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Controlling the intracellular processes ⋆

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Abstract:
In this work we propose new approach to the cancer treatment. We postulate to consider cancer as a malfunction of the cells regulatory unit, and use control engineering and systems theory approaches to describe that malfunction. In our approach mathematical models were used for the description of system and its malfunction. Then we postulate to deliver a secondary control signal to the malfunctioning cells which role is to omit the damaged regions and return the cell to its normal parameters. This should results in a programmed death of the cancer cells. In this paper we introduce the cell as an object of control, describe p53 signaling pathway as an example of cellular regulatory unit, then discuss its possible malfunctions which result in cancer and describe available secondary control signals. Finally we present the results of our *in silico* experiments, which show the efficiency of the proposed secondary controls.

1. INTRODUCTION

Modern medicine faces the problem of numerous genetic diseases including cancer, for which no effective treatment exists due to the variability and high complexity of the processes involved. Despite the fact that cell populations are heterogenic, and significant differences exist between organisms of the same species, the personalized and targeted therapies are still uncommon. The main reason is that our knowledge of the intracellular mechanisms involved in cancer development is still very imprecise. Cancer is usually considered on an organism, tissue or cell level while we propose to consider it as a malfunction of the intracellular regulation processes. In our approach the cell is introduced as a chemical reactor where genes, transcripts and proteins are equivalent to specific substrates and their products. Their mutual interactions and dependencies between them form a complex system with many positive and negative feedback loops called signaling pathways. Appropriate mathematical description of this system allows applying control engineering and systems theory methods, such as bifurcation or sensitivity analysis, in order to determine the best target and most efficient therapy protocol. In the proposed approach cancer state is represented by changes is specific model parameters. This leads to the alterations of the equilibrium point types and/or shifts of the bifurcation points, compared to the normal (healthy cells) state. Because in contrast to the mechanical objects the dysfunction of such system cannot be repaired physically we propose to use additional secondary control signals which role will be to omit the defective part of the signaling pathway and by that sensitize the control object to the primary control signal or even replace that control. From the control-engineering point of view this means that the role of the secondary control is to return the equilibrium points type and/or bifurcation points to the original state.

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2. DESCRIPTION OF THE CONTROL OBJECT

2.1 General description

The intracellular processes can be sumarized in three main blocks (Fig. 1)

- Gene switching and transcription - gene activation occurs when a protein called transcription factor attaches to the promoter region in the DNA, which initiates gene transcription. Gene deactivation is caused by spontaneous or forced transcription factor detachment from the promoter region or attachment of the gene's repressor. Active gene produces mRNA of the corresponding protein. As a result one active gene can produce several to several thousand mRNA copies in a short period of time.
- Translation - After transcription mRNA is transported from the nucleus to the cytoplasm where it is translated in ribosomes into a protein. Once again one mRNA may be used to produce several or hundreds of protein molecules. The mechanism of the transcription and translation includes powerful amplification; single gene activation event may result in the production of hundreds of thousands of protein molecules.
- Proteins interaction - mature proteins can interact with each other. The simplest form of such interaction is the formation and dissolution of protein complexes. Some proteins can also modify others by phosphorylation, acetylation or sumoylation which usually changes the target protein functions e.g. allows nuclear translocation, changes its stability of initiates transcription of genes coding other proteins.

Signal transduction in cells is not unidirectional. Usually several feedback loops, positive and negative, are involved in the regulatory modules. Common negative feedback loops such as for example transcription factor-induced activation of a gene coding for its own inhibitor, provides

