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COMPUTATIONAL APPROACHES FOR DRUG REPOSITIONING AND COMBINATION THERAPY DESIGN

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Heterogeneous high-throughput biological data become readily available for various diseases. The amount of data points generated by such experiments does not allow manual integration of the information to design the most optimal therapy for a disease. We describe a novel computational workflow for designing therapy using Ariadne Genomics Pathway Studio software. We use publically available microarray experiments for glioblastoma and automatically constructed ResNet and ChemEffect databases to exemplify how to find potentially effective chemicals for glioblastoma — the disease yet without effective treatment. Our first approach involved construction of signaling pathway affected in glioblastoma using scientific literature and data available in ResNet database. Compounds known to affect multiple proteins in this pathway were found in ChemEffect database. Another approach involved analysis of differential expression in glioblastoma patients using Sub-Network Enrichment Analysis (SNEA). SNEA identified angiogenesis-related protein Cyr61 as the major positive regulator upstream of genes differentially expressed in glioblastoma. Using our findings, we then identified breast cancer drug Fulvestrant as a major inhibitor of glioblastoma pathway as well as Cyr61. This suggested Fulvestrant as a potential treatment against glioblastoma. We further show how to increase efficacy of glioblastoma treatment by finding optimal combinations of Fulvestrant with other drugs.

Keywords: Systems biology; MedScan; drug repositioning; glioblastoma; enrichment analysis; angiogenesis.

1. Introduction

With high attrition rate of drugs and with drying out drug pipeline, repositioning of the existing drugs and combinatorial therapies become highly appealing for

drug development. It is well documented that drugs already approved for treatment of certain diseases can be effective against other similar conditions. The cost reduction for drug repositioning is enormous since an approved drug already passed most safety tests. Examples of successful drug repositioning include Ceftriaxone, an antibiotic adopted to treat Amyotrophic Lateral Sclerosis[1]; Fumagillin, an antiamebic compound later shown to have an anti-angiogenic effect in cancer[2]; NSAID drugs which demonstrated some efficiency against Alzheimer's disease[3]; and retinoic acid which showed efficiency against Acute Promyelocytic Leukemia[4] (see Ref. 5 for a comprehensive review of repositioned drugs).

In this paper, we suggest a novel workflow for selecting drug candidates and designing drug cocktails from the pool of drugs available on the market. Our approach uses Pathway Studio[6,7] software, ResNet database[7] and ChemEffect[8] database from Ariadne Genomics. Both databases are automatically constructed and updated using MedScan natural language processing (NLP) technology.[9] The information extracted by MedScan is imported into a relational database and stored as a network of molecular interactions, where each interaction is annotated with corresponding references. This allows using graph navigation tools for computational analysis and drug selection. Pathway Studio and databases generated by MedScan technology proved to be important research tools for studying cellular processes and interpreting high-throughput profiling data. Originally, MedScan information extraction technology was designed to extract information about protein interactions and regulatory events. This information constitutes the core of the ResNet database.[10] Recently, MedScan was extended to extract information about drug actions and toxicity. This information is stored in the new database called ChemEffect.[8]

We describe two workflows for finding new application of existing drugs (drug repositioning). The first workflow is based on the knowledge of the disease available from scientific literature. To find the drugs that are potentially active against the disease, the consensus disease pathway is constructed. This is done manually using information from existing reviews and collection of canonical pathways available in Pathway Studio. Constructed disease pathway is then used to search ChemEffect database for chemicals inhibiting multiple proteins in the pathway.

Our second workflow is data-oriented. It involves analysis of gene expression data and unique algorithm available only in Pathway Studio called Sub-Network Enrichment Analysis (SNEA),[11] which finds the network entities (proteins, complexes or functional classes) with significant expression changes downstream. We show that SNEA results can suggest novel therapeutic targets that have not yet been described in the literature for the treatment of disease. These targets can be added to the disease pathway and also used to find chemicals inhibiting them from the ChemEffect database.

