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Molecular modeling of novel anticancer agents for treatment of ovarian carcinoma by utilizing chlorambucil for optimizing properties

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ABSTRACT

Epithelial ovarian cancer is a lethal and most common malignancy of the female enital area. Although advanced-stage ovarian cancer is responsive to multiple cytotoxic drugs, less that 25 % of these patients are alive and disease free after five years of diagnosis. This clinical reality justifies the origin and examination of new drug designs. This work presents nitrogen mustard alkylating agents developed from an optimized molecular modeling analysis of chlorambucil. Thirteen analogs of chlorambucil are selected to introduce potential anticancer agents that have pharmaceutical properties advantageous for clinical application. Important molecular properties such as Log P, polar surface area, formula weight, molecular volume, and violations of the Rule of 5 were determined. For all agents the polar surface area ranged from 23.547 A^2 to 86.788 A^2. No agent showed violations of the Rule of 5 or had greater than 11 rotatable bonds. There was a wide variance in Log P values which ranged from -0.908 to 4.958. Many agents showed a numerically low polar surface area suitable for blood-brain barrierpenetration ($\leq$ 90 A^2), but not accompanied by a supporting value of Log P. The broad range in Log P values may enhance the efficacy of these agents for application because of diverse lipid by-layer permeation. With the alkylating nitrogen mustard group these agents possesses an aromatic ring and either an amide or carboxyl substituent. Molecular properties were analyzed by multivariate methods such as cluster analysis, K-means cluster analysis, discriminant analysis, and ANOSIM (analysis of similarity) which revealed underlying subtle and clinically important relationships among these analogs. Multiple regression analysis produced a mathematical expression relating various molecular properties and useful in predicting additional novel drug designs analogous to this series.

KEYWORDS

Ovarian carcinoma;
Chlorambucil;
Anticancer;
Multivariate.

INTRODUCTION

Ovarian cancer is not widespread but is the deadliest of all female cancers. In the most advanced stage cure is achieved in only 5 percent of cases. Survival of epithelial ovarian cancer is directly correlated to the stage of the disease[1]. The absence of symptoms during early-stage I and II is related to the high mortality rate[1]. Al-

though advanced-stage ovarian cancer is responsive to multiple cytotoxic drugs, less than 25 % of these patients are alive and disease free five years after diagnosis[1]. Causes of this low survival statistic is thought to be primarily due to drug resistance[1]. This fact provides suitable justification to pursue design of novel drug designs. Single agents found active in ovarian cancer treatment include alkylating agents (chlorambucil, melphalan, cyclphos-mamide), platinum compounds (ie. cisplatin), anthracyclines (ie. doxorubicin), methotrexate, 5-fluorouracil, and taxanes[1].

Chlorambucil is a bifunctional alkylating chemotherapy drug used in the treat-ment of ovarian cancer, leukemia, breast cancer, and various autoimmune diseases[2]. An alkylating agent is capable of replacing a hydrogen atomwith an alkyl group at physiological conditions of 37^0C and pH 7.4[3]. At physiological pH the relative rates of nucleophilic substitution are in order of thiolate>amino>phosphate>carboxylates[3]. Chlorambucil actively binds to DNA as well as RNA[2]. Studies on DNA have shown that nitrogen mustards find the following order in terms of reactivity: N-7 of guanine>N-3 of adenine>N-1 of adenine>N-1 of cytosine (although phosphate groups, N-3 of cytosine, and O-6 of guanine can be alkylated)[3]. Chlorambucil can induce DNA intrastrand or interstrand cross-linking by either S_N1 or S_N2 type reactions[3]. The differences in effectiveness of alkylating agents is considered to be due to variance in pharmaceutical factors, membrane transport properties, lipid solubility, detoxification mechanisms, ability to penetrate the blood-brain barrier, and enzymatic repair of alkylated DNA[3]. These findings further substantiate the legitimacy of developing new drugs for treatment.

