Design of Anticancer Agents Utilizing Streptozocin for *In Silico* Optimization of Properties and Pattern Recognition Identification of Group Features

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Abstract: Streptozocin has been shown to be useful in the clinical treatment of malignant neuroendocrine tumors of the pancreas. The poor prognosis for patients having malignant tumors of pancreas suggests the investigation and development of new therapeutics. Nine analogs to streptozocin are determined by *in silico* physicochemical analysis and generation of structures by modeling from functional group isosteres. In these analogs is preserved the alkylating nitrosourea moiety, however, the covalently bonded substituent has significant hydrogen bonding sites and may include a ring structure. Analogs retain a broad range in lipophilicity, having a range of Log P from -2.798 (hydrophilic) to 3.001 (lipophilic). Standard deviation of molecular masses is only 12.6% of the group mean, so a small alteration in size occurs which is also reflected by only a 15.5% deviation in molecular volumes. Streptozocin and seven analogs show zero violations of the Rule of 5 which suggests favorable bioavailability. All compounds showed at least seven hydrogen bond acceptors with a strong positive correlation between hydrophilicity to the total number of hydrogen bond acceptors and donors. Analysis of similarity (ANOSIM) and discriminant analysis determined that streptozocin is highly similar to all nine analogs. However hierarchical cluster analysis and K-means cluster analysis were able to elucidate patterns of associations and differentiation among the ten compounds. This study demonstrates the efficacy of utilizing *in silico* optimization and pattern recognition to elucidate potential anticancer drugs.

INTRODUCTION

Pancreatic cancer is a deadly malignant tumor of the pancreas which kills tens of thousands in the United States and Europe each year. Approximately 95% of all pancreatic cancers are adenocarcinomas and 5% include serous cystadenomas, acinar cell cancers, and neuroendocrine tumors. The neuro-endocrine system is the interface between the messenger nervous system and hormone system acting as messenger to regulate physiological activity. Gastro entero pancreatic neuroendocrine tumors, or GEP-NETs, are cancers at the nervous-endocrine system interface and include carcinoids (slow growing) and pancreatic endocrine tumors. Islet cell tumors or endocrine pancreatic tumors (EPTs) are thought to originate in the islets of Langerhans of the pancreas. Roughly 2% of pancreatic tumors belong to the GEP-NET class.

Streptozocin is a glucosamine-nitrosourea that is an alkylating antineoplastic agent that falls with in the nitrosourea class of drugs and is particularly toxic to insulin producing beta cells of the pancreas [1]. Streptozocin itself has a molecular structure that resembles glucose and can be transported into the cell by glucose transport proteins such as GLUT2 [1]. Beta cells of the pancreas have a large number of GLUT2 glucose transporters such that induced toxicity is substantial, whereas other glucose transporters do not recognize streptozocin [2, 3]. This fact played a significant role in the application of streptozocin in the treatment of pancreatic islet cell cancer [4]. Streptozocin is mutagenic, teratogenic, carcinogenic, and can cross the placenta [1-3]. The typical dose of streptozocin is administered intravenously because it has poor oral absorption. Streptozocin degrades to a methylcarbonium ion with only a small fraction of administered dose excreted in the kidneys.

Streptozocin has been studied as part of a combination chemotherapy for advanced cancer of the pancreas [5]. Response rates for treatment of pancreatic cancer using streptozocin, fluorouracil, and semustine together were greater than that achieved with treatment by melphalan alone [5]. Pancreatic islet cell carcinoma treated with a combination regimen of streptozocin with fluorouracil showed a reduction in the size of the enlarged metastatic tumor, with improved liver enzymes, and accompanied with only mild toxic reaction [6]. Other investigators have observed considerable success with streptozocin and doxorubicin combination chemotherapy for malignant neuroendocrine pancreatic tumors [7]. Up to 60% of patients receiving streptozocin/doxorubicin regimen gave a favorable response and survival benefit [7]. Other studies provide supporting results, showing that streptozocin, doxorubicin, and fluorouracil in combination brought about a response rate of 39% with longer progression free survival and overall survival for treatment of islet cell cancer (pancreatic endocrine carcinoma) [8]. A study was performed comparing combination chemotherapy of a streptozocin-doxorubicin pair with a streptozocin-fluorouracil pair [9]. The relative tumor regression rate was 69% and 45%, respectively, demonstrating the superiority of the streptozocin-doxorubicin combination [9]. In addition the streptozocin-doxorubicin regimen had a significant advantage in long term survival [9]. These encouraging results clearly support

