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Highlights

- 3000 previously identified virtual fentanyl-like structures generated in 2019 were assessed
- Five years have passed, and some of the virtual predicted compounds have now been identified and reported
- Validation of the previously reported structure-based approach for designer fentanyl-like molecules

Evidence-based successful example of a structure-based approach for the prediction of designer fentanyl-like molecules

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Keywords

QSAR; fentanyl; μOR; opioid binding affinity; designer fentanyl-like molecules; novel synthetic opioids; new psychoactive substances

Abstract

In 2019, we published three innovative quantitative structure-activity relationship models (QSAR) for predicting the affinity of mu-opioid receptor (μOR) ligands. The three different models were then combined to produce a consensus model used to explore the chemical landscape of 3000 virtual fentanyl-like structures, also generated by us by a theoretical scaffold-hopping approach to explore potential novel active substances and predict their activity. Interestingly, five years have passed, and some of the virtual predicted compounds have been identified/reported to e.g. the Early Warning System or the United Nations Office on Drugs and Crime, thus confirming our warning hypothesis that new emerging drugs from our screen would find way to the dark market.

1. Introduction

Narcotic analgesics, of which morphine is the prototype, work by targeting proteins called opioid receptors (OR), the activation of which can result in a range of pharmacological actions that are utilised to treat various health conditions [1-4]. Their pharmacological activity was understood long before morphine was discovered, resulting int *Papaver somniferum* preparations being widely used as medicines since the time of ancient civilizations [5]. Regretfully, in paralell to the therapeutic application the poppy plant was being used recreationally even during that time, and this societal sickness continues to exist now, posing a serious threat to society everywhere [5]. Synthetic opioid deaths from opioid overdoses are on the rise, adding to the well-known social problem of the North America opiod crisis [6-8]. The Drug Enforcement Administration (DEA) reports that fentanyl analogues are becoming more and more common in the street drug market due to their low cost, easiness of synthesis, and high potency. The strong μOR agonist fentanyl is responsible for the traditional pharmacological effects of this family of drugs, and minor alterations to the molecule's central core (4-anilidopiperidine, Figure 1) may provide ligands with greater potency, putting the user at serious risk. The variation in potency among fentanyl analogues poses a serious risk to public health, with certain derivatives, like carfentanyl, being 10,000 times more potent than morphine. This is of particular concern for both regular/tolerant users who could easily incur in the consumption of a lethal dose while trying to overcome the tolerance associated with opioid usage, and for occasional users who could overdose being unaware of what they are consuming [8-10]. It is noteworthy that numerous structural alterations to the initial fentanyl chemical scaffold do not impact its basic function or binding capabilities to the mu-opioid receptor (μOR). Consequently, a vast chemical space of potentially physiological abusive fentanyl analogues exists [11, 12]. Due to a substance's potential for abuse, the DEA in the USA has the authority to schedule it to a legislative state; however, to assess such potential a thorough examination is required, hence, to establish if scheduling is needed may take up to two years. To speed up and support the scheduling process and to identify and characterise the hazards associated with unclassified fentanyl-like structures, the Centre for Drug Evaluation and Research created a docking-based virtual screening method in 2018 [13]. Docking-based virtual screening is a computational method used in drug discovery to predict how small molecules (like potential drugs) interact with a target protein, helping identify promising candidates for further testing. It simulates the interaction with the receptor of these molecules into the protein's binding site and predicts their affinity and potential as drug candidates. Unfortunately, a structure-based docking methodology such as the one developed by the Center for Drug Evaluation and Research, even if speeding up the classification process, still has some limitations as the calculation requires time and it could be computationally expensive if the molecules to analyze are in huge numbers. Differently, a ligand-based method such as the one proposed by us is normally faster, and once the library of structure analogues, i.e. conformers, are generated, a prediction value is easy to obtain. In 2019 we were the first to develop a ligand-based quantitative structure-activity relationship models (QSAR) for the classification of designer fentanyl-like structures [14] using Forge software[15]. Ligand-based quantitative structure-activity relationship (QSAR) models analyze the relationship between the chemical structure of molecules (ligands) and their biological activity. By correlating structural features with observed biological effects, QSAR helps predict the activity of new compounds. In order to make pattern recognition and prediction easier in the chemical and biological sciences, QSAR models and other AI and non-AI based computational tools are widely utilized [16-22]. We concluded our paper [13] by stating that the proposed ligand-based tool could be considered by the DEA, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), and other regulatory bodies to speed up the classification of novel fentanyl-like NPS. Moreover, the three different models were then used to explore the chemical landscape of 3000 virtual fentanyl-like structures, also generated by us by a theoretical scaffold-hopping approach [15] (from the positions highlighted in Figure 1) to explore potential novel active substances and predict their activity. Scaffold hopping is a strategy in drug discovery that involves identifying and replacing the core structural framework (or scaffold) of a molecule while maintaining its desired biological activity. This approach helps generating novel