Using glioblastoma as an example, we show that it is possible to combine the outcomes of these two approaches to select a most likely drug candidate from the collection of drugs available in ChemEffect database. The selected candidate,

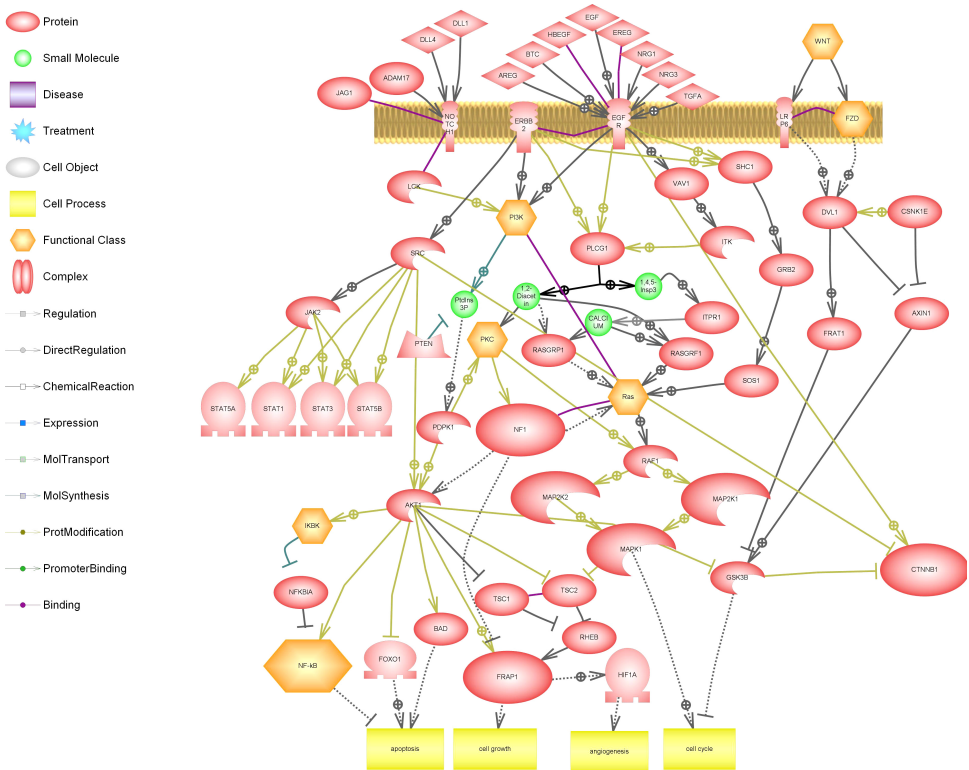


Fig. 1. Glioblastoma consensus pathway as a combination of EGFR-H1FA, EGFR-STAT, Notch and Wnt pathways. Proteins playing important role in glioblastoma according to review articles are enlarged.

Fulvestrant, is a known and approved drug with documented side effects which is known to be an effective inhibitor of several components of canonical glioblastoma pathway (Fig. 1).[12–15] Fulvestrant is also known to inhibit Cyr61 protein which was found as a novel key expression regulator of glioblastoma identified by SNEA. However, its potential action against glioblastoma disease has not been discussed yet.

The efficacy of anti-cancer therapies can be improved by using a combination of several drugs.[16,17] We show how one can rationally design combination therapy by computational optimization of efficacy towards disease and by minimization of potential side effects that may arise from drug–drug interactions. Our workflow uses ChemEffect database to eliminate the least optimal drug combinations in several consecutive selection steps based on predicted efficacy and toxicity of drug combinations.