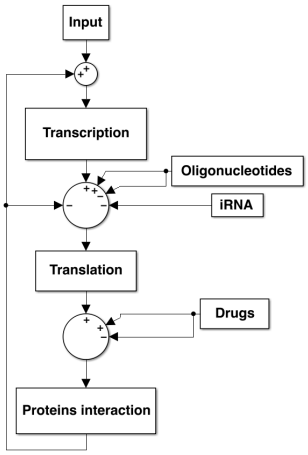

Fig. 1. Intracellular processes and the possible secondary control signals

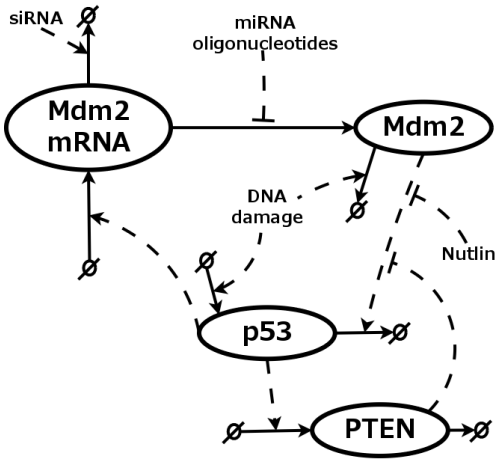

Fig. 2. Simple scheme of the p53 signaling pathway and the possible secondary control signals. Solid lines stand for the transitions e.g. production or degradation, while dashed for influences. Arrows represent possitive influence while hammerhead negative.

homeostasis steady state for the cell. Negative feedback in conjunction with significant delays might lead to the protein level oscillations in the cell. There are also proteins responsible for mRNA degradation which also constitute a negative feedback. Positive feedback loops which amplify signal such as transcription factor-induced activation of a gene responsible for the inhibition of transcription factor inhibitor may introduce bistability into the system. Bistability allows the cell to make decisions such as inducing apoptosis or proliferation after DNA damage (Puszynski et al. [2008]). The interesting fact is that the positive feedback loops inside the cell usually work through a double negation, in which the negative feedback is blocked.

2.2 p53 signaling pathway

One of the most important signaling mechanisms in the eukaryotic cells is the p53 signaling pathway, responsible for maintaining genome integrity. It activates when the integrity of DNA is violated and its function is to stop the cell cycle, initiate the DNA damage repair processes and when the repair is not possible or inefficient to trigger the programmed cell death called apoptosis (Vogelstein et al. [2000]). The distortions in the p53 protein function or in the p53 regulatory module are observed in the majority of known cancers, resulting in the inability to trigger apoptosis in the malignant cells.

Fig. 2 presents a simple scheme of the p53 signaling pathway. In healthy cells p53 is kept at a low level by its inhibitor Mdm2, which in turn is transcriptionally dependent on the p53, thus constituting a negative feedback loop. Another protein which transcription is positively regulated by p53 is PTEN which through the long feedback loop (simplified on the Fig. 2) involving PIP3 and Akt, blocks the p53 degradation by Mdm2. This constitutes the positive feedback loop which works through double negation, where p53 inhibits the action of its own inhibitor. DNA damage induces amplification of the Mdm2, degradation and p53 stabilization. Stable p53 in turn enhances Mdm2 production resulting in the p53 and Mdm2 protein levels oscillation. At this stage cell cycle blockade occurs as well as initiation of the DNA damage repair processes. Meanwhile elevated levels of the p53 induce PTEN production and long, positive feedback loop action. This loop works as a clock. If the DNA damage is not repaired fast enough Mdm2induced p53 degradation is blocked and p53 stabilizes at high level, resulting in apoptosis initiation.

Extended description of the p53 signaling pathway can be found in Puszynski et al. [2008]

2.3 Possible dysfunctions of the p53 signaling pathway

In normal cells we can distinguish two major steady states as long as the p53 dynamics are considered: proliferation attractor and apoptotic attractor (Fig. 3A). The first one represents the normal cell life. DNA damages push the cell away from proliferation attractor. If it is small enough and the DNA repair processes are efficient the cell returns to the first attractor, otherwise it is drawn towards the apoptotic attractor. When the DNA damages cannot be repaired the proliferation attractor becomes a repellor and all the damaged cells should die (Fig. 3B). Around half of the known cancer types express malfunctioning p53 protein which is not considered in this work. In the remaining cancers, p53 signaling pathway is modified and by that do not plays its apropriate role. For example in the human breast cancer cells (MCF-7) PTEN gene methylation causes its dysfunction and by that lack of PTEN production (Krawczyk et al. [2007]). Without PTEN the positive feedback loop is broken therefore the negative feedback between p53 and Mdm2 cannot be blocked and even extensive DNA damage does not lead to apoptosis. Because DNA repair in MCF-7 is also not possible the cells exhibit limit cycle (Fig. 3C). Another example of the p53 signaling pathway modification found in many cancer types is overexpression of the p53 inhibitor Mdm2, caused by amplification of the Mdm2 gene number. For example bone osteosarcoma cells (SJSA-1) have 50 copies of the Mdm2 gene instead of normal 2 (Oliner et al. [1992]). This results in a shrinkage of the attraction area of the apoptotic attractor which in turn results in a higher resistance of the cancer cells to the DNA damage, making them harder to kill (Fig. 3D).