compounds with improved properties or different pharmacological profiles compared to the original molecule. Interestingly, almost five years have passed since our publication and some of the virtual predicted compounds have been identified/reported to e.g. Early Warning System (EWS)/United Nations Office on Drugs and Crime (UNODC), thus confirming our warning hypothesis (literally, "*the newly identified libraries may potentially aid the interpretation of toxicological analyses where the presence of novel synthetic opioids is postulated*") that new emerging drugs from our screen would find way on the (dark) market.

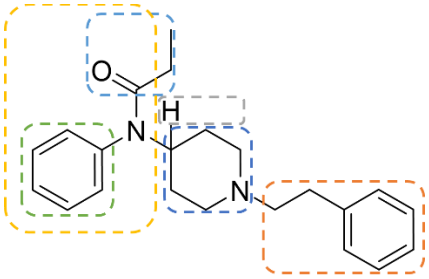


Figure 1. Fentanyl structure and positions studied in the scaffold-hopping.

2. Material and methods

The original models [14] for the ligand-based evaluation were made as follows. All the compounds' chemical structures used to develop the QSAR models were obtained from the ChEMBL database. Datawarrior was used for the selection of the molecules, and only those with affinity data on the human μOR (ID: CHEMBL233) were included in the analysis. In particular the selection of compounds was limited to those in which the displacement of the radioligand [^{3}H]DAMGO from the human μOR was utilised to determine all of the K_i values. The resulting 115 structures, all the fentanyl-like 3D-optimized structures were imported into the software Forge (v10.4.2, Cresset, New Cambridge House, Hertfordshire, UK) [15] to set the field-based 3D-QSAR model and the 2D k-Nearest Neighbor (kNN) models. Of the 115 structures, 21 molecules were utilised as an external validation (test set) to assess the models, and 94 molecules were chosen at random as a training set. The range of pK_i values for the compounds in the training and test sets was 10.1 to 5.3. Each fentanyl like molcule was aligned on the the previously reported active conformation of fentanyl (as the reference molecule) [23]., and field points (negative and positive electrostatic, van der Waals shape, and hydrophobic description of the molecules) were generated using the extended electron distribution (XED) force field included in Forge. The Extended Electron Distribution (XED) force field is a molecular modelling method that accounts for both bonded and non-bonded interactions using electron density distribution, providing accurate descriptions of molecular structures and properties. A force field is a computational model that describes the interaction energies and forces between atoms or molecules in a system. As an alternative to the 3D-field QSAR, other two QSAR models were developed at the same time using Forge kNN (k-nearest neighbors) method. The kNN is a simple machine learning algorithm used for classification and regression tasks based on similarity to the k closest data points in a training set and it is well-known, robust and has an effective distance learning approach [24, 25]. The two kNN models were developed using two different 2D-fingerprint similarities: the ECFP6 and the FCFP6 circular fingerprint descriptors. kNN stands for k-Nearest Neighbors. ECFP6 and FCFP6 are circular fingerprint descriptors used in cheminformatics for molecular representation. ECFP6 represents extended connectivity fingerprints of up to 6 bonds, while FCFP6 represents functional connectivity fingerprints of up to 6 bonds. For the calculation of the different models, Forge uses the SIMPLS algorithm [26, 27]. All the generated models showed both good predictive and descriptive capabilities, demonstrated by the high r^2 and q^2 values [14] for both the training and the cross-validated training sets. Among the three different models, the presence of the 3D-descriptors included in the 3D-field model clearly increased the quality of the description, as demonstrated