We used glioblastoma brain cancer data to illustrate our workflows. Glioblastoma is the most common and most aggressive form of primary brain tumor in humans. The median survival rate is less than 12 months. Surgical resection is

typically followed by a quick recurrence in close proximity of the original site. No effective treatment currently exists and the results of clinical trials of proposed treatments have been largely disappointing.[16,17] Glioblastoma is one of the most vascularized cancers and induces intensive angiogenesis to provide proper blood supply. Another prominent feature of glioblastoma cells is the resistance to apoptosis.[18] In addition, the effectiveness of traditional chemotherapies is limited by their inability to cross the blood–brain barrier. Multiple pathways are involved in progression of glioblastoma and, respectively, a number of therapeutic strategies have been designed to suppress glioblastoma development. These include inhibition of growth factors and their receptors by antibodies and using kinase inhibitors to block the activity of downstream signaling cascades. However, the efficacy of anti-survival pathways therapies remains very modest in glioblastoma. This is probably due to the glioblastoma's lack of dependence on any single molecular pathway for survival. It is possible that combining the inhibitors of multiple survival pathways or different points along the same pathways may offer therapeutic advantage over the single-point acting drugs.

2. Targeting Multiple Proteins in the Consensus Pathway

To demonstrate how literature-derived information can be used for drug repositioning, we mined ResNet and ChemEffect databases to compile a detailed molecular pathway (disease model) of glioblastoma. We used available review publications[19,20] to obtain the names of signaling pathways and key proteins affected in glioblastoma. Several receptor signaling cascades are known to be involved in glioblastoma progression, including abnormally increased EGFR, PDGFR and VEGFR signaling, and downstream PI3K/Akt, Wnt/GSK3B, Ras/MAPK, NFkappaB and p53-dependent apoptotic pathways.[19,20] We have merged together the collection of canonical signaling pathways (EGFR-H1FA, EGFR-STAT, Notch and Wnt) in Pathway Studio to compile a canonical glioblastoma pathway (Fig. 1). Additional key proteins which are known to be mutated or have altered activity in glioblastoma[19,20] were added to the pathway by linking them with other pathway components using implemented shortest path algorithm.

We then used search tools available in Pathway Studio in order to find small molecules from ChemEffect database which have reported negative effects on the proteins from the glioblastoma pathway. We found more than 750 drugs inhibiting in this sense at least one protein from the glioblastoma pathway. It is conceivable that targeting multiple targets in disease pathway should be more efficient in treating the disease.[16,17] Therefore, we sorted all drugs by their out-degree (number of negatively regulated proteins). Fifty-four chemicals from the initial set were known from literature to suppress five or more proteins on the glioblastoma pathway (Supplementary Table 1). Twenty-four of them have been reported in the literature to have some effect on glioblastoma cell lines. Overall, we found that drugs reported to have some effect against glioblastoma have increased out-degree towards proteins

in glioblastoma pathway. On average one reported anti-glioblastoma drug inhibited seven proteins in the glioblastoma pathway while all drugs inhibited on average only two targets. All found drugs were saved and used later for the rational design of combination therapy.

3. Finding Novel Therapeutic Targets in Glioblastoma

To find additional novel therapeutic targets for glioblastoma, we have analyzed publicly available glioblastoma expression dataset GDS2428[21] using SNEA.[11] We first calculated differential expression between glioblastoma samples from GDS2428 and averaged "normal brain" expression profile. This "normal brain" profile was constructed from publicly available expression profiles in NCBI GEO. The GPL96 platform-based normal brain samples were downloaded from GEO, z-transformed,[22] clustered to remove outliers and then averaged. We also performed z-transformation of expression values in glioblastoma samples from GDS2428 experiment and then calculated differential expression of glioblastoma versus normal brain values to perform SNEA analysis.

SNEA is a variation of gene set enrichment analysis algorithm. Unlike GSEA[23] which uses predefined collection of gene sets, SNEA uses the entire literature-extracted expression network stored in the database to generate collection of sub-networks used as gene sets for GSEA statistical test. Each sub-network represents immediate downstream neighbors of every expression regulator in the global network. Thus, SNEA determines the activity of expression regulators based on the differential expression of its targets and favors (assigns lower p-value) those which have more significant expression changes downstream.