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the contention that the design of novel antineoplastic drugs utilizing streptozocin as a parent modeled compound may render effective agents for use in the clinical treatment of pancreatic islet cell (neuroendocrine) carcinoma.

MATERIALS AND METHODOLOGY

Molecular Modeling and Assembly of Constructs

Numerical values of molecular properties and molecular modeling were accomplished utilizing ACD/Chem Sketch modeling v. 10.00 (Advanced Chemistry Development, 110 Yonge Street, Toronto Ontario, M5C 1T4 Canada). Other properties; polar surface area, violations of Rule of 5, molecular volume, number of oxygens/nitrogens/amines/hydroxyls, etc were determined using Molinspiration (Molinspiration Chemiformatics, Nova ulica 61, SK-900 26 Slovensky Grob, Slovak Republic). The determination of drug-likeness for all compounds was accomplished for GPCR (G protein-coupled receptor) ligand activity, ion channel modulator activity, kinase inhibition, and nuclear receptor ligand activity by Molinspiration. Visualization of 3-dimensional scaffolding was achieved by utilizing SPARTAN modeling (Wavefunction, 18401 Von Darman Avenue, Irvine CA 92612 USA).

Pattern Recognition Analysis

To identify underlying associations/patterns within the molecular properties numerical matrix various pattern recognition techniques were applied. Included in this application is hierarchical cluster analysis accomplished by KyPlot v. 2.0 Beta 15 (copyright Koichi Yoshioka 1997-2001). Discriminant analysis, and non-hierarchical K-means cluster analysis were performed by PAST v. 1.80 (copyright Oyvind Hammer, D.A.T. Harper 1999-2008).

Numerical Analysis of Multivariate Matrix Field

Statistical analysis of all numerical data was performed by Microsoft EXCEL (EXCEL 2003, copyright 1985-2003). Multiple regression analysis of molecular property values was accomplished by GraphPad Instat v. 3.00 for Windows 95 (GraphPad Software, San Diego California USA). Correlation analysis for Pearson r was done for descriptors indicated and was accomplished by EXCEL (2003) and PAST.

RESULTS

Streptozocin is currently utilized in the clinical treatment of pancreatic islet cell carcinoma. The molecular structure of streptozocin is presented in Fig. (**1**), and was used as the parent (to be modeled) compound for development of mo-