Unlike cervical and breast cancer there is presently no effective method for screening for ovarian cancer[4]. Known risk factors include, family history, advanced age, nulliparity, early menarche, and late menopause[4]. Chlorambucil has been combined with thiotepa as a double alkylator therapy for advanced stage ovarian cancer[5]. Combination chlorambucil and carboplatin chemotherapy was found to be well tolerated (ie. no nephrotoxicity and no neurotoxicity) by stage 1C, II, III, and IV patients with ovarian cancer[6], that prolonged survival for patients having small volume residual disease[6]. Chlorambucil was found beneficial to patients having platinum resistant end stage epithelial ovarian cancer[7], also with minimal toxicity as found in other studies. Treatment of ovarian carcinoma that is resistant to chlorambucil is particularly problematic because the alternative combination therapy utilizing cisplatin, adriamycin, and cyclphosphamide has marked toxicity that has induced drug related patient deaths[8]. These findings suggests that drug design utilizing chlorambucil as a parent model may yield additional compounds having favorable pharmaceutical properties. Chlorambucil has also been utilized beneficially in combination with rituximab to treat ocular adnexal lymphomas[9]. These findings clearly support the endeavor to investigate new drug designs for the treatment of ovarian cancer.

EXPERIMENTAL

Molecular modeling methods and determination

Several software approaches to advanced molecular modeling were utilized to develop novel molecular species analogous to chlorambucil. Visualization of two dimensional structure constituents was accomplished by Molinspiration (Molinspiration Cheminformatics, Liscie udolie 2, SK-841 04 Bratislava, Slovak Republic) and Molsoft (Molsoft L.L.C., 3366 North Torrey Pines Court, Suite 300, La Jolla, CA 92037). Additional modeling support and determination of various molecular properties was accomplished by utilizing ChemSketch (Advanced Chemistry Development, 90 Adelaide Street West, Toronto Ontario, M5H 3V9 Canada).

Pattern recognition and statistical analysis

Various operations to achieve pattern recognition within the numerical data matrix of molecular properties was accomplished in addition to descriptive statistics determination. Underlying relationships of the molecular properties for all compounds presented in this work were ascertained using cluster analysis and K-means cluster analysis (PAST v. 1.28, copyright Hammer and Harper 1999-2004; KyPlot v. 2.0 beta 15 copyright Koichi Yoshioka 1997-2001). Data analysis of similarity, ANOSIM, was accomplished by PAST v. 1.28. Differentiation of numerical values by group was achieved utilizing discriminant analysis was done by PAST v. 1.28. Relationships of causality for various molecular properties was determined by path analysis

