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Noninvasive *in vivo* whole body luminescent analysis of luciferase labelled orthotopic prostate tumours

Nadia El Hilali, Nuria Rubio, Jerónimo Blanco *

Dpto. Patología Molecular y Terapéutica, (IIBB), Centro de Investigación y Desarrollo (CSIC), 08034 Barcelona; Spain

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Abstract

Light photons refracted through living tissues can be used to noninvasively monitor the proliferation of cells expressing bioluminescent markers. We demonstrate the use of a luminometer for noninvasive *in vivo* whole body luminometric analysis (*in vivo* WBLA) of luciferase-expressing prostate tumours growing orthotopically in nude mice, and thus hidden from visual inspection. In this procedure, the intraperitoneally (i.p.) inoculated luciferin, the luciferase substrate, reaches the tumours rapidly and the light photons generated by the tumour are recorded by placing the anaesthetised mice in the detection chamber of a luminometer, over the detector slot. We show that the number of recorded light photons is proportional to the tumour mass and to the luciferase activity recorded *in vitro*. The procedure is applied to demonstrate the use of paclitaxel as an antineoplasic agent with its well characterised antiproliferative activity.

Keywords: Noninvasive monitoring, Luciferase; Prostate cancer; Orthotopic tumours; Tumour therapy; Nude mice

1. Introduction

Animal models are an important tool for the study of tumour biology and an obligatory step for the development and screening of new antineoplastic therapies. Generally, experimental tumours are inoculated subcutaneously (s.c.), due to the ease with which subsequent growth and a possible drug effect can be followed visually. Recently, it has been recognised that organ-specific orthotopic implantation reproduces better the characteristics of tumour growth and metastasis capacity [1–3]. Thus, the orthotopic organ has become the preferred implantation site for experimental tumours. However, in the case of primary tumour growth assessment, the frequently hidden nature of orthotopic organs precludes direct monitoring, forcing the researcher to sacrifice multiple animals for each time-point in order to significantly assess the effect of drugs.

Traditional models of tumour spread, based on counting metastasic nodules [4], the survival time of experimental animals [5] or the histological analysis of colonised tissue sections to count tumour cell colonies [6] are semi-quantitative and costly. Recently, the introduction of luciferase as a tumour cell reporter has allowed very sensitive and quantitative detection [7] of the tumour cell burden in experimental animal tissue homogenates [8,9]. Cancer cell lines permanently expressing either the firefly luciferase [10] (Luc) or the green fluorescent protein [11] (GFP) have also been used successfully to noninvasively monitor local tumour growth and the development of metastasis in different organs of living mice [12]. In these approaches, highly sensitive low light video equipment is used to capture and quantify the small fraction of photons that are

* Corresponding author. Present address: Centro de Investigación Cardiovascular, CSIC Sant Antoni María Claret 167, 08025, Barcelona, Spain. Fax: +93 556 5559.
E-mail addresses: Jblanco@csic-iccc.santpau.es, jbfpmt@cid.csic.es (J. Blanco).

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emitted by photoprotein tracers and escape through living tissues, providing spatial and temporal information about tumours [13,14]. In this context, a significant advantage of bioluminescent over fluorescent markers is the almost total lack of background noise due to the absence of endogenous chemioluminescent reactions in mammals [15]. However, due to the high light absorption by tissues and to the spatial arrangement of the sample organs relative to the light gathering equipment, *in vivo* Bioluminescent imaging is less sensitive than luminometric analysis of tissue homogenates [16].

Tumour images are not always required to follow the development of luciferase-labelled tumours in living animals and the researcher would often be satisfied with repeated noninvasive bulk measurements of light activity to monitor changes in tumour size in the same animal. Such measurements could be obtained by "*in vivo* Whole Body Luminometric Analysis" (*in vivo* WBLA) using a sensitive luminometer to detect photons that transverse the living animal tissues and escape to the exterior following luciferin inoculation. Such an alternative approach, would also reduce the number of experimental animals, while still maintaining the high data consistency and significance resulting from repeated measurements in the same animal.

