Biomedicine & Pharmacotherapy 150 (2022) 112993

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Anti-tumor effects of cryptotanshinone ($C_{19}H_{20}O_3$) in human osteosarcoma cell lines

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ARTICLE INFO

Keywords:
Osteosarcoma
Cancer modeling
Differential equations
Cryptotanshinone
Chemotherapy
Gene regulatory networks
Cancer pathways

ABSTRACT

Osteosarcoma is the most prevalent malignant bone tumor and occurs most commonly in the adolescent and young adult population. Despite the recent advances in surgeries and chemotherapy, the overall survival in patients with resectable metastases is around 20%. This challenge in osteosarcoma is often attributed to the drastic differences in the tumorigenic profiles and mutations among patients. With diverse mutations and multiple oncogenes, it is necessary to identify the therapies that can attack various mutations and simultaneously have minor side-effects. In this paper, we constructed the osteosarcoma pathway from literature and modeled it using ordinary differential equations. We then simulated this network for every possible gene mutation and their combinations and ranked different drug combinations based on their efficacy to drive a mutated osteosarcoma network towards cell death. Our theoretical results predict that drug combinations with Cryptotanshinone ($C_{19}H_{20}O_3$), a traditional Chinese herb derivative, have the best overall performance. Specifically, Cryptotanshinone in combination with Temsirolimus inhibit the JAK/STAT, MAPK/ERK, and PI3K/Akt/mTOR pathways and induce cell death in tumor cells. We corroborated our theoretical predictions using wet-lab experiments on SaOS2, 143B, G292, and HU03N1 human osteosarcoma cell lines, thereby demonstrating the potency of Cryptotanshinone in fighting osteosarcoma.

1. Introduction

Osteosarcoma is a mesenchymal tumor characterized by the production of the osteoid by tumor cells [1]. It emanates in the metaphyses of long bones such as the tibia and femur and has the highest incidence in adolescent patients. Although accounting for less than 1% of all cancers diagnosed in the US, the overall survival rates of osteosarcoma have saturated at around 60% and the survivability is highly unsatisfactory in patients with relapses and metastasis [2].

Cell proliferation needs various nutrients and energy to keep the cell cycle dynamics going, and aberrant cell proliferation is a defining characteristic of cancer [3]. To understand this out-of-control cell growth in cancer, scientists have been studying various biological scales such as genetic deviations, physical stresses, and metabolic pathways [4]. In this vein, identification of growth factors and growth factor signal transduction pathways was momentous in understanding the mechanisms that initiate and drive cell proliferation [5]. In healthy cells, these processes are well-regulated, whereas cancerous cells often have significant genetic mutations that aberrantly enhance these pathways, allowing the cells to proliferate uncontrollably and form a tumor. These mutations mainly occur as oncogenes and tumor suppressor genes and often contribute to tumor growth [6].

Gene regulatory networks (GRNs) characterize the interactions between genes and other molecules and understanding them have been aiding the scientific community in elucidating the key mutations and pathways responsible for cancer [7]. As a result, oncogenes and tumor suppressor genes have been long studied in relation to cancer, however, the sheer number of these mutations and possibilities makes it challenging to use a naive or brute-force approach in designing therapy. Hence, researchers have been developing various mathematical and computational techniques to model tumor growths and their therapeutic responses [8]. GRNs have been modeled using various techniques, such

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https://doi.org/10.1016/j.biopha.2022.112993
Received 8 February 2022; Received in revised form 12 April 2022; Accepted 17 April 2022
Available online 21 April 2022

as Bayesian networks [9], Boolean networks [10], probabilistic boolean networks [11], and differential equations [12]. These models have shown significant success in demonstrating the fundamental pathways and mechanisms of cell proliferation and improving treatment strategies in cancer.

In this paper, we describe a methodology using ordinary differential equations to theoretically assess the effectiveness of various drugs and their combinations in killing osteosarcoma cells. We first make use of the biological literature to construct a GRN model of the osteosarcoma cancer pathway. Then, using the methodology of differential equations, we simulate the osteosarcoma pathway and rank the drug combinations. Finally, we validate our theoretical results using experiments on multiple human osteosarcoma cell lines and discuss the biological significance of our results.

2. Osteosarcoma pathway

Biological pathways and networks oversee various cellular processes necessary for the survival of an organism. In a regulated environment, cells receive decisive signals, primarily from growth factors, that determine the fate of a cell in different scenarios [13]. Specifically, growth factors can influence if a particular cell should proliferate or undergo programmed cell death, and oncogenes often override the actions of these growth factors and force cell proliferation [14].

Different growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin growth factor (IGF) promote various pathways and regulate cell growth, differentiation, and apoptosis [5]. Among these, the epidermal growth factor, the epidermal growth factor receptor (EGFR), and the insulin-like growth factor 1 receptor (IGF1R) signaling play a key role in the development of bone and in the prognosis and progression of different cancers [15]. EGFR along with IGF1R and other growth factors bind with their receptors and form an integrated system and administer crucial cellular pathways such as the Janus kinase pathway, the Phosphoinositide 3-kinase pathway, and the Mitogen-activated protein kinase pathway.