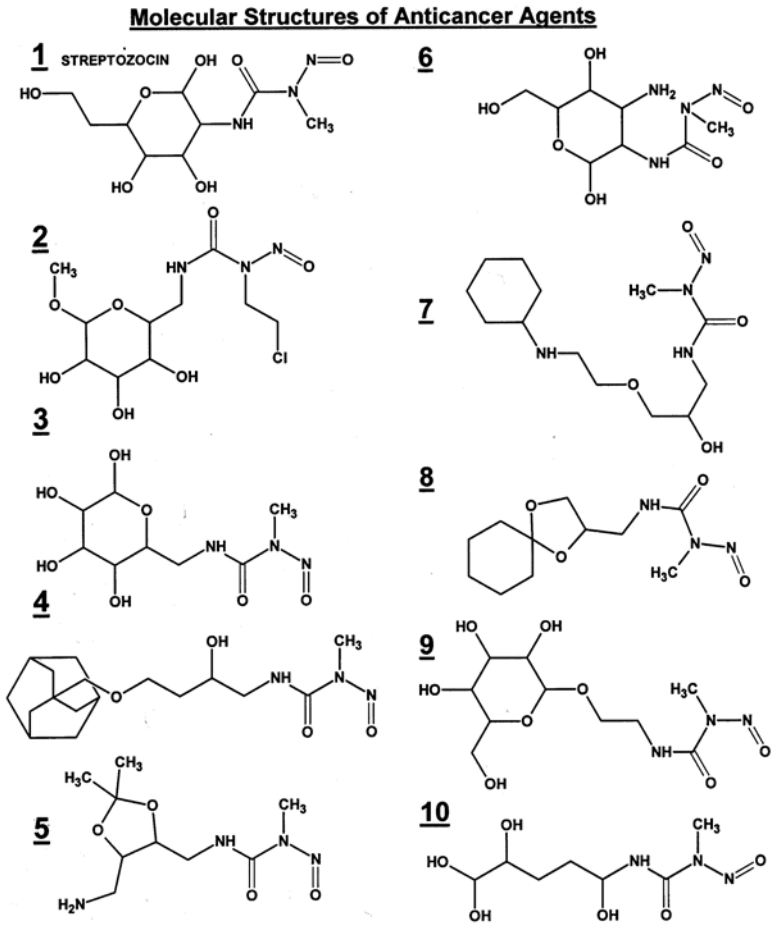

Fig. (1). The molecular structures of the parent compound streptozocin and nine chemical analogs are presented here for comparison. Note that all compounds, excepting number 10, have a ring substituent. The nitrosourea moiety comprises the alkylating functional group of the structure. Many compounds have substantial hydrogen bonding donor/acceptor sites. The secondary amide nitrogen atom of all compounds must have a hydrogen attached.

lecular analogs that may have effectiveness in cancer treatment. The nine analogs derived are also presented in Fig. (**1**) and have numerous features in common with the parent streptozocin. They include as follows by inspection: i) A nitrosourea alkylating moiety; ii) A secondary amide group having a hydrogen covalently bonded to the nitrogen atom; iii) A non-alkylating substituent; iv) Except for analog 10, all drugs have a ring substituent; v) The non-alkylating substituent has hydrogen donor/acceptor capability.

Various molecular properties that have substantial pharmacological importance were determined for the parent streptozocin and the nine analogs. These properties (descriptors) include: Log P (lipophilicity), polar surface area (PSA), number of heavy atoms, formula weight, number of rotatable bonds, hydrogen bonding centered atoms (nitrogens, oxygen), molecular volume, and violations of Rule of 5. All drugs together showed a broad range in lipophilicity, with a range of Log P from -2.798 (hydrophilic) to 3.001 (lipophilic). Standard deviation of molecular mass is 12.6% of the mean, therefore a small alteration in size occurs which is also reflected by only a 15.5% deviation in molecular volume. Streptozocin and seven analogs show zero violations of the Rule of 5, analogs 6 and 9 showed one violation. All compounds showed at least seven hydrogen bond acceptors with a strong correlation between hydrophilic tendency to the total number of hydrogen bond acceptors and donors. Correlation of drug by properties determined for the ten compounds was determined using Pearson r and produced very high drug-to-drug correlation based on the properties presented in Table **1**. All inter-drug correlation r values were greater than 0.9500. The outcome of Analysis of Similarity (ANOSIM) presented a value of 0.1081. In addition to Pearson r and ANOSIM for correlation and similarity, respectively, underlying relationships among the ten drugs can be investigated by use of non-hierarchical K-means analysis. This was done for two cluster outcome and should the following clustering based on similarity: Cluster 1) Drug 1 (streptozocin), 3, 5, 6, 8, and 10; Cluster 2) Drug 2, 4, 7, and 9. For three cluster outcome the clustering results were as follows: Cluster 1) Drug 1 (streptozocin), 3, 5, 6, 8, and 10; Cluster 2) Drug 2, 7, and 9; Cluster 3) Drug 4 only. Finally, for the four cluster outcome the clustering results were as follows: Cluster 1) Drug 2 and 9; Cluster 2) Drug 4 and 7; Cluster 3) Drug 5 and 8; Cluster 4) Drug 1 (streptozocin), 3, 6, and 10.