Our current experiments demonstrate the double use of a luminometer, an affordable and readily available tool, to noninvasively monitor the growth of internal tumours and to quantify, at the end of the experiments, the number of luciferase-labelled tumour cells in primary tumour homogenates. Using this procedure, we show that paclitaxel (Taxol®), a microtubule-active drug that interacts with tubulin or microtubules increasing their polymerisation [17,18] was effective in reducing the size of luciferase-expressing PC-3M.-Sluc human prostate tumours growing orthotopically in nude mice.

2. Materials and methods

2.1. Cells and cell culture

Highly metastatic androgen-independent human prostate cancer cells PC-3MM2 were obtained from Dr I. Fidler (M.D. Anderson Cancer Center, University of Texas) and were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12, supplemented with 10% v/v foetal bovine serum (FBS).

2.2. Transfection

PC-3M.Sluc cells were generated by transfection of PC-3M cells with the pRC/cytomegalovirus (CMV) expression vector (Invitrogen, San Diego, CA) containing the firefly (*Photinus pyralis*) luciferase gene coding region, and were cloned as described in Ref. [16].

2.3. Experimental tumorigenesis

Six-week-old male BALB/c homozygous nude mice (nu/nu) were purchased from IFFA-CREDO (L'Arabresle, France) and maintained in a specific pathogen-free environment throughout the experiment. Animals were kept for at least one week in the facility before experimental manipulation.

To generate intramuscular tumours, PC-3M.Sluc (1×10^6) luciferase-expressing tumour cells in 100 μl of culture media without FBS were injected intra-muscularly (i.m.) into each of the mouse thighs.

For surgical manipulation to generate orthotopical tumours, mice were anaesthetised by an intraperitoneal (i.p.) injection of a mixture of droperidol (Roche, Basel, Switzeland) 6 mg/kg and midazolam (Rovi S.A, Madrid, Spain) 12 mg/kg and then inoculated in each prostatic lobe with 5×10^5 luciferase-expressing tumour cells suspended in 50 μl of culture media without FBS as described in Ref. [1].

2.4. Cytostatic treatment

Five days after inoculation, mice were randomly separated in to two groups. Treated animals were inoculated i.p. daily with 10 mg/kg paclitaxel (Taxol® Bristol-Myers Squibb, New York, NY) dissolved in dehydrated ethanol and Cremophor, and diluted in 0.9% w/v NaCl solution during an 11 day period. Control animals were inoculated with 0.9% w/v NaCl solution following the same treatment protocol. Mice were sacrified after the treatment period, necropsies were performed and primary tumours harvested to determine the luciferase activity in the tissue homogenates.

2.5. Time course of light emission and in vivo WBLA

Immediately following anaesthesia and i.p inoculation of 150 μl of an aqueous solution of D-luciferin 100 mg/kg, mice bearing PC-3M.Sluc prostate tumours were placed in the detection chamber of a Turner Design luminometer model 20/20 with the abdomen directly facing the detector slot. Luminometer readings were registered during a 60 s interval, every minute during a 17 min interval. The optimum time range for light production was from 5 to 9 min and the interval of time between 6–8 min after the luciferin injection was chosen as the standard for the *in vivo* determinations. The measurements of light were expressed as Relative Light Units (RLUs). In all of the determinations, the background signal, from mice bearing no tumours was substracted from the readings.

2.6. Luminometer detail

The Turner Designs 20/20 Luminometer is a compact luminometer designed for use in molecular and cellular biology assays using firefly luciferase. The instrument is equipped with a photomultiplier tube designed to detect light in the 370–630 nm range. Photons entering the detector are converted into electrons and amplified by the photomultiplier resulting in a signal directly proportional to the number of incident photons. Luminescence is not indicated in physical units of measurement, but rather in ''RLU'', that are digitally displayed.

The sample chamber is designed with a holder adaptable to accept cuvettes of different sizes and a lid ensuring perfect light closure. In our experiments, the sample adaptor was removed and the mice were accommodated in the sample chamber placing the tumour site directly over the detector slot. Measurements were carried out at ambient temperatures.

