phase. After the reaction was completed, the mixture was placed in the freezer for 24 hours, and the resulting precipitate was filtrated and dried. Purification of the synthesized compounds was carried out using a combination of crystallization with either ethanol or methanol and column chromatography, using toluene:ethyl acetate (10:1) solvent system.

Analytical Methods

Melting point (°C) determination of compounds were carried out using a FP62 capillary melting point apparatus (Mettler Toledo, Columbus, OH, USA) and were uncorrected. The reactions were controlled by TLC on aluminium sheets 20x20 cm silica gel 60 F254 (Merck, Darmstadt, Germany) plates. Compounds **4**, **5**, **6** and **7** were purified by column chromatography using silica gel 60 mesh (Merck, Darmstadt, Germany) as stationary phase and toluene:ethyl acetate (10:1) solution as mobile phase.

Infrared (IR) spectra were recorded on a Spectrum One FT-IR spectrometer (PerkinElmer, Waltham, MA, USA; Version 5.0.1), by applying potassium bromide (KBr) as a background, and the frequencies were shown in cm^{-1}.

^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Mercury-500 FT-NMR spectrometer (Varian Inc., Palo Alto, CA, USA) using tetramethylsilane (TMS; Sigma-Aldrich, St. Louis, MO, USA) as the internal reference. Deuterated dimethyl sulfoxide (DMSO-d6) or deuterated chloroform ($CDCl_3$) were used as solvents, and the chemical shifts were reported in parts per million (ppm).

Full FT-IR and NMR results are provided in Supplementary Material.

BIOLOGICAL STUDY

Cell Culture

MCF-7 and HUVEC cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA; Cat. No.11875093) with 10% (v/v) fetal bovine serum, 1% streptomycin-penicillin at 37°C in an incubator containing 5% CO_2.

Cytotoxic Activity

To assess the viability of MCF-7 and HUVEC cell lines in response to compound treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was conducted. Cells were seeded into 96-well plates at a density of 3,500 cells/well in 100 µL of medium. After a 24-hour incubation, the cells were treated with six different concentrations (6.25, 12.5, 25, 50, 100, and 200 µM) of the compounds, each diluted in 100 µL of medium. Control wells received the same volume of medium without the compounds.

After 72 hours of incubation, 10 µL of 5 mg/mL MTT in phosphate-buffered saline (PBS) was added to each well. After 3 hours of incubation at 37°C, formazan crystals were dissolved in 150 µL of 2-propanol and incubated at room temperature for an additional 30 minutes. Absorbance values were determined at 570 nm using a Multiskan Ascent microplate reader (Thermo Electron Corporation, Vantaa, Finland). Tamoxifen was used as a reference drug to compare the cytotoxic activity on cells.

The absorbance of the control group (no compound) was considered as 100%. The percentage inhibition (%) of cell proliferation was determined with the following equation:

$$\text{Percentage inhibition (\%)} = (\text{Compound}_{Abs}\text{-Blank}_{Abs}) \times 100/(\text{Control}_{Abs}\text{-Blank}_{Abs}).$$

Cytotoxic measurement parameter IC_{50}, inhibitory concentration by 50%, was obtained using curve fitting method on excel and R^2 values were considered. Each compound was studied with 5 replicates in two independent experiments. Graphs were generated and statistics were calculated using Microsoft Excel.

The SI measures a drug's or compound's activity specifically against targeted cancer cells compared to healthy cells. The SI is calculated as the ratio of the IC_{50} value of the compound in healthy cells to its IC_{50} value in cancer cells. An SI value greater than 1 indicates a desirable level of selectivity toward cancer cells (15).

RESULTS

Chemical data

The desired compounds were synthesized by Mannich reaction between 3-methylindole, and various substituted piperidine and piperazine derivatives, following a procedure adapted from previously published literatures (16). The general synthetic procedure of the ten target compounds is illustrated in the scheme below (Scheme 1).

The FTIR, ^{1}H-NMR, and ^{13}C-NMR spectra of the newly synthesized compounds furnished compelling evidence regarding their structural characteristics. The IR spectra revealed key functional group vibrations, with aromatic and aliphatic C-H stretching bands observed between 3061-2828 cm^{-1}, indicating the presence of both aromatic and aliphatic hydrogens in the compounds. The aromatic C=C stretching bands were observed within the range of 1618-1459 cm^{-1}. In ^{1}H-NMR spectra, the indole CH_3 singlet peaks were detected at 2.35 ppm, which is consistent with the methyl group attached to the indole ring. Additionally, piperidine hydrogens showed peaks between 1.72-2.92 ppm, while the piperazine hydrogens were detected between 2.53-3.45 ppm, reflecting the incorporation of these heterocyclic rings into the structure. The N-CH_2-N singlet peaks recorded at 4.82-4.90 ppm further corroborate the presence of indole-piperidine and piperazine linkages. Aromatic hydrogens in the indole and phenyl rings were observed between 6.95-7.60 ppm.

3-Methyl-1-[(4-phenylpiperidin-1-yl)methyl]-1H-indole (1)

Yield: 40 %. mp: 108°C. IR (KBr, cm^{-1}): 1465 (aromatic C=C), 2948 (aliphatic C-H), 3020 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 1.78-1.87 (m, 4H, piperidine), 2.31-2.42 (m, 6H, CH_3 + 3H piperidine), 3.11 (d, 2H, piperidine, J = 11.2 Hz), 4.90 (s, 2H, N-CH_2-N), 6.98 (s, 1H, indole), 7.16-7.17 (dd, 1H, indole, J_1 = 7.9 H_Z, J_2 = 0.9 H_Z), 7.20-7.25 (m, 4H, phenyl), 7.28-7.33 (m, 2H, 1H indole + 1H phenyl), 7.49 (d, 1H, indole, J = 8.25 H_Z), 7.61 (d, 1H, indole, J = 7.85 Hz). ^{13}C-NMR (DMSO, δ ppm): 9.65 (CH_3), 33.32, 42.18, 51.62 (piperidine-C), 67.96 (CH_2), 109.88, 110.61, 118.88, 118.89, 121.63, 126.21, 126.46, 126.82, 128.45, 128.78, 137.45, 146.13 (Ar-C).

1-[(3-Methyl-1H-indol-1-yl)methyl]-4-phenylpiperidin-4-ol (2)

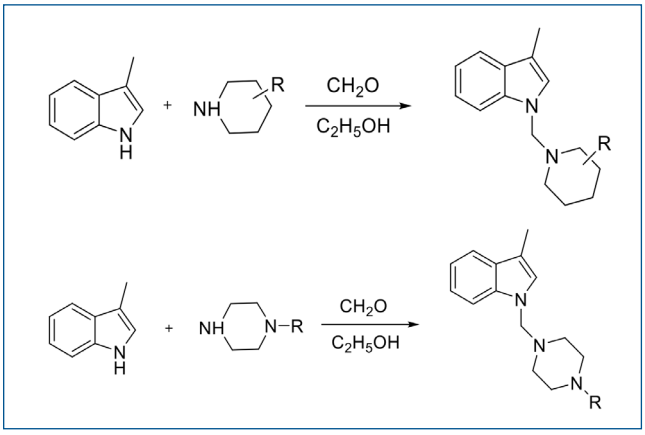
Yield: 42%. mp:129°C. IR (KBr, cm^{-1}): 1459 (aromatic C=C), 2912 (aliphatic C-H), 3053 (aromatic C-H), 3292 (O-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 1.75-1.78 (dd, 2H, piperidine, J_1 = 14.1 H_Z, J_2 = 2.4 H_Z), 2.17 (t, 2H, piperidine, J = 11.4 H_Z), 2.36 (s, 3H, CH_3), 2.70 (t, 2H, piperidine, J = 11.35 H_Z), 2.88 (d, 2H, piperidine, J = 10.95 H_Z), 4.86 (s, 2H, N-CH_2-N), 7.01 (s, 1H, indole), 7.14-7.17 (dd, 1H, indole, J_1 = 14.9 Hz, J_2 = 0.75 H_Z), 7.23-7.30 (m, 2H, phenyl), 7.35-7.38 (m, 2H, phenyl), 7.47-7.51 (m, 3H, indole + phenyl), 7.60 (d, 1H, indole, J = 7.8 H_Z). ^{13}C-NMR (DMSO, δ ppm): 9.64 (CH_3), 38.29, 46.97 (piperidine-C), 67.88 (CH_2), 71.12 (C-OH), 109.83, 110.79, 118.90, 121.61, 124.48, 126.29, 127.09, 128.39, 128.90, 137.35, 148.20 (Ar-C).

1-[(3,5-Dimethylpiperidin-1-yl)methyl]-3-methyl-1H-indole (3)

Yield: 39%. mp: 87°C. IR (KBr, cm^{-1}): 1461 (aromatic C=C), 2916 (aliphatic C-H), 3056 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 0.86 (d, 6H, piperidine $(CH_3)_2$, J = 5.9 H_Z), 1.70-1.73 (dd, 6H, piperidine, J_1 = 12.8 H_Z, J_2 = 5.1 H_Z), 2.36 (s, 3H, CH_3), 2.92 (d, 2H, piperidine, J = 6.6 H_Z), 4.83 (s, 2H, N-CH_2-N), 6.95 (s, 1H, indole), 7.14 (t, 1H, indole, J = 7.8 H_Z), 7.22-7.28 (m, 1H, indole), 7.45 (d, 1H, indole, J = 8.2 H_Z), 7.59 (d, 1H, indole, J = 7.85 H_Z). ^{13}C-NMR (DMSO, δ ppm): 9.64 (CH_3), 19.58 $(CH_3)_2$, 31.29, 41.75, 58.68 (piperidine-C), 67.94 (CH_2), 109.93, 110.43, 118.75, 118.77, 121.57, 126.49, 128.72, 137.43 (Ar-C).

3-Methyl-1-[(4-methylpiperidin-1-yl)methyl]-1H-indole (4)

Yield: 43%. mp: 46°C. IR (KBr, cm^{-1}): 1460 (aromatic C=C), 2920 (aliphatic C-H), 3054 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 0.92 (d, 3H, CH_3, J = 5.7 H_Z), 1.28-1.26 (m, 3H, piperidine), 1.64 (d, 2H, piperidine, J = 9.4 H_Z), 2.19 (t, 2H, piperidine, J = 10.85 H_Z), 2.35 (s, 3H, CH_3), 2.95 (d, 2H, piperidine, J = 11.3 H_Z), 4.82 (s, 2H, N-CH_2-N), 6.95 (s, 1H, indole), 7.15 (t, 1H, indole, J = 7.15 H_Z), 7.28-7.22 (m, 1H, indole), 7.45 (d, 1H, indole, J = 8.2 H_Z), 7.59 (d, 1H,



Scheme 4.1. General synthesis procedure of compounds.

indole, J = 7.8 H_Z). ^{13}C-NMR (DMSO, δ ppm): 9.62 (CH_3), 21.86 (piperidine-CH_3), 30.35, 34.18, 51.25 (piperidine-C), 67.99 (CH_2), 109.88, 110.47, 118.77, 118.80, 121.52, 126.47, 128.72, 137.44 (Ar-C).

1-{4-[bis(4-fluorophenylmethyl)piperazin-1-yl]methyl}-3-methyl-1H-indole (5)

Yield: 56%. mp: 145°C. IR (KBr, cm^{-1}): 1504-1599 (aromatic C=C), 2920 (aliphatic C-H), 3060 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 2.36-2.42 (m, 7H, 3H CH_3 + 4H piperazine), 2.62 (s, 4H, piperazine), 4.20 (s, 1H, CH), 4.85 (s, 2H, N-CH_2-N), 6.93-6.97 (m, 5H, 4H phenyl + 1H indole), 7.13-7.16 (dd, 1H, indole, J_1 = 14.8 H_Z, J_2 = 0.75 H_Z), 7.21-7.24 (m, 1H, indole), 7.28-7.31 (m, 4H, phenyl), 7.41 (d, 1H, indole, J = 8.2 H_Z), 7.60 (d, 1H, indole, J = 7.8 H_Z). ^{13}C-NMR (DMSO, δ ppm): 9.62 (CH_3), 50.58, 51.55 (piperazine-C), 67.32 (CH_2), 74.40 (CH), 109.75, 110.71, 115.29, 115.46, 118.88, 118.91, 121.65, 126.41, 128.80, 129.17, 129.23, 137.41, 138.14, 138.16, 160.82, 162.77 (Ar-C).