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is a pivotal signaling network and has been involved in multiple cancers [16]. Cells when exposed to cytokines activate the Janus kinase which phosphorylates its target STAT proteins. STAT proteins are able to form dimers and heterodimers with other STAT proteins or molecules. STATs then transfer into the nucleus and activate genes that control different biological processes such as cell proliferation and immune regulation [17]. In healthy cells, the JAK/-STAT pathway is regulated by different inhibitory proteins. However, abnormal activity in the JAK/STAT pathway and constitutive activation of JAKs and STATs have been linked to oncogenesis or tumorigenesis in different human cancers including osteosarcoma. STAT3 is a member of the STAT gene family and was the first identified oncogene in the STAT family. Aberrantly active STAT3 has been shown to promote tumor progression in numerous malignancies including breast, ovarian, pancreatic, colon, and osteosarcoma [18]. Consequently, inhibitors of STAT3 such as HO-3867 and Cryptotanshinone (CPT) have shown promising anti-tumor activity by curtailing the proliferative effects of STAT3 and the JAK/STAT pathway [19].

The phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that are fundamental in supervising many aspects of cell survival and proliferation [20]. Different growth factors and regulators stimulate PI3K which acts as a catalyst in the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the inner side of the plasma membrane. PIP3 recruits and activates multiple proteins such as the serine/threonine kinase (Akt). Activated PI3K/Akt pathway controls cell growth and protein synthesis by stimulating crucial downstream proteins such as the mammalian target of rapamycin (mTOR) and the pro-survival transcription factor nuclear factor-KB (NFKB) [21]. Hence, the PI3K/Akt/mTOR signaling pathway supervises cell survival, growth, and proliferation, which are pivotal for the onset of oncogenesis. The PI3K/Akt/mTOR pathway is one of the most vital tumorigenic pathways and mutations in this pathway have been identified in almost all human cancers including osteosarcoma [22]. With 100% of advanced-stage osteosarcoma patients having altercations in the PI3K/Akt/mTOR pathway, therapeutic studies on osteosarcoma cells with LY294002 (PI3K inhibitor) and Temsirolimus (mTOR inhibitor) have demonstrated some success [23].

Growth factors and other upstream genomic signals activate another crucial pathway, the mitogen-activated protein kinase/extracellular signal-regulated (MAPK/ERK) pathway [24]. Upon activation of the MAPK proteins, ERK translocates to the nucleus, and phosphorylation of ERK leads to the activation of proteins responsible for triggering cell proliferation. The MAPK/ERK signaling cascade, sometimes also referred to as the Ras/Raf/MEK/ERK pathway, is an integral pathway and is tightly regulated under healthy conditions by phosphatases and feedback from other pathways [25]. However, in a cancerous network, mutations in the MAPK pathway can over-activate the pathway and promote an environment conducive to tumor growth. These mutations often develop in epithelial growth factor receptor (EGFR), signal transducers (RAS), and other downstream kinases in the MAPK/ERK pathway. Therefore, clinical trials with inhibitors of MEK, such as U0126, have shown significant success in inhibiting the effect of mutations in the MAPK pathway and thereby constraining cell proliferation [26].

Most human cancers are highly heterogenous and one of the key characteristics of cancer cells is genetic instability [27]. This instability exists both at the chromosomal level (resulting in amplifications and deletions) and the single nucleotide level (resulting in point mutations). Both of these abnormalities often lead to mutations, and there is increasing evidence that indicates most human cancers, including osteosarcoma, contain multiple mutations in the JAK/STAT, PI3K/Akt, and MAPK/ERK pathways [28]. As a result, targeted therapy using, say PI3K/Akt inhibitors, are generally futile on cancer cells with mutations in the JAK/STAT pathway. Hence, there is a growing interest to identify therapies that can inhibit multiple mutations simultaneously.

In Fig. 1, we constructed the gene regulatory pathway of osteosarcoma using the above-discussed information from the literature. The JAK/STAT, PI3K/Akt, and MAPK/ERK pathways interact with each other and trigger the genes responsible for cell proliferation or anti-apoptosis. The gene interactions are represented by black arrows and drug inhibitions are represented by red arrows. We now discuss the methodology we used in this paper to arrive at therapies that can target multiple pathways in osteosarcoma.

3. Materials and methods

3.1. Differential equations

Biological pathways and systems have cause-and-effect relationships between different genes and molecules that oversee important processes, including cell metabolism, signal transduction, and cell proliferation [29]. In an effort to elucidate these processes, gene regulatory networks have been effective in modeling signaling pathways and analyzing them. Various computational techniques such as boolean models, probabilistic boolean models, continuous linear models, bayesian networks, and ordinary differential equations (ODE) have been developed for regulatory network analysis [30–32]. ODE modeling is a class of qualitative models that captures the instantaneous change in the level of each molecule as a function of other molecules. We illustrate the modeling of ODEs with the help of an example below.

