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ON THE POTENTIALITIES OF NUCLEAR GAMMA RESONANCE (MÖSSBAUER EFFECT) SPECTROSCOPY AS A NEW, LOW DOSE APPROACH TO CANCER RADIATION THERAPY

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The extension of a new, low dose, Mössbauer spectroscopy approach to cancer radiation therapy to "easy" isotopes other than Iron-57 is outlined. New in vitro studies using a number of cancer cell lines are reported.

1. Introduction

High doses (1 to 10^2 Gy, 10^2–10^4 rad) are common to conventional cancer radiation therapy since only a small fraction of the total of the absorbed radiation

Table 1
The effect of treatment with drug ^{57}Fe(III)·Bleomycin and *M*ōssbauer radiation on cell line *HTB26* (*H*uman *B*reast-*C*ancer *T*issue) (from ref. [1])

Experiment	Proliferation time (hrs)	Proliferation relative to no-treatment		
		No-treatment	Drug alone	MIRAGE (vel = 1.5 mm/s)
1	192	100	79	32
2	193	100	71	24
3	168	100	82	0
4	168	100	67	17
5	144	100	88	25
6	120	100	92	33
7	120	100	100	20
		mean =	83	21
		std dev =	11	10

Table 2
Effect of drug ^{57}Fe(III)·Bleomycin (no radiation) on C3H mice bearing spontaneous mammary adenocarcinomas (from ref. [1])

Initial [a] tumor volume (mm^3)	Final tumor volume (mm^3)	Final vol/ initial vol
320	1950	6.09
117	3415	29.2
62	893	14.3
266	4713	17.7
130	5936	45.7
356	2028	5.70
520	1972	3.79
255	2924	11.5
236	1349	5.72
220	3024	13.7
94	2371	25.2
90	190	2.12
116	480	4.14

avg = 14 ± 12 (std dev)
[a] Tumor volume was calculated as follows: $(L \times W^2)/2$.

leads to double strand breaks in rapidly developing DNA. Mills et al. [1] have recently reported a new low dose (10^{-5} Gy) therapy based on NGR. The essence of this approach is as follows: (I) A cytotoxic drug or other chemical agent known to intercalate between the base pairs of DNA or otherwise adduct to DNA is labeled with an NGR active isotope, e.g. ^{57}Fe ligated by bleomycin [2,3]. (II) The Mössbauer spectrum of (I) so labeled is determined. (III) Micromolar doses of I are administered to cell cultures (in vitro) or actively growing mouse tumors (in vivo). (IV) The latter are irradiated at a *constant Doppler velocity* corresponding to one of the known NGR resonances of labeled (I). Significant cell growth diminution and tumor ablation are observed. (1) This is evident from consideration of tables 1, 2, and 3 (all taken from ref. [1]) where "MIRAGE" is an acronym for *M*össbauer *I*sotopic *R*esonant *A*bsorption of *G*amma *E*mission.

2. Rationale

The rationale for this approach is based in part on the high magnitude of NGR cross sections compared to other mechanisms (photo-electric and compton scattering) for γ-ray attenuation in tissue, $\sigma_{NGR}/\sigma_{photo} \approx 200/1$. In addition, there are the facts that the base pairs holding DNA strands together are joined by hydrogen bonds ($\sim 0.1 \sim 0.5$ eV) while the chemical bonds of the polynucleotide itself are ~ 4 eV. On the other hand, the Auger electrons with energies ~ 5 keV

Table 3
Effect of ^{57}Fe(III)·Bleomycin with Mössbauer radiation on C3H mice bearing spontaneous mammary adenocarcinomas (from ref. [1])

Initial [a] tumor volume (mm^3)	Final tumor volume (mm^3)	Final vol/ initial vol
579	291	0.503
515	0.5	0.001
256	41	0.163
259	104	0.402
157	187	1.19
157 [b]	1403	8.9
62	97	1.56
811	578	0.713
304	1062	3.49
277	593	2.14
226	128	0.566
236	663	2.81
243	374	1.54
82	193	2.35

avg = 1.9 ± 2.2 (std dev).
[a] Tumor volume was calculated as follows: $(L \times W^2)/2$.
[b] Tumor was difficult to immobilize because of its location on the chest wall.

for ^{57}Co and ^{57}Fe and the large positive charge build-up accompanying the cascade initiated by internal conversion on re-emission of NGR γ-rays are more than required for scission of hydrogen bonds and the all important sugar-phosphate links of DNA chains. This leads to lethal multiple *double strand* breaks.