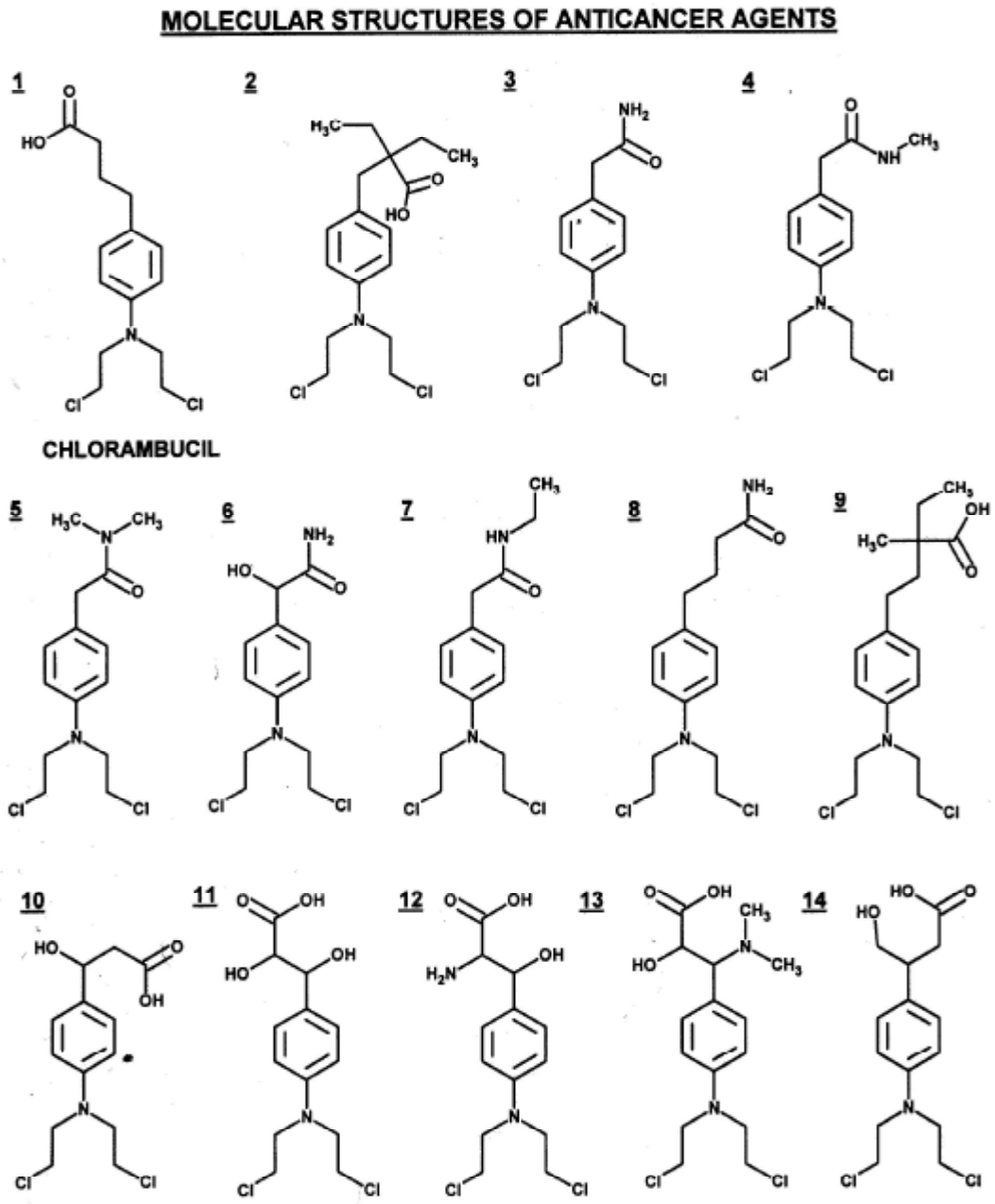

Figure 1

utilizing OpenStat 3 v. 3.5.2 (Open Stat 3, copyright William G. Miller March 2004). Multiple regression analysis utilizing various properties was determined by GraphPad Instat v. 3.05 (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, Copyright 1992-1998 GraphPad Software www.graphpad.com). Correlations of numerical values and descriptive statistics was done by EXCEL (Microsoft Office Excel 2003, copy right 1985-2003 Microsoft Corporation).

RESULTS AND DISCUSSION

Less than 25% of patients diagnosed with advance-stage ovarian cancer are alive and free of disease five years later[1]. Acquired and intrinsic drug resistance is considered to be primarily responsible for this outcome[1]. This state of affairs supports the need to determine new drug designs for clinical application. The development of analogues to chlorambucil is the focus of this work and begins with known structure and phar-

maceutical activity of this bifunctional alkylating antitumor agent. The aromatic ring within chlorambucil is a rigid component of it's overall molecular structure. Retained in the analogues studied here, the aromatic ring has the bifunctional nitrogen mustard groupin para position to a constituent having either a carboxyl functional group or a amide group. Amide functional groups are subject to a slower rate of Phase I hydrolysis than that of ester groups[10]. Carboxyl groups undergo Phase I oxidation at their α- and β-carbon atoms provided methylene groups are adjacent to the carboxyl group[10]. Under Phase II metabolism the carboxyl groups are conjugated with an amino acid[10]. The aromatic ring and bifunctional nitrogen mustard group is retained in all analogues, however the substituent para to the alkylating moiety is altered by rigidity (ie. number of rotatable bonds), size (formula weight), and atoms for hydrogen bonding (ie. hydrogen bond donator –NH or –OH, or acceptor oxygen and nitrogen). In this manner the important pharmaceutical properties of Log P, polar surface area, and molecular volume can be altered to determine beneficial activity for the clinical treatment of ovarian carcinoma.