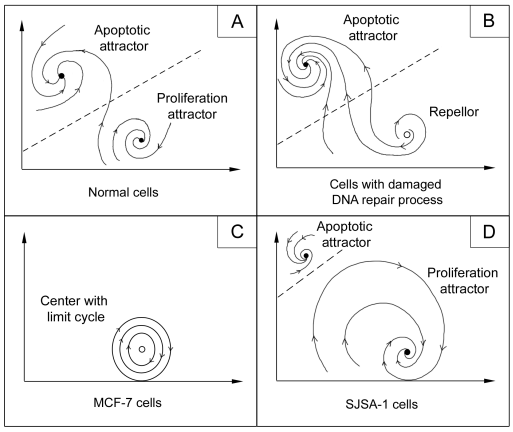

Fig. 3. Scheme of the equilibrium points type in healthy cells [A] and their changes in the cells with damaged DNA repair [B] or cancer states [C] [D]

3. SECONDARY CONTROL SIGNALS

As mentioned malfunctions in the p53 signaling pathway can be considered as a change of the equilibrium points type and/or the bifurcation points location. Despite the modern genetic engineering achievements which allow for the gene number and functionality modifications *in vitro* we are still far away from their widespread usage *in vivo*. Because "mechanical" repair of the damaged regions in the signaling pathway is not possible, assuming DNA damage as the primary input signal, insufficient to cause cancer cells apoptosis, we can try to find additional control signals to omit damaged regions and restore the original behavior of the cells (DNA repair or apoptosis). On the Fig. 1 we presented possible, secondary control signals shortly described in the next subsections.

3.1 Small RNA (iRNA)

Small RNAs regulate almost all aspects of cell life, including their development, growth, differentiation, proliferation, apoptosis, stress response, cell metabolism and cell signaling. Among others the class of small RNAs includes small interfering RNAs (siRNA) and microRNA (miRNA). Small RNAs incorporate two main mechanisms involving either miRNA or siRNA particles which in nucleus become a part of a multiprotein complex called RISC, responsible for the gene silencing processes. Unlike miRNAs siRNAs do not naturally occur in the human cells but their high specificity and the ability to almost entirely silence the expression level of a single target gene has proven their usefulness as a controlling agent in many therapeutic studies (Bumcrot et al. [2006]).

MiRNAs are known to be involved in many genetic disorders since alterations in their expression profile were shown to be connected with a wide range of diseases, mainly cancer (Soifer et al. [2007]). It was also shown that up-/down-regulation of different miRNAs can lead to modified sensitivity to chemo- and radiotherapy (Hummel et al. [2010]). The natural mechanism of miRNA-based gene expression regulation involves translation inhibition, mRNA destabilization or both. miRNA target sites usually include a perfectly complementary seed region (6-8 nt long) and some possible mismatches outside of it. Small specificity level of miRNA sequence recognition motifs, which in many cases allows one specific miRNA to interact with transcripts of hundreds of distinct genes, results in a widespread impact on the protein synthesis (Baek et al. [2008]).

SiRNA target recognition mechanism is highly specific, requiring nearly all of the 20-30 siRNA nucleotides to be complementary to the targets transcript sequence. Only then the cleavage mechanism based on Ago2 protein, which is a part of the RISC complex, can be activated resulting in a target mRNA degradation (Li et al. [2007]).