2.7. Noninvasive in vivo WBLA to monitor tumour growth

In vivo measurement of luciferase activity in prostate tumours of control- and Paclitaxel-treated mice was performed every 5 days, and at days 4, 7, 13 and 17 in the case of i.m. tumours. Prior to observation, the animals were anaesthetised by an i.p injection of a mixture of droperidol (Roche, Basel, Switzeland) 6 mg/kg and midazolam (Rovi S.A, Madrid, Spain) 12 mg/kg and then i.p. inoculated with 150 μl of an aqueous solution of D-luciferin 100 mg/kg. The mice were then positioned on the sample chamber of a Turner Designs luminometer model TD 20/20, with the abdomen (orthopic tumours) or thigh (i.m tumours) directly facing over the detector slot. Light emission was recorded during a 60 s time period starting 6 min after the luciferin inoculation. The *in vivo* WBLA measurements were expressed as RLUs.

2.8. Cell and tissue homogenates

Lysates from cultured cells were prepared by performing one freeze-thaw cycle in RLB (Reporter Lysis Buffer, Promega Corporation). Tumour extracts were prepared by mechanical homogenisation of the tissue in RLB at a 1:1 ratio (weight/volume), using a Ultra-Turrax T-25 tissue homogeniser (janke and Kunkel, Staufen, Germany), followed by centrifugation at 25000*g* for 45 min and 4 °C to remove the insoluble particles.

2.9. In vitro luciferase assay

Luciferase activity in cell extracts or tissue homogenates was measured by chemiluminiscence, using the standard luciferase assay kit (Promega Corporation, Madison, WI). Production of light was measured using a Turner Designs luminometer model TD 20/20, after the addition of 100 μl of luciferase assay reagent (Promega corporation) to 20 μl of cell lysate or tissue homogenate. Light detector measurements are registered as RLUs, then expressed in tumour cell equivalents (TCEs) as described below.

2.10. Counting of tumour cell equivalents and sensitivity of detection

TCE convention was adopted for practical reasons as it provides an approximate idea of the number of tumour cells present in the tissue homogenates. To calculate the number of TCEs present in a given mouse tissue, first, the background light, in RLUs, generated by a blank sample consisting of an aliquot of the same tissue homogenate devoid of luciferase-expressing cells, was determined. Second, the blank value was subtracted from the RLUs produced by a 20 μl aliquot of tissue homogenate and the resulting number (the amount of RLUs due to the tumour cells present) was then divided by the slope (RLU/tumour cell) of a standard curve, generated in the corresponding tissue homogenate using known numbers of light-emitting tumour cells. Tissue homogenates in which the luciferase activity of the 20 μl aliquot exceeded the detector range (9999 RLU/min) were adapted to the counter range by diluting with RLB. Minimun assay sensitivity was defined as the number of TCEs required to generate a quantity of light equivalent to two standard deviations (SD) of the background chemiluminescence. Measurements equal or below the defined minimum assay sensitivity were considered equal to zero TCEs.

2.11. Statistical analysis

The significance of the difference between the treated and control groups was determined using the Student's *t* test.

3. Results

3.1. Time course of in vivo light emission after i.p. inoculation of luciferin

In order to establish the optimal time interval for accurate *in vivo* WBLA, mice bearing orthotopic PC-3M.Sluc tumours were inoculated i.p. with D-luciferin immediately following anaesthesia, and the light emitted by the tumour recorded as described in Section 2. Light

produced by the tumours was detected in the first minute following inoculation, and increased rapidly during the following 5 min (Fig. 1). Two separate measurements were carried out. Light emission reached a maximum value at 6 min and remained stable during the period between 6 and 10 min post-luciferin administration. The interval between 6 and 8 min post-D-luciferin injection was therefore chosen to record the light emitted from the luciferase-labelled tumours in our experiments.