by the high value of r^2 (0.99) for the training set. In order to enlarge the chemical landscape evaluation of fentanyl-like compounds, a bioisosteric and fragment replacement software tool (Spark v10.4.0, Cresset, New Cambridge House, Hertfordshire, United Kingdom) was adopted to produce a scaffold-hopping analysis and to generate a virtual library of μOR ligands [28, 29], investigating different portions of the original structure of fentanyl as reported in Figure 1, were each colour represent a different studied part. In particular, the molecule was divided into six different parts and 500 new virtual molecules were generated for each substitution pattern for a total of 3000 analogues. Subsequently, each ligand was evaluated by exploiting the predictive capabilities of the 3D-field and 2D-kNN QSAR models. For each case, the replacement was performed using the same dataset of fragments already reported by us [30]. For this work the chemical structures of all the 3000 molecules were retrieved from the supplementary material of our previously published research [14]. The molecules were compared with a dataset of all reported opioids from NPS*finder*® [31]. The two-dimensional structures of the dataset were built using Marvin Sketch (18.24, ChemAxon Ltd, Budapest, Hungary). The protonation states of the molecules were calculated assuming a neutral pH. Datawarrior (6.0.0, Idorsia Pharmaceuticals Ltd, Allschwil, Switzerland) [32, 33] was used for handling the selection of the molecules with fentanyl-like structures among the entire downloaded dataset. The similarity analysis was performed using the default FragFp descriptor in DataWarrior. The FragFp descriptor in DataWarrior is a method for molecular representation that encodes structural information into a binary fingerprint format. It dissects molecules into smaller fragments, such as functional groups, rings, and specific bond arrangements. The FragFp descriptor is particularly useful in cheminformatics for tasks such as virtual screening, similarity searching, and clustering of chemical compounds. By capturing key structural features, FragFp enables efficient comparison of molecular structures and helps identify molecules with similar characteristics or biological activities. This can aid in the discovery of potential drug candidates or in understanding structure-activity relationships in chemical datasets. Per default Datawarrior calculates a FragFp descriptor of the first structure column within the data table. This descriptor can be used to calculate similarities between molecules. The FragFp similarity between two molecules is the number of fragments that both molecules have in common divided by the number of fragments being found in any of the two molecules. The cut-off similarity was set to 95%.

3. Results and Discussion

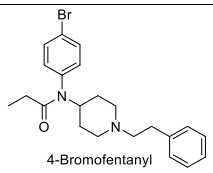
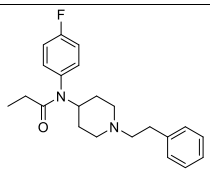
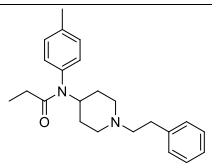
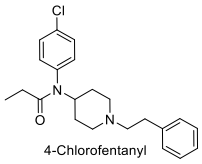
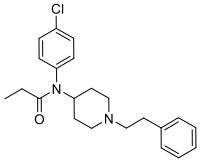
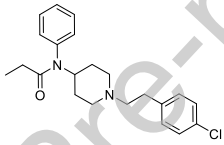
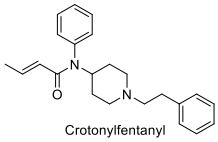
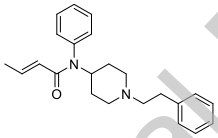
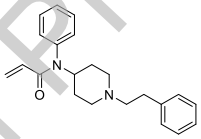
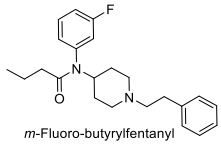
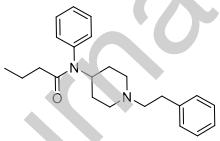
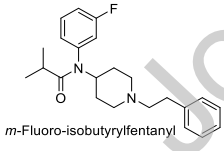
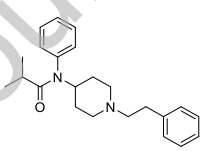
The main objective of this paper was to fast-screen our 3000 hypothesized molecules against the up-to-day reported opioids in NPS finder to discover whether some of these substances were predicted by our model before they were identified on the dark market. To achieve this, the structures were imported, and a pair analysis was conducted using the FragFp descriptor in DataWarrior. The FragFp similarity between two molecules is the number of fragments that both molecules have in common divided by the number of fragments being found in any of the two molecules. The whole set of compounds was screened against the NPS*finder*® retrieved database, and only the compounds with more than 95% FragFp similarity were further analysed. The total compounds with >95% FragFp similarity were 80 (see supplementary materials, DataWarrior file). Theese 80 compounds were evaluated, and among them, 11 compounds were found to have beein officially identified/reported as NPS after being predicted by our scaffold hopping excercise The 11 compounds are summarized in Tables 1 and 2, where the reported/identified NPS is compared with the matching structure from our 3000 fentanyl-like analogues dataset . While some of the compounds do not bear 100% similarity with the actual reported NPS, i.e. a FragFp score less than 1.00, some of them are identical, with a 1.00 FragFp reported score. Compound id 16054, 4-Bromofentanyl reported in 2020, was not exactly identified by our analysis, but two very similar compounds, namely 4-Fluorofentanyl and 4-Methylfentanyl, were identified. Compound id 16063, 4-Chlorofentanyl reported in 2021, was precisely identified, and another analogue was also proposed with the chlorine substituent in the other aromatic ring (0.96 similarity). Compound id 16067, Crotonylfentanyl, reported by DEA in 2020, was also identified in our