3.2 Oligonucleotides

Oligonucleotides are short strands of nucleotides, commonly made in a laboratory by a solid-phase chemical synthesis. They can be manufactured with any sequence and are therefore widely used in biology for polymerase chain reaction (PCR), DNA sequencing and molecular probe development. They can be constructed to be similar to specific miRNA or siRNA but they have also one advantage over them. Oligonucleotides may be complementary to the specific miRNA or to the miRNA target site on a specific mRNA. The first leads to the miRNA degradation while the second blocks miRNA-mRNA interactions and by that prevents the mRNA degradation without the mRNA translation blockage (Wang [2011]). In contrast to the siRNA and miRNA oligonucleotides may serve not only as a negative secondary input to the system but also a positive one, although acting through double negation.

3.3 Nutlin

Nutlin family of molecules is an example of a drug type secondary control signal. This type usually works on the level of individual molecules influencing their activity or interactions. Such influence can be positive (enhancing) or negative (inhibiting activity or interactions). As mentioned apropriate cell functioning requires p53 level enhancement after DNA damage, while transcriptionally dependent on the p53 Mdm2 molecules inhibit p53. Mdm2 uses three mechanisms which lead to the p53 blockage - by binding to it and impairing its ability to activate transcription, by favoring its nuclear export and enhancing its proteosomal degradation (Chene [2003]).

Nutlin-3 is a cis imidazoline derivative which prevents the Mdm2-p53 interactions. Nutlin-3 binds to the p53 pocket located on the surface of the Mdm2, preventing p53 from binding and therefore leading to p53-dependant pathway stabilization in defective, Mdm2 rich, cell conditions (Vassilev et al. [2004]). Since it acts on the p53 regulatory module, indirectly by the Mdm2 suppressor, it loses its high functional efficiency in the p53 deficient cells. The most beneficial effects of Nutlin-3 were observed in the cells highly overexpressing Mdm2 although it may also increase p53 module efficiency in tumors with normal Mdm2 expression (Tovar et al. [2006]).

Previous studies have shown that p53-MDM2 interaction blockage by the use of Nutlin-3 can lead to p53 activation and tumor growth inhibition, becoming a novel therapeutic strategy (Chene [2003] and Shangary et al. [2009])

with potential clinical relevance that was already observed in vivo (Vassilev et al. [2004] and Tovar et al. [2006]). The biggest advantage of Nutlin-3 is its non-genotoxic p53 activation that does not introduce DNA damage or post-translational p53 modifications, making it a viable alternative to the current cytotoxic chemotherapy drugs. Despite its ability to restore p53 in wild-type p53 tumor cells, high Nutlin-3 concentrations were also shown to inhibit cell proliferations even in cells lacking p53 (Shangary et al. [2009]).

Non-cancer cells indicate the absence of Nutlin-3 toxic effects experiencing only the inhibition of cell cycle but not the cell death, yet Nutlin-3 sensitivity and specificity still requires additional research in order to describe its precise mechanism of intercellular action. Among recent studies concerning other Nutlin-3 dependent mechanisms there were several reports showing up-regulation of various p53 related genes such as Notch1 (Secchiero et al. [2009]) or p21 (Secchiero et al. [2007]).

4. METHODS

We used our previous mathematical model of the p53 signaling pathway (Puszynski et al. [2008]) and modified it separately for each of the three secondary control signals: siRNA, miRNA with oligonucleotides and Nutlin. We combined miRNA with oligonucleotides because of the previously mentioned reasons. Our model is based on Haseltine-Rawling postulate Haseltine et al. [2002] where the changes in amount of all molecules are described by ODE and gene switching by the Gillespie algorithm. This allows us to conduct fast model simulations, while maintaining its stochasticity required for a single cell response simulation and determination of apoptotic fraction. The modifications for siRNA and miRNA/oligonucleotides, required changes only in the Mdm2 mRNA equation and respectively one or three additional equations for siRNA amount inside the cell for active and inactive miRNA as well as inactive Mdm2 mRNA. A more detailed description of the siRNA modification can be found in Puszynski et al. [2012] and miRNA /oligonucleotide in Lalik et al. [2014]. Below we present the modified Mdm2 mRNA equation for the second case:

$$\begin{aligned}&\frac{d}{dt}Mdm2_{mRNA} = s_1 * G_{Mdm2} - d_1 * Mdm2_{mRNA}\\&-k_1 * Mdm2_{mRNA} * (1 + miRNA) * \frac{1}{1 + Oligo}\\&+k_2 * iMdm2_{mRNA}\end{aligned} \quad (1)$$

where $iMdm2_{mRNA}$ is mRNA inactivated by miRNA or oligonucleotides and miRNA represents both miRNA and miRNA silencing oligonucleotides. Individual terms in the equation describe the mRNA transcription from an active gene, degradation, inactivation and inactive mRNA spontaneous activation respectively. It is worth mentioning that Mdm2 mRNA is under control of at least 2 known miRNA (miR-194 and miR-1207) so even without additional miRNA or oligonucleotides some part of the mRNA is inactivated or degraded.

The addition of Nutlin required extensive model modifications resulting from the simplifications made to the original model. It assumed that the p53 poli-ubiquitination caused by Mdm2 is fast and irreversible. Because Nutlin disrupts p53-Mdm2 interaction and ubiquitilation is a reversible process it was necessary to represent each stage of the ubiquitination, especially the creation of p53-Mdm2 complexes and dissolution represented with appropriate equations. Moreover the model takes into account pharmacokinetics and pharmacodynamics of the Nutlin itself. The exact description of the modified model is beyond the scope of this work and can be found in Puszynski et al. [2014].

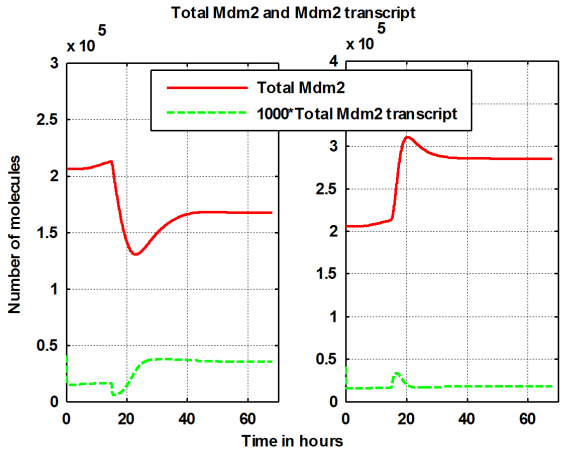

Fig. 4. Total number of Mdm2 and Mdm2 transcript molecules after transfection with 10μM of miR-194 (left) or 10μM of anty-miR-194 oligonucleotides (right). Transfection starts at t=15h.

The majority of parameters for all model variations were taken from the *in vitro* or *in vivo* (Nutlin pharmacokinetics) experiments results found in the literature (e.g. Zhang et al. [2011]). The remaining parameters were fitted "by hand" to achieve the appropriate model behavior.

5. RESULTS

First we analyzed the impact of Mdm2 mRNA-specific miRNA and oligonucleotides on the p53 signaling pathway and cells viability. We used oligonucleotides with sequences of miR-194 (m194) and miR-1207 (m1207), which are known to regulate Mdm2 mRNA, and oligonucleotides complementary to them (anti-m194 and anti-m1207 respectively). Anti-m194 and anti-m1207 bind to the appropriate miRNAs and by that prevent mRNA deactivation or degradation. Our *in vitro* as well as numerical experiments show that the miR-194 or m194 transfection inactivates part of the Mdm2 mRNA resulting in a decreased number of Mdm2 protein molecules (Fig. 4 left). Please note that because of the negative feedback between p53 and Mdm2, reduced level of Mdm2 results in the p53 level increase, and therefore increased production of Mdm2 mRNA. Similarly transfection with anti-m194 or anti-m1207 results in Mdm2 mRNA stabilization and by that Mdm2 level elevation, following p53 level drop and decreased Mdm2 mRNA production (Fig. 4 right).