3.2. *In vivo* monitoring of tumour development and the effect of paclitaxel treatment

To monitor primary tumour development, a group of four mice were inoculated with 1×10^6 PC-3M.Sluc cells in each thigh and analysed for light emission *in vivo* at the indicated times (Fig. 2(a)). For *in vivo* WBLA, the mice were anaesthetized, inoculated i.p. with 150 μl of an aqueous solution of D-luciferin, 100 mg/kg and placed in the detection chamber with the thigh placed directly over the detector slot. Both tumours were counted sequentially. The graph in Fig. 2(a) shows the progressive increase in the light emission resulting from tumour growth. Light emitted by the tumours was easily measured by day 4 and earlier. In contrast, tumours could not be detected by visual inspection until day 10. Similar results were produced with another group of animals (data not shown).

To determine if *in vivo* WBLA could be used to monitor growth of internal tumours, 8 immunodepressed mice were orthotopically inoculated with 5×10^5 PC-3M.Sluc human prostate tumour cells in each prostatic lobe. At day 5 after the orthotopic implantation, the animals were randomly divided into two equally sized groups, control and treated, and inoculated i.p. daily with either 10 mg/kg paclitaxel (treated) or 100 μl of saline solution (control), according to the treatment protocol shown in Fig. 3. *In vivo* WBLA was performed as described in Materials and methods section, by placing the mice in the detection chamber of the luminometer with the abdomen directly over the detector slot.

Fig. 2(b) shows the total amount of light recorded from 4 control and 3 treated mice (a fourth mice from the treated group died at the end of the experiment, just before the last determination) at the indicated times, expressed as RLUs. Light from small, non-palpable tumours was already detectable in the first measurement at day 10. In control mice, the light emission capacity of the tumours increased linearly with time. Except for the first measurement, where there was no significant difference between control and treated animals, $P_1 = 0.26$, in the second and third measurements paclitaxel-treated animals produced not only significantly lower amounts of light, but the significance of the difference between the control and treated groups increased with the duration of the treatment, $P_2 = 0.043$ and $P_3 = 0.035$, respectively.

3.3. Relationship between the amount of *in vivo* recorded light and tumour mass

At the end of the paclitaxel treatment, following the last *in vivo* WBLA, the animals were immediately sacrificed and the prostate tumours harvested and weighed. As was also the case for the *in vivo* WBLA values, the mass of the paclitaxel-treated tumours was significantly lower ($P = 0.0028$) than that of the control tumours, as can be observed from the clearly separated

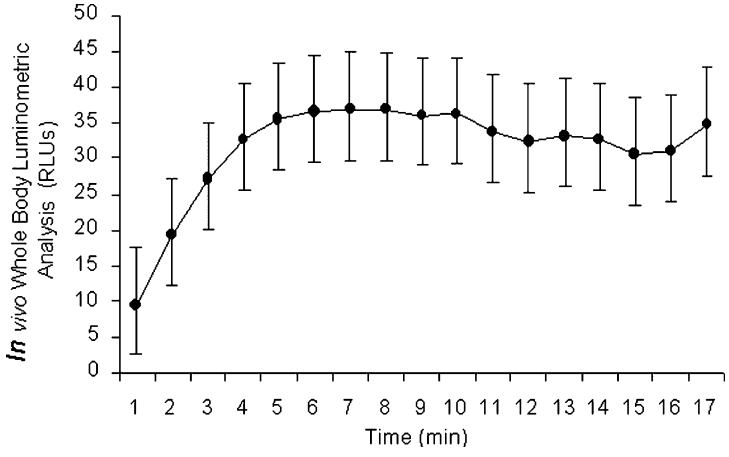

Fig. 1. Time course of light emission *in vivo*: The graph shows light intensity, recorded by *in vivo* whole body luminescent analysis (WBLA), versus time post-intraperitoneal (i.p.) luciferin inoculation, from luciferase-labelled prostate tumours, expressed in relative light units (RLUs). The datapoints represent the means ± standard deviations (SD) ($n = 2$). The maximum at 6–8 min post-substrate inoculation was chosen as the optimum interval for light recording.