3-Methyl-1-{4-[(4-trifluoromethylphenyl) piperazin-1-yl]methyl}-1H-indole (6)

Yield: 64%. mp: 147°C. IR (KBr, cm^{-1}): 1462-1618 (aromatic C=C), 2838 (aliphatic C-H), 3027 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 2.35 (s, 3H, CH_3), 2.73 (s, 4H, piperazine), 3.30 (s, 4H, piperazine), 4.86 (s, 2H, N-CH_2-N), 6.89 (d, 2H, phenyl, J = 8.75 H_Z), 6.99 (s, 1H, indole), 7.16 (t, 1H, indole, J = 7.1 H_Z), 7.25-7.28 (m, 1H, indole), 7.44-7.48 (m, 3H, 2H phenyl + 1H indole), 7.60 (d, 1H, indole, J = 7.85 H_Z). ^{13}C-NMR (DMSO, δ ppm): 9.61 (CH_3), 47.90, 50.60 (piperazine-C), 67.60 (CH_2), 109.74, 111.07, 114.66, 119.01, 119.10, 120.49, 120.75, 121.78, 123.63, 125.78, 126.07, 126.35, 126.38, 126.40, 128.97, 137.24, 153.19 (Ar-C).

1-{4-[(4-Bromophenyl)piperazin-1-yl]methyl}-3-methyl-1H-indole (7)

Yield: 47%. mp: 146°C. IR (KBr, cm^{-1}): 1586 (aromatic C=C), 2828 (aliphatic C-H), 3038 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 2.35 (s, 3H, CH_3), 2.72 (s, 4H, piperazine), 3.17 (t, 4H, piperazine, J = 4.6 H_Z), 4.85 (s, 2H, N-CH_2-N), 6.75-6.76 (dd, 2H, phenyl, J_1 = 6.95 H_Z, J_2 = 2.15 Hz), 6.98 (s, 1H, indole), 7.14-7.17 (dd, 1H, indole, J_1 = 14.9 Hz, J_2 = 0.85 Hz), 7.23-7.28 (m, 1H, indole), 7.33-7.34 (dd, 2H, phenyl, J_1 = 6.95 Hz, J_2 = 2.2 Hz), 7.45 (d, 1H, indole, J = 8.25 Hz), 7.60 (d, 1H, indole, J = 7.8 Hz) ^{13}C-NMR (DMSO, δ ppm): 9.61 (CH_3), 48.92, 50.31 (piperazine-C), 67.62 (CH_2), 109.76, 111.00, 111.95, 117.79, 118.98, 119.05, 121.73, 126.09, 128.96, 131.86, 137.25, 150.24 (Ar-C).

1-{4-[(4-methoxyphenyl)piperazin-1-yl]methyl}-3-methyl-1H-indole (8)

Yield: 50%. mp: 115°C. IR (KBr, cm^{-1}): 1513 (aromatic C=C), 2831 (aliphatic C-H), 2934 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 2.35 (s, 3H, CH_3), 2.75 (s, 4H, piperazine), 3.11 (t, 4H, piperazine, J = 4.6 H_Z), 3.78 (s, 3H, O-CH_3), 4.85 (s, 2H, N-CH_2-N), 6.83-6.90 (m, 4H, phenyl), 6.99 (s, 1H, indole), 7.14-7.17 (dd, 1H, indole, J_1 = 14.9 H_Z, J_2 = 0.85 H_Z), 7.23-7.28 (m, 1H, indole), 7.47 (d, 1H, indole, J = 8.25 H_Z), 7.60 (d, 1H, indole, J = 7.85 H_Z). ^{13}C-NMR (DMSO, δ ppm): 9.62 (CH_3), 50.60, 50.63 (piperazine-C), 55.56 (O-CH_3), 67.67 (CH_2), 109.81, 110.89, 114.42, 118.44, 118.94, 118.98, 121.68, 126.16, 128.95, 137.29, 145.66, 153.92 (Ar-C).

1-{4-[(2,3-dichlorophenyl)piperazine-1-yl]methyl}-3-methyl-1H-indole (9)

Yield: 56%. mp: 110°C. IR (KBr, cm^{-1}): 1448-1579 (aromatic C=C), 2832 (aliphatic C-H), 3061 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 2.36 (s, 3H, CH_3), 2.78 (s, 4H, piperazine), 3.08 (s, 4H, piperazine), 4.86 (s, 2H, N-CH_2-N), 6.94-6.95 (dd, 1H, phenyl, J_1 = 7.55 H_Z, J_2 = 2 H_Z), 7.00 (s, 1H, indole), 7.13-7.18 (m, 3H, indole + phenyl), 7.24-7.28 (m, 1H, indole), 7.47 (d, 1H, indole, J = 8.25 H_Z), 7.61 (d, 1H, indole, J = 7.85 H_Z). ^{13}C-NMR (DMSO, δ ppm): 9.64 (CH_3), 50.62, 51.15 (piperazine-C), 67.74 (CH_2), 109.80, 110.97, 118.64, 118.96, 119.02, 121.72, 124.69, 126.15, 127.43, 127.57, 128.98, 134.05, 137.26, 151.15 (Ar-C).

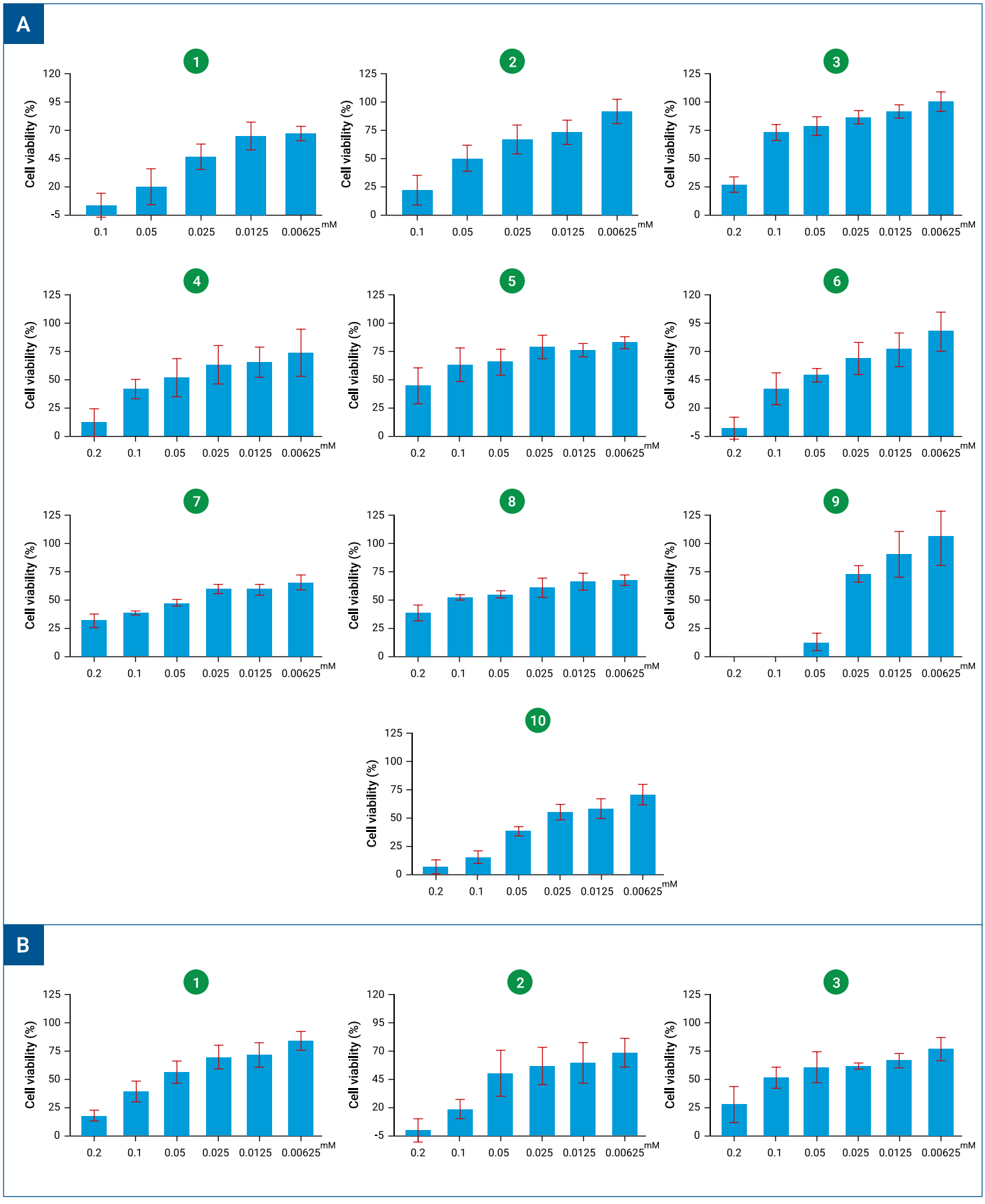
tert-Butyl-4-[(3-methyl-1H-indol-1-yl)methyl] piperazine-1-carboxylate

Yield: 35%. mp: 83°C. IR (KBr, cm^{-1}): 1421-1461 (aromatic C=C), 1693 (C=O), 2916 (aliphatic C-H), 2932 (aromatic C-H). ^{1}H-NMR ($CDCl_3$, δ ppm): 1.43 (s, 9H, tert-butyl), 2.34 (s, 3H, CH_3), 2.53 (s, 4H, piperazine), 3.45 (s, 4H, piperazine), 4.83 (s, 2H, N-CH_2-N), 6.95 (s, 1H, indole), 7.16 (t, 1H, indole, J = 7.5 H_Z), 7.22–7.28 (m, 1H, indole), 7.41 (d, 1H, indole, J = 8.2 H_Z), 7.59 (d, 1H, indole, J = 7.85 H_Z). ^{13}C-NMR (DMSO, δ ppm): 9.59 (CH_3), 28.39 (CH_3)$_3$, 43.09, 50.28 (piperazine-C), 67.67 (CH_2), 79.72 (O-CH), 109.72, 110.98, 118.97, 119.04, 121.74, 126.10, 128.90, 137.22 (Ar-C), 154.53 (C=O).

The chemical structures of all molecules, synthesized are given in Supplementary Material.

Biological activity

The anticancer activity of the target compounds was examined against breast cancer cell line MCF-7 and non-cancerous human endothelial cell line HUVEC using MTT assay to investigate their selective cytotoxicity effect. Tamoxifen was used as standard drug. Among the investigated derivatives, compounds **1**, **2**, **3**, **9** and **10** exhibited selective inhibitory effect on MCF-7 cells with SI values of 3.21, 1.08, 2.90, 1.48 and 2.29 respectively, suggesting that these compounds are acting selectively on cancerous cells. Also, these compounds exerted a greater selective cytotoxic activity than tamoxifen that exhibited an SI value of 1.15. The IC_{50} values of these compounds on MCF-7 cell line were determined as follows: 27, 53, 35, 32 and 31 μM. These compounds' IC_{50} values on HUVECs were obtained as 85, 57, 100, 48 and 71 μM.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70
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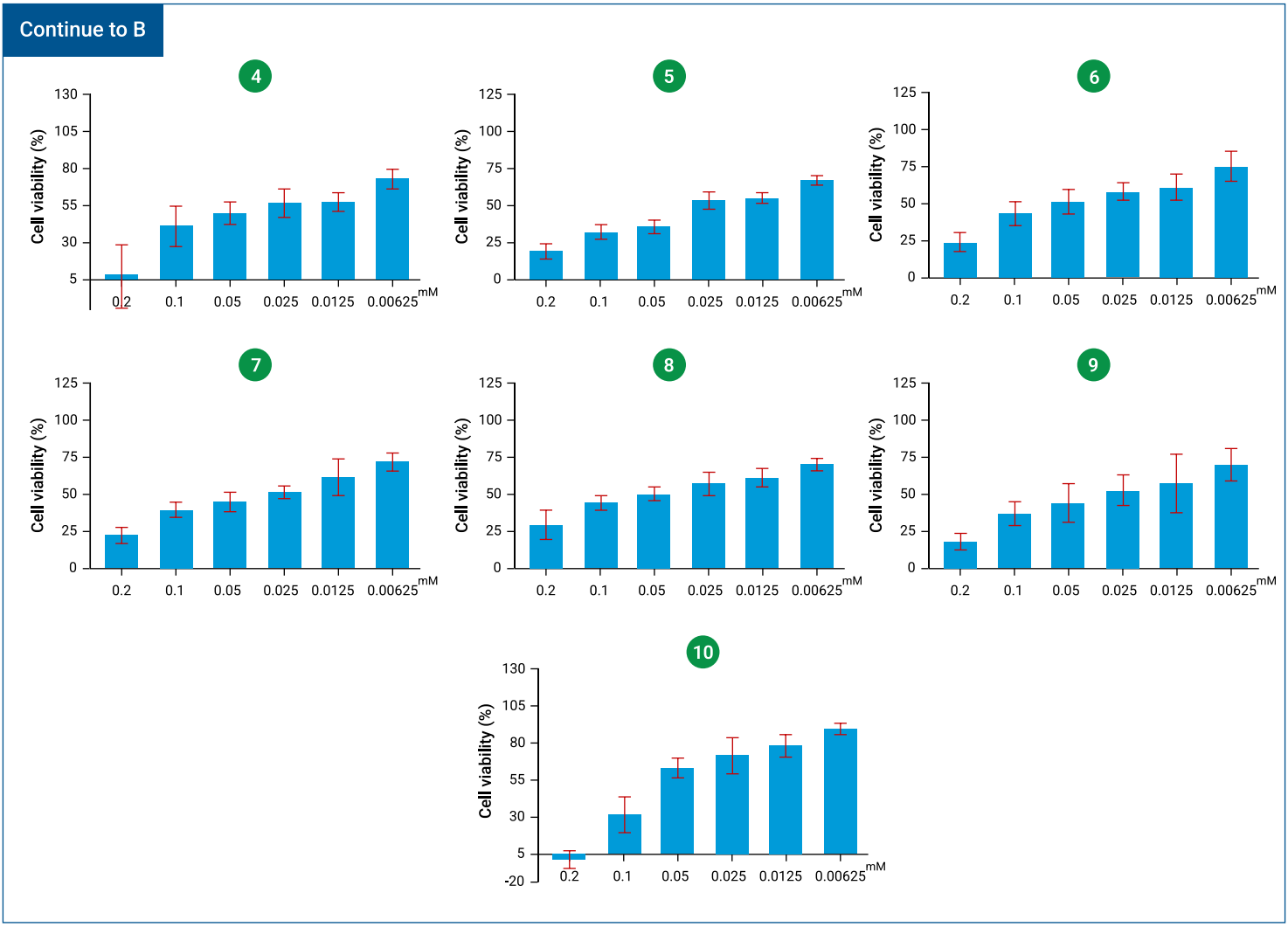