To derive an equation for supporting the design and description of antineoplastic nitrosoureas that are related to streptozocin, multiple regression was accomplished for formula weight as dependent variable and Log P, PSA, and number of atoms as independent variables. The outcome equation as follows:

formula weight = -28.150 + (2.748)(Log P) + (0.3283)(PSA) + (13.984) (number of atoms).

The value of R squared was 0.9643, therefore the model explains 96.43% of variance in the formula weight.

Subtle associations among the ten drugs can be elucidated by applying a more sensitive determinate of similarity utilizing hierarchical cluster analysis. Utilizing the numerical matrix presented in Table **1** it is possible to reveal then underlying relationships not apparent by inspection. The outcome of cluster analysis is seen in Fig. (**2**), where drugs 1 to 10 are grouped into clusters determined to indicate the highest degree of similarity (based on numerical values of desired molecular properties). The drugs are grouped as pairs: 1 (streptozocin) and 3, 5 and 8, 2 and 9, 4 and 7, with drugs 6 and 10 associated with 1 and 3 by supernode (higher cluster having greater distance (Euclidean) from the rudimentary clusters).

Discriminant analysis (DA) was performed on all drugs using properties of Table **1**. DA is useful in identifying descriptors that distinguish subjects from among a multivariate matrix. This pattern recognition is also sensitive and can identifying subjects that may be outliers from within a multivariate collection. DA was carried out and outcome is presented in Fig. (**3**) as a 2-way plot wherein the closer proximity between subjects suggests greater similarity. Clearly visible is the strong clustering of all ten compounds into very close proximity and therefore showing very high similarity.

Table 1. Molecular Properties of Streptozocin and Analogs

Compound	Log P	Polar Surface Area (Angstroms^2)	Number of Atoms	Formula Weight	Number of Oxygens and Nitrogens	Number of Amines and Hydroxyls	Number of Rotatable Bonds	Molecular Volume (Angstroms^3)	Violations of Rule of 5
1 Streptozocin	-2.088	151.917	18	265.222	10	5	3	219.350	0
2	-0.647	140.923	21	327.721	10	4	6	267.454	0
3	-1.869	151.917	18	265.222	10	5	3	219.350	0
4	3.001	91.233	24	339.436	7	2	8	324.502	0
5	-0.14	106.262	17	246.267	8	3	4	223.496	0
6	-2.798	157.712	18	264.238	10	6	3	222.618	1
7	1.484	103.25	21	302.375	8	3	9	291.627	0
8	2.234	80.209	18	257.29	7	1	3	235.422	0
9	-1.885	161.151	21	309.275	11	5	6	261.936	1
10	-1.597	142.683	16	237.212	9	5	6	204.351	0

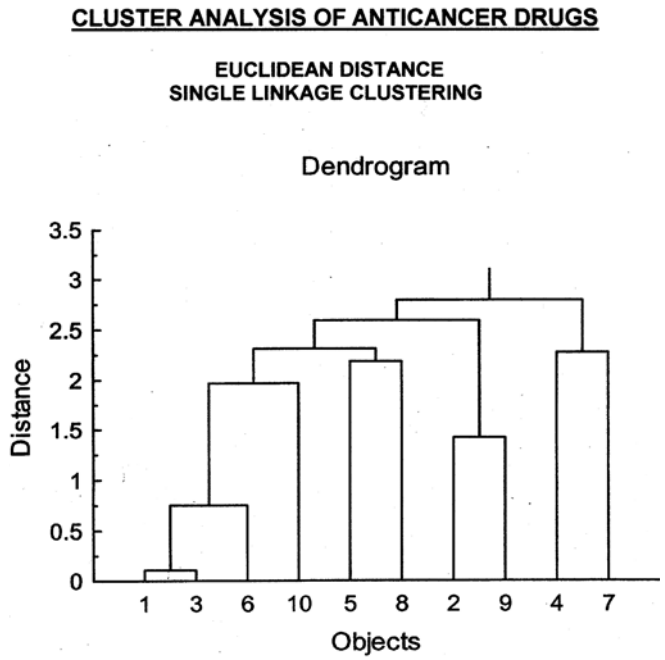

Fig. (2). Hierarchical cluster analysis results distinguish the analogs for parent by molecular properties. Useful in estimating potential significant physicochemical divergence from the parent streptozocin (1) and analogs. Streptozocin is most similar to analog 3 but related to analogs 6 and 10 (see Table **1**). Remaining analogs are paired within clusters as follows: 5 and 8, 2 and 9, 4 and 7.