Suppose we have a simple toy network of five genes where genes A and B independently activate gene C, gene C activates gene D which finally activates gene E as shown in Fig. 2. Furthermore, let us assume that gene A is inactive and gene B is mutated and always active. We also have drugs Drug1 and Drug2 that inhibit genes C and D respectively, and our goal is to rank the two drugs based on their efficacy to inhibit gene E.

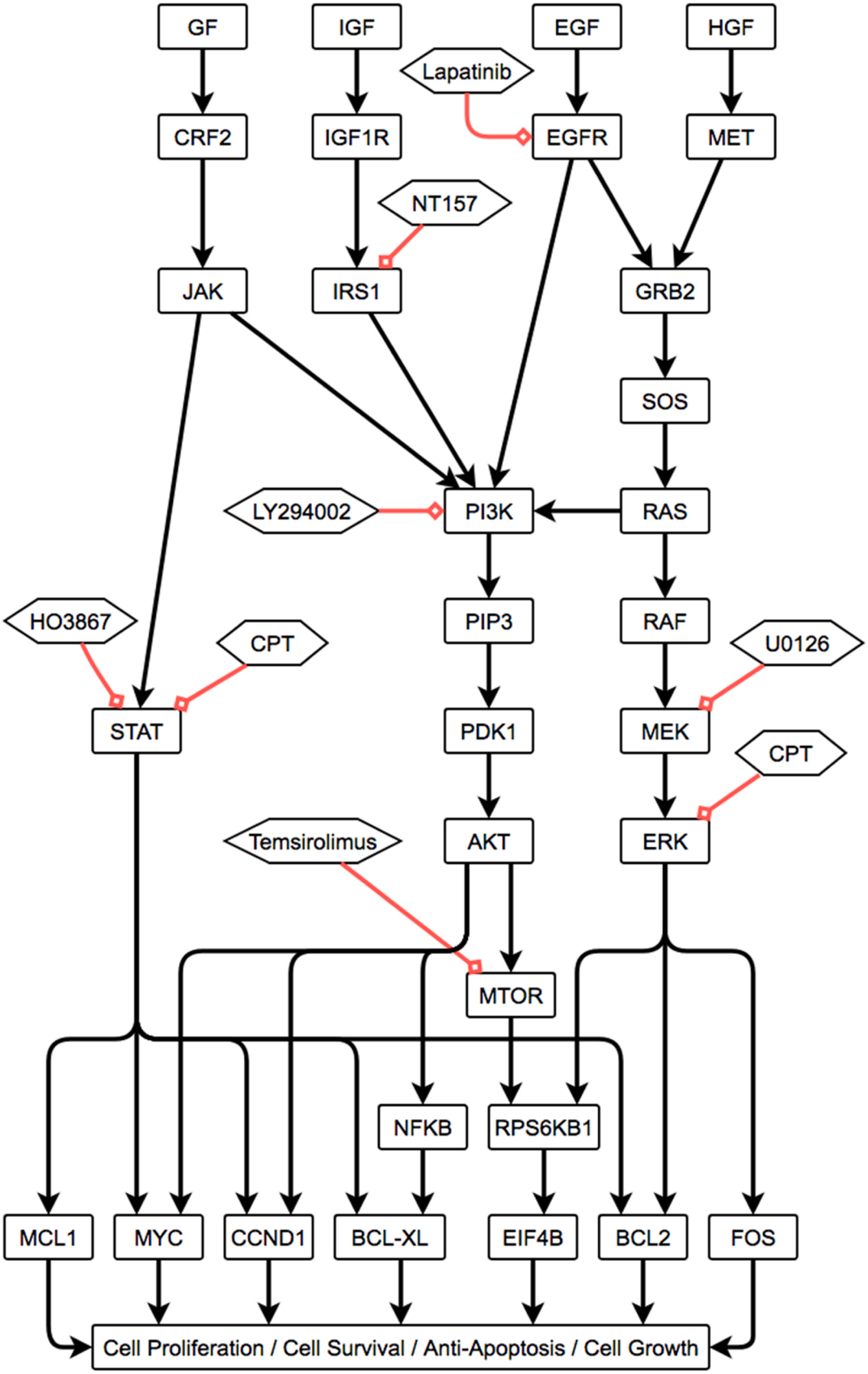

Fig. 1. Osteosarcoma cancer pathway. The JAK/STAT, PI3K/Akt, and MAPK/ERK pathways interact with each other and activate the genes and anti-apoptotic factors crucial for cell proliferation, growth, and anti-apoptosis. The gene interactions are represented by black arrows and drug inhibitions are represented by red arrows.

