Fractionated Irradiation of Five Human Lung Cancer Cell Lines and Prediction of Survival According to a Radiobiology Model

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Abstract. *Background: This study evaluates a predictive radiobiology model by measurements of surviving fraction (SF) by the clonogenic assay or the extrapolation method and the proliferation rate in vitro. It is hypothesized that incorporating proliferation to intrinsic radiosensitivity, measured by SF, to predict radiation responsiveness after fractionated irradiation adds to the model's accuracy. Materials and Methods. Five lung cancer cell lines with known SF after 1 Gy (SF1), and also SF2 and SF5, were irradiated with three different fractionation regimes; 10×1 Gy, 5×2 Gy or 2×5 Gy during the same total time to achieve empirical SF. In addition, the SF1, SF2 and SF5 after fractionated irradiation was calculated for each cell line based on the already known single fraction SF and with or without a proliferation factor. The results were compared to the empirical data. Results and Discussion: By using the clonogenic assay to measure radiosensitivity, prediction of radiosensitivity was improved after fractionated radiotherapy when proliferation was used in the radiobiology model. However, this was not the case in the cell lines where the extrapolation method was used to calculate SF. Thus, a radiobiology model including intrinsic radiosensitivity, measured by the clonogenic assay, as well as proliferation, is better at predicting survival after fractionated radiotherapy, compared to the use of intrinsic radiosensitivity alone.*

An understanding of the radio-responsiveness of an individual tumour is of potential clinical importance so that radiotherapy can be optimised by adjusting the administered dose and/or fractionation schedule (1).

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Measurement of survival in the clonogenic assay has become one of the most commonly used and 'gold standard' radiobiological techniques for evaluating radiosensitivity (2). However, since the technique is not optimal in cell lines with a low clonogenic capacity, it would be advantageous to develop alternative measurements of radiation responsiveness in different types of cancer. One such method involves the measurement of the surviving fraction (SF) based on tumour cell proliferation after irradiation, *i.e.* the extrapolation method (3).

Proliferation also has an important role in determining tumour response to fractionated radiotherapy, particularly in studies that investigate problems with protracted treatment times and tumour control (4, 5).

A model to predict outcome after radiotherapy based on intrinsic radiosensitivity but also including proliferation has previously been presented (6). The current study hypothesises that incorporating proliferation to intrinsic radiosensitivity SF in a formula predicting radiation responsiveness adds to its accuracy. The study therefore evaluated measurements of SF by the clonogenic assay and extrapolation method, and assesses their usefulness in the radiobiology model, with a particular focus on the problem of individual radiation responsiveness and the importance of proliferation with an *in vitro* set-up of fractionated irradiation.

Empirical SF was measured after fractionated irradiation in five different cell lines. The effect of irradiation was furthermore predicted for each cell line taking into account individual single fraction SF. Calculations were performed with or without the doubling time collected through earlier *in vitro* studies. These results were then compared with the empirical results.

Materials and Methods

Tumour cell lines. The investigated cell lines have all been established, characterized and reported previously (7-10). U-1285 is a small cell lung cancer (SCLC) cell line that grows unanchored in loose aggregates. Single-dose irradiation of this cell line has a

typical SCLC survival curve with no shoulder (11). U-1906 E is also an SCLC cell line that grows anchored to the bottom of the dish. It has similar characteristics to the classic SCLC phenotype, *i.e.* no shoulder. This cell line has transformed several times from the radioresponsive 'early' variant U-1906 E to a 'late' more radioresistant variant with a distinct shoulder, U-1906 L (10). U-1752 is a squamous cell carcinoma cell line that grows very tightly anchored to its dish. Its single-dose irradiation survival indicates an extreme radioresistant profile. Finally, U-1810 is a large cell carcinoma cell line. This cell line has demonstrated an extraordinarily high radioresistant profile (11).

The cell cultures were kept in RPMI-1640 medium supplemented with 10% calf serum, streptomycin and penicillin in an incubator with 100% humidity, 37˚C and 5% CO_2. The cells were grown in 5 ml Falcon dishes and, when the experiment was repeated with cell lines U-1752 and U-1810, the cells were grown in Falcon cell culture flasks.