SNEA found majority of sub-networks to contain mainly downregulated genes. In order to find possible drug target candidates we have focused our attention on the processes which are activated in glioblastoma. First, we took the sub-networks with the smallest SNEA p-values (< 0.05) that were enriched by up-regulated genes (60% or more overexpressed genes) indicating that their regulator is activated. The list of the protein activation SNEA seeds is presented in Table 1.

We found that most of the activated regulators were related to cell-cycle regulation, which we considered as trivial result for analysis of cancer samples. Other regulators are related to the apoptosis and extracellular matrix events. We found two major up-regulated regulators related to angiogenesis. Since this process is known to be specifically important for glioblastoma,[17] we focused further analysis on the angiogenesis-related regulators. One of the regulators was an immediate-early gene Cyr61 (Fig. 2), another was Angptl6. We did not find any information in ChemEffect database about drug action on angiopoietin-related growth factor Angptl6, therefore it could not be considered as potential target for drug repositioning. Cyr61 is an extracellular matrix protein which modulates activity of extracellular growth factors and their receptors. It is also known to interact with integrins and activate a number of cancer-related cascades, including AKT, FAK and Wnt pathways.[24]

Table 1. Glioblastoma activation SNEA seeds.

Gene set seed	General function	# of downstream genes
AIFM1	Apoptosis	8
ANGPTL6	Angiogenesis	6
BBC3	Apoptosis	5
BUB1	Cell cycle	6
BUB1B	Cell cycle	9
CDC25A	Cell cycle	10
CYR61	Angiogenesis	20
E2F1	Cell cycle	150
E2F3	Cell cycle	37
E2F4	Cell cycle	45
E2F6	Cell cycle	15
HIPK2	Apoptosis	16
LGALS7	Cross-linking of ECM	5
LOX	Cross-linking of ECM	10
MAD2L1	Cell cycle	11
MCM7	Cell cycle	8
NOV	Cross-linking of ECM	8
OAZ1	Polyamine biosynthesis	7
SFN	Apoptosis	10

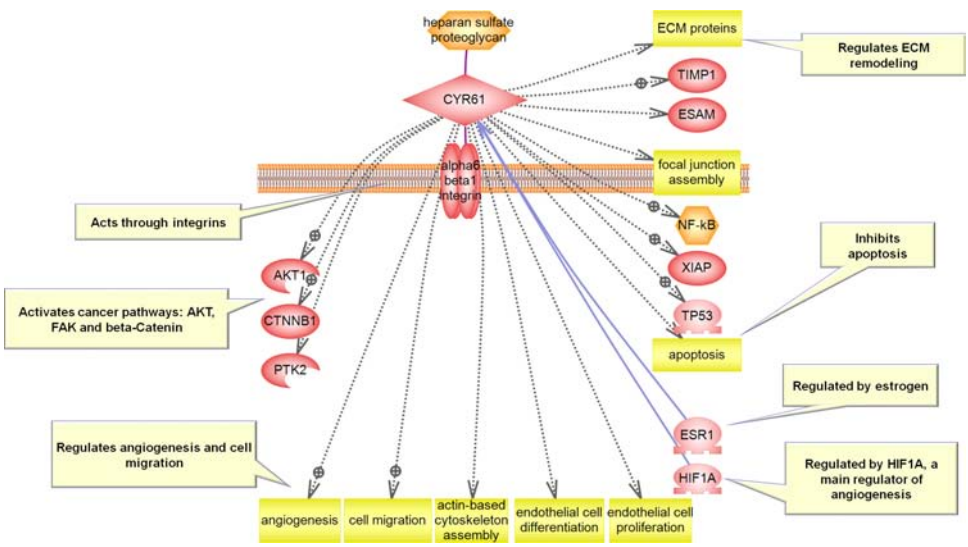


Fig. 2. Functional analysis of Cyr61. Solid arrows here – expression relations, dotted arrows – general regulation relations, solid line – physical binding.