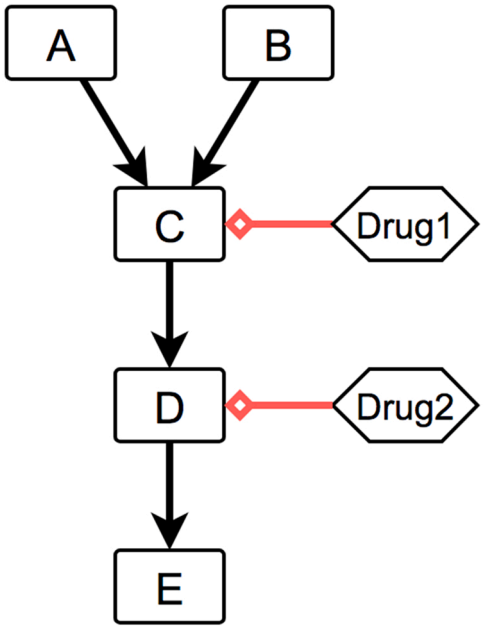

Fig. 2. Toy gene regulatory network of five genes and two drugs. Genes A and B independently activate gene C, gene C activates gene D which finally activates gene E. Drug1 and Drug2 inhibit genes C and D respectively.

We now write the differential equations for this toy network.

With gene A inactive, the differential equation for A is trivial and can be written as,

$$\frac{dA}{dt} = 0$$

With gene B mutated and assuming exponential proliferation, the differential equation for B can be written as,

$$\frac{dB}{dt} \propto B \Longrightarrow \frac{\mathrm{dB}}{\mathrm{dt}} = \mathrm{k_1 B}$$

If Drug1 is inactive, change in the level of gene C is proportional to its input genes A and B, but negatively proportional to gene C since it activates gene D. But if Drug1 is active, gene C is inhibited and change in the level of gene C is zero. Assuming a binary model for the drugs where drug = 0 represents inactive and drug = 1 represents active, the combined differential equation for C can be written as,

$$\frac{dC}{dt} = (k_2 A + k_3 B - k_4 C)(1 - \mathrm{Drug1})$$

Similarly, if Drug2 is inactive, gene D is activated by gene C and gene D activates gene E, but if Drug2 is active, the change in the level of gene D is zero. Hence, the combined differential equation for D can be written as,

$$\frac{dD}{dt} = (k_5 C - k_6 D)(1 - \mathrm{Drug2})$$

Finally, gene E is activated by its input gene D. Hence, the differential equation for E can be written as,

$$\frac{dE}{dt} = k_7 D$$

We can now solve these linear equations with appropriate initial conditions and plot their graphs. We can then quantify these graphs using statistics such as the area under the curve (AUC) and compare the two drugs based on their efficacy to inhibit gene E (or influence the statistic).

Using this methodology of differential equations, we modeled the osteosarcoma pathway discussed in Fig. 1 and inferred the drug combinations that effectively drove a mutated network towards cell death. The detailed codes and the implementation of this methodology are publicly available online at https://github.com/hashwanthvv/osteo . We then validated our theoretical results using experiments on osteosarcoma cell lines.

3.2. Experimental setup

Cell culture and sample preparation: We performed experiments on SaOS2, 143B, G292, and HU03N1 human osteosarcoma cell lines exposed to different drug combinations in a 384-well microtiter plate (Greiner Bio-One 781091). We used an imaging media (IM) of 70% M-199 (11825015), 30% RPMI-1640 (11875085) with 10% FBS (16000044), 14 mM Glutamax (35050061), 20 mM Hepes (15630080), 7 mM of sodium pyruvate (11360070), 1% Penicillin Streptomycin (15240062), 0.5 μM Vybrant Dye-Cycle Violet Stain (V35003), 0.7 g of glucose (A2494001), and CellTox Green Cytotoxicity Assay (G8742) at 1:5000 dilution. For each experiment, we used 30 μl aliquots of each single cell line at a density of 75000 cells/well to each well pre-coated with 10 μg/ml of Rat Tail Collagen Type I (354249). We cultured the cells at 37 °C for 20 h in 5% CO_2 incubator.

Treatment and imaging: In three replicate wells, we treated the cells with single drug and drug combinations. We used the following drugs and their combinations in our experiments: U0126 (10 μM), HO-3867 (10 μM), NT157 (10 μM), Lapatinib Ditosylate (5 μM), LY294002 (10 μM), Temsirolimus (10 μM), and Cryptotanshinone (20 μM). The chemical structures of these drugs are shown in Fig. 3. After adding IM media (30 μl) comprising twice the concentration of a single drug or drug combinations over the microtiter plate, we scanned the control and treated cells every hour for 24 h using an ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices) sampling at three distinct imaging sites.

Next, we performed imaging analysis over seven-days and analyzed the images using the SDC morphological toolbox in Matlab. We then extracted various cellular measurements such as nucleus mean intensity, nucleus size, and CellTox green mean intensity for each individual cell line. Using these morphological features and a two-step data processing method, we classified the cells as living or dead and computed the fraction of dead tumor cells and plotted them.

4. Results

4.1. Theoretical results

We applied the methodology of differential equations to the osteosarcoma pathway and ranked the drug combinations according to their efficacy to kill osteosarcoma tumor cells.