This gives us a great tool for the adjustment of cellular sensitivity to IR (ionizing radiation) (Fig. 5). Cells viability after the IR exposure depends on the radiation dose. If the radiation is lower than a certain threshold (1.9 Gy in our case) then the induced DNA damage is

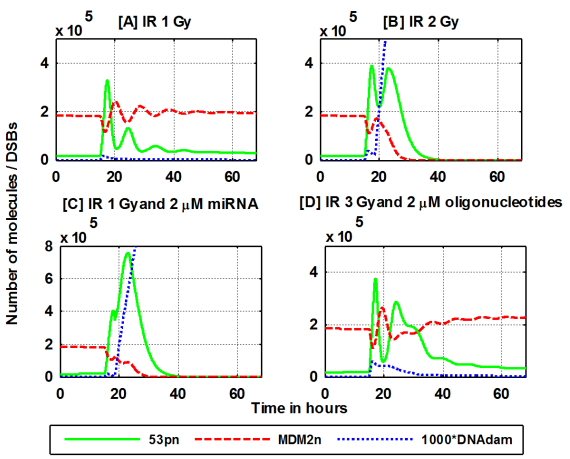

Fig. 5. Time courses of the nuclear phosphorylated p53 (green), nuclear Mdm2 (red) and DSBs number (blue) in normal cells. Cells stimulation starts at t=15h.

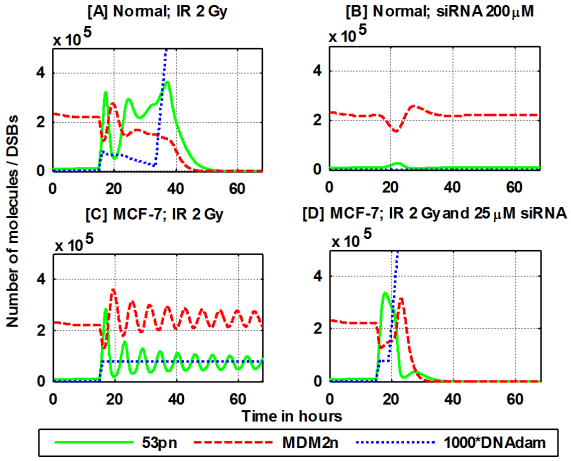

Fig. 6. Time courses of the nuclear phosphorylated p53 (green), nuclear Mdm2 (red) and DSBs number (blue) in normal and MCF-7 cells. Cells stimulation starts at t=15h.

small enough to be quickly repaired allowing the cell to survives (Fig. 5A). If the IR dose exceeds the threshold then the positive feedback loop blocks p53 degradation and p53 reaches the level in which apoptosis is initiated (Fig. 5B). Please note that during the apoptosis DNA is cut by DFF (DNA Fragmenting Factor) nucleases which terminates all transcription processes. These processes are reflected by our model as DSBs number follows up to infinity and mRNA as well as protein number drops to 0. Even if the IR dose of 1 Gy is insufficient to trigger the apoptosis, additional stimulation by 2 μM of miR-194 or m194 for 4h changes the cells fate (Fig. 5C). Similarly 3 Gy of IR causes cell death but when followed by 2 μM of anti-m194 it becomes insufficient to trigger apoptosis (Fig. 5D).

The miR-194, miR-1207, m194 and m1207 oligonucleotides as well as Mdm2 mRNA specific siRNA can be used not only for the control of normal but also cancer cells. For example, as mentioned before, MCF-7 cells have a broken positive feedback loop and therefore cannot commit apoptosis. Our *in silico* experiments show that normal cells die after exposure to 2 Gy IR (Fig. 6A) while MCF-7 exhibit undamped oscillations (Fig. 6C). By the use of a secondary control, in a form of 4h siRNA pulse, we are able to change the cells response to radiation. Even 25 μM of Mdm2 mRNA-specific siRNA, provided right after IR is enough to trigger apoptosis in the MCF-7 cells (Fig. 6D) while in the absence of IR even the dose as high as 200 μM is not sufficient to significantly change the cells behavior (Fig. 6B). In the time when this paper was prepared, we were conducting an experimental *in vitro* verification of the *in silico* results for the siRNA case, and the initial results were in a good agreement with our simulations.