Irradiation. Irradiation was performed after plating, with a linear accelerator operating at 8 or 16 MeV with a dose rate of 4 Gy/min. The dishes were covered by a 1 cm bolus to afford sufficient build-up for relevant dose homogeneity.

All the fractionation schedules were delivered at 10 Gy in five consecutive days but in three different fractionation sizes: Schedule 1:1 Gy twice a day with six hours between radiations, to a total of 10 Gy; Schedule 2: 2 Gy once a day during five days; Schedule 3:5 Gy on day 1 and again on day 5.

A second set of irradiation of tumour cell lines U-1752 and U-1810 was performed in a slightly different way, due to a change of experimental apparatus. The procedure involving the radiotherapy and the handling of the cell lines was the same when the experiment was repeated. Irradiation was performed with ^{137}Cs in a Gamacell 40 Exactor with a dose rate of 1.29 Gy/min in the range 0-1 Gy and a dose rate of 1.23 Gy/min with 2 Gy and above.

For this set of experiments, all fractionation schedules were delivered at 8 Gy in four days in three different fractionation sizes continuing from day 2 to day 5 in the same week as the first set of experiments: Schedule 1: 1 Gy twice a day with at least six hours apart, to a total of 8 Gy; Schedule 2: 2 Gy once a day for four days; Schedule 3: 4 Gy on day 2 and again on day 5.

Evaluation of the irradiation effect

The clonogenic assay. The clonogenic assay method has been described elsewhere (11). Before irradiation, 5×10^3 cells were suspended in a mixture of RPMI-1640 medium (with serum and antibiotics as described above) and agarose (Sigma type VII LTG agarose) to a final concentration of 0.33% agarose and then seeded on plastic dishes (Falcon). Before seeding, the dishes were covered with 2 ml of 1% agarose mixed with RPMI-1640 medium 1:1 (with serum and antibiotics) to a final concentration of 0.5% agarose. After irradiation, the cells were incubated for three weeks. After two to three weeks, when the cultures were in good condition, the final cell counting was performed. It was observed that after three weeks the number of large clones did not increase and the viability of certain clones started to decrease. Since the outline of the cell membranes of the U-1285 and U-1906 tumour cells were difficult to observe, 0.125 mm was used as the minimum diameter of a large clone. Such a clone was estimated (by counting the stained nuclei) to contain at least 50 cells. The number of clones in relation to the non-irradiated control cells was plotted on a log linear diagram and the radiobiological parameters were extrapolated graphically by eye. In the studies with single-dose irradiation, four investigations were performed in duplicate or triplicate. In the studies with fractionated irradiation, two investigations were performed in triplicate.

The extrapolation method. The extrapolation method has been described previously (11, 12) and was used for the cell lines U-1752 and U-1810 due to their low clonogenic capacities. In brief, 5×10^5 cells were placed in culture dishes (Falcon, 50 mm in diameter) and after irradiation the cells were regularly evaluated with an inverted light phase microscope over a period of five to six weeks. At a confluence of 50-100%, the cells were sub-cultivated 1:2 or 1:4 depending on the density of the culture.

At sub-cultivation, the non SCLC cell lines were treated first with EDTA (Triplex III, Merck) in phosphate-buffered saline (PBS) then trypsin (0,25% w/v in PBS) and after that were gently sucked up and down with a pipette to loosen and dissociate the cells. The cells were counted and the density was adjusted as to cover fewer than 25% of the dish and an appropriate volume saved for further cultivation. Staining with trypan-blue regularly demonstrated that less than 5% dead cells were present. Growth of the irradiated cells was delayed, the length of the delay depending on dose and sensitivity of the cell line. After the time lag, the cultures started to grow exponentially. The extrapolated total number of cells was obtained by multiplying the cell number by the dilution factor.

The growth after each treatment, single dose or fractionated, was plotted on a log-linear diagram with time on the linear scale and number of cells on the log scale. The number of surviving cells was obtained by extrapolation of the exponential part of the curve to the interception of the y-axis. The SF was calculated as the quotient between the extrapolated value of each individual irradiated culture and the untreated culture. The result after each dose schedule was derived from two to four investigations performed in duplicate and each cell dish was studied twice a week for at least five weeks.