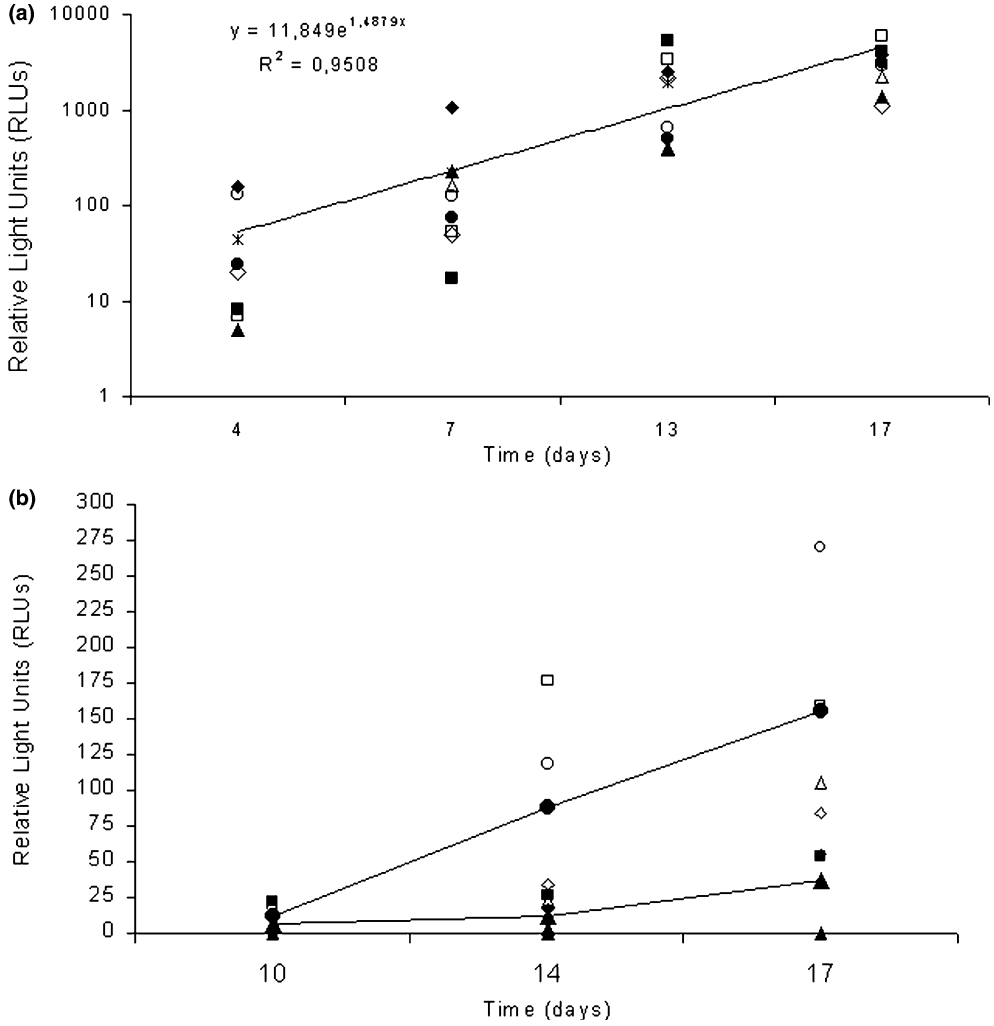

Fig. 2. *In vivo* WBLA of tumour growth: (a) *In vivo* monitoring of intramuscular (i.m.) tumour growth: Mice were inoculated (i.m.) with 1×10^6 PC-3M.Sluc cells in each thigh. After a period of time for tumour development, measurements were performed as described in Section 2, at the indicated days. Light emitted by i.m. tumours, is expressed as relative light units (RLUs). Each symbol represents an individual tumour. Solid line represents the fitting function described by the corresponding equation, symbols represent average values ($n = 8$). (b) *In vivo* monitoring of tumour growth in control- and paclitaxel-treated mice: The graph shows the effect of paclitaxel on orthotopic prostate tumours. Empty symbols represent individual control mice; solid symbols represent individual treated mice. Solid lines with corresponding symbols represent average values ($n = 4$). Day 10, $P > 0.05$; days 14 and 17, $P < 0.05$.

distributions of value in Fig. 4(a). The graph also shows that the relationship between the amount of light recorded by *in vivo* WBLA and the mass of the tumours can be described by a power function, $R^2 = 0.88$.