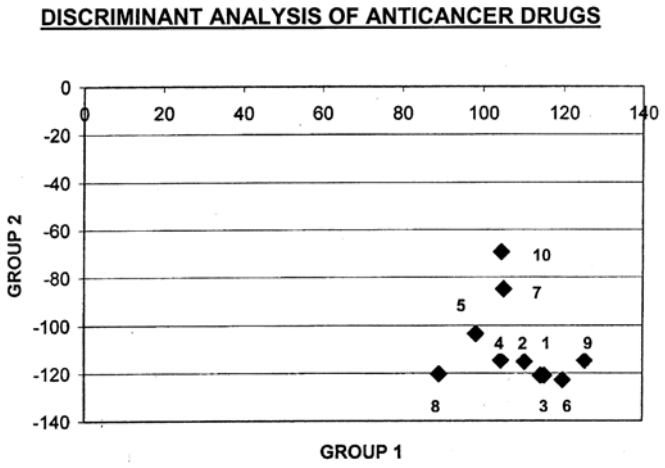

Fig. (3). Results for discriminant analysis presented within a 2-way plot indicate clearly that streptozocin (1) and analogs are very similar and are positioned in close proximity to each other. Plotted along discriminant group 1 and 2, these compounds are highly clustered and hence quite similar.

Activity of all nine analogs and streptozocin were rigorously analyzed under four criteria of known successful drug activity in the areas of GPCR ligand activity, ion channel modulation, kinase inhibition activity, and nuclear receptor ligand activity (see Materials and Methodology). Results are shown for all ten compounds in Table **2** by means of numerical assignment. Numerical values of streptozocin (drug 1) are presented in column 1 and have consistent negative values placing streptozocin outside the numerical range of values for current therapeutic agents used in each category (clinical drugs have values from zero to two). Likewise analogs 2 through 10 have consistent negative values in all categories and numerical values conforming and comparable to that of streptozocin. Pearson r correlation was calculated to show extremely high correlation of streptozocin to drugs 2, 3, 4, 5, 6, 7, 8, and 10 ($r > 0.8400$) and very high correlation to drug 9 ($r > 0.7600$). Therefore it is readily seen that analogs 2 through 10 are expected to have similar activity to streptozocin based upon these four rigorous criteria (GPCR ligand, ion channel modulator, (kinase inhibitor, and nuclear receptor ligand).

DISCUSSION

Streptozocin has been found to enhance the clinical treatment of advance pancreatic neuroendocrine carcinoma when administered in combination chemotherapy [5-9]. Streptozocin is a nitroso-urea alkylating agent with a heteroatom ring substituent having four hydrogen bond donors and one acceptor. That portion of the molecule is hydrophilic and contributes significantly to the Log P value of -2.088 and polar surface area of 151.917 A^2 (see Table **1**). Previous studies have shown that this PSA value seriously impedes oral bioavailability [10]. Only for analogs 4 and 8 (see Fig. (**1**) and Table **1**) do the values of PSA become less than 100 A^2 to be 91.233 A^2 and 80.239 A^2, respectively, suggesting intestinal absorption of about 50% and 75%, respectively. Clearly than a change in PSA could have substantial affect on administration of analogs 4 and 8 that may be beneficial to the patient. To support this contention, note that analogs 4 and 8 have zero violations of the Rule of 5. The Rule of 5 is a set of parameters devised to aid the screening of potential drug "hits" identified through processes such as high throughout screening [11]. Applying the Rule of 5 increases the probability that a potential chemotherapeutic will have favorable bioavailability. The criteria are as as follows [11]: A) Not more than 5 hydrogen bond donors; B) Not more than 10 hydrogen bond acceptors; C) Formula weight less than 500; and D) Log P less than 5. Two or more violations of the Rule of 5 suggests the probability of problems in bioavailability [11]. All analogs excepting 6 and 9 have zero violations of the Rule of 5. The small deviation from average molecular mass, number of heavy atoms (19.2 ± 2.4), and molecular volume for all ten compounds suggests adherence to preservation of atom type and functional group presence in these *in silico* optimized designs.