The method was somewhat modified when applied after the second set of irradiation of U-1752 and U-1810 cells. The experiment was repeated in these two cell lines as a control to verify that the cell cultures had not become overcrowded in first experiment. For the repeat experiments, 1×10^6 cells were originally seeded in cell culture flasks (Falcon 100×75 mm), thus giving more space for growing cells. Triplicate flasks were used for each fractionation schedule and also as control. After irradiation was completed, cell numbers were counted twice a week for three weeks and at the same time sub-cultivated, preserving 20% to 30% of cells (U-1752) or 5% to15% (U-1810) on each occasion in order for the concentration of cells not to become too confluent. At sub-cultivation, the cells were treated with EDTA (Triplex III, Merck) in PBS and then with trypsin (0.25% w/v in PBS) to loosen them and after that, if needed, gently sucked up and down in a pipette to dissociate the cells. The data was calculated and plotted in Microsoft Excel 2003.

The fractionated radiotherapy prediction model. The model has been described elsewhere (6). It assumes a constant SF during the entire course of fractionated irradiation as is the case in isodose calculations according to the alpha-beta method. Other deterministic conditions are that all cell death is accomplished by the radiation and that the proliferation rate is constant and exponential throughout the fractionated treatment.

Table I. *Surviving fraction (SF) in the five cell lines after single-dose and fractionated irradiation.*

		1285†	1906E†	1906L†	1752‡	1810‡
SF	1 Gy	0.51	0.77	0.85	0.94	0.95
single dose	2 Gy	0.24	0.42	0.64	0.9	0.89
	4 Gy	-	-	-	0.76§	0.73§
	5 Gy	0.02	0.07	0.14	0.65	0.62
SF measured	10×1 Gy	0.0036	0.076	0.92	0.53	0.76
after fractionated	5×2 Gy	0.01	0.022	0.74	0.56	0.68
irradiation	2×5 Gy	0.014	0.023	0.21	0.28	0.58
	8×1 Gy	-	-	-	0.81	0.51
	4×2 Gy	-	-	-	0.38	0.68
	2×4 Gy	-	-	-	0.36	0.68

§Calculated; †clonogenic assay; ‡extrapolation method.

Table II. *Calculated surviving fraction (SF) in the five cell lines after fractionated irradiation.*

		1285	1906E	1906L	1752	1810
SF calculated	10×1 Gy	0.0032	0.196	0.61	0.46	0.50
with doubling	5×2 Gy	0.002	0.033	0.31	0.51	0.48
time correlation	2×5 Gy	0.001	0.013	0.11	0.39	0.35
coefficient	8×1 Gy	-	-	-	0.54	0.58
	4×2 Gy	-	-	-	0.59	0.56
	2×4 Gy	-	-	-	0.54	0.49
Doubling time (h)		61	61	54	60	52
SF calculated	10x1 Gy	0.0012	0.073	0.2	0.54	0.6
without	5×2 Gy	0.0008	0.013	0.11	0.59	0.56
doubling time	2×5 Gy	0.0004	0.005	0.04	0.42	0.34
correlation	8×1 Gy	-	-	-	0.61	0.66
coefficient	4×2 Gy	-	-	-	0.66	0.63
	2×4 Gy	-	-	-	0.58	0.53

The calculations were performed according to the following formulae:

$$C_N \times C_0 \text{ x } SF^N \times P^{N-1}$$

where C_N is the number of cells after N irradiation treatments, C_0 equals the original number of cells immediately before first treatment, SF is the surviving fraction of cells for each dose and cell line and P is the proliferation factor describing exponential growth:

$$P = e^{\ln 2(t-m)/Td}$$

where t is the time interval between fractions, m is the mitotic delay set to 1.5 h/Gy and Td is the doubling time of the cell population, which might imply the potential doubling time if all cell kill is afforded by irradiation.

For each cell line, the SF after fractionated irradiation was calculated by using the known SF after a single fraction and proliferation data and entered in the model above.

These results were then compared to the empirical results after fractionated irradiation as described above, where SF was measured after completion of all fractions.

Results

Survivals after single-dose and fractionated irradiation are detailed in Table I. The SCLC cell line U-1285 was the most radioresponsive after single-dose, as well as after fractionated, irradiation. SF was considerably lower after fractionated than after single-dose irradiation in both U-1285 and U-1906E.