3.4. Relationship between in vivo WBLA and luminometric quantification of tumour homogenates

The tumours harvested after paclitaxel treatment were homogenised and analysed using the luminometer to determine their total contents in TCEs, which is defined as the number of tissue culture cells that would produce the same amount of light *in vitro*. The relationship between *in vivo* WBLA and the number of TCEs detected *in vitro* in the tumour homogenates can also be described by a power function, with a determination coefficient of $R^2 = 0.9125$ (Fig. 4(b)).

The effect of paclitaxel was manifested by the significance of the difference in the value of the three

Table 1
Significant results

	In vivo WBLA (RLUs)	*In vitro* luciferase activity (TCEs)	Tumour weight (mg)
P value	0.0348	0.0547	0.0028

Significances of the differences between control and treated groups for the three different measured magnitudes. *P* value by Student's *t* test. $n = 3$ for treated group; $n = 4$ for control group.

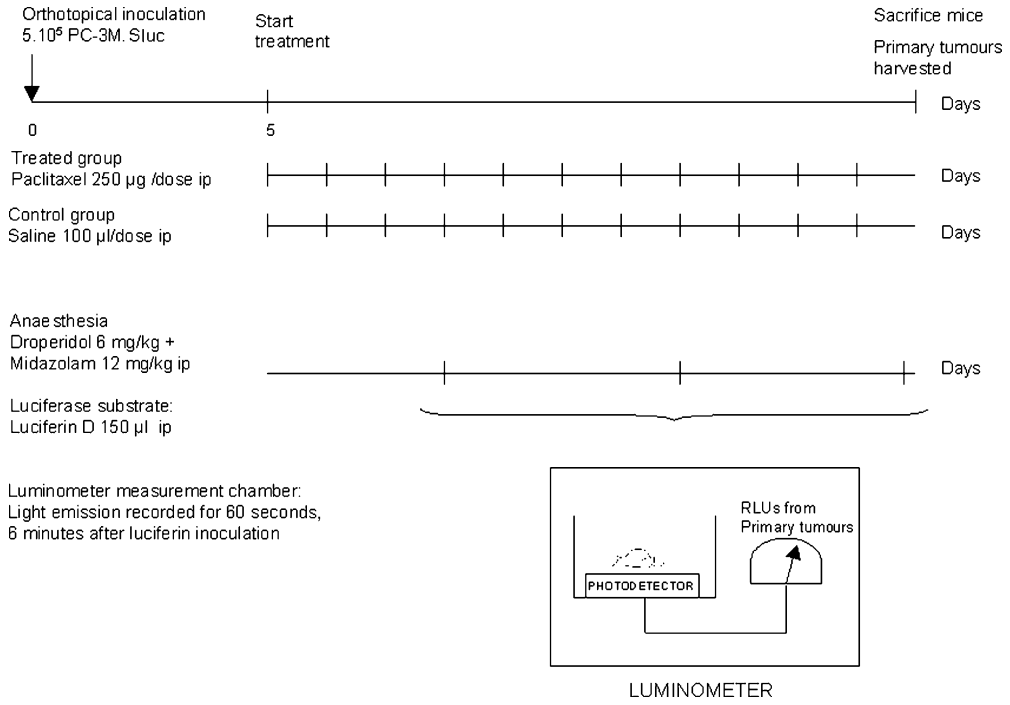

Fig. 3. Protocols for treatment and *in vivo* whole body luminometric analysis (*in vivo* WBLA). The line diagram represents the protocol followed for the administration of treatments (paclitaxel and saline solution). Vertical bars correspond to each day of treatment administration, starting at day 5 post-inoculation of tumour cells. The lower panel of the figure illustrates the mouse in the luminometer receptacle and the steps followed for *in vivo* WBLA. Immediately following i.p administration of anaesthesia and D-luciferin, the animals were placed in the detector chamber with the abdomen directly facing the detector slot. At 6 min post-luciferin administration, the light emitted by the tumour was recorded as relative light units (RLUs).

magnitudes, *in vivo* WBLA, $P_1 = 0.0348$; TCEs in tumour homogenates, $P_2 = 0.0547$ and tumour mass, $P_3 = 0.0028$, between the control and treated tumours (Table 1). As expected, the difference between the control- and paclitaxel-treated mice for the ratios: *in vivo* WBLA/tumour mass, TCEs/tumour mass and *in vivo* WBLA/TCEs was non-significant since the three parameters are, more or less, direct estimates of the tumour cell number.