The non-alkylating substituent of chlorambucil (compound **(1)**, Figure 1) or ($-CH_2CH_2CH_2COOH$) was utilized as the parent structure for search and prediction of similar constructs by utilizing analog search (see Experimental) followed by property determination. Compound analogues **(2 to 14)** (Figure 1) are generated by replacing the substituent found in the para position relative to the bifunctional alkylating group of chlorambucil. notably the superseding substituents have either a carboxyl or amide functional group. The resulting compounds are shown in figure 1 and express molecular properties sufficiently diverse to permit differentiation by pattern recognition methods and categorization of expected pharmaceutical activity. The superseding moieties also bring into the new structure methyl groups, hydroxyl groups, and amine groups that well affect the Log P and polar surface area properties while retaining the DNA and RNA alkylation activity.

Previous studies have shown that structure modifications of drugs are paramount to activity and potency modifications[3]. Variation in the efficacy of alkylating agents is thought to result from differences in pharmaceutical factors[3]. There-fore the variation of substituent structure in analogues 2 to 14 can be assumed to be largely responsible for alteration in molecular properties for the discussion to follow.

Molecular properties of compounds **(1 to 14)** presented in figure 1 are ascertained and presented in Table 1. Pattern recognition methods and descriptive statistical analysis of the data matrix revealed underlying inter-relationships as well as beneficial pharmaceutical potential. Of the **(14)** compounds (inclusive of chlorambucil) the highest correlation ($r>0.9000$) occurred between values of polar surface area (PSA) and number of –OH and –NH (nOHNH), or formula weight (FW) and molecular volume. Other distinctly correlated properties ($r>0.8000$) lies between FW and number of rotatable bonds (nRotB), or number of atoms (nATOMS) and nRotB. These correlations suggest significant intrafamily characteristics inherent to alkylating agents targeting cervical cancer. Interestingly the range in values of Log P (range=5.866) and PSA(range= $63.241A^2$) indicate additional pharmaceutical efficacy which will be discussed now. Previous studies have determined a strong relationship of intestinal drug absorption and values of PSA[11,12,13]. Essentially drug structures having a PSA of greater than $140A^2$ were less than 10% absorbed within the intestinal tract[12,13]. However those drug structures have PSA of less than $60A^2$ were found to be greater than 90% absorbed within the intestinal tract[13], a characteristic inclusive of compounds **(1,2,3,4,5,7,8, and 9)**. This finding is a strong beneficial property of these drug designs. No member of 1 to 14 has a PSA value greater than $140A^2$. In addition, other work has shown that drugs having PSA of less than $90A^2$ have a high probability of piercing the blood-brain barrier[14], thereby 1 to 14 shows potential expression of brain targeting antitumor activity. Focusing on Log P values reveals additional useful characteristics for clinical application.

Drugs having Log P less than 0 are amenable to intravenous injection (drug 12). Another characteristic of drugs 1 to 14 has important pharmaceutical ramifications and consideration on the potential for these constructs for clinical application. All compounds 1 to 14 have zero violations of the rule of 5. The rule of 5 was developed to assist the screening of novel drug designs for potential clinical usage. This criteria states that a chemical structure has increased probability of poor

TABLE 1

	Desriptors								
	Formula								
Compound	Log P	PSA	nON	Weight	nOHNH	nATOMS	Number violations	nRotB	Volume
1 Chlorambucil	3.465	40.537	3	304.22	1	19	0	9	268.51
2	4.504	40.537	3	346.30	1	22	0	10	318.14
3	2.162	46.332	3	275.18	2	17	0	7	238.18
4	2.536	32.336	3	289.21	1	18	0	7	255.86
5	2.339	23.547	3	303.23	0	19	0	7	272.80
6	1.171	66.560	4	291.18	3	18	0	7	246.22
7	2.912	32.336	3	303.23	1	19	0	8	272.78
8	2.950	46.332	3	303.23	2	19	0	9	271.78
9	4.958	40.537	3	360.33	1	23	0	11	334.94
10	1.956	60.746	4	306.18	2	19	0	8	259.76
11	1.040	80.992	5	322.19	3	20	0	8	267.80
12	-0.908	86.788	5	321.20	4	20	0	8	271.07
13	0.246	64.003	5	349.26	2	22	0	9	305.69
14	0.781	60.755	4	320.22	2	20	0	9	276.56

PSA= polar surface area(Angstroms^2); nOH=number of oxytrogens; nOHNH= number of hydroxyls and amines; nATOMS= number of atoms; Number violations=violation of rule of 5; nRotB= number of rotatable bonds; Volume= molecular volume(Angstroms^2)