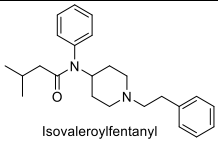
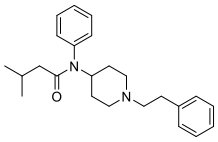
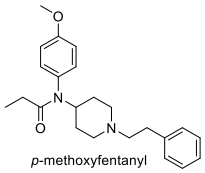
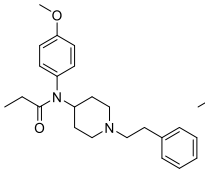
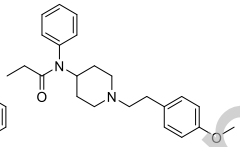
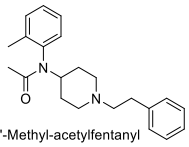
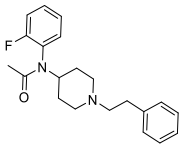
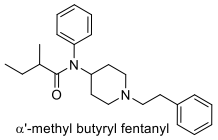
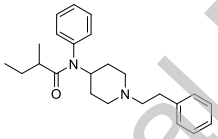
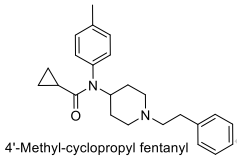
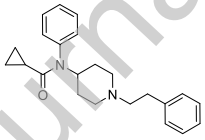
set of compounds and a derivative with an acrylic group (0.97 similarity) was also proposed. Compounds with id 16078 and 16093, *m*-Fluoro-butyrylfentanyl reported in 2019 and *m*-Fluoro-isobutyrylfentanyl reported in 2020, were virtually identified by us without the fluorine atoms giving two compounds with high similarity index of 0.96 in both cases. Compound id 16116, isovaleroylfentanyl reported in late 2019, was also precisely predicted as it was compound id 16122, *p*-methoxyfentanyl, initially reported in 2020, but already postulated by us. Compound 16146, 2'-Methyl-acetylfentanyl reported in 2022, was not exactly identified but a very similar compound with a fluorine substituent instead or the methyl was theorized by us before the actual identification. Compound 16154, α'-methyl butyryl fentanyl reported in 2019, was also exactly identified in our dataset. A highly similar compound (cyclopropyl fentanyl) was virtually identified in our set of compounds instead of compound 16157, 4'-Methyl-cyclopropyl fentanyl reported by DEA in 2019. And finally, pivaloylfentanyl, compound id 16182 reported in 2020, was already present in our set of postulated fentanyl-like molecules a year before its original report. The study successfully predicted several opioid-based NPS before their identification on the dark market, demonstrating the efficacy of the predictive model in fast-screening potential opioid substances. Despite not achieving 100% FragFp similarity with some identified NPS, the model still managed to identify structurally similar analogues, indicating its utility in predicting potential emerging substances. The identification of these compounds before their appearance on the market highlights the importance of proactive approaches for drug regulation and monitoring. The predicted compounds exhibit structural similarities to existing fentanyl analogues, suggesting that they may exert similar pharmacological effects. By comparing the predicted compounds with known fentanyl analogues, researchers and regulatory agencies can anticipate emerging trends in the design and synthesis of potent opioids, enabling more effective regulatory responses.

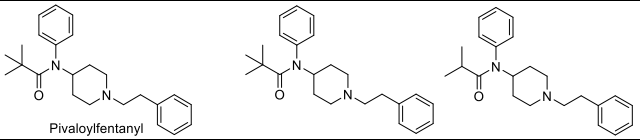
Table 1. Predicted compounds compared to reported NPS.

Reported NPS	Predicted Compound
4-Bromofentanyl	4-Fluorofentanyl
	4-Methylfentanyl
4-Chlorofentanyl	4-Chlorofentanyl
	Chloro-substituted analog (See Table 2)
Crotonylfentanyl	Crotonylfentanyl
	Acrylic-substituted analog (See Table 2)
m-Fluoro-butyrylfentanyl	Butyrylfentanyl
m-Fluoro-isobutyrylfentanyl	Isobutyrylfentanyl
Isovaleroylfentanyl	Isovaleroylfentanyl
p-methoxyfentanyl	*p*-methoxyfentanyl
2'-Methyl-acetylfentanyl	Similar compound with fluorine substituent (See Table 2)
α'-methyl butyryl fentanyl	α'-methyl butyryl fentanyl
Cyclopropyl fentanyl	Cyclopropyl fentanyl
	Similar compound (See Table 2)
Pivaloylfentanyl	Pivaloylfentanyl