Referring to Fig. 1, we have growth factors (GF, IGF, EGF, and HGF), drugs (Lapatinib, Temsirolimus, HO3867, U0126, LY294002, NT157, and Cryptotanshinone), and anti-apoptotic factors (MCL1, MYC, CCND1, BCL-XL, EIF4B, BCL2, and FOS). Anti-apoptotic factors are reporter genes that can be used to track the activity of cell proliferation or cell growth [33]. Higher activity of these anti-apoptotic factors has been linked to enhanced cell proliferation and tumor progression. To quantify this cell growth, we plotted the combined activity of these anti-apoptotic factors and computed the area under the curve (AUC). In cells with inactive growth factors and no mutations, these anti-apoptotic factors are inactive, and the AUC will be clearly zero. However, in cells with mutations, these anti-apoptotic factors are active despite inactive growth factors and the AUC will be non-zero. Hence, our goal is to drive this mutated network (AUC ‡ 0) towards cell death (AUC = 0) using an appropriate therapeutic intervention.

Given the harmful side-effects of therapeutic drugs, we used a maximum of two drugs per combination (from seven drugs) in our experiments. Keeping the growth factors absent, for each drug combination and mutation in the osteosarcoma pathway, we computed the AUC

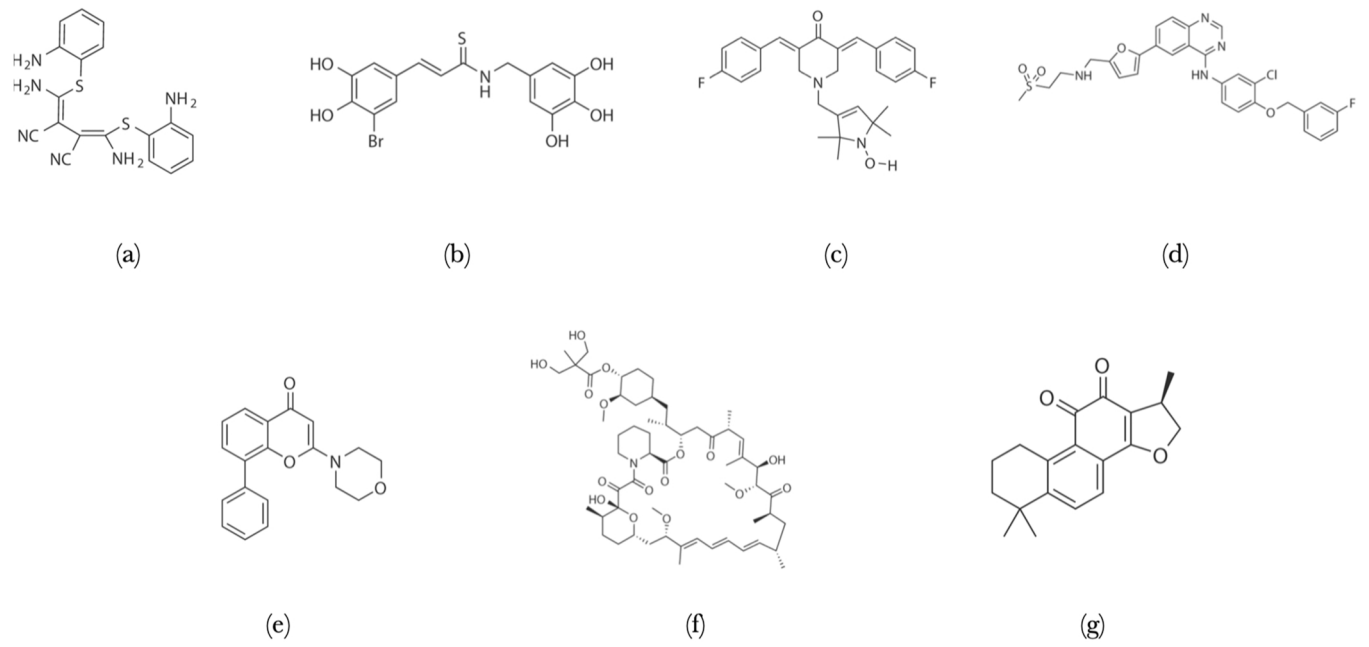

Fig. 3. Chemical structures of the drugs: (a) U0126 (b) NT157 (c) HO-3867 (d) Lapatinib (e) LY294002 (f) Temsirolimus (g) Cryptotanshinone.

of the anti-apoptotic factors and normalized them with no therapeutic intervention as reference. A higher AUC value corresponds with a higher aberration from the ideal output (AUC = 0) and possibly a higher risk of tumorigenesis, whereas a lower AUC value corresponds with a lower aberration from the ideal output and possibly a lower risk of tumorigenesis.

Advanced cancers exhibit multiple mutations; hence, we additionally computed the AUC values for multiple mutations occurring simultaneously. Due to a large number of combinations, we selected a combination of two mutations occurring simultaneously with each of STAT3, PI3K, and RAS mutations. Hence, we tabulated the AUC values of single mutations, mutations with STAT3 mutation, mutations with PI3K mutation, and mutations with RAS mutation for each drug combination and constructed a heatmap of these values in Fig. 4. From the figure, the drugs Lapatinib, Temsirolimus, HO3867, U0126, LY294002, NT157, and their combinations have relatively high AUC values (shade of red), whereas Cryptotanshinone and its combinations have relatively low AUC values (shade of green). These values signify the substantial effect of Cryptotanshinone and its combinations on inhibiting osteosarcoma tumor cells over a wide array of mutations. Specifically, Cryptotanshinone in combination with Temsirolimus can inhibit the JAK/STAT, MAPK/ERK, and PI3K/Akt pathways simultaneously and stymie cell growth, whereas the drug combinations excluding Cryptotanshinone are only able to handle specific individual mutations.