Even more interestingly, Cyr61 was shown to induce angiogenesis[25] and inhibit apoptosis.[26] Consistent with its angiogenic activity, Cyr61 transcription is regulated by HIF1A,[27] a key angiogenesis-inducing transcription factor, and also by estrogen receptor ESR.[28]

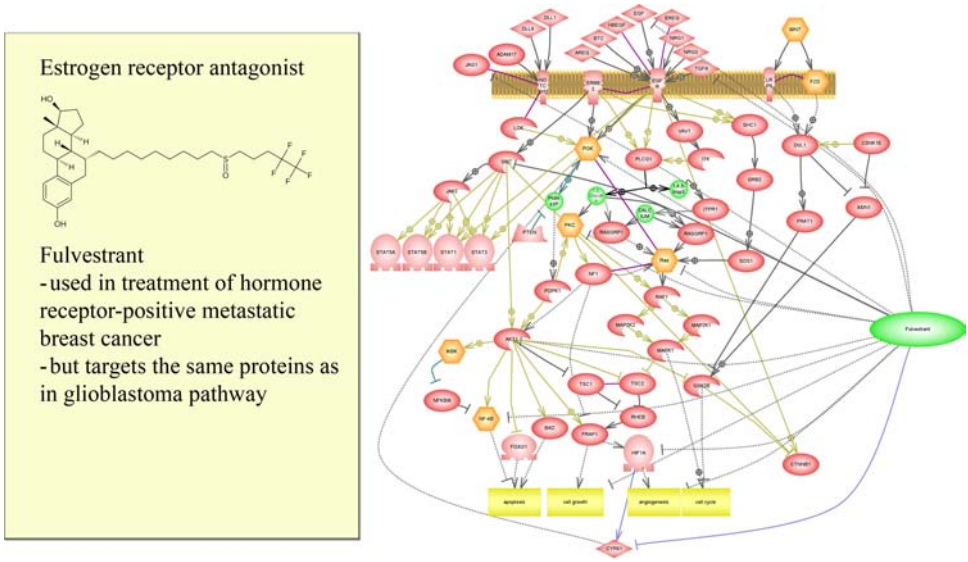


Fig. 3. Fulvestrant repositioning. Fulvestrant inhibits Cyr61 as well as several other proteins from glioblastoma pathway. Cyr61 is regulated by HIF1A, and is shown to affect PI3K pathway, thus activating glioblastoma positive feedback loop. For legend please refer to Fig. 1.

For all these reasons, we concluded that Cyr61 represents a novel therapeutic target for glioblastoma treatment. Its role in glioblastoma can be deduced by incorporating Cyr61 into the consensus glioblastoma pathway. Cyr61 appears to provide positive feedback loop self-activating glioblastoma pathway, since there are known regulations HIF1A → Cyr61 and Cyr61 → PI3K, where HIF1A and PI3K are the active members of glioblastoma pathway (see Figs. 1–3).

We then searched ChemEffect database for compounds known to down-regulate Cyr61. Found chemicals were compared with compounds inhibiting more than five targets in the consensus glioblastoma pathway indentified by the first workflow. We found one drug present in both analysis results — Fulvestrant, an estrogen receptor antagonist, which can negatively regulate Cyr61[28] and is also known to inhibit eleven targets in the glioblastoma pathway.

4. Developing Fulvestrant-Containing Drug Cocktails for Glioblastoma

Fulvestrant is an FDA-approved drug for treatment of ER positive metastatic breast cancer in postmenopausal women. According to relations in ChemEffect, Fulvestrant also negatively regulates eleven components of the glioblastoma pathways (Fig. 3, Supplementary Table 2), including Src,[12] Ras/MAPK pathway[13,14] and Akt1.[15] These properties make Fulvestrant an attractive candidate for anti-glioblastoma therapy. Even though Fulvestrant cannot cross the blood–brain

barrier,[29] that may not be necessary for glioblastoma since the blood–brain barrier appears to be compromised in this disease.[29] On the other hand, Fulvestrant is one of many estrogen receptor antagonists available on the market and therefore other drugs from this class capable of penetrating the blood–brain barrier may be effective against glioblastoma as well.