4. Discussion

Bioluminescent proteins are convenient tumour cell tracers that allow *in vitro* quantification of small numbers of metastatic cells colonising target organs. Since a small fraction of the photons generated by such photoproteins *in vivo* transverses living tissues, tumour growth can be easily and noninvasively recorded in images that provide spatial and quantitative information about the tumours [16]. It has been recognised that bioluminescent imaging (BLI) technologies have advantages over traditional methods, such as tumour mass determination and histological analysis of tissue sections for tumour monitoring. The possibility of obtaining real-time information by repeated noninvasive imaging of the same animal results in significant gains in data consistency and savings in animal resources, a situation that is particularly relevant during the monitoring of internal tumours not accessible to direct visual observation.

Since tumours are composites of a variety of host cells and other stromal components that may vary considerably during tumour development [5], tumour mass or size are only indirect measurements of the tumour cell number. Procedures based on imaging light photons emitted by the tumours, as reporters of an enzymatic activity directly proportional to the number of tumour cells are generally more reliable indicators of the tumour cell number. However, the need for expensive imaging equipment can be a significant limitation for the widespread use of photon-based noninvasive methods.

In the present work, we demonstrated the use of a luminometer for non-invasive *in vivo* WBLA to measure the light generated by luciferase-labelled tumours. Our results showed that human prostate tumours PC-3M.Sluc, growing orthotopically in the abdominal cavity of nude mice and therefore inaccessible to direct visual observation, can be monitored effectively using a widely available and relatively inexpensive bench-top luminometer. This has the potential advantage of being able to continuously monitor the growth behaviour of each tumour in the same animal over time minimising