Figure 2. Cell viability graphs of MCF-7 (A) and HUVEC (B) cells treated with the target compounds. SD±data mean. At least 2 independent study with 5 technical replicates.

The compounds tested, exhibit dose dependent cytotoxic effect on both MCF-7 and HUVEC cell lines (Figure 2). The effect of the compounds was more prominent even at low concentrations when administered on MCF-7 compared to HUVECs. This suggested that the breast cancer cell line MCF-7 was more vulnerable to applied compounds.

DISCUSSION

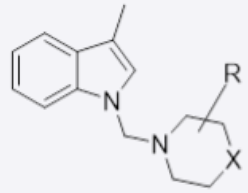
Literature has extensively investigated the role of the indole scaffold in development of anticancer agents (17-20) and the cytotoxic activity of piperidine and piperazine derivatives (21,22).

Even though, the indole derivatives have been described as quite potent molecular structures targeting cancer cells, only a few studies have investigated the cytotoxic activity of 3-methyl indole (23). Therefore, in this work, we synthesized hybrid molecules with the general structure of 1-[(substituted-1-piperidinyl)methyl)]-3-methyl-1H-indole and 1-[(4-(substituted-1-piperazinyl) methyl)]-3-methyl-1H-indole, and investigated their cytotoxic activities using MTT assay, the results indicated that compounds **1**, **2**, **3**, **9** and **10** exhibited selective inhibitory effect on MCF-7 cells suggesting that these compounds are acting on cancerous cells, selectively.

CONCLUSION

Ten novel N-substituted 3-methylindole derivatives were synthesized with moderate yields. Their structural elucidation was confirmed using IR, ^{1}H-NMR, ^{13}C-NMR spectroscopic methods. The target compounds were investigated for their anticancer activity against MCF-7 and HUVEC cell lines. Among the tested compounds, compounds **1**, **2**, **3**, **9** and **10** exhibited selective inhib-

Table 1. Cytotoxicity and selectivity of the target compounds on MCF-7 and HUVEC cell lines.



Compound No.	X	R	HUVEC		MCF-7		SI ≥ 1
			IC_{50}	R^2	IC_{50}	R^2	
1	C	4-phenyl	**85**	**0.95**	**27**	**0.92**	**3.21**
2	C	4-hydroxy-4-phenyl	**57**	**0.95**	**53**	**0.95**	**1.08**
3	C	3,5-dimethyl	**100**	**0.95**	**35**	**0.98**	**2.90**
4	C	4-methyl	47	0.95	141	0.95	0.33
5	N	Bis(4-fluorophenyl) methyl	33	0.82	158	0.94	0.21
6	N	4-trifluoromethylphenyl	74	0.91	78	0.94	0.95
7	N	4-bromophenyl	56	0.85	61	0.85	0.93
8	N	4-methoxyphenyl	74	0.92	105	0.95	0.71
9	N	2,3-dichlorophenyl	**48**	**0.91**	**32**	**0.99**	**1.48**
10	N	4-*tert*. butoxy carbonyl	**71**	**0.97**	**31**	**0.85**	**2.29**
Tamoxifen	-	-	11	0.98	10	0.99	1.15

itory effect on MCF-7 cells with SI values of 3.21, 1.08, 2.90, 1.48 and 2.29 respectively. The IC_{50} values of these compounds were of 27, 53, 35, 32 and 31 μM. Comparing to the standard, compounds showed moderate cytotoxic activity. Moreover, in future studies, these compounds can be further structurally modified and investigated for their biological activity to obtain remarkable agents.

Ethical Approval: N.A.

Informed Consent: N.A.

Peer-review: Externally peer-reviewed

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Optimization of ROS Measurement in PANC-1 Cells

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Abstract

Objective: Accurate measurement of reactive oxygen species (ROS) is essential for understanding oxidative stress-related cellular responses. Among the available detection methods, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is widely used due to its sensitivity and ease of application. However, signal variability due to differences in incubation time can impact data reliability. This study aimed to optimize the incubation time of H_2DCFDA for accurate detection of intracellular ROS levels in PANC-1 cells using flow cytometry.

Materials and Methods: PANC-1 cells were cultured under optimized conditions and incubated with 10 µM H_2DCFDA from 5 minutes to 90 minutes. Following incubation, cells were detached, washed, and analyzed using flow cytometry (FITC channel). Three independent biological replicates were performed for each time point.

Results: A time-dependent increase in intracellular ROS fluorescence was observed. At 5 minutes, 19.79% of cells were ROS-positive, which increased from 30.65% to 65.70% until 25 minutes, respectively. After that point, ROS-signal was saturated and measured approximately 90-95%, like positive control. The strongest fluorescence signal, was detected at 30 minutes, indicating a peak in probe oxidation and ROS detection efficiency.

Conclusion: This study demonstrates that H_2DCFDA provides reliable time-sensitive ROS detection in PANC-1 cells, with 30-minute incubation offering optimal signal intensity without additional chemical induction. However, variations in ROS dynamics among different cell types underscore the need for cell-specific optimization of assay conditions.

Keywords: H2DCFDA, ROS assay, flow cytometry, oxidative stress, PANC-1

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INTRODUCTION

Oxidative stress is a condition that arises when the production of reactive oxygen species (ROS) during cellular metabolism exceeds the antioxidant defense capacity (1). ROS comprise a variety of reactive molecules and free radicals, including superoxide anion ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydroxyl radical (•OH), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2) (2). Under physiological conditions, low levels of ROS play regulatory roles in intracellular signaling pathways; however, excessive ROS can lead to oxidative damage of lipids, proteins, and DNA contributing to inflammation, tissue injury, and organ dysfunction (3). Furthermore, oxidative stress plays a key role in the pathogenesis of various chronic diseases, including cancer progression, atherosclerosis, neurodegenerative disorders, and diabetes (1). Recent studies have shown that although a certain amount of well-regulated ROS production contributes to immune homeostasis, uncontrolled elevation of ROS levels may cause cellular damage. As a result, the biology of ROS and oxidative stress has become an intensive area of investigation across multiple disciplines such as cancer research (2).

One One of the most used techniques for detecting intracellular ROS levels is the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). H_2DCFDA, non-fluorescent, is a cell-permeable compound that is hydrolyzed by intracellular esterases and, upon oxidation, in the presence of ROS, is converted into the highly fluorescent product DCF. The H_2DCFDA method is advantageous due to its relative ease of use, high sensitivity, and low cost (3). When combined with flow cytometry, fluorescence intensity can be quantified at a single-cell level, enabling precise identification of cells undergoing elevated oxidative stress within heterogeneous populations (2).

However, the H_2DCFDA-based ROS detection method has some important drawbacks. The H_2DCFDA probe does not respond equally to all types of ROS; for instance, it does not react directly with superoxide anions ($O_2^{\bullet -}$) but can be oxidized indirectly in the presence of catalytic metal ions such as iron particularly Fe (II). As a result, fluorescence signals may not always reflect actual ROS levels under certain experimental conditions. Furthermore, H_2DCFDA can be oxidized by other reactive species, including nitric oxide (NO) and peroxynitrite, and its fluorescence signal may also be influenced by changes in intracellular antioxidant levels, such as glutathione (4). To ensure accurate ROS measurement, factors such as light sensitivity of the fluorophore, esterase variability among cells, and mechanical stress during cell handling must be carefully controlled (5). Taken together, while H_2DCFDA serves as a valuable and dynamic indicator of oxidative activity, careful consideration of its limitations is essential for accurate interpretation of data.

The biological effects of ROS depend not only on their concentration but also on the duration of exposure. While transient increased ROS levels may serve as physiological signals, prolonged and widespread ROS accumulation is often associated with pathological outcomes. Besides, the kinetics of ROS production, persistence, and clearance are critical for the accurate interpretation of experimental results. Inadequate temporal optimization may lead to misinterpretation, for instance, mistaking a transient ROS spike for sustained oxidative stress. Hence, precise control and optimization of temporal parameters in ROS measurements are essential for elucidating underlying mechanisms and identifying effective therapeutic targets (6). In this regard, the duration of incubation is a key variable when using fluorescent probes such as H_2DCFDA for ROS detection. Short incubation periods may yield insufficient signal intensity, whereas excessively long exposures can result in signal saturation, photobleaching, or loss of cellular viability, all of which compromise data reliability (7). Moreover, metabolic activity, esterase expression, and ROS dynamics vary across cell types, necessitating specific optimization for each experimental system. Researchers can ensure that ROS measurements accurately reflect the biological state of the cells and remain within the linear detection range of the probe by systematically calibrating incubation times (8).

Since oxidative stress plays an important role in cellular physiology as well as in pathophysiology, the accurate, sensitive, and consistent measurement of ROS levels is essential. However, the technical limitations and interpretability of ROS detection protocols can significantly impact the reliability of experimental outcomes. In many studies, H_2DCFDA incubation time is used as a fixed value, even though it's not always tested for the specific cell type or experimental setup. However, this can compromise data accuracy—short incubations may yield weak signals, whereas extended durations can lead to fluorescence saturation, phototoxicity, or cell stress. In this context, we optimized the H_2DCFDA incubation time for PANC-1 cells using flow cytometry, as incubation conditions can strongly influence baseline ROS measurements. As no ROS-inducing agent was used in this study, it is particularly important to tailor the protocol to each cell type to ensure reliable detection of intrinsic ROS levels.

MATERIALS AND METHODS

Cell Culture

PANC-1 pancreatic cancer cells (American Tissue Cell

Culture [ATCC], Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) at 37°C. For passages, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution is used.

Preparation of H2DCFDA

To prepare the stock solution, 0.0097 g of H_2DCFDA powder (MedChemExpress, HY-D0940) was accurately weighed and dissolved in 2 mL of dimethyl sulfoxide (DMSO) with the aid of ultrasonic agitation, yielding a 10 mM concentration. For experimental use, the working solution was freshly prepared by diluting the stock in 1X phosphate-buffered saline (PBS) to achieve a final concentration of 10 µM. This solution was then applied directly to the cells for ROS detection.

Incubation with H_2DCFDA

Intracellular ROS levels were assessed using the fluorescent probe H_2DCFDA, 24 hours after seeding the cells at approximately 70% confluency, with or without treatment. Cells were incubated with 2 mL of a 10 µM H_2DCFDA solution prepared in 1X PBS for 5-10-15-20-25-30-40-50-60 and 90 minutes at 37°C in the dark. As a positive control, cells were also treated with 500 µM H_2O_2 for 30 minutes. After incubation, cells were detached by trypsinization and collected in 5 mL of 1X PBS. The suspension was then centrifuged at 300 g for 3 minutes at room temperature. The resulting cell pellet was gently resuspended in 100 µL of 1X PBS, and samples were immediately analyzed by flow cytometry to measure ROS-associated fluorescence. Three biological replicates were carried out.