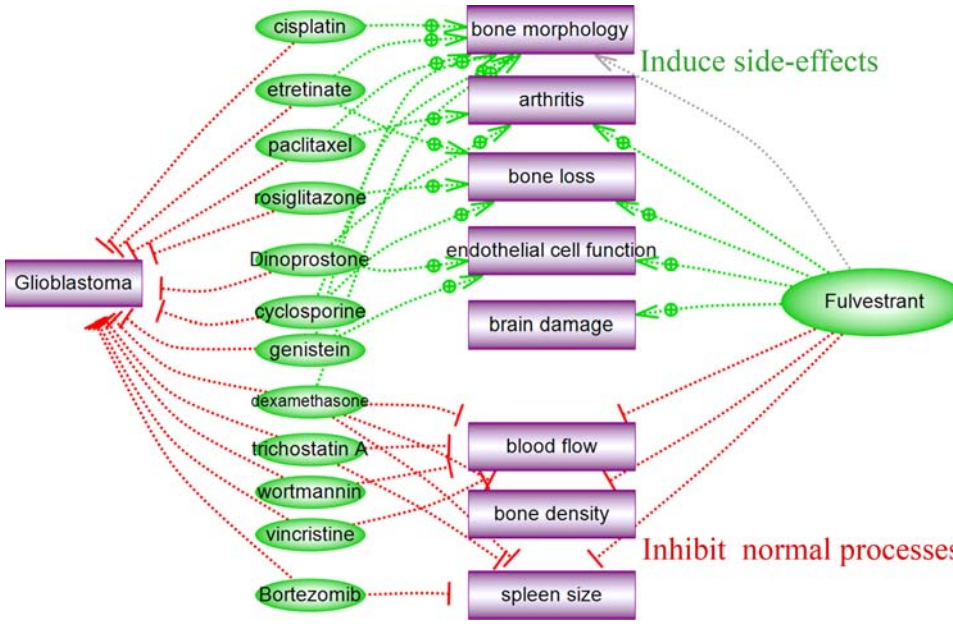
To find other drugs that could work in combination with Fulvestrant, we considered 24 compounds (Supplementary Table 1), which have been reported in the literature to have some effect on glioblastoma and which inhibit multiple components of glioblastoma pathway. In order to select the best drugs that can be used in combination with Fulvertsrant, we have eliminated the least optimal combinations based on several criteria applied consecutively. The description of such consecutive elimination is as follows.

(1) We first eliminated two drugs that inhibit mostly the same components of glioblastoma pathway as Fulvestrant (Tamoxifen and Iressa) and hence are less likely to provide additional gain to Fulvestrant efficacy towards glioblastoma pathway.
(2) Then we eliminated drugs that have the same side effects as Fulvestrant (Bortezomib, Cisplatin, Cyclosporine, Dexamethasone, Dinoprostone, Etretinate, Genistein, Paclitaxel, Rosiglitazone, Trichostatin A, Vincristine, Wortmannin) to minimize the possibility of toxicity induced by drug–drug interaction [Fig. 4(a)]. The data about side effect are readily available in ChemEffect database and can be easily searched, compared and visualized.
(3) The remaining ten compounds were examined for the total number of known side effects (Supplementary Table 3). The chemicals with the least number of side effects were selected as candidates for the combinational therapy with Fulvestrant: 4-Hydroxymidazolam, geldanamycin and rottlerin [Fig. 4(b)].