3. Extension to other NGR active isotopes

In the remainder of this paper, the advantages and problems of extending this method of cancer radiation therapy to other NGR isotopes are briefly considered in light of the constraint that while the NGR source may be cooled, the patient must necessarily remain at ambient T. New in vitro studies completed subsequent to the work of ref. [1] are also reported.

Conventional radiation therapy works through a combination of (1) direct photon induced DNA strand scissions along with the radiation production of (2) chemical free radicals that, for instance, are capable of hydrogen atom abstraction and ultimate destabilization of the DNA backbone. As such this therapy requires high radiation doses owing to the small absorption cross sections involved. It is emphasized that the present approach is based in part on (3) Coulombic effects accompanying much higher probability resonant absorption and re-emission of Mössbauer gamma rays. This occurs in NGR drugs already

intimately associated with the DNA of neoplastic tissue. The low dose effect of ref. [1] may, in fact, be a synergistic combination of (2) and (3).

Thus the "Mössbauer drug" must be relatively non-labile with respect to the NGR isotope and hopefully have a high binding constant and frequency (drug/DNA base pair quotient) of attachment to developing DNA. As these are "chemistry problems," (albeit important), they will not be considered further herein save to say that the chemistry of such NGR-isotope delivery systems is already well established. There are, for example, the porphyrins which tend (for reasons not yet fully understood) to selectively, strongly bind to the DNA of neoplastic tissue and numerous other cytotoxic pharmaceuticals (e.g. mitomycin) that have been extensively investigated in a cancer chemotherapy context. In the present context, one must derivatize these with a Mössbauer active isotope.

There then remains the choice of appropriate NGR isotope. Firstly, for deep-seated tumors, one requires an energetic gamma ray of high half depth ($D_{1/2}$) but not so energetic as to lead to a vanishing recoil-free fraction. The latter would preclude ambient temperature (*absorber*) resonant absorption. Secondly one desires a convenient isotope half life for practicable clinical use along with a high natural abundance to increase efficiency. Failing the latter, the isotope should be readily commercially available for isotopic enrichment of the delivery drug. Finally, since the mechanism of the present approach is believed, in part, to be related to coulombic effects arising from internal conversion and Auger cascade (all occurring within a very small time interval), one desires a large total internal conversion coefficient, α_T or a large $\sigma_{NGR\text{-}Auger}$ where $\sigma_{NGR\text{-}Auger} = \sigma_{NGR} \times \alpha_T$ (table 4). The Mössbauer active isotopes embodying in one way or another the forgoing criteria are given in table 4. These also allow for significant resonance effects with either ambient temperature absorber and source or at least source cooling (77 or 4.2 K). We conclude this section by stating that we are currently pursuing the development of these isotopes in the present context in our laboratories. In addition to in vitro and in vivo studies, investigations of P-32

Table 4
Parameters [a] of some "easy" NGR isotopes

	E (keV)	α_T	I.A. (%)	$T_{1/2}$	$\sigma_{NGR\text{-}Auger}$ (cm^2)	$D_{1/2}$ (cm)
^{125}Te	35.46	13.65	6.99	60D	3.63×10^{-18}	6.9
^{161}Dy	25.66	2.90	18.88	6.9D	2.76×10^{-18}	2.2
^{119m}Sn	23.9	5.1	8.58	245D	7.14×10^{-18}	1.8
^{151}Eu	21.5	28.6	47.8	87D	6.81×10^{-18}	1.5
^{121}Sb	37.2	11.1	57.25	50Y	2.16×10^{-18}	8.1
^{129}I	27.8	5.1	0	34D	1.99×10^{-18}	3.0
^{127}I	57.6	3.8	100	109D	7.83×10^{-19}	21.3
^{57}Fe	14.4	9.0	2.1	270D	2.30×10^{-17}	0.5

[a] *Mössbauer Effect Data Index*, J.G. Stevens and V.E. Stevens (IFI/Plenum, 1974).

labeled linear DNA and closer circular (super coiled) DNA are also underway. We end this article with a detailed consideration of some recent in vitro studies using human and mouse cancer cell lines.