4.2. Experimental results

To validate the significance of Cryptotanshinone in deterring cell proliferation, we ran experiments on multiple osteosarcoma cell lines using the fluorescent protein reporter imaging method discussed earlier.

In Fig. 5, we plotted the cell-killing produced by selected drug combinations in SaOS2, 143B, G292, and HU03N1 human osteosarcoma cell lines. The curves represent the fraction of tumor cells killed (apoptotic fraction) over time. The untreated cell lines (horizontal curves in Fig. 5a) serve as a reference, and it is clear that Cryptotanshinone and its combinations effectively kill the tumor cells within 24 h (p-value < 0.0001). In Fig. 5b, we further see that Lapatinib is inefficient in killing the cells by itself, but with the addition of Cryptotanshinone, the cell-killing improves drastically (p-value < 0.0001). Collectively, our experiments corroborate the efficacy of Cryptotanshinone and its combinations in inhibiting tumor progression in osteosarcoma.

5. Discussion

Osteosarcoma is derived from primal mesenchymal cells and mainly affects the long bones in both humans and canines [34]. Several factors such as radiation, viruses, chemical agents, and hereditary diseases have been linked to triggering osteosarcoma. Although the 5-year survival rate in patients with localized osteosarcoma is around 70%, the long-term survival in patients with metastasis is less than 20% even with intrusive therapies [35]. This poor prognosis in patients with metastatic osteosarcoma is often associated with multiple genetic abnormalities and the difficulty in analyzing their marked complexity at the time of diagnosis [36].

Cell proliferation is fundamental for the growth and meticulous functioning of an organism. However, in cancer cells, genetic mutations, and epigenetic alterations in signaling pathways often trigger out-of-control cell proliferation and initiate tumorigenesis [37]. Hence, it is only natural to consider these pivotal transduction molecules and pathways as attractive targets for cancer therapy. Recent advances in genomics and high-throughput technologies studies have played a momentous role in understanding the key signaling pathways and oncogenes involved in most cancers [38]. Specifically, the JAK/STAT, PI3K/Akt/mTOR, and MAPK/ERK pathways play a central role in proliferation and apoptosis, and these pathways have been aberrantly active in numerous cancers including pancreatic, colorectal, breast, prostate, ovarian, skin, bone, and lung [18], [21], [25], [39], [40].

The JAK/STAT, PI3K/Akt/mTOR, and MAPK/ERK pathways interact with each other, and mutations in these pathways activate transcription factors that regulate transcription of anti-apoptotic and cell survival genes including BCL2, BCL-XL, and MCL1. Although researchers have been investigating several therapeutic agents to inhibit these anti-apoptotic and cell survival genes, there has been a marginal success in the last 30 years [41]. Hence, there has been an interest to target upstream genes instead that can cut-off the downstream phosphorylation and transcription of cell survival genes that supervise cell proliferation. Specifically, studies on STAT3 have shown its adjuvant role in chemotherapy. STAT3 is a crucial transcription factor involved in cell growth, proliferation, and apoptosis. After stimulation of cytokines, the Janus Kinase phosphorylates STATs, and two STAT molecules dimerize and