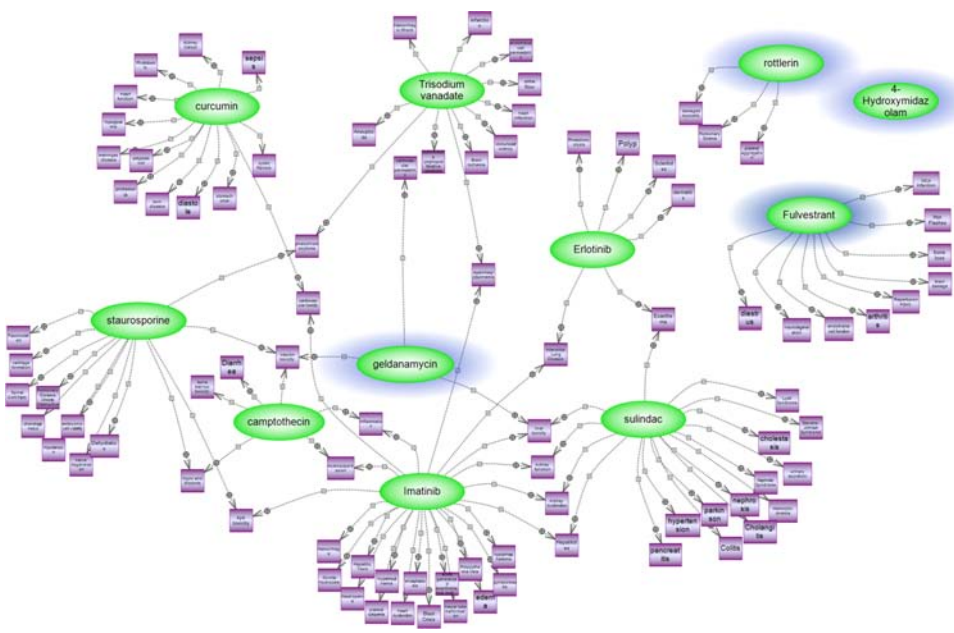
While inhibiting several targets in glioblastoma pathway in addition to Fulvestrant, every selected drug also provides relatively low toxicity risk. The side effects of predicted optimal drug cocktail are presented in Fig. 4(b). Inhibition of additional targets in glioblastoma pathway by these drugs is shown in Fig. 5. Selected compounds and their targets from glioblastoma pathway are highlighted. One can see that the combination of these drugs potentially provides a stronger inhibition of glioblastoma-related pathways compared with Fulvestrant alone and that selected drugs do not synergize with Fulvestrant side effects while having small number of side effects overall.

5. Discussion

To design new drugs or to reposition existing drugs, it is absolutely necessary to use as much available information about the specific disease as possible. This information must include disease-specific pathways, interactions between disease-related proteins and compounds, indirect links between possible disease drugs and their



(a)



(b)

Fig. 4. Optimization of drug mixture by reducing the toxicity risk. (a) Chemicals with the same side effects as Fulvestrant. (b) Chemicals with small number of side effects that also do not synergize with Fulvestrant toxicity are highlighted. List of references can be found in Supplementary Table 3.