Flow Cytometry Acquisition and Analysis Parameters

Flow cytometric analysis was performed using a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). H_2DCFDA was excited using a 488 nm argon-ion laser, and emission was detected in the FITC channel (FL1; 530/30 nm bandpass filter). A minimum of 10,000 events were collected per sample. Instrument settings, including forward scatter (FSC), side scatter (SSC), and FITC voltages, were optimized using untreated (unstained) control cells. Data acquisition and analysis were conducted using FlowJo™ software v10 (BD Biosciences, Ashland, OR, USA).

RESULTS

Flow cytometric analysis using H_2DCFDA staining revealed a time-dependent increase in intracellular ROS levels. Histograms obtained from the FITC-A channel demonstrated a progressive rightward shift in fluorescence intensity with increasing incubation time. At 5 minutes, 19.79% of the cells exhibited ROS positivity, which

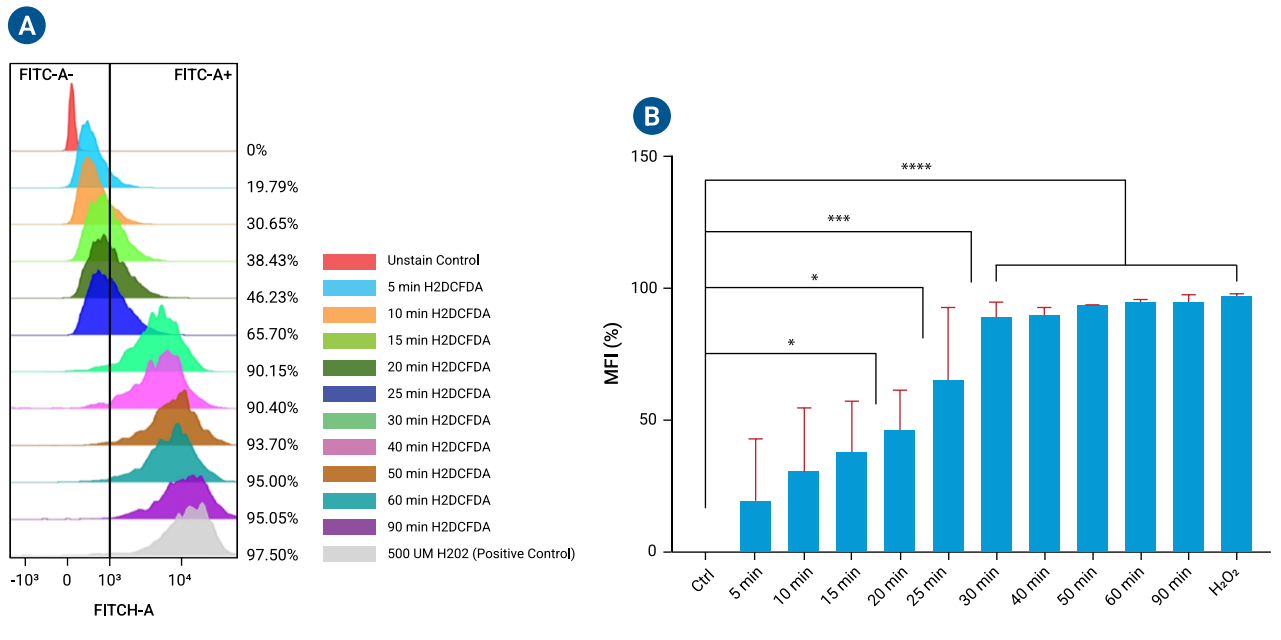

FIGURE 1. (A) Representative FITC-A histograms of PANC-1 cells stained with H_2DCFDA for various incubation durations (5 to 90 minutes), along with negative (unstained) and positive (H_2O_2-treated) controls. (B) Quantitative analysis of ROS levels presented as mean fluorescence intensity (MFI), normalized to the control group. Data are expressed as mean ± standard deviation (SD) of three independent experiments. Statistical comparisons were performed relative to the unstained control group.
*p<0.05, ***p<0.01.

increased to 30.65%, 38.43%, 46.23%, 65.70% and %90.15 at 10, 15, 20, 25 and 30 minutes, respectively. ROS signal levels appear to plateau, indicating that the probe reaches saturation and no additional ROS accumulation is detected beyond 30 minutes, significantly (p>0.05) (Figure 1). All statistical comparisons were performed relative to control samples that were not treated with H_2DCFDA.

DISCUSSION

In this study, intracellular ROS levels were measured in PANC-1 cells using H_2DCFDA-based flow cytometry method to detect the most appropriate incubation time. The findings showed that there was a gradual increase in FITC-A fluorescence signal depending on the incubation time and the ROS positive cell ratio increased significantly especially at the 30th minute (Figure 1). After that point, fluorescence signal reached a saturation point and there was no significant difference for the following time points. These results suggested that H_2DCFDA is a suitable indicator for monitoring intracellular oxidative stress levels in a time-sensitive manner.

This increase in ROS levels indicates that cells are exposed to increasing oxidative stress over time and suggests that H_2DCFDA accurately reflects dynamic ROS changes. The 19.79% positivity detected at 5 minutes shows that H_2DCFDA is sensitive to ROS even in short-term incubations, while the 90.15 % positivity reached at 30 minutes provides quantitative information about the oxidative status of the cells (Figure 1). This linear increase supports the fact that H_2DCFDA is oxidized by increasing ROS over time and becomes fluorescent DCF, which can be measured by flow cytometry, as reported in the literature (9,10). According to our results, ROS levels could be measured even at incubation times as low as 5 minutes, but 30 minutes is generally accepted as the standard time in the existing literature. For instance, Kim and Xue (11) measured ROS levels in colorectal cancer cells by incubating them with a 10 μM H_2DCFDA solution at 37°C for 30 minutes. Also, Bode et al. (3), used H_2DCFDA in measuring ROS by incubating different cells with it for 30 minutes. Our results also demonstrated that a 30-minute incubation time is suitable for PANC-1 cells, as it provided a high ROS signal compared to control cells. Notably, no significant difference was found between the 25- and 30-minute time points, suggesting that saturation occurs over 30 minutes. Moreover, no significant differences were observed among the extended incubation times beyond 30 minutes, further supporting the notion of signal saturation.

On the other hand, the concentration of H_2DCFDA is also a critical factor affecting ROS detection. Wu and Yotnda (12) recommend using a starting dose of 10 μM for the determination of ROS in cancer cells to evaluate the toxicity. In our study, a 10 μM solution was used, which is consistent with the initial recommendations. Soares et al. (13) optimized ROS detection in human blood samples, using a 120 μM solution and an incubation time of 30 minutes. Gonzalez and Salido (14) worked on pancreatic acinar cells to detect ROS levels, with a 10 μM H_2DCFDA solution and incubation time of 40 minutes. These studies demonstrate that incubation time and concentration can significantly affect the results, depending on the cell type.

However, some limitations of using H_2DCFDA should be considered. H_2DCFDA is not equally sensitive to all ROS species; for example, it does not directly respond to $O_2^{\bullet -}$ but can be indirectly oxidized *via* metal ions. This means that in some experimental conditions the measured fluorescence signal may not fully reflect the true ROS concentration. Furthermore, H_2DCFDA also reacts with other reactive species such as NO and peroxynitrite, which may reduce the specificity of the signal (4). In addition, since intracellular antioxidant levels such as glutathione may also affect the signal, standardization of experimental conditions is of great importance. Some studies report that trypsinizing adherent cells and then incubating them with DCFH-DA in suspension can yield higher ROS signals. However, this approach can disrupt cell integrity due to enzymatic disaggregation, which can affect the ROS signal. Incubation with H_2DCFDA directly on adherent cells, as in our protocol, yields results that are closer to those obtained with standard ROS measurement methods in the literature (15,16).

This study demonstrates that H_2DCFDA is a highly sensitive and applicable method for the detection of ROS; however, potential limitations should be considered in the interpretation of the data. In particular, standardization of cell culture duration, incubation time and measurement conditions will increase the comparability and biological significance of the obtained signals. In this respect, the study provides a reliable experimental protocol that can be used in oxidative stress studies and provides a methodological contribution to H_2DCFDA-based analyses in the literature.

CONCLUSION

In conclusion, the need for cell type-specific optimization in the assessment of ROS levels with H_2DCFDA is clear. Among the most important limitations of our study is the lack of a healthy cell line. However, the optimizations for the time and dose used were performed to determine the appropriate conditions for PANC-1 cells. Therefore, it is crucial for each study to experimentally determine the conditions specific to its own cell line to obtain reliable and comparable results.

Ethical Approval: N.A.

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RESEARCH ARTICLE | Yeditepe Journal of Health Sciences, 13(2):91-8

Antibiotic Use Behaviors and Influencing Factors Among Adult Patients Attending Family Health Centers: A Theory of Planned Behavior Approach

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Abstract

Objective: This study aimed to assess antibiotic use behaviors among adults attending family health centers in a district of İstanbul, using the *Theory of Planned Behavior*. The study also examined socio-demographic variables influencing attitudes, subjective norms, and intentions related to antibiotic use.

Materials and Methods: A cross-sectional survey was conducted among 151 literate adults aged 18 and above. A structured questionnaire measured demographic data and antibiotic-related knowledge, attitudes, subjective norms, and intentions. Data were analyzed using Mann-Whitney U and Kruskal-Wallis tests due to non-normal distribution.

Results: Of the participants, 64.2% were female and the mean age was 43.88 (standard deviation [SD] = 15.04). Most participants reported using antibiotics upon physician recommendation (92%) and refrained from using antibiotics without a prescription (90.7%). However, 27.8% kept leftover antibiotics at home. Gender was not significantly associated with attitude or intention scores, but men had significantly higher subjective norm scores than women (p=0.01), indicating greater influence by others. Households with more members showed higher susceptibility to social influence. Participants who believed injectable antibiotics were more effective had significantly more favorable attitudes toward antibiotic use (p=0.001). Keeping antibiotics at home and requesting antibiotics from physicians were both significantly associated with stronger intentions to use antibiotics without medical advice.

Conclusion: While general awareness of appropriate antibiotic use was high, misconceptions such as favoring inject-

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ables and keeping antibiotics in reserve persisted. Gender and household size influenced social norms, and behavioral intentions were shaped by access and past practices. Interventions should prioritize educational programs, early health literacy efforts, and physician-patient communication to reduce misuse.

Keywords: Antibiotic use, public health, theory of planned behavior, social norms, health literacy, medication misuse

INTRODUCTION

The irrational use of drugs (IUDs) continues to pose a significant threat to global public health. According to the World Health Organization (WHO), approximately half of all medications worldwide are prescribed, dispensed, or sold inappropriately, and a similar proportion of patients do not adhere to their prescribed treatments (1). The IUDs encompasses a range of problematic practices, including polypharmacy, overprescription of injectable medications, use of expensive alternatives despite equally effective, affordable options, underuse of essential medications, inappropriate self-medication, and particularly the excessive and improper use of antibiotics (1-3). These behaviors not only undermine treatment outcomes but also increase the risk of adverse drug reactions, contribute to the inefficient use of healthcare resources, and erode public trust in healthcare systems.

Antibiotic misuse is one of the most urgent dimensions of IUDs. A retrospective study examining antibiotic use, resistance, and trade dynamics in Türkiye and European Union (EU) countries between 2005 and 2015 revealed that Türkiye, which ranked second among EU nations in antibiotic consumption in 2005, became the leading country by 2015 (3). In a context where antibiotic use is alarmingly frequent, the need to promote rational antibiotic practices becomes even more pressing. The WHO defines rational antibiotic use as the selection of the appropriate agent, dose, duration, and route of administration, tailored to the patient's diagnosis and disease severity, and based on clinical evidence (4). However, irrational patterns such as polypharmacy, overprescription, unnecessary use without clear clinical indications, and consumption without physician oversight remain widespread in Türkiye (5-7).

The consequences of such misuse are profound. Inappropriate antibiotic use leads to increased healthcare costs, elevated morbidity and mortality rates, and the emergence of antibiotic resistance, a global crisis with far-reaching implications (8). Particularly in low- and middle-income countries, these challenges are exacerbated by factors such as limited access to healthcare services, poor sanitation infrastructure, lack of awareness, and a tendency toward self-medication (9,10). Moreover, literature suggests that the most common reason for antibiotic use in the general population is upper respiratory tract infections, many of which are viral in origin and do not require antibiotic therapy (11).