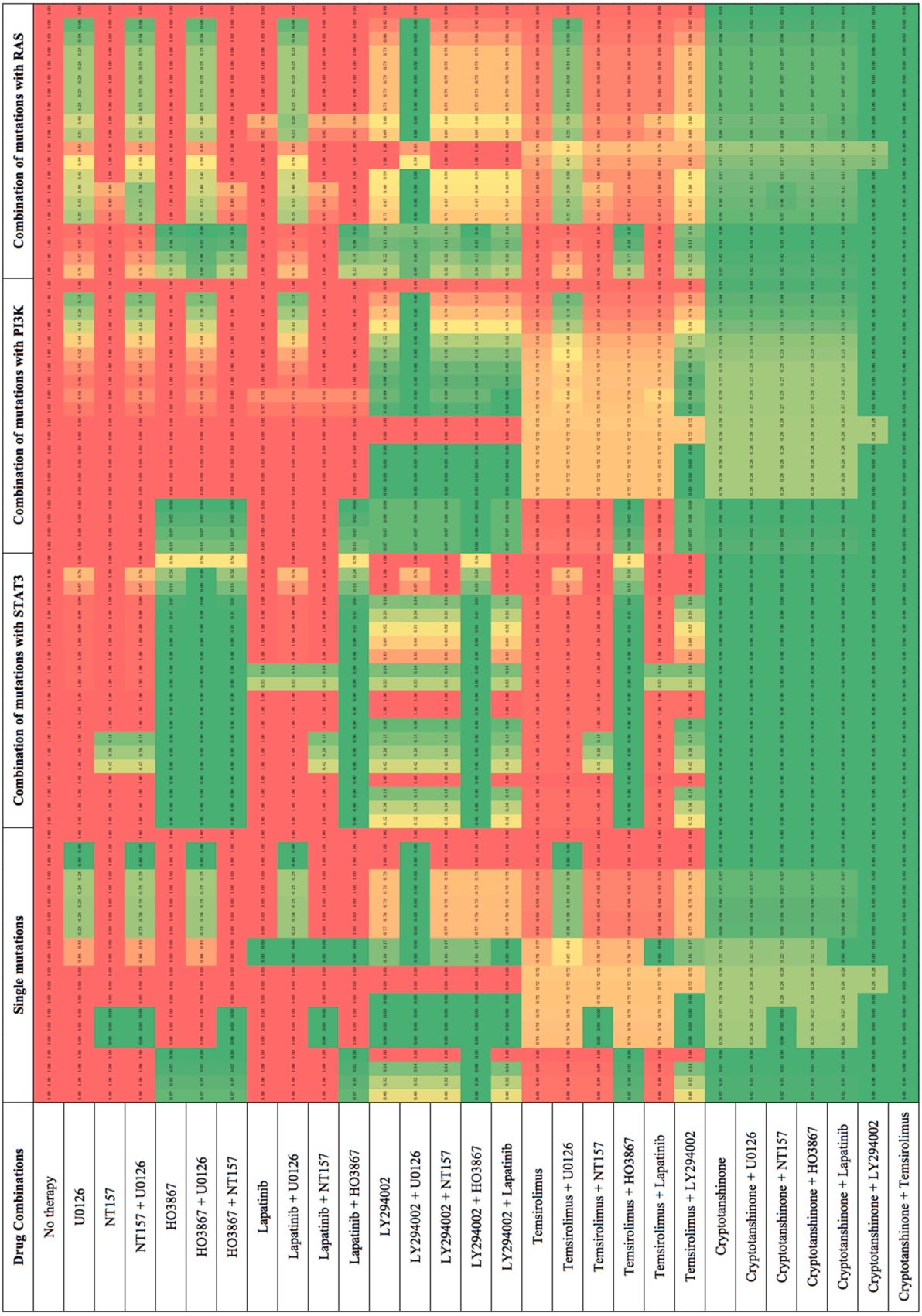

Fig. 4. Heatmap of AUC values. We tabulated the AUC values for each drug combinations across different mutations and their combinations with STAT3, PI3K, and RAS. From the heatmap, we see that Cryptotanshinone, and its combinations have relatively low AUC values and other drug combinations have relatively high AUC values.