In U-1906L, on the other hand, the fractionated irradiation SF was slightly higher than after the corresponding single doses. This demonstrated that proliferation must have taken place to such an extent that repeated cell kill from irradiation was overcompensated.

In the squamous cell line, U-1752, and the large cell lung cancer cell line, U-1810, fractionated irradiation accomplished slightly higher cell kill compared to single- dose irradiation.

The 5 Gy fractionation schedule was the most efficient in the three most radioresistant cell lines, thus comparatively less efficient in the most radioresponsive cell line.

Prediction of survival after fractionated treatment was performed both with and without a proliferation factor. The doubling time of the different cell lines during normal culturing conditions was used, implying subcultivation twice a week. The data are presented in Table II. In the cell lines U-1285, U-1906L and U-1906E there was a better prediction of SF when calculations were performed with a doubling time, except for 10×1Gy in U-1906E This did not reach statistical significance ($p \leq 0.05$), possibly due to a small number of measurements. The significance of the difference between calculations with and without a proliferation factor using Student's *t*-test was $p=0.08$. In the U-1752 and U-1810 cell lines, prediction was the same with and without a proliferation factor *i.e.* doubling time. The data are summarised in Figure 1.

The effect of irradiation was studied according to the extrapolation method in the U-1810 and U-1752 cell lines due to their low clonogenic capacity. This method requires repeated cell counting and in both these cell lines an exponential growth was observed during the irradiation period. When applying the model it resulted in a lower number of cells than was found during irradiation (Table III). This is also expressed as a lower SF when using the model with a proliferation correlation coefficient as shown in Table II. Due to the pronounced proliferation during the experiment the experiment was repeated slightly different as explained above in these two cell lines in order to exclude the possibility that overcrowding influenced the results. No major changes were found, although once again a marked proliferation was observed in these two cell lines throughout the experiment. The resultant calculations gave a total number of cells during the entire duration of experiment and it was therefore possible to calculate a doubling time. When the cell doubling time for the first two weeks after

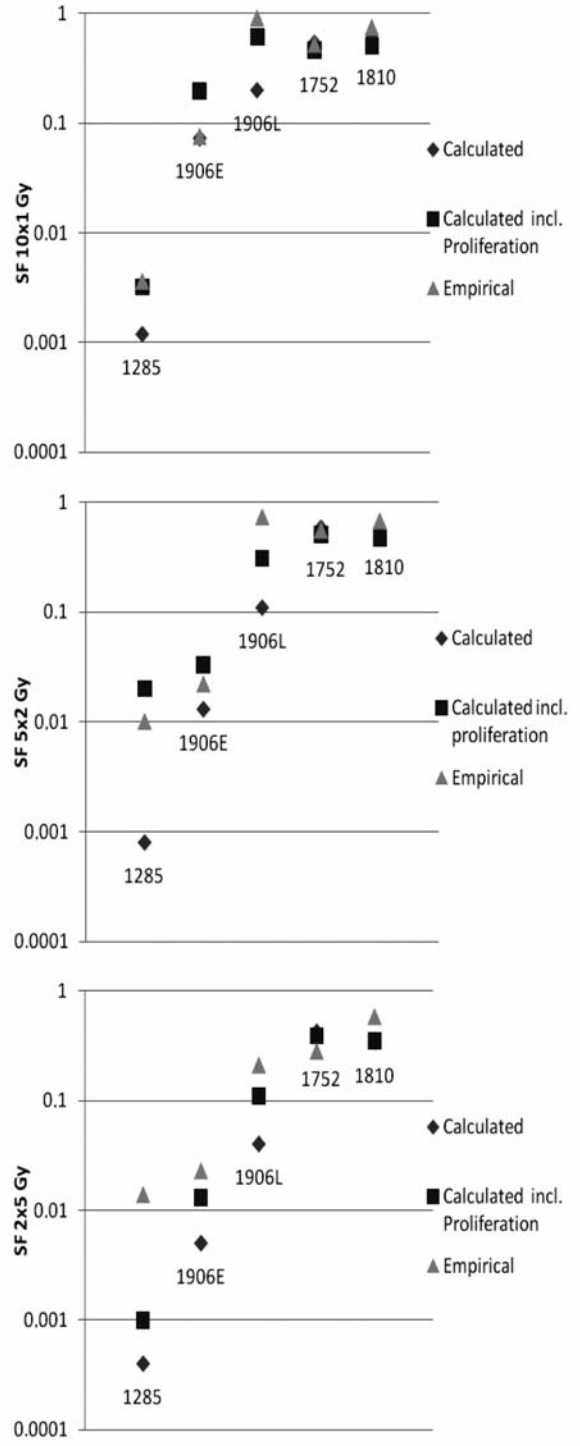

Figure 1. *Correspondence between calculated SF with or without doubling time correlation coefficient and empirical SF after fractionated irradiation.*