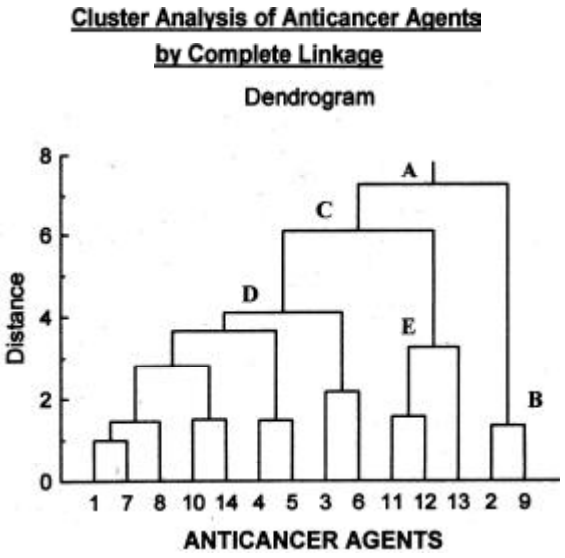

Figure 2: Hierarchical cluster analysis results utilizing complete linkage method with standard Euclidean distance. Drugs 2 and 9 are clearly distinct from all remaining drugs. Drug 7 is considered most similar to chlorambucil (drug 1). Initial super cluster A is initially separated in cluster C and B, where C is further sub-divided into clusters D and E. Drugs 11,12, and 13 fall into cluster E, whereas cluster D contains 1,7,8,10,14,4,5, 3, and 6. Drugs falling into identical clusters are considered to be most similar

absorption and permeation if the following parameters exist[15]: (1) There are more than 5 hydrogen bond donors; (2) The molecular weight is greater than 500; (3) The Log P is greater than 5; (4) There are more than 10 hydrogen bond acceptors (sum of nitrogens and oxygens). All structures presented here show zero violations of the rule of 5, indicating that 1 to 14 would expect to have favorable permeation following administration *in vivo*.

To determine subtle and underlying relationships within the data matrix of TABLE 1 it is appropriate to apply various multivariate methods of analysis, that will determine pattern identification also. Associations by properties will help determine anticipated similarities of pharmaceutical activity as well as analogy in clinical application. A widely-used method for pattern recognition is cluster analysis, which arranges sets of cases (drugs) into clusters such that cases within a particular cluster are more similar to each other than cases found in other clusters[16,17]. A vertical dendrogram is presented in figure 2 showing results of hierarchical divisive clustering using complete linkage (ie. Distance between clusters is defined as the distance between the farthest pair of points within any two clusters). Beginning as super cluster A the drugs 1 to 14 are further divided into classifications that ultimately designates each drug to another that has the greatest similarity. Secondary clusters C and B divide drugs 2 and 9 from all the remaining. Formation of tertiary clusters D and E further separate drugs 11,12, and 13 from 1(chlorambucil), 7,8, 10,14,4,5,3, and 6. Drug 7 is shown to be highest similarity to chlorambucil which is supported by comparison of (chlorambucil versus 7): Log P: 3.465 to 2.912; FW: 304.22 to 303.23; nON: 3 to 3; nATOMS: 19 to 19. Drugs 11,12, and 13 are among the highest in for-

mula weight, number of atoms, and PSA. By similar observation drugs 2 and 9 found in secondary cluster B have sufficient similarity by properties to be distinguished from the remaining 12 members. By this approach it can be determined which constructs may act analogously in pharmaceutical activity.

Discriminant analysis (DA) has several purposes, including[16,17]: (1) Classify cases into groups; (2) To determine the relative importance of independent variables in classifying a dependent variable; (3) To test whether cases are classified as predicted; (4) Determine an efficacious way to distinguish between known groups. Using properties of Table 1 the DA result will show how drugs 1 to 14 are distinguished into two groups of highest dissimilarity. The results conclude that group 1 consists of 1(chlorambucil), 7,8,9,13, and 14; whereas group 2 has drugs 2,3,4,5,6,10,11, and 12. Notably drug 7 is determined to be more similar to chlorambucil as was found in cluster analysis.