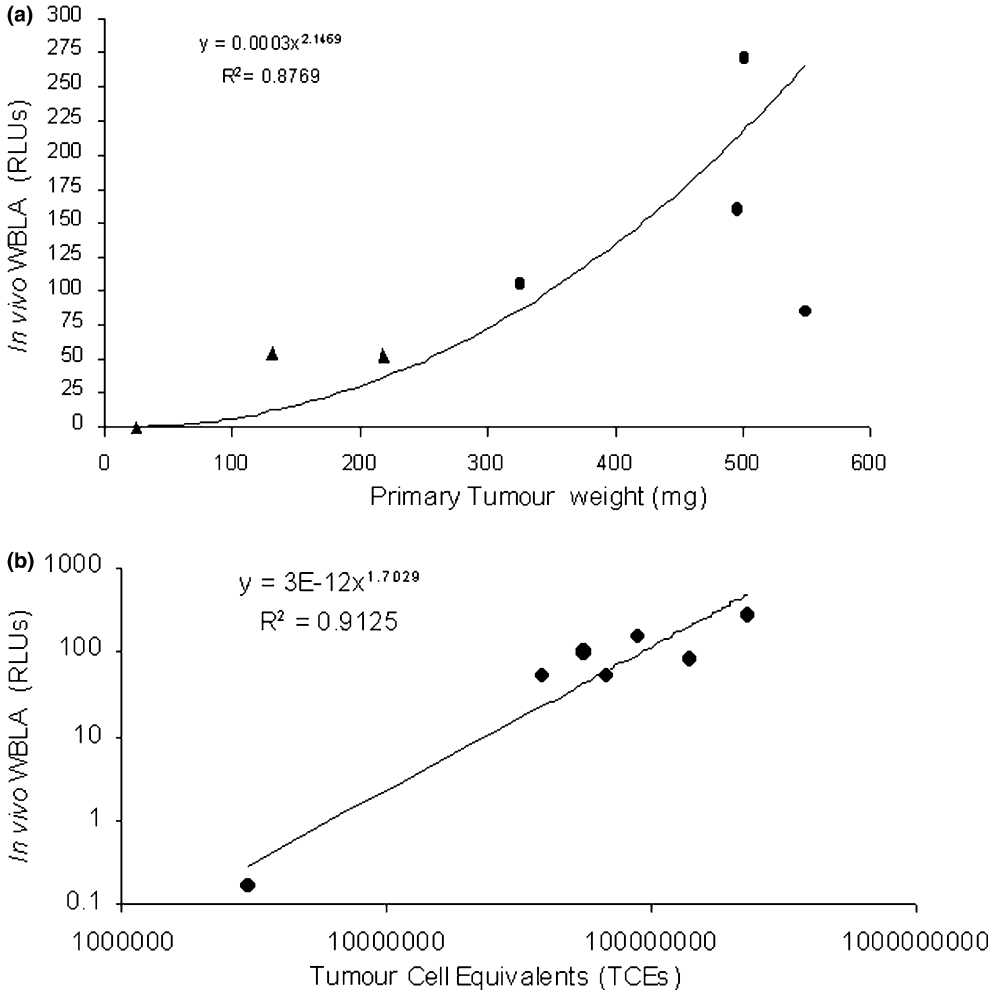

Fig. 4. Relationship between *in vivo* WBLA and tumour mass or number of tumour cell equivalents (TCEs), at the end of the experiment. The solid line shows the fitting functions described by the equations in the insets. (a) Relationship between primary tumour weight (mg) and *in vivo* WBLA (RLUs). (▲) treated mice and (●) control mice. (b) Relationship between TCEs from tumour homogenates and *in vivo* WBLA (RLUs).

the effects of inter-animal and sample variations and resulting in better data consistency and reproducibility using a smaller number of animals.

We show that luciferin inoculated i.p. readily reaches prostate tumours in live animals and that the luciferase-labelled cells in the tumours generate light photons, with an intensity that is at its maximum at 6 min post-inoculation and that can be measured by placing the live animal in the sample chamber of a luminometer.

In vivo WBLA showed a significant reduction ($P = 0.035$) of the light produced by the paclitaxel-treated tumours, relative to controls, that was proportional to the treatment duration, in accordance with the known antiproliferative effects of paclitaxel.

By the end of the treatment, paclitaxel-treated tumours were significantly smaller than control tumours, according to any one of the three different measuring criteria. *In vivo* WBLA measurements were also well described by power functions of either tumour mass $R^2 = 0.88$, or, more importantly, of the luciferase activity in tumour homogenates $R^2 = 0.91$. Although the relationship between tumour mass and luminescence measurements appears to be described better by a power function than by a linear one, we can only speculate on the causes producing such a deviation from linearity. In the first place, *in vivo* measurements are affected by the inhomogeneities in the physical properties of the tissues in the path of light, such as differences in tissue densities. More difficult to evaluate, but also likely to be important and interesting, are the effects due to the biological behaviour of the tumours themselves. While tumour mass is a measurement of all the tumour components, including stroma and tumour-induced vasculature, luminescence measurements account only for the number of tumour cells. Thus, it is possible that the observed departures from linearity between total tumour mass and total number of tumour cells, as measured by luminescence, may reflect important differences between the growth dynamics of the stromal component, which is provided by the host, and the strictly tumoral

(luminiscent) component during tumour development (data not shown).

External recording of photons is well suited for monitoring cell proliferation. What is more, our results indicate that *in vivo* WBLA can be standardised by luminometric analysis of tumour homogenates and tumour mass measurements after the animals are sacrificed.

Our results show that proliferation of luciferase-expressing tumours and the changes resulting from treatment with antineoplasic agents can be effectively and noninvasively monitored using a luminometer. Use of this low cost *in vivo* WBLA procedure should allow the gathering of cell proliferation data repeatedly in the same animal and with little harm, from tumours that are hidden visually, thereby improving data consistency and saving resources.

Conflict of interest statement

None declared.

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