The WHO has declared antimicrobial resistance a critical threat to the sustainability of modern medicine and public health worldwide (12). In light of this, understanding the factors that drive antibiotic use at the community level is essential for designing effective interventions. Public knowledge and beliefs play a key role in shaping these behaviors. Antibiotic resistance is a global health concern, and inappropriate use of antibiotics remains widespread. To better understand the psychological and social determinants of antibiotic use, the present study was informed by the *Theory of Planned Behavior* (TPB). According to TPB, an individual's behavior is shaped by three main constructs: attitudes (personal evaluation of the behavior), subjective norms (perceived social pressure to perform or not perform the behavior), and perceived behavioral control (perception of ease or difficulty in performing the behavior). Together, these constructs influence behavioral intention, which is considered the most immediate predictor of actual behavior. Previous studies have successfully applied TPB to explore health-related behaviors such as medication adherence, vaccination uptake, and antibiotic use, highlighting its usefulness in identifying modifiable targets for behavioral interventions (13,14). By integrating this framework, our study aims to provide a more comprehensive understanding of the factors influencing antibiotic use intentions within the Turkish population."

This study aims to explore antibiotic use behaviors among adult patients attending family health centers (FHCs) located in a district of İstanbul. Using the TPB as a conceptual framework, which emphasizes the influence of attitudes, subjective norms, and behavioral intentions on individual actions, the study seeks to identify determinants of both appropriate and inappropriate antibiotic use. By addressing these behavioral drivers, the findings aim to inform future strategies for promoting rational antibiotic use in primary care settings.

MATERIALS AND METHODS

This study was designed as a descriptive cross-sectional survey. It was conducted among literate adults aged ≥18 years attended three FHCs located in a district of İstan-

bul. A structured questionnaire consisting of 44 items was administered to participants. The questionnaire included 22 multiple-choice questions, 3 open-ended questions, and 19 items based on a 5-point Likert scale.

The survey instrument comprised three main sections: 1) sociodemographic information (e.g., gender, age, economic status, and monthly household income), 2) questions regarding antibiotic use habits, and 3) the validated and reliable Antibiotic Use Scale developed by Atik and Doğan (15). The inclusion of sociodemographic variables was intended to explore their potential influence on antibiotic-related behaviors.

All participants completed the survey anonymously. Trained research assistants conducted the data collection through structured face-to-face interviews in private consultation rooms at the FHCs. No identifying information (e.g., names, contact details) was recorded, and participants were reassured that their responses would remain confidential. This approach was chosen to maximize response accuracy while ensuring anonymity, as some items concerned potentially sensitive behaviors such as using antibiotics without a prescription. The study employed convenience sampling to recruit participants.

Data were analyzed using SPSS Statistics for Windows, Version 25 (IBM Corp., Armonk, NY, USA). Frequency and percentage distributions were calculated, and Mann-Whitney U and Kruskal-Wallis tests were employed to assess statistical significance. A p-value of <0.05 was considered statistically significant.

The 5-point Likert items were scored as follows for positively worded statements: “Strongly Agree” = 5, “Agree” = 4, “Somewhat Agree” = 3, “Disagree” = 2, and “Strongly Disagree” = 1. Reverse scoring was applied for negatively worded items.

The scale was developed in line with the TPB, aiming to interpret individuals’ antibiotic-related behaviors based on three core components: attitude, subjective norms, and behavioral intention (15). According to the TPB, individuals consider the consequences of their actions, form intentions accordingly, and then act to achieve desired outcomes.

Attitude was assessed through statements reflecting participants' emotional and cognitive evaluations of antibiotic use. For example:

- “I feel more comfortable when I take antibiotics.”
- “Being ill makes me very unhappy; I want to take antibiotics to recover quickly.”

Participants rated their level of agreement on a scale from 1 (Strongly Disagree) to 5 (Strongly Agree). Higher mean scores indicated more favorable attitudes toward antibiotic use.

Subjective norms were evaluated by examining perceived social pressures or influences regarding antibiotic use. Statements included:

- “If a friend offers me antibiotics for a cold or flu, I would accept.”
- “I see no harm in using antibiotics based on someone’s recommendation without seeing a physician.”

High scores in this section suggested that participants were more likely to be influenced by social factors in their antibiotic-related decisions.

Behavioral intention was considered the most direct predictor of antibiotic use. It reflects the individual's commitment to engaging in such behavior. Statements included:

- “I keep leftover antibiotics for future use.”
- “If I experience similar symptoms, I would not hesitate to reuse antibiotics without a prescription.”

Again, responses were rated on a 5-point Likert scale, and higher average scores indicated stronger intentions to use antibiotics independently.

The study was conducted in accordance with the principles of the Declaration of Helsinki. It was approved by the Marmara University Clinical Research Ethics Committee on December 3, 2021 (Decision No: 09.2021.1336) and by the Provincial Directorate of Health. All participants were informed about the purpose of the study and the ethics approval, and verbal consent was obtained prior to participation.

RESULTS

Data was obtained from 151 participants. Of the participants, 64.2% were female and the mean age was 43.88 (standard deviation [SD]=15.04) (Table 1). Among the participants, 88.74% (n=134) reported that no one in their household was a regular antibiotic user, while 11.26% (n=17) reported having at least one regular antibiotic user in their household.

Regarding antibiotic use in the past year due to infections, the vast majority (92%, n=58) stated that they used antibiotics based on a physician’s recommendation. Additionally, 90.7% of the participants reported that they would not use antibiotics without a physician’s prescription, while 9.2% admitted to using antibiotics without a prescription.

Table 1. Demographic characteristics of participants.

	n (%)
Sex	
Male	54 (35.8)
Female	97 (64.2)
Last graduated school	
Literate	5 (3.3)
Primary school	38 (25.2)
Secondary school	13 (8.6)
High school	53 (35.1)
University	42 (27.8)
Income	
More than expenses	53 (35.1)
Equal to expenses	69 (45.7)
Less than expenses	29 (19.2)
Insurance	
Public health	97 (63.8)
Private	13 (8.6)
Other	11 (7.5)
None	30 (20.1)
Marital status	
Single	29 (19.2)
Married	112 (74.2)
Divorced / Others	10 (6.6)
Total	151 (100.0)

A total of 72.19% (n=109) of participants stated that they did not keep antibiotics at home as a backup, whereas 27.81% (n=42) reported keeping backup antibiotics. When asked about the source of these backup antibiotics, the most common responses were leftover antibiotics from previous treatments and antibiotics prescribed earlier by a physician.

Furthermore, 79% of the participants indicated that they would not request antibiotics from their physician, while 21% stated that they had asked their physician to prescribe antibiotics. Regarding perceptions of antibiotic efficacy, 69% (n=98) believed that injectable antibiotics are more effective than oral ones.

A large proportion of participants (74.8%, n=113) reported that they read the package leaflet of medications. When asked about possible side effects of antibiotics, 62.9% mentioned kidney damage, 53.8% stomach upset, 48.3% liver damage, 43.37% diarrhea, and 41.7% allergic skin rashes (Table 2).

To investigate the relationship between participants' intention scores (based on the Antibiotic Use Scale) and their behavior of requesting antibiotics from a physician, the normality of the data was assessed using Quantile–Quantile (Q-Q) plots and the Kolmogorov-Smirnov test. The data were found not to follow a normal distribution.

The median intention score for participants who did not request antibiotics was 1.2, while it was 2.0 for those who

Table 2. Participants' views on the adverse effects of antibiotics.

Adverse Effects	n (%)
Nephrotoxic	
Yes	95 (62.9)
No	56 (37.1)
Diarrhea	
Yes	66 (43.7)
No	85 (56.3)
Allergic rash may occur as a side effect	
Yes	63 (41.7)
No	88 (58.3)
Gastrointestinal irritation	
Yes	88 (58.3)
No	63 (41.7)
Hepatotoxic	
Yes	73 (48.3)
No	78 (51.7)
Total	**151 (100)**

Table 3. Analysis of gender differences in attitude, subjective norms, and intention scores.

Sex	Attitude		Subjective Norm		Intention	
	Median (IQR)	*p*	Median (IQR)	*p*	Median (IQR)	*p*
Female	4.6 (2.6)	0.449	1.6 (0.9)	0.01	1.0 (1.6)	0.057
Male	4.8 (2.05)		1.8 (1.45)		1.9 (2.0)	

IQR: Interquartile range.

did request antibiotics. This difference was analyzed using the Mann-Whitney U test and was found to be statistically significant (p=0.044).

DISCUSSION

In this study our findings reveal that while many patients adhere to prescription-only antibiotic use, misconceptions still persist. Our data revealed that 90.7% of participants reported not using antibiotics without a physician's prescription. This suggests a high level of awareness among individuals regarding the importance of prescription-based antibiotic use. In a thesis conducted in Denizli, 65% of participants responded similarly (16). These findings are also comparable to those of Gül et al. (17), who evaluated the knowledge and attitudes of Ankara residents regarding self-medication with antibiotics. While demographic characteristics in both studies were similar (mean age=37.1 years; 70% with high school or university education), our results differed in terms of behaviors such as keeping antibiotics at home and requesting them from physicians. In our study, 27.81% of participants reported keeping antibiotics at home, and 21% stated that they had requested antibiotics from a physician. In contrast, Gül et al. (17) reported these rates as 64.9% and 64%, respectively. This discrepancy may be due to differences in gender distribution or regional antibiotic practices.

Significant progress has been made in Türkiye to promote rational drug use, largely due to initiatives under the National Rational Drug Use Action Plan (18). Contributions from universities, healthcare professionals, and the pharmaceutical sector have supported governmental efforts to enhance public awareness regarding the

Table 4. Comparison of attitude, subjective norms, and intention scores by participants' beliefs and behaviors regarding antibiotic use.

		Attitude		Subjective norm		Intention	
		Median (IQR)	*p*	Median (IQR)	*p*	Median (IQR)	*p*
IM antibiotics more effective than oral[a]	Yes	5.0 (2.8)	**0.003**	1.8 (1.4)	0.068	1.4 (2.0)	**0.034**
	Not Sure	4.1 (1.8)		1.4 (0.9)		0.8 (1.3)	
	No	4.8 (2.8)		1.2 (1.8)		0.8 (1.2)	
Keeping antibiotics at home[b]	Yes	4.9 (2.5)	0.223	1.6 (1.4)	0.697	2.3 (1.3)	**<0.001**
	No	4.6 (2.4)		1.6 (1.0)		0.8 (1.4)	
Requested antibiotics from physician[b]	Yes	4.5 (2.9)	0.920	1.6 (1.3)	0.789	2.0 (2.1)	**0.044**
	No	4.6 (2.2)		1.6 (1.2)		1.2 (1.6)	

IM: Intramuscular, **IQR:** Interquartile range.
[a]Kruskall-Wallis test.
[b]Mann-Whitney U test

rational use of medications. Additionally, a nationwide study conducted in 2016 revealed that the prevalence of non-prescription drug use in the general population was 80.48% (19).

When evaluating knowledge of the purpose of antibiotics, 84.4% of participants in our study stated that antibiotics are used to treat infections and inflammation, indicating strong awareness of their proper use. In comparison, only 58% of participants in Gökçe's study (16) answered this correctly. Understanding the purpose of antibiotics is essential for preventing antibiotic resistance across populations.

Regarding the statement, 'I would not hesitate to use previously prescribed antibiotics without a prescription if I experienced similar symptoms,' 52.3% of participants strongly disagreed. In a study by Artantaş et al. (20), evaluating adult patients visiting family medicine clinics in Ankara, 75% of participants reported they would use the antibiotic previously prescribed by their physician in cases of similar symptoms. Despite similar demographics between the two studies, the differing responses may stem from regional variations or potential social desirability bias affecting participants' transparency.

Although education, occupation, and income did not significantly affect behaviors. However, the persistence of inappropriate practices like storing antibiotics and self-prescription indicates behavioral reinforcement beyond formal knowledge.

In our study, 74.8% of participants reported reading the drug information leaflet. When asked about the appropriate time to stop using antibiotics, 59.6% answered "as advised by the physician." In comparison, Karakurt et al. (21) found that 83.6% of university students in Erzincan read drug leaflets, while 45.8% stopped using medication based on physician advice and 47.9% stopped when symptoms were resolved. This discrepancy may be attributed to differences in age and sample size. It may also suggest that younger populations require more structured education on responsible medication use.