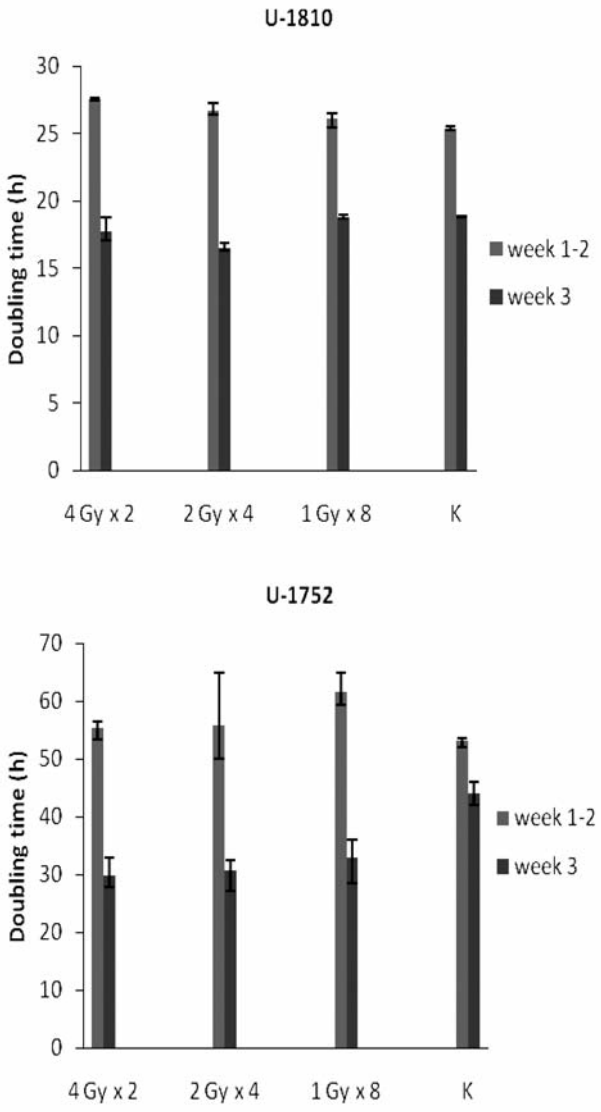

Figure 2. *Doubling time the first two weeks compared with the third week during subcultivation in different fractionation regimes and control.*

irradiation was compared with the cell doubling time for the consecutive week, an increased proliferation was seen in all fractionations in both cell lines (Figure 2).

Discussion

Irradiation induces DNA damage that might be repaired, conserved or lead to apoptosis. Factors determining radioresponsiveness *in vitro* are the inherent characteristics of the cell, as well as external factors such as the culture medium and the growth conditions of cells. Inherent radioresponsiveness depends on many genetic factors governing the degree of damage induction, repair capacity and the capacity of the cell to survive despite damage. Fractionated irradiation presents a still more complicated situation. Induction and accumulation of sub-lethal damage, redistribution of cell cycle and proliferation during the

Table III. *Cell growth during irradiation demonstrated by relative cell number on day 4 and 7 respectively.*

Cell line	Irradiation dose	Empirical		Calculated	
		Day 4	Day 7	Day 4	Day 7
U-1752	Control	1.37	6.03	1.51	3.46
	10×1 Gy	1.02	3.99	0.73	1.57
	5×2 Gy	1.22	3.54	0.75	1.72
	2×5 Gy	1.02	2.55	0.54	1.23
U-1810	Control	2.59	5.67	1.99	5.61
	10×1 Gy	2.06	3.51	1.04	2.71
	5×2 Gy	1.98	4.71	1.00	2.52
	2×5 Gy	1.61	2.64	0.61	1.74

treatment period are important extra factors to consider, not to mention the problems of hypoxia and re-oxygenation.