The reason why miRNA, oligonucleotides or siRNA stimuli were only considered as a secondary control to the IR is that alone they are unable to induce cellular death. The main reason is that they do not have a sufficient mRNA silencing efficiency. Typically they are able to lower mRNA amount to around 75% or at best case to 90-95% of the base level, which still leaves Mdm2 protein at a level high enough to block the p53 increase, and its ability to trigger apoptosis. Nutlin works differently, targeting the protein

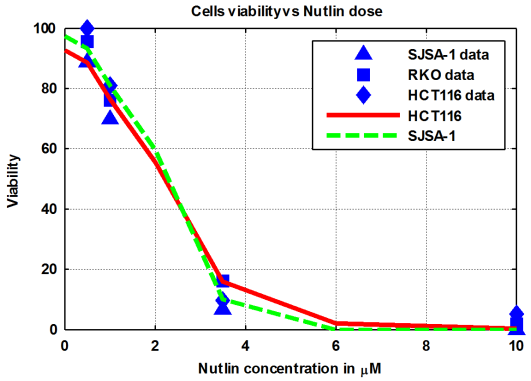

Fig. 7. Dependence of cells viability on Nutlin-3 dose after their *in vitro* treatment. Cells viability was measured 48h after the treatment started. Blue markers are taken from Vassilev et al. [2004] while red and green lines present the model simulations result

interaction level. A high enough Nutlin dose is able to block the p53-Mdm2 interaction in all HCT116 cells with normal Mdm2 expression level, and even in SJSA-1 cells which have 25-fold Mdm2 gene amplification resulting in strong Mdm2 overexpression (Fig. 7).

Nutlin as a secondary control in the p53 signaling pathway has also a one major advantage over siRNA, miRNA or nucleotides - its *in vivo* application is easy. It can be dosed orally or injected like normal drugs while others are recognized by the body defense mechanism as virus-originating DNA and quickly removed, therefore requiring special delivery mechanisms like nanocapsules (Davis [2009]) and/or additional chemical stabilization. We implemented Nutlin pharmacokinetics after the oral dosage in mice given by Zhang et al. [2011], and examined how the viability of HCT116 and SJSA-1 cells depends on the total dose and delivery schedule (Fig. 8). The given Nutlin total dose was delivered as a single dose or split to 4 equal doses delivered within 24 or 6 hour breaks between them. The results show that a single drug administration is better than metronomic for small total doses, while for high total doses metronomic administration proves to be

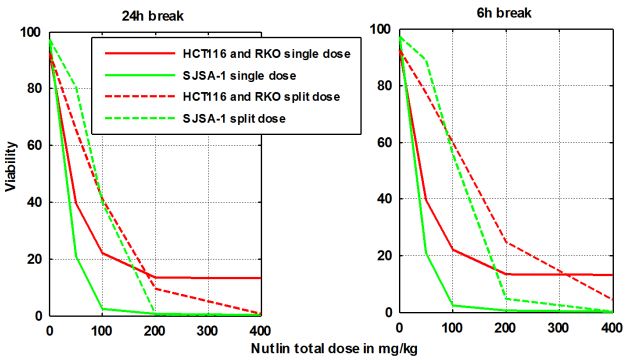

Fig. 8. Cells viability dependence on the Nutlin-3 dose, after the oral dosage. Cells viability was measured 48h after the treatment started.

more efficient. Moreover they indicate that 24 hour split is more efficient than 6 hour and that Nutlin is more effective in the Mdm2 overexpressed cells rather than in cells with normal Mdm2 level.

6. CONCLUSION

In this work we explored the impact of possible secondary control signals on the p53 signaling pathway. We showed that by their usage it is possible to change the response of a regulatory unit. The next step will be to use a sensitivity analysis in order to determine the additional possible targets for the secondary control. Then the optimization methods may be used to find the best possible control sequence. In the near future such approaches can result in the development of personalized therapies, in which the model parameters will be determined on the basis of data obtained from the patients. The model analysis will be used then to find the best targets and determine the best secondary control for the therapy.

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