When asked about the side effects of antibiotics in a multiple-choice format, the most commonly selected answer was kidney damage (62.9%), followed by stomach discomfort (58.3%). Similarly, Kenesarı and Özçakar (21) found that mothers most often cited kidney damage and stomach discomfort both before and after a brief educational intervention (22). These findings support the results of our study, given the similar age and education levels of participants.

Beliefs regarding the efficacy of injectable antibiotics were also assessed. A total of 69% of our participants believed that injectable antibiotics are more effective than oral forms. In Gökçe's study (16), only 13% of participants stated that there was no difference between injectable and oral antibiotics. This suggests a persistent misconception in the general population, highlighting the need to educate patients that injectable antibiotics should only be used when deemed necessary by a healthcare professional and should not be requested indiscriminately. The perception that injectable antibiotics are more effective contributes to positive but potentially risky attitudes. Social influence is stronger among men and those from larger households, indicating a need for targeted interventions.

Lastly, 92% of participants in our study stated that they used antibiotics only upon their physician's recommendation. This finding indicates a high level of awareness regarding the importance of using antibiotics only under medical supervision.

Limitations

This study has some limitations that should be acknowledged. First, although the study assessed behavioral intentions regarding antibiotic use, actual behavior was not objectively verified through prescription records or pharmacy data. This restricts the ability to explore potential discrepancies between reported intentions and real-world practices.

Second, the sample was drawn from only three FHCs, and more than half of the participants (62.9%) had a high school or university education. This relatively high educational level and limited geographic coverage may limit the representativeness of the sample and reduce the generalizability of the findings to the broader Turkish population.

Third, contextual factors should be considered when interpreting the results. In Türkiye, antibiotics are legally restricted and cannot be purchased without a prescription. This regulatory environment may have influenced participants' reported behaviors and intentions, potentially leading to underreporting of non-prescription use.

Fourth, although the study utilized the Antibiotic Use Scale, the Methods section does not provide sufficient detail regarding the scale's items, subdimensions, and scoring system. This may reduce transparency and replicability in future studies.

Finally, as with all self-reported data, the findings are subject to social desirability bias. Participants may have provided responses that aligned with socially acceptable practices rather than their actual behaviors, especially concerning sensitive issues such as self-medication with antibiotics. Despite these limitations, the study provides

valuable insights into behavioral intentions surrounding antibiotic use, offering useful implications for public health interventions and policies.

CONCLUSION

This study highlights that the majority of participants show appropriate attitudes and behaviors toward antibiotic use, including reliance on physician prescriptions and awareness of potential side effects. However, misconceptions still persist, particularly regarding the perceived superiority of injectable antibiotics and the tendency to keep antibiotics at home for future use.

Comparative findings from similar regional studies suggest that variations in behavior may be influenced by factors such as age, education level, gender distribution, and regional healthcare practices. While overall awareness appears to be increasing, targeted public health interventions and educational campaigns remain necessary, especially to correct misinformation about antibiotic administration routes and to discourage self-medication practices.

Improving antibiotic literacy among all age groups is crucial for combating antimicrobial resistance and promoting rational drug use. Future research should consider larger and more diverse populations to further explore sociocultural factors that influence antibiotic-related behavior.

Ethical Approval: The study was approved by the Marmara University Clinical Research Ethics Committee on December 3, 2021 (Decision No: 09.2021.1336) and by the Provincial Directorate of Health.

Informed Consent: All participants were informed about the purpose of the study and the ethics approval, and verbal consent was obtained prior to participation.

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Author Contributions: Concept – Ş.P., N.E.L., A.G.T., N.S., S.Ş., F.E.B., M.E., E.K.; Design – Ş.P., N.E.L., A.G.T., N.S., S.Ş., F.E.B., M.E., E.K.; Supervision – Ş.P., N.E.L., A.G.T.; Fundings – Ş.P., N.E.L., A.G.T.; Data Collection and/or Processing – Ş.P., N.E.L., A.G.T., N.S., S.Ş., F.E.B., ME, E.K.; Analysis and/or Interpretation – Ş.P., N.S., S.Ş., F.E.B., M.E., E.K.; Literature Review – Ş.P., N.E.L.; Writer – Ş.P., Ö.T.; Critical Reviews – Ş.P., Ö.T.

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RESEARCH ARTICLE | Yeditepe Journal of Health Sciences, 13(2):99-104

The Effect of Cisplatin on *GATA2* and *GATA6* Gene Expression in Head and Neck Cancer Cell Lines

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Abstract

Objective: Head and neck squamous cell carcinoma (HNSCC) constitutes over 90% of malignancies in the head and neck region and remains a significant clinical burden due to high mortality and resistance to therapy. Cisplatin is a commonly used chemotherapeutic agent in HNSCC treatment; however, its effectiveness is often limited by resistance. This study aimed to evaluate the impact of cisplatin on *GATA2* and *GATA6* expression in two HNSCC cell lines.

Materials and Methods: The HNSCC cell lines, HSC3 and SCC47, were exposed to varying concentrations of cisplatin to assess cytotoxic effects, with cell viability evaluated using the MTS assay. Based on the results, the half-maximal inhibitory concentrations (IC_{50}) were determined as 2.18 μM for HSC3 and 5.6 μM for SCC47 at 48 hours post-treatment. Subsequent experiments involved treating each cell line with its corresponding IC_{50} dose for 48 hours. Total RNA was then isolated using the TRIzol reagent, and complementary DNA (cDNA) was synthesized for downstream analysis. Quantification of *GATA2* and *GATA6* gene expression was performed via quantitative PCR (qPCR) using TaqMan probes, with *ACTB* as the housekeeping gene. Relative gene expression levels of *GATA2* and *GATA 6* were calculated using the comparative ΔΔCt method.

Results: *GATA6* expression was significantly upregulated (approximately 3-fold) following cisplatin treatment, whereas *GATA2* levels remained unchanged compared to untreated controls in HSC3 cells. In contrast, SCC47 cells showed a modest increase in both *GATA2* and *GATA6* expression; however, these changes did not reach statistical significance.

Conclusion: Cisplatin modulates the expression of *GATA2* and *GATA6* in a cell line-dependent manner in HNSCC. The observed upregulation of *GATA6* in the more cisplatin-sensitive HSC3 line may be associated with treatment response. However, this association remains correlative, and further functional studies are required to establish causality. These preliminary findings warrant additional investigation to clarify whether *GATA2* and *GATA6* could serve as potential biomarkers or therapeutic targets in cisplatin-treated HNSCC.

Keywords: HNSCC, cisplatin, *GATA2*, *GATA6*, gene expression

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INTRODUCTION

Accounting for over 90% of malignancies in the head and neck region, head and neck squamous cell carcinoma (HNSCC) poses a substantial global burden, marked by high morbidity and mortality rates. According to 2022 data, approximately 946,456 new cases and over 482,001 deaths occur annually (1).

The most frequently affected anatomical regions include the oral cavity, larynx, and pharynx. The development of HNSCC is commonly associated with factors such as alcohol and tobacco consumption, exposure to environmental carcinogens, and high-risk human papillomavirus (HPV) infections (2). In the treatment of head and neck cancers, surgical resection is frequently complemented by radiotherapy and chemotherapy (3). Among the most common chemotherapeutic agents used in these combination therapies is cisplatin. Cisplatin functions by forming intra-cellular DNA cross-links, which subsequently block replication and trigger apoptosis (4). However, while cisplatin demonstrates efficacy in some patients, a significant proportion develop either primary or acquired resistance to the treatment (5,6). Elucidating these resistance mechanisms is crucial for the development of targeted therapies.

GATA transcription factors are a family of zinc finger DNA-binding proteins that regulate the development of various tissues by modulating gene transcription, either through activation or repression. This tightly coordinated regulation enables GATA factors to couple cellular differentiation with the cessation of proliferation and the enhancement of cell survival. Given their critical roles in maintaining tissue homeostasis, it is not surprising that dysregulation of GATA genes has been implicated in the pathogenesis of several human cancers (7).

In recent years, transcription factors have been extensively investigated in cancer biology due to their critical roles in regulating processes such as cell proliferation, differentiation, metastasis, and drug resistance (8). In this context, the GATA family of transcription factors has garnered significant attention due to its wide-ranging biological functions, spanning embryonic development, regulation of immune responses, and involvement in cancer pathogenesis (9). This family comprises six members, *GATA1* through *GATA6*, each characterized by tissue-specific expression patterns and functions (10).

While *GATA2* is classically known for its crucial role in the differentiation and maintenance of hematopoietic cells (11), recent reports indicate its expression in epithelial-derived tumors and its potential to regulate cell proliferation and metastasis (12). *GATA6* is expressed in epithelial tissues such as the digestive system, lung, and pancreas, where it can act as either a tumor suppressor or a tumor promoter in tumor development (13,14).

The effects of GATA family members on head and neck cancer are not yet fully understood. However, preliminary data suggest that alterations in the expression levels of these genes may influence tumor cell behaviors, including proliferation, invasion, and drug response (15). This study investigated the effect of cisplatin treatment on the expression levels of *GATA2* and *GATA6* genes in two distinct HNSCC cell lines, namely HSC3 and SCC47. This research is expected to contribute to the understanding of whether *GATA2* and *GATA6* are associated with treatment response in head and neck cancers and may support their future consideration as potential biomarkers.

MATERIALS AND METHODS

Cell Culture

The HNSCC cell lines, HSC3 and SCC47, were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; high glycose) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Routine screening for mycoplasma contamination was performed on all cell lines using a PCR-based detection kit (Mycoplasma PCR Detection Kit; Applied Biological Materials, Richmond, BC, Canada).

Cytotoxicity Assay

Cytotoxicity of cisplatin was evaluated using the MTS assay. HSC3 and SCC47 cells were seeded into 96-well plates at a density of 2500 cells per well and incubated overnight to allow for cell adhesion. After that cells were treated with varying concentrations of cisplatin (0–20 µM). Untreated cells served as negative controls, while wells containing medium only were used as blanks. Following incubation periods of 24 to 72 hours, 12 µL of MTS reagent (CellTiter 96® AQueous One Solution; Promega, Madison, WI, USA) was added to each well, followed by 2 hours of incubation in the dark at 37°C. Absorbance was measured at 490 nm using microplate reader,

RNA Isolation After Cisplatin Treatment

HSC3 and SCC47 cells were seeded into 6-well plates at a density of 80,000 cells per well and incubated overnight to allow for adhesion. The next day, cells were treated with cisplatin at their respective IC_{50} concentrations and incubated for 48 hours. Following treatment, cells were harvested by trypsinization and centrifugation. Total

RNA was extracted from the resulting cell pellets using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoPhotometer (Implen, Munich, Germany).

Quantitative Real-Time PCR (qPCR)

Complementary DNA (cDNA) synthesis was performed using 1000 ng of total RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was subsequently conducted on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan® probes specific for *GATA2* and *GATA6*. β-actin (ACTB) served as the endogenous control for normalization. All reactions were carried out in technical triplicates, and relative gene expression levels were determined using the comparative ΔΔCt method.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism, version 8.0 (GraphPad Software, San Diego, CA, USA). Results are presented as mean ± standard deviation (SD). A p-value <0.05 was considered statistically significant.