Hierarchical analysis does not require the determination of number of final clusters prevenient. Whereas non-hierarchical cluster analysis requires a determined number of clusters prior to arrangement of cases within those clusters. K-means cluster analysis is such a non-hierarchical cluster analysis that will be applied to resolve precise differentiation of cases (drugs) into clusters of highest similarity. An initial determination having three final clusters yielded the following results: (Cluster 1) Drug 2, 9, 13; (Cluster 2) Drug 1,3,4,5,6,7,8,10; (Cluster 3) Drug 11,12,14. Further refinement of analysis for increase resolution was done into four clusters, yielding the following results: (Cluster 1) Drug 2,9,13; (Cluster 2) Drug 1,3,4,5,7,8; (Cluster 3) Drug 11, 12; (Cluster 4) Drug 6, 10, 14. These findings indicate analogous conclusions to that of cluster analysis and DA whereas groupings of drugs are thought to have congruent pharmaceutical activity. Notably drug 7 is considered most similar to chlorambucil by K-means cluster analysis (four cluster resolution). In addition, to pattern recognition within the data matrix values it is possible to evaluate the over-all similarity of the cases via all properties simultaneously by applying Analysis of Similarity or ANOSIM. This test compares distances between groups with distances within groups themselves[17]. A large positive value of R up to 1.0000 signifies dissimilarity between cases. ANOSIM was applied to the data matrix of TABLE 1 and yielded a result an R of 0.1336. This low value of R indicates all cases (drugs) are substantially similar to each other. This result obtained after consideration of all numerical values inclusively. Application of Path Analysis (PA) was accomplished here to discern the relationship of several highly important parameters that significantly influence pharmaceutical activity. The influence of Log P, PSA, nON, and nOHNH on values of formula weight inclusive of 1 through 14 can be ascertained by PA and yielding path coefficients that function the same as standardized regression coefficients[18]. To produce the formula weights of all drugs the four properties above have the following path coefficients(correlations), respectively: 1.231, -0.098, 1.657, and -0.322. Therefore Log P and number of oxygens and nitrogens (nON) have the greatest affect on formula weight. A finding useful in designing drugs of similar make-up as 1 to 14.

To assist further design of drugs alike the cases 1 to 14 a multiple regression analysis of Table 1 yields a mathematical algorithm which can be utilized to predict numerical values of properties for hypothetical analogs. The following regression equation was obtained for determination of formula weight (FW) utilizing Log P, PSA, nON, nOHNH, nRotB, and volume (Vol): FW = 49.412+0.3607(Log P)+0.6090(PSA)+3.912 (nON)+6.632 (nOHNH)+0.0295(nRotB)+0.8330 (Vol). Value of R indicates this model accounts for 99.97% of variance. Based on the standardized regression coefficients of the model the properties of PSA, nON, nOHNH, and volume present the greatest contribution to the model, an important observation when predicting formula weight of analogous constructs.

Affects induced by variation on any combination of these properties on the overall suitability of a proposed drug construct can be determined.

CONCLUSION

Thirteen novel drug designs were developed here by extensive examination of the major non-alkylating substituent of chlorambucil and generating a family of isosteres. Isosteric substitution within the chlorambucil parent structure generated 13 analogs that showed favorable pharmaceutical properties such as zero violations of the Rule of 5. These constructs are therefore

predicted to have effective absorbance and permeation. All constructs 1 to 14 possess PSA values indicating likelihood of penetrating the blood-brain barrier for activity against brain tumors. Constructs 1, 2, 3, 4, 5, 7, 8, and 9 have PSA of less than $60A^2$ with an expected intestinal absorbance of greater than 90%. Extensive analysis by pattern recognition methods identified underlying relationships of these analogs to chlorambucil. Hierarchical cluster analysis and K-means cluster analysis indicated clearly that drugs 2 and 9 are highly distinct from chlorambucil, but drug 7 is strongly similar. ANOSIM analysis recognized significant similarity among 1 to 14 when all molecular properties are examined simultaneously. This work shows distinctly the efficacy of isosteric substitution on the parent frame structure of chlorambucil to generate a useful library of potential anticancer drugs for the clinical treatment of ovarian cancer.

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