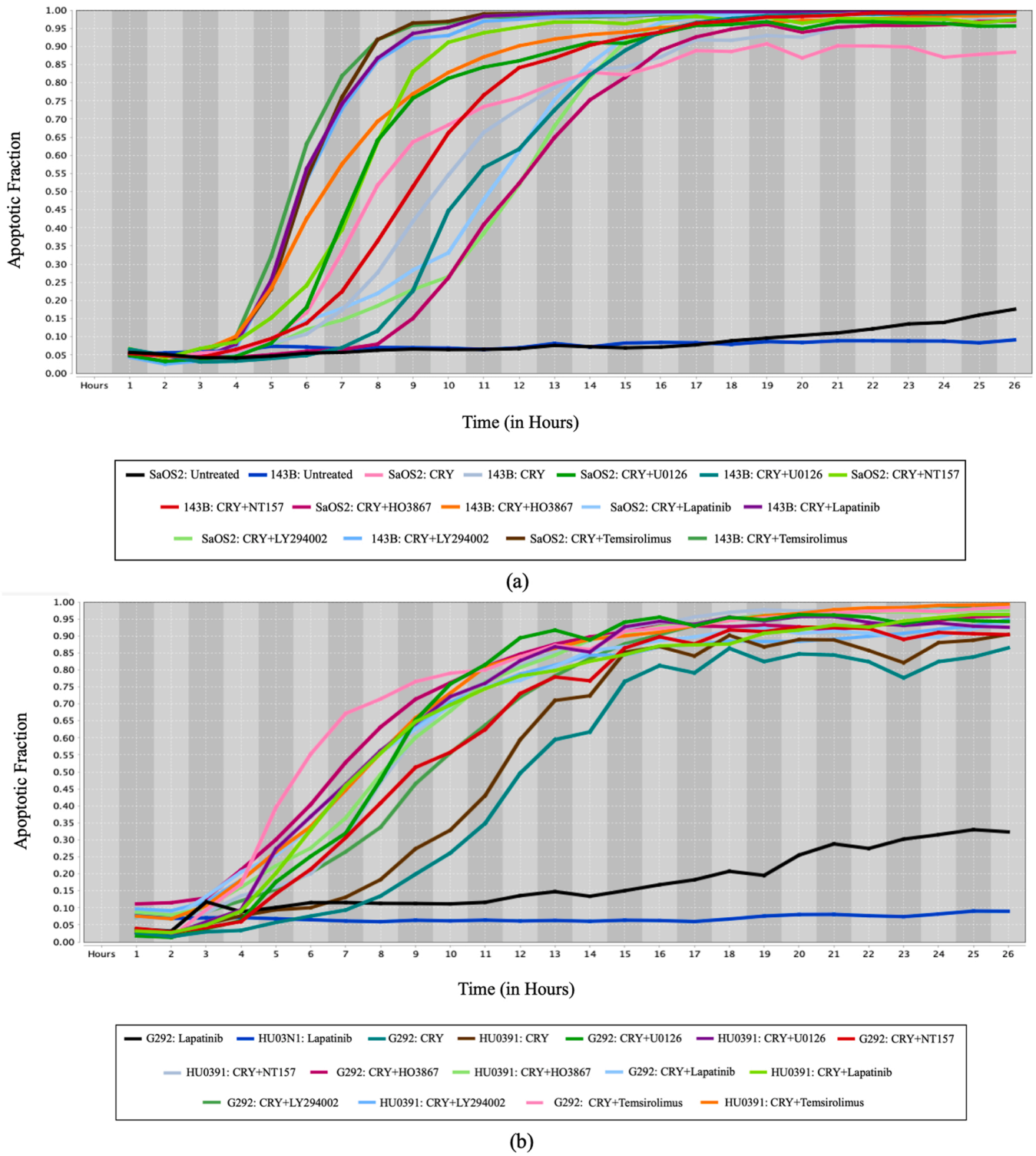

Fig. 5. Apoptosis fraction versus time (in hours) for selected drug combinations in SaOS2, 143B, G292, and HU0391 human osteosarcoma cell lines. The cell-killing produced by Cryptotanshinone, and its combinations is significantly higher than the control group (p-value < 0.0001). The cell lines and drug combinations in the legend from left to right are (a) SaOS2: Untreated cell line, 143B: Untreated cell line, SaOS2: Cryptotanshinone (CRY), 143B: CRY, SaOS2: CRY+U0126, 143B: CRY+U0126, SaOS2: CRY+NT157, 143B: CRY+NT157, SaOS2: CRY+HO3867, 143B: CRY+HO3867, SaOS2: CRY+Lapatinib, 143B: CRY+Lapatinib, SaOS2: CRY+LY294002, 143B: CRY+LY294002, SaOS2: CRY+Temsirolimus, 143B: CRY+Temsirolimus. (b) G292: Lapatinib, HU0391: Lapatinib, G292: Cryptotanshinone (CRY), HU0391: CRY, G292: CRY+U0126, HU0391: CRY+U0126, G292: CRY+NT157, HU0391: CRY+NT157, G292: CRY+HO3867, HU0391: CRY+HO3867, G292: CRY+Lapatinib, HU0391: CRY+Lapatinib, G292: CRY+LY294002, HU0391: CRY+LY294002, G292: CRY+Temsirolimus, HU0391: CRY+Temsirolimus.

translocate to the nucleus and promote the transcription of target genes. Consequently, various techniques have been employed to inhibit STAT3 and develop therapeutic agents to constrain tumorigenesis [42].

Therapeutic drugs bind with receptors on enzymes in cells and prevent the function of target protein(s) or gene(s). With multiple genetic mutations and up-regulated pathways, it is imperative to design combination therapy that can simultaneously intervene multiple pathways [43]. However, the naive approach of testing all possible drug combinations experimentally is not a rational approach. As a result, different computational models have been developed to come up with drug combinations that are promising and advantageous over others.

6. Conclusion

In this paper, we applied a mathematical modeling approach using differential equations to the osteosarcoma pathway. Using this technique, we simulated our network with multiple mutations and ranked the drug combinations according to their efficacy to kill the osteosarcoma cells. Our simulations revealed the anti-tumor effects of Cryptotanshinone ($C_{19}H_{20}O_3$) and its combinations across diverse mutations. We confirmed our theoretical results with experiments on SaOS2, 143B, G292, and HU03N1 human osteosarcoma cell lines using various drug combinations. Cryptotanshinone is a naturally occurring compound derived from a traditional Chinese herb and has shown significant success in achieving cell death in several human cancer cell lines [44–46]. Cryptotanshinone mainly attacks STAT3 by blocking the STAT−STAT dimerization, and this inhibition arrests cells in the $G_1 - G_0$ phase of the cell cycle [47]. This results in further inhibition of anti-apoptotic and cell survival genes and impedes cell proliferation. We conclude that our findings establish the efficacy of Cryptotanshinone on osteosarcoma and we believe these results provide a foundation for in-vivo experiments and clinical development of this drug.

CRediT authorship contribution statement

H.V., A.D., and M.B. designed the research study. A.D. acquired funding. H.V. organized and revised the manuscript. C.S., J.H., R.L., M. B., T.M., and H.M.W-R performed the experiments. H.V. analyzed the data and wrote the manuscript with input from all the authors. All authors contributed to this research and reviewed the manuscript

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

This work was supported in part by the National Science Foundation, USA Grants ECCS-1609236 and ECCS-1917166 and in part by the TEES-AgriLife Center for Bioinformatics and Genomic Systems Engineering (CBGSE) College Station, TX, USA. Startup Funds.

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