The cellular and molecular biological processes after fractionated irradiation are extremely complicated, however, the effect of fractionated irradiation is considered to be fairly predictable looking from a population-based point of view, otherwise there would be little logical use of fractionated irradiation in the clinical setting. This implies that it is possible to predict how many patients can be cured in a group of patients with the same diagnosis who are given a certain radiotherapy treatment. However in practice, prediction and optimisation of the anti tumoural effect leave a lot to be desired. There is currently no means by which individual response to radiotherapy can be. Nonetheless twenty years ago, it was stated by Fletcher, introducing the first ESTRO Regaud lecture by Lester Peters, that the question whether it is possible to predict radiotherapy effect on tumours by a test is the Holy Grail of radiotherapy (13, 14).

Standard fractionation schedules with standard total doses are, as a rule, used by radiotherapists in curative settings and modifications are only performed to a certain extent. Lower doses are, as a rule, given for malignant lymphomas and seminomas and higher doses are given for squamous cell carcinomas. Few studies have performed characterisation of radioresponsiveness in individual tumours, which is not only an uncertain task but also consumes a much time and resources.

The importance of proliferation during fractionated irradiation is evident and many studies with modified fraction schedules have been performed (15-18). The standard alpha-beta isodose concept does not consider proliferation, even if such a factor could be added. An analysis by Withers *et al*. indicated that accelerated proliferation does not begin until three to four weeks after the beginning of irradiation (4). However, the study of Saunders *et al*. (15), with accelerated hyperfractionated irradiation from the start of treatment according to the CHART regimen, demonstrated an advantage of this approach in lung cancer and is comparable to results of other radiotherapy regimes in carcinoma of the head and neck (16). Studies from other groups have presented partly conflicting results, yet the impression is that dose and fractionation are important issues and that the balance between tumour control and treatment toxicity is delicate.

The concept behind the experiments performed in this study was to investigate if adding proliferation as a parameter enhances the accuracy of a model using inherent radiosensitivity, SF, to predict SF after fractionated irradiation. This was tested in different cell lines with different radiobiological characteristics.

When searching databases for studies of SF after fractionated irradiation measured empirically, only a few, dated studies can be found. Earlier results have demonstrated that the inherent radioresponsiveness of tumour cell lines varies considerably within most categories of human tumours. The current results with a very radiosensitive (U-1285), a moderate sensitive (U-1906E), a moderate resistant (U-1906L) and two very resistant cell lines (U-1752 and U-1810) indicate that the individual inherent radio- responsiveness is a major factor determining the effect of fractionated irradiation.

The fraction size was of some importance and the most radioresponsive SCLC cell line (without a shoulder in its radiosensitivity curve) was more sensitive to low-dose than to high-dose fractions. In the two non SCLC cell lines (with large shoulders), the high-dose fraction regimes were more successful.

Pre-treatment tumour SF has been found to be an independent prognostic factor in a clinical material. In a study by West *et al*. it was found that SF2=0.42 was a threshold point for local control in cervical carcinomas treated with irradiation (19), and a similar result was found for head and neck carcinomas in another study (20).

In the two very radioresistant cell lines, U-1752 and U-1810, predicted survival was equally accurate without use of a proliferation factor. The results were at first confusing, since it was observed that these cell lines proliferated during the treatment period. It was even possible to demonstrate an accelerated proliferation a couple of weeks after irradiation (Figure 2). The basis of the extrapolation method is to measure proliferation after irradiation. SF at any given time in a treated cell line is related to unirradiated control during normal proliferative conditions. Proliferation is, at any given time along the extrapolated growth curve, represented by the steepness of the curve. If a proliferation factor is introduced, this should consequently be done in both treated and control cell lines. Accordingly, when using the extrapolation method, there will be little difference in predicting SF with or without a proliferation factor.

In a previously published study applying the model to clinical material of patients with head and neck carcinomas, a capacity to predict local control in patients treated with fractionated radiotherapy was demonstrated (21).

The current observations lead one to conclude that adding proliferation to intrinsic radiosensitivity in this model seems to add to the model's accuracy to predict SF after fractionated irradiation, as was demonstrated in the cell lines where SF was measured by the clonogenic assay method.

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