RESULTS

Cisplatin-Induced Cytotoxicity in HSC3 and SCC47 Cells

Cisplatin treatment induced a dose- and time-dependent reduction in cell viability in both HSC3 and SCC47

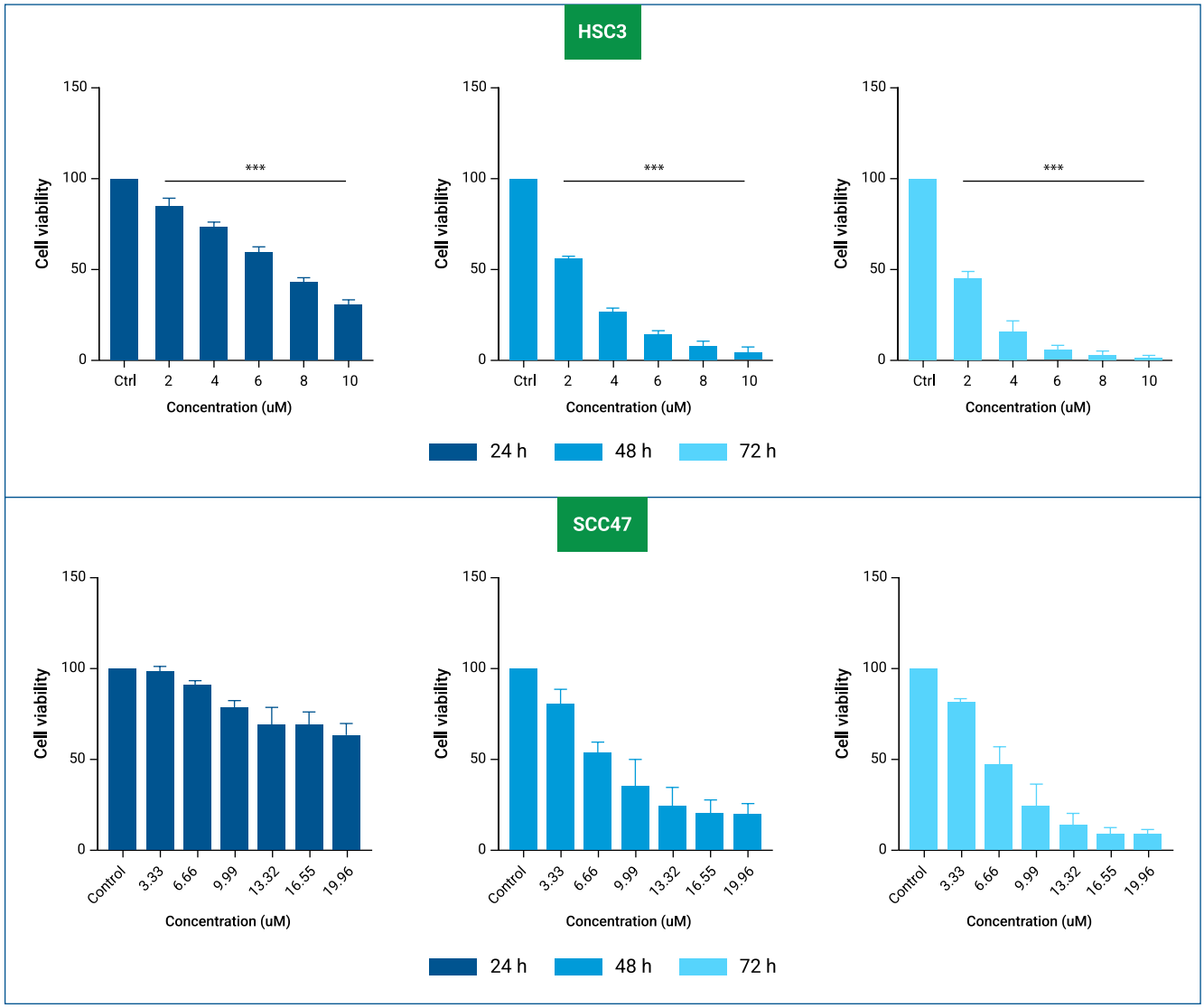

Figure 1. Effect of cisplatin on cell viability in HSC3 and SCC47 cells. Cells were treated with increasing concentrations of cisplatin (0–20 μM) for 24, 48, and 72 hours, and cell viability was measured using the MTS assay. Data represent the mean ± SD of three independent biological replicates (p<0.05, p<0.01, p<0.001).

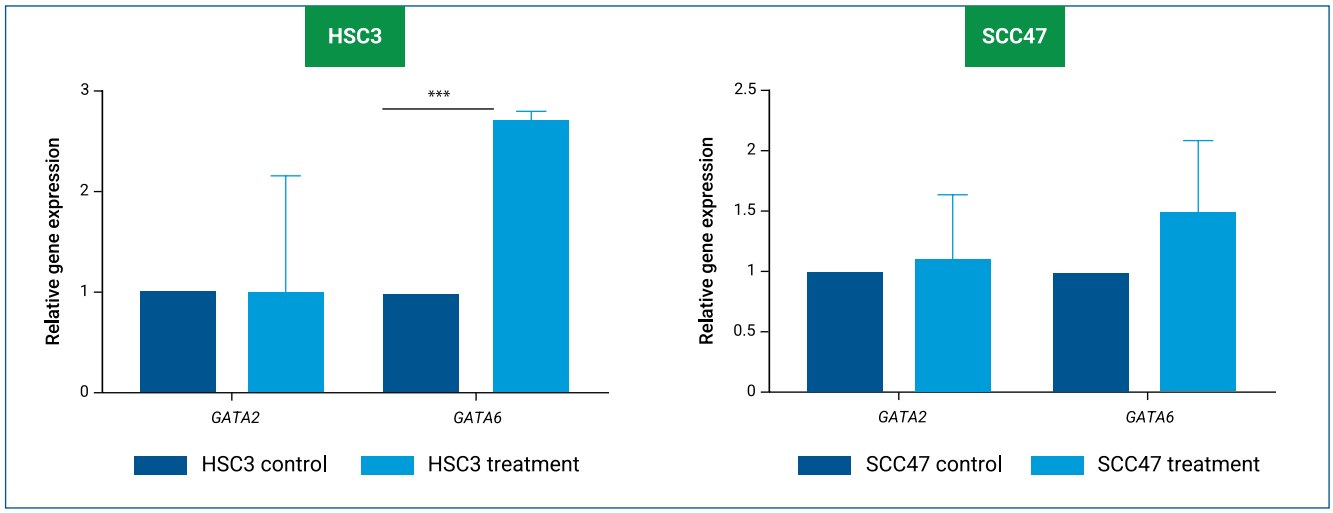

Figure 2. Relative expression of *GATA2* and *GATA6* following cisplatin treatment in HSC3 and SCC47 cells. HSC3 cells were treated with cisplatin at 2.18 µM for 48 hours, whereas SCC47 cells were treated at 5.6 µM for 48 hours (corresponding to their respective IC_{50} values). Gene expression was quantified by qRT-PCR, normalized to ACTB, and calculated using the $2^{\wedge -\Delta\Delta Ct}$ method. Data represent the mean ± SD from three independent biological replicates, each performed in technical triplicates. Statistical significance was determined using an unpaired two-tailed Student's *t*-test compared with untreated controls ($p<0.05$, $p<0.01$, $p<0.001$).

HNSCC cell lines. In HSC3 cells, significant cytotoxicity was observed at concentrations of ≥4 µM. Specifically, at 10 µM, cell viability decreased to below 20% after 72 hours of treatment ($p<0.001$) (Figure 1). The half-maximal inhibitory concentration (IC_{50}) of cisplatin in HSC3 cells was calculated as 2.18 µM at 48 hours. For SCC47 cells, a more gradual decline in viability was observed, with the IC_{50} determined to be 5.6 µM at 48 hours. At the highest tested concentration (19.98 µM), cell viability dropped below 15% after 72 hours (Figure 1).

Differential Effects of Cisplatin on *GATA2* and *GATA6* Expression in HSC3 and SCC47

To assess the impact of cisplatin, gene expression analysis was performed 48 hours after treatment with the respective IC_{50} doses, revealing distinct expression patterns of *GATA2* and *GATA6* between the two cell lines. In HSC3 cells, *GATA6* expression was markedly upregulated, showing an approximately 3-fold increase compared to untreated controls, while *GATA2* levels remained largely unchanged (Figure 2). In contrast, SCC47 cells exhibited a slight increase in both *GATA2* and *GATA6* expression following cisplatin exposure; however, these changes were not statistically significant (Figure 2).

DISCUSSION

In this study, we demonstrated that cisplatin induces a time and dose-dependent cytotoxic effect on the HNSCC cell lines HSC3 and SCC47, with IC_{50} values of 2.18 µM and 5.6 µM, respectively. These results align with previous reports highlighting variability in cisplatin sensitivity among HNSCC cell lines. This variability may be attributed to intrinsic molecular factors such as p53 status, DNA repair capacity, apoptotic threshold, and HPV status. Notably, SCC47 is HPV-positive, whereas HSC3 is HPV-negative, a difference that may contribute to their differential responses to cisplatin (16).

Beyond its direct cytotoxic effects, cisplatin modulated the expression of *GATA2* and *GATA6*, transcription factors involved in cell differentiation, proliferation, and stress response (17). In HSC3 cells, *GATA2* expression remained largely unchanged following cisplatin exposure, while *GATA6* was significantly upregulated, suggesting a possible role in the cellular response to DNA damage. In SCC47 cells, both *GATA2* and *GATA6* showed a modest increase in expression; however, these changes did not reach statistical significance. This may reflect a less pronounced transcriptional response or differences in regulatory sensitivity compared to HSC3.

These findings demonstrate that cisplatin reduces cell viability in a cell line-dependent manner and differentially modulates the expression of key transcription factors. Notably, *GATA6* exhibited a substantial increase in the more cisplatin-sensitive HSC3 cells, suggesting a possible role in stress adaptation or the cellular response to DNA damage. An alternative interpretation is that *GATA6* upregulation may reflect the activation of pro-apoptotic pathways or an attempt to drive differentiation, thereby sensitizing HSC3 cells to cisplatin. Based on this, we propose two testable hypotheses: first, *GATA6* may fa-

cilitate the transcription of apoptotic regulators in response to cisplatin-induced DNA damage, lowering the apoptotic threshold in HSC3. Second, *GATA6* may promote partial differentiation programs that reduce cellular plasticity and survival capacity, thereby enhancing cisplatin cytotoxicity. In contrast, *GATA2* expression remained unchanged in HSC3, while both *GATA2* and *GATA6* showed modest, non-significant upregulation in SCC47 cells. These distinct expression patterns point to a cell-specific transcriptional response to cisplatin and underscore the potential of *GATA2* and *GATA6* as biomarkers or therapeutic targets in HNSCC.

Previous studies have implicated *GATA2* in both tumor suppression and progression, contingent on the cellular context. Notably, previous research in colorectal cancer has shown that high *GATA2* expression is significantly correlated with poor disease-free survival and increased recurrence risk, highlighting its potential role as a prognostic biomarker (18). In contrast to findings in colorectal cancer, reduced expression of the hematopoietic transcription factor *GATA2* has been associated with poor prognosis in hepatocellular carcinoma (HCC) patients following surgical resection (19). This suggests that the prognostic role of *GATA2* may vary depending on tumor type and tissue context.

GATA6 is a member of the evolutionarily conserved GATA transcription factor family, which regulates gene expression by binding to GATA-specific motifs located within promoter regions (20). *GATA6* has been implicated in many cancer types, exhibiting context-dependent functions. In certain malignancies, such as gastric, colorectal, and breast cancers, as well as cutaneous T-cell lymphoma, it acts as an oncogenic driver, contributing to tumor progression (21-24).

In line with our findings that cisplatin induces differential *GATA6* expression in HNSCC, recent studies in oral squamous cell carcinoma (OSCC) provide mechanistic insights into *GATA6*'s oncogenic roles. Notably, *GATA6* has been shown to bind the *FN1* promoter and upregulate fibronectin-1 expression, thereby promoting proliferation, invasion, and migration in OSCC models; these effects were reversed upon *FN1* overexpression following *GATA6* knockdown (25).

Conversely, in other tumor types, including astrocytoma and HCC, *GATA6* has been shown to exert tumor-suppressive effects (26,27). Collectively, our findings indicate that *GATA2* and *GATA6* show differential expression in response to cisplatin in a gene and cell line-dependent manner. While these observations highlight a potential association between GATA factors and treatment sensitivity, further functional validation is required before they can be considered reliable biomarkers or therapeutic targets in HNSCC.

This study has several limitations. Only two HNSCC cell lines (one HPV-positive and one HPV-negative) were analyzed, which limits generalizability, particularly given the known differences in p53 pathway status. The study is correlative and lacks functional validation, and although ACTB was used as a housekeeping gene, inclusion of additional reference genes would strengthen the qPCR analyses. Finally, the mechanisms underlying GATA modulation remain unclear and warrant further investigation.

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Informed Consent: N.A.

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RESEARCH ARTICLE | Yeditepe Journal of Health Sciences, 13(2):105-9

Impact of Anti-HLA-DR51/52/53 Antibody Positivity on Predicting Flow Cytometry Crossmatch Results in Kidney Transplant Candidates

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Abstract

Objective: Genetic differences between the patient and the donor and the immune system cells response to these differences are among the causes of allograft rejection. Human leukocyte antigen (HLA)-DR, DP, DQ antigens have been shown to be expressed in renal epithelial cells, and in addition, a marked increase in HLA class-II expression has been reported in rejected renal allografts. Human leukocyte antigen-DR contributes to the rejection process due to its role in the activation of $CD4^+$ T cells. Polymorphism in the HLA-DRB3 (DR52), DRB4 (DR53), and DRB5 (DR51) loci is weak, and they are not encoded by the same loci as HLA-DRB1. In addition, these loci are inherited together with the alleles of the DRB1 locus encoding HLA antigens. In our study, we aimed to investigate the correlation between anti-HLA DR51/52/53 antibody positivity detected alone or in combination in the sera of patients on the kidney transplant waiting list and flow cytometry crossmatch (FCXM) positivity.