Pattern recognition methods offer great advantages for the mechanism of drug design, especially in both biological and physiochemical calculation and collation of data having computer databases, molecular modeling systems, and property prediction packages [12-14]. K-means cluster analysis is a non-hierarchical clustering method where the outcome number of clusters must be designated by the investigators [15]. Highest resolution determined at four cluster outcome, the clustering results were as follows: Cluster 1) Drug 2 and 9; Cluster 2) Drug 4 and 7; Cluster 3) Drug 5 and 8; Cluster 4) Drug 1 (streptozocin), 3, 6, and 10. Initial inspection reveals that streptozocin is determined to be most similar to analogs 3, 6, and 10, an outcome identical to hierarchical cluster analysis (Fig. **2**). Viewing the molecular structures

Table 2. Drug-Likeness Scores Based on Activity

Activity Of Drug-Likenss	1	2	3	4	5	6	7	8	9	10
GPCR ligand	-1.62	-1.87	-1.45	-0.78	-1.23	-1.33	-0.56	-1.22	-1.30	-1.87
Ion Channel Modulator	-1.16	-1.61	-1.13	-0.57	-0.57	-1.17	-0.38	-0.96	-1.31	-0.96
Kinase Inhibitor	-1.66	-1.80	-1.90	-1.14	-1.64	-1.60	-0.96	-1.88	-1.56	-1.71
Nuclear Receptor Ligand	-1.90	-2.24	-2.41	-1.23	-2.47	-2.16	-1.76	-1.88	-2.27	-2.28

Drug 1 = Streptozocin.

(Fig. **1**) of 1, 3, 6, and 10 reveals that only analog 10 does not have a ring substituent but an aliphatic chain on which there are located four hydrogen bond donors (-OH). Each of 1, 3, 6, and 10 have Log P values less than zero and PSA greater than 140 A^2. Consistency of isosteres along with Log P and PSA suggests analogs 3, 6, and 10 may be a useful alternative to streptozocin (Note that there is also high consistency among 1, 3, 6, and 10 in number of heavy atoms and molecular volume). ANOSIM implements a statistical test whether there is a considerable difference between two or more groups of sampling data [16]. A large value of R of up to 1.000 signifies dissimilarity among groups whereas a low R approaching zero signifies high similarity [16]. The R value of 0.1081 signifies that streptozocin and these nine analogs are highly similar, a outcome substantiated by results of discriminant analysis (see Fig. **3**).

Hierarchical cluster analysis displays outcome as a horizontal or vertical dendrogram depicting the assembly of subjects within clusters so that highest similarity lies with subjects found in identical clusters and lowest similarity among more distant clusters [14]. Dendrograms can provide a distance measurement along one axis to represent relative separation among the clusters. Viewing Fig. (**2**) is apparent that streptozocin (1) is most similar to analog 3 (also suggested by K-means cluster analysis and discriminant analysis), but associated with analog 6 and 10 by branching to a supernode. The remaining analogs are paire as 5 and 8, 2 and 9, 4 and 7. Again, analogs 3, 6, and 10 are determined to be possible alternatives to streptozocin.