Table 2. Structures of predicted compounds compared to the structure of reported NPS.

id from NPS*finder*®	Structure	Most similar structure/structures	Calculated similarity	Data reported
16054	 4-Bromofentanyl	 	0.98 and 0.98	Reported by NPS discovery in Dec 2020[a]
16063	 4-Chlorofentanyl	 	1.00, 0.96	Reported by the DEA July 2021[b] and by NPS discovery December 2020[c]
16067	 Crotonylfentanyl	 	1.00, 0.97	Reported by DEA in Feb 2020[d]
16078	 *m*-Fluoro-butyrylfentanyl		0.96	Reported by the DEA Feb 2019[e]
16093	 *m*-Fluoro-isobutyrylfentanyl		0.96	DEA last report in May 2020[f]

16116	 Isovaleroylfentanyl 	1.00	Identified/reported Sept 2019[g]
16122	 *p*-methoxyfentanyl  	1.00, 0.96	Reviewed by the DEA in 2020[h]
16146	 2'-Methyl-acetylfentanyl 	0.97	Recoded by the DEA in 2022[i]
16154	 α'-methyl butyryl fentanyl 	1.00	Reported by DEA in April 2019[j]
16157	 4'-Methyl-cyclopropyl fentanyl 	0.96	Reported by NMSlab in Nov 2019[k] and by DEA on the 30th of september[l]

16182	 Pivaloylfentanyl	1.00, 0.96	Reported on Jan 2020[m]

[a] collected in March 2020 https://bitnest.netfirms.com/www.forensicscienceeducation.org/Bromofentanyl_121720_CFSRE-Toxicology_Report.pdf
[b] https://bitnest.netfirms.com/www.swgdrug.org/para-chlorofentanyl%20hydrochloride.pdf,
[c] date of collection March 2020 https://bitnest.netfirms.com/www.forensicscienceeducation.org/Chlorofentanyl_121720_CFSRE-Toxicology_Report.pdf
[d] https://bitnest.netfirms.com/www.swgdrug.org/Z-Crotonyl%20fentanyl.pdf
[e] https://bitnest.netfirms.com/www.swgdrug.org/meta-fluorobutyryl%20fentanyl.pdf
[f] https://bitnest.netfirms.com/www.swgdrug.org/meta-fluoroiosbutyryl%20fentanyl.pdf
[g] https://bitnest.netfirms.com/www.swgdrug.org/Isovaleryl%20fentanyl.pdf
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4. Conclusions

In conclusion, our work, initiated more than five years ago, has proven to be prescient in its foresight into the emergence of novel mu-opioid receptor (μOR) ligands. The publication of three groundbreaking Quantitative Structure-Activity Relationship (QSAR) models in 2019 paved the way for a comprehensive exploration of the chemical landscape of 3000 virtual fentanyl-like structures. These virtual compounds were generated using a theoretical scaffold-hopping approach, a method developed by our team to explore potential novel active substances and predict their activity. Remarkably, the passage of these years has provided us with valuable insights into the practical implications of our research. Some of the virtual compounds predicted through our models have been later identified and reported by authoritative bodies, underscoring the predictive power of our approach. Of course, natural limitations of QSAR modelling must be considered i.e. they might perform well within the dataset used for training and testing but could struggle to accurately predict the activity of compounds outside of the used dataset. Our research anticipated the emergence of new drugs and as we already suggested in our original paper it should be used to the early identification and reporting of these substances to regulatory agencies. This temporal validation of our work reinforces the critical role that predictive modeling, specifically QSAR, plays in understanding the chemical space and anticipating the development of potentially harmful compounds. The success in identifying virtual compounds that have materialized in the streets underscores the practical relevance of our research. Moreover, the result of this study could also help understand adverse reactions and the planning of preventive strategies for tackling the opioid crisis [34]. As we reflect on the past five years, our models have not only stood the test of time but have also demonstrated their applicability in real-world scenarios, serving as valuable tools for drug discovery and regulatory efforts. Looking ahead, the synergy between computational modeling and experimental validation will continue to be pivotal in advancing our understanding of drug design and development. The evolving landscape of novel substances demands a proactive and multidisciplinary approach, and our work stands as a testament to the importance of foresight and innovation in addressing emerging challenges in the field of pharmacology and drug discovery. We invite the scientific community [35-39] and regulatory agencies to further analyze the already published warning compounds and to further consider similar dataset produced by us for cannabinoids [30], serotoninergic acting compounds [40] and benzodiazepines [41].

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