Materials and Methods: In our study, the panel reactive antibody (PRA) identification and FCXM test results of 200 patients who tested positive for PRA screening between 2019 and 2023 at the Tissue Typing Laboratory of İstanbul Faculty of Medicine were retrospectively analysed.

Results: Of the patients included in the study, PRA screening tests were positive (n=200), and antibodies against at least one antigen belonging to the anti-HLA-DR51/52/53 subgroups were detected in 55.5% (n=111) of these patients in the PRA identification test. All alleles in the DR51, DR52, DR53 subgroups were found to be associated with FCXM-B positivity. In addition, DR16 (p=0.017) correlated with FCXM-T positivity both alone and in combination with DR15 (p=0.019), while in the DR52 subgroup, the simultaneous positivity of DR13, DR14, DR17, and DR18 (p=0.027) was significantly correlated with FCXM-T positivity.

Conclusion: The findings obtained in our study suggest that HLA-DR51/52/53 antibodies may be effective in predicting FCXM positivity.

Keywords: Donor specific antibody, flow cytometry crossmatch, HLA-DRB3, HLA-DRB4, HLA-DRB5

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INTRODUCTION

Human leukocyte antigen (HLA) -DR is one of the class II HLA proteins and consists of heterodimeric α and β chains. The HLA-DR alpha chain is encoded by *HLA-DRA*. There are four different genes that can encode the β chain, the most common of which is *HLA-DRB1*. Other genes that can encode the β chain are *HLA-DRB3*, *HLA-DRB4*, or *HLA-DRB5*. These genes are generally expressed at lower levels than *HLA-DRB1*, but they may be present in a specific haplotype depending on the associated *HLA-DRB1* gene (1-3). The *HLA-DRB3/4/5* genes are closely related to the *HLA-DRB1* locus (4). It has been reported that they show a strong linkage disequilibrium (5). Although the expression levels of the *HLA-DRB3*, *HLA-DRB4*, and *HLA-DRB5* loci are lower than those of *HLA-DRB1*, they can be detected by serological methods and are therefore referred to as the HLA-DR52, -DR53, and -DR51 antigens, respectively (6).

HLA-DRB3 (i.e., DR52) is linked with the DR11, DR12, DR13, DR14, DR17(3), and DR18(3) antigens, while *HLA-DRB4* (i.e., DR53) is linked with the DR4, DR7, and DR9 antigens. DRB5 (i.e., DR54) is linked with the DR15(2) and DR16(2) antigens. (7,8) The genes linked to *HLA-DRB1* and *HLA-DRB3/4/5* are shown in Table 1 (9).

HLA-DR plays a critical role in the rejection process, particularly due to its effect on the activation of $CD4^+$ T cells (9). Our hypothesis is that anti-HLA-DR 51/52/53 antibody positivity may be effective in predicting flow cytometry crossmatch (FCXM) outcome. In our study, we aimed to investigate the effect of anti-HLA DR51/52/53 antibody positivity on flow FCXM results.

MATERIALS AND METHODS

Between 2019 and 2023, 200 patients on the kidney transplant waiting list who tested positive for panel reactive antibody (PRA) screening were included in the study. The PRA identification and FCXM test data of the patients, which were studied simultaneously, were analyzed retrospectively.

Initially, all patients underwent PRA screening tests. Panel reactive antibody identification tests were performed on 200 patients who tested positive for PRA screening tests to detect donor specific antibody (DSAs) using the Lifecodes® kit (Immucor, Norcross, GA, USA) according to the manufacturer's instructions. The PRA tests were performed using the Luminex® 200 system (Luminex Corp., Austin, TX, USA). In addition, FCXM tests were performed on all patients and donors. Panel reactive antibody identification and FCXM tests were conducted simultaneously at İstanbul University İstanbul Faculty of Medicine Department of Medical Biology, Tissue Typing Laboratory. In PRA screening and identification tests, anti-HLA antibodies with a mean fluorescence intensity (MFI) ≥1000 were considered positive (10).

Crossmatching tests were performed using the FCXM method. Lymphocyte cells to be used in the FCXM test were isolated from peripheral blood samples obtained from patients and donors via density gradient centrifugation method. Two-color flow cytometry was performed. CD3-PC5 monoclonal antibodies were used to label T cells, and CD19-PE monoclonal antibodies were used to label B cells. Immunoglobulin (IgG) antibodies were labeled with IgG-FITC secondary antibody (11).

Statistical Analysis

All statistical analyses were performed using SPSS Statistics for Windows, version 21.0 (IBM Corp.; Armonk, NY, USA). Results for continuous variables are calculated as mean (SD). The $\chi 2$ test was used to compare categorical data. Gender ratios are expressed as percentages. The p-value <0.05 is considered statistically significant.

RESULTS

The mean age of the 200 patients included in the study was 52.7 ± 7.6 years, whereas the mean age of their donors was 45.6 ± 8.8 years. The female-to-male ratio among patients was 54.5% (n=109) to 45.5% (n=91). In the PRA screening, one or more antigens belonging to the anti-HLA-DR51/52/53 subgroups were detected in the sera of 55.5% (n=111) of the 200 patients who tested positive. Among the DR51 subgroup antibodies, DR15 (p=0.007) and DR16 (p=0.011) were independently correlated with B cell FCXM positivity, while the combination of these antibodies was also correlated with B cell FCXM positivi-

Table 1. Linked genes between *HLA-DRB1* and *HLA-DRB3/4/5 (9).*

Gene	Coding protein	Linked HLA-DR antigen
DRB3	Beta chain of DR52	DR11, DR12, DR13, DR14, DR17(3), DR18(3)
DRB4	Beta chain of DR53	DR4, DR7, DR9
DRB5	Beta chain of DR51	DR15(2), DR16(2)
None	None	DR1, DR8, DR10

HLA: Human leukocyte antigen.

ty (p=0.006). Additionally, DR16 was found to be correlated with FCXM-T positivity both alone (p=0.017) and in combination with DR15 (p=0.019) (Table 2).

Table 2. Correlation of DRB5 (DR51) antibody and FCXM positivity.

Antigen	FCXM-T cell positivity	FCXM-B cell positivity
DR15	p=0.123	p=0.007
DR16	p=0.017	p=0.011
DR15 + DR16	p=0.019	p=0.006

HLA: Human leukocyte antigen; **FCXM:** Flow cytometry crossmatch.

Table 3. Correlation of DRB3 (DR52) antibody and FCXM positivity.

Antigen	FCXM-T cell positivity	FCXM-B cell positivity
DR11	p=0.050	p<0.001
DR12	p=0.015	p=0.038
DR14	p=0.068	p=0.003
DR17	p=0.103	p=0.016
DR18	p=0.103	p=0.016
DR13 + DR14 + DR17 + DR18	p=0.027	p=0.263

HLA: Human leukocyte antigen; **FCXM:** Flow cytometry crossmatch.

Table 4. Correlation of HLA-DRB4 (DR53) antibody and FCXM positivity.

Antigen	FCXM-T cell positivity	FCXM-B cell positivity
DR4	p=0.048	p=0.064
DR7	p=0.266	p=0.026
DR9	p=0.218	p=0.001
DR4 + DR9	p=0.667	p=0.024

HLA: Human leukocyte antigen; **FCXM:** Flow cytometry crossmatch.

In the DR52 subgroup, DR12 alone was correlated with FCXM-T positivity (p=0.015), while DR11 (p<0.001), DR12 (p=0.038), DR14 (p=0.003), DR17 (p=0.016) and DR18 (p=0.016) were individually correlated with FCXM-B positivity. In addition, the association of DR13, DR14, DR17 and DR18 was correlated with FCXM-T positivity (p=0.027) (Table 3).

In the DR53 subgroup, DR4 alone (p=0.048) was found to be correlated with FCXM-T positivity, while DR7 (p=0.026) and DR9 (p=0.001) were individually correlated with FCXM-B positivity. The association of DR4 and DR9 was correlated with FCXM-B positivity (p=0.003) (Table 4).

DISCUSSION

Patients with a history of sensitivity, such as recurrent pregnancies, blood transfusions, and secondary transplants, may develop antibodies against HLA antigens. The presence of antibodies with high MFI levels against donor class I and class II HLA antigens in the patient's serum prior to kidney transplantation is a significant risk factor for antibody-mediated rejection after transplantation (12). Recent studies have shown that donor-specific antibody (DSA) development is associated with antibody-mediated damage and poor graft outcomes, and that HLA class II DSA has a greater effect than class I (13,14). In one study, it was shown that anti-class II DSA may be highly associated with humoral rejection, and it was reported that the determination of anti-class II DSA may be important in the overall assessment of immunological risk in kidney transplant patients (15). In another study, PRA positivity was detected in 20.4% of patients with chronic kidney disease on the waiting list, and the rates of anti-HLA antibodies against HLA class II were reported to be higher (16).

The HLA-DR51 antigen encoded by the *HLA-DRB5* gene, the HLA-DR52 antigen encoded by the *HLA-DRB3* gene, and the HLA-DR53 antigen encoded by the *HLA-DRB4* gene are thought to be weaker than other HLA-DR antigens encoded by *HLA-DRB1* genes (9,17). However, it is known that HLA-DR51, -DR52, and -DR53 antigens are always associated with DR antigens. Anti-HLA-DR53 antibody is a risk factor for acute mediated rejection (AMR) and resistance to elimination (18). Katsuma et al. (19) reported a subclinical AMR case in which the MFI of DR53 increased after intensive immunosuppressive therapy. This case report highlights that high MFI values for HLA-DR53 may be significant in the early diagnosis of subclinical AMR. The study also points out that mismatch in HLA-DR 51, 52, and 53 alleles may be associated with subclinical AMR.

Our study retrospectively evaluated 200 class II PRA-positive patients and found that 55.5% (n=111) were posi-

tive for at least one antigen belonging to the anti-HLA-DR51/52/53 subgroups. Similar to the findings in the literature, the results of our study suggest that HLA-DR51, -DR52, and -DR53 antigens should be included in HLA-DR typing analysis because they are always associated with DR antigens. Additionally, anti-HLA-DR51, -DR52, and -DR53 (-DR51/52/53) antibodies can be evaluated as DSA for antibody analysis. One study showed that mismatches in the HLA-A, -B, and -DRB1 loci, as well as HLA-DR51/52/53 antigen mismatches, also have an effect on HLA allosensitization. In the relevant study, it was determined that the risk of allosensitization is 3, 2; 3, 4; 3,5; and 3,9-fold higher for HLA-A, -B, -DRB1, and -DR51/52/53 mismatches, respectively (9). Another study reported that antibody-mediated rejection occurred more frequently in the group with possible anti-HLA-DR51/52/53 DSA (20). All of this literature data emphasizes the importance of considering these antibodies during HLA-DR51/52/53 typing and DSA evaluation for all donors and recipients.

The FCXM test is a highly sensitive cell-based method that predicts graft rejection. In our study, in addition to the studies in the literature, the correlation between anti-HLA-DR51/52/53 DSA positivity and T cell and B cell FCXM positivity was investigated, and it was found that all DR51/52/53 donor-specific antibodies, either alone or in combination, showed a correlation with B cell FCXM positivity. In addition, it has been found that DR4, DR16, DR11, and DR12 alone, and DR15 and DR16 in combination, may be associated with T cell FCXM positivity. The results of our study suggest that FCXM positivity may predict the presence of HLA-DR52/53/54 antibodies, which are an important risk factor for rejection. Therefore, considering these antibodies during HLA-DR51/52/53 typing and DSA assessment for all donors and recipients may contribute significantly to transplant success and post-transplant immunological follow-up.

However, recent studies have shown that DSA do not always prevent organ transplantation, and therefore, broader studies to understand the subtypes of anti-HLA antibodies and their activity will be very important.

Ethical Approval: This study was approved by the Ethics Committee of İstanbul University on February 3, 2023 (Decision No. 2022/1707).

Informed Consent: N.A.

Peer-review: Externally peer-reviewed

Author Contributions: Concept –D.K., F.O.; Design – H.S.Ç., D.K.; Supervision – F.O., Ç.K.Ç.; Data Collection and/or Processing – D.K., H.S.Ç.; Analysis and/or Interpretation – D.K., F.O., Ç.K.Ç.; Literature Review – D.K., H.S.Ç.; Writer – D.K., H.S.Ç.; Critical Reviews – F.O., Ç.K.Ç.;

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