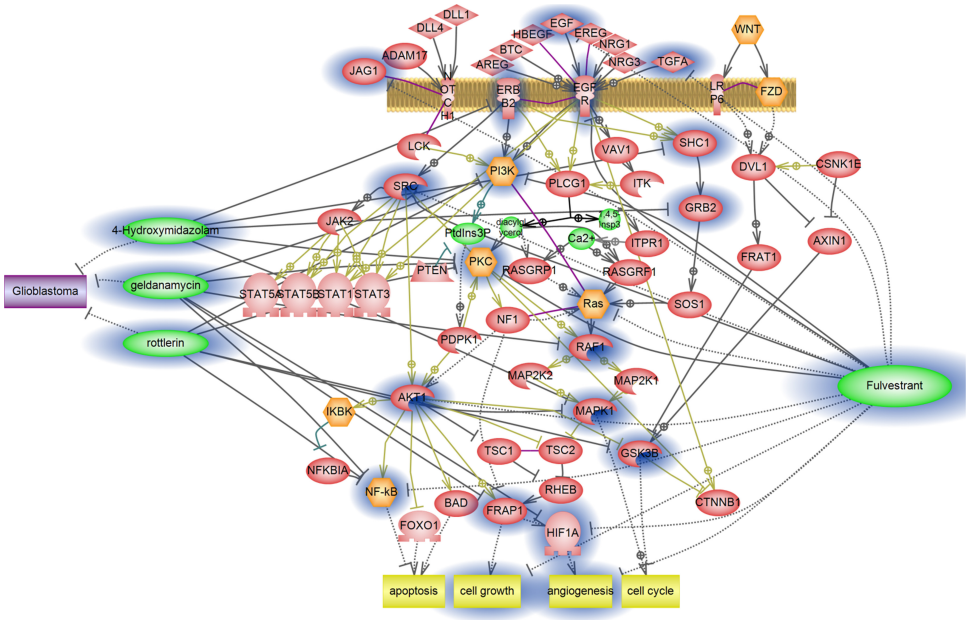


Fig. 5. Proposed glioblastoma drug cocktail and its targets in glioblastoma pathway.

toxicity, and disease-specific microarray data. The main source of knowledge about protein function and about effects of small molecules and their toxicity is scientific publications. This is why the use of literature-based databases such as ResNet and ChemEffect, which reflect the knowledge from freely available full-text articles or PubMed articles, provides a significant advantage to *in silico* drug design.

We have shown the workflow for drug repositioning using Pathway Studio software, ResNet and ChemEffect databases. Glioblastoma data were used as an example. We first built disease pathway on the bases of available reviews to support further hypothesis generation and data analysis. Then we identified known compounds affecting multiple proteins from the disease pathway. Using experimental data we identified potential new drug target for glioblastoma and then found chemicals that are known to inhibit this novel target. We show that two approaches produce converging results: (1) the novel target identified from high-throughput experiment can be added to the consensus disease pathway; (2) the drug (Fulvestrant) inhibiting novel target also inhibits multiple targets in a disease pathway.

We also suggested the workflow to design combination therapy based on complementarity of compound targets in a disease pathway that potentially increase therapeutic efficacy and based on reduction of toxicity risk from drug–drug interaction. Our workflow for combination therapy design can be scaled to design statistical algorithm that computationally tests all possible combinations of drugs targeting disease pathway to find optimal combinations of two or more drugs. Automation

of this process is necessary due to the large number of possible drug combinations that is impossible to test in clinical trials or even experimentally in *in vitro* disease models.

The workflows described in the paper can be generalized to other diseases as long as a model for the disease exists, however the exact steps may vary and depend on the available knowledge. The disease pathway can be built using the information available from scientific literature as we did in this paper for glioblastoma or by analyzing gene expression and other high-throughput data. We used glioblastoma pathway only as an example to show that current scientific literature provides sufficient amount of information on drug action to enable prioritizing of drugs based on their efficacy towards the disease model. We also show that there is also enough information to rationally design drug cocktails based on the complementation of individual drug efficacies towards disease model and based on the minimization of potential side effects due to drug–drug interactions. Our sole purpose of the paper was to suggest principals for computational algorithms for drug selection and for rational design of drug cocktails. Because glioblastoma was used only as an example, we do not want to claim the validity of our findings with respect to Glioblastoma model and Fulverstrant.

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Anton Yuryev received his Ph.D. from Johns Hopkins University where he discovered proteins physically linking transcription and mRNA processing in eukaryotic cells. During his postdoc at Novartis Pharmaceuticals he showed that mammalian protein kinase could be imported into mitochondria. At the birth of Bioinformatics Dr. Yuryev started working as Senior Scientist at InforMax and then continued at Orchid Bioscience as Senior Bioinformatics Analyst. Dr. Yuryev has published 40 scientific publications and authored several algorithms for primer design and pathway analysis. He is now the Senior Director of Application Science at Ariadne Genomics. His research focuses on studying topological and evolutionary properties of biological networks and developing algorithms and workflows for pathway reconstruction, for analysis of high-throughput data and for drug discovery.

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Prior to his appointment, Mazo was Scientific Project Manager at InforMax where he launched several gene expression and PPI products and Research Scientist at CLONTECH where he developed the first commercial retroviral system.

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