When given two sets of multivariate data, discriminant analysis constructs an axis that functions to maximize the differences between the two sets [17]. Discriminant analysis (DA) can investigate differences among groups, determine the most facile manner to distinguish groups, and asses the relative importance of independent variables [17]. Inspection of the 2-way axis presented in Fig. (**3**) representing outcome by DA show clustered subjects (drugs) along group 1 axis and group 2 axis. Although the plot shows all drugs in proximity, analog 3 is near identical to streptozocin (1) with analog 6 and 2 closest. Supporting ANOSIM and K-means cluster analysis outcome, streptozocin is still highly similar to analogs 1 to 9 but determined to have highest similarity to analog 3 and 6. No critical schism among the parent compound and structural analogs is discerned by DA. The contribution of Log P, PSA, and number of atoms have been shown to play an important role in establishing similar drug likeness (multiple regression R squared 0.9643) however the Pearson r correlation coefficient greater than 0.9500 (for all drugs) show considerable association of each drug by all descriptors that were calculated (Table **1**). Consequently it is clear that construction of nitrosoureas analogous to streptozocin produces viable alternatives that have many consistent features in hydrogen bond donor/acceptor sums, PSA, molecular weight and volume. Altogether an *in silico* construction of analogous structures, controlled by restrictions in functional group and isosteric encompassment, produced potential chemotherapeutic tools for clinical treatment of pancreatic related carcinoma. Pattern recognition methods elucidated and resolved underlying associations of these analogs to the parent compound based upon the numerical values of various critical pharmaceutical properties.

When evaluating the expected bio-activity of novel drug structures it is very useful to apply a screening criteria to populations of the potential drug candidates. A filtering of potential drug constructs can be achieved by implementing Bayesian statistics in comparing molecular structures of known successful active molecules to novel constructs. All ten compounds were analyzed in this manner (see Materials and Methodology) under the criteria of GPCR ligand activity, ion channel modulation, kinase inhibition, and nuclear receptor ligand activity giving results presented in Table **2** that are very highly correlated to streptozocin (no Pearson r value is less than 0.7600). Also, the numerical values of the activities determined for analogues 2 through 10 are consistent and comparable to that of streptozocin.

This further supports the outcome that analogs 2 through 10 are substantially alike streptozocin and with congruent drug-likeness.

CONCLUSION

Utilizing streptozocin as parent, nine molecular analogs were contrived that retained the alkylating moiety and thereby the antineoplastic activity. The analogs were sufficiently varied from the parent to possess a broad range of lipophilic character, revealed by Log P, yet retain adequate features to have Pearson r correlation of greater than 0.9500 among each other (based on properties presented in Table **1**). This observation supported by an ANOSIM value of 0.1081, indicating substantial similarity. In addition to these outcomes, discriminant analysis determined that all drugs were highly analogous within the descriptors applied, with discriminant scores showing close proximity clustering within a 2-way plot.

All compounds excepting analogs 6 and 9 were determined to express zero violations to the Rule of 5, hence an indication of favorable bioavailability based on drug like-

ness. The considerable number of hydrogen donor/acceptor atoms incurred significant hydrophilic character into the majority of these drugs (supported by Log P values less than 0). Non-hierarchical K-means clustering at highest resolution showed the parent streptozocin to be closest to analogs 3, 6, and 10. Multiply regression analysis determined an equation relating formula weight to descriptors of Log P, polar surface area, and number of heavy atoms to design/predict other anticancer nitrosoureas (with emphasis on targeting pancreatic islet cells carcinoma).

A more sensitive hierarchical cluster analysis algorithm processed numerical values of descriptors to resolve the association among streptozocin and analogs. The outcome paired streptozocin more closely to analog 3 but with association with analog 6 and 10 at greater Euclidean distance within the dendrogram. Other analogs were paired 5 and 8, 2 and 9, concluding with 4 and 7. This study demonstrated the efficacy of modeling analogous compounds to a successful parent antineoplastic agent based on molecular isosteres and structural analogy. Pattern recognition methods elucidated underlying associations within the numerical values of the descriptors that are vital in inspecting drug likeness and bioavailability. Comparing relative activity scores of streptozocin to those of analogs 2 through 10 utilizing four drug classes (GPCR ligand, ion channel modulator, (kinase inhibitor, and nuclear receptor ligand) showed all compounds are very highly correlated with expected similar bio-activity. Molecular modeling and pattern recognition analysis are very useful tools for designing novel chemotherapeutics for the treatment of neuroendocrine tumors of the pancreas.

ABBREVIATIONS

GEP-NETs	=	Gastro entero pancreatic neuroendocrine tumors
EPTs	=	Endocrine pancreatic tumors
PSA	=	Polar surface area
ANOSIM	=	Analysis of Similarity
DA	=	Discriminant analysis

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