4. New Mössbauer experiment

Experiments were performed to further confirm that the Mössbauer effect is responsible for an increased killing effect of magnitude 10^5 relative to conventional radiotherapy. It was found that milliRad levels of radiation in addition to ^{57}Fe-Bleomycin increased the relative killing effect by 4 to 20 fold in a resonance radiation dose/response fashion. It was further found that iron alone or with radiation has no effect on cell survival and that radiation and Bleomycin together demonstrated no effect on survival when the effect of Bleomycin alone was subtracted. The experiments were performed on a variety of cancer cell lines at Bristol-Meyers Corporate Research, Walingford, Conn., using a fresh preparation of Fe-57 bleomycin drug solution. The room temperature Mössbauer spectrum of the ethanol induced precipitate from such solutions is shown in fig. 1. The experiments of ref. [1] were based on constant velocity irradiations at +1.5 mm/sec. The latter value was chosen via extrapolation of the low temperature data of ref. [3] to ambient temperature. The spectrum of fig. 1 is unusual in that slow paramagnetic relaxation broadening effects are clearly evident. This feature

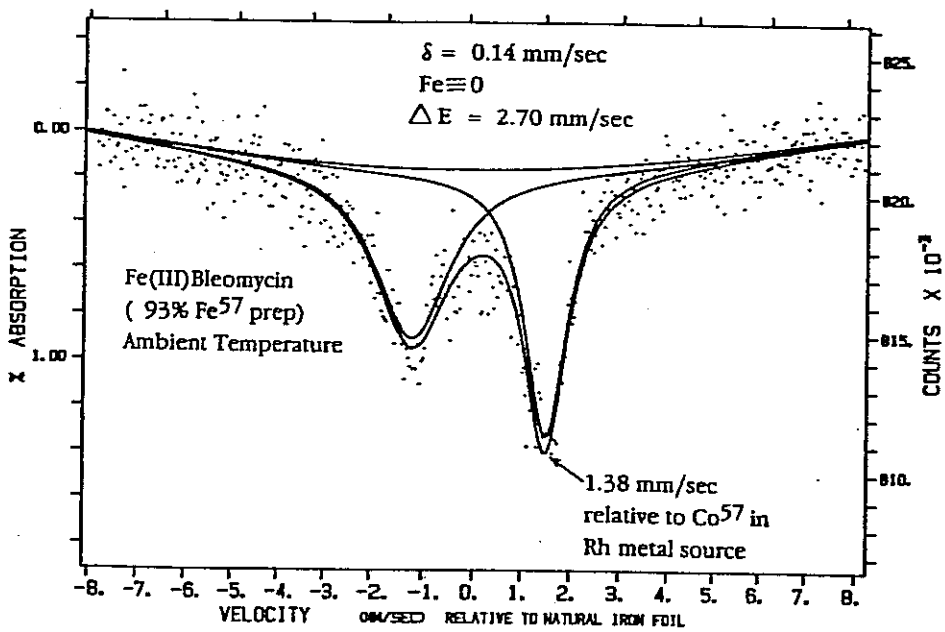

Fig. 1. Room temperature Mössbauer spectrum of ethanol induced precipitate of Fe(III)-Bleomycin.

is not that common for low-spin Fe III at room temperature. In any event, it is clear that irradiations at constant velocities near ~ 1.4 mm/sec should have a strong resonant absorption effect. We chose to bracket this value in the experiments that follow, where constant velocity irradiations were performed at +1.3 and +1.5 mm/sec. Finally, we point out that previous critiques [4,5] of the NGR approach [1] to cancer radiation therapy are largely if not wholly addressed in our recent rebuttal [6] and the present article.

5. Experimental approach

These in vitro experiments utilized the B16 cell line (mouse melanoma) and the A549 cell line (human lung cancer). We used 96 well microtiter plates because they allowed dose response curves to be generated. We evaluated the cell lines without any treatments; with cells alone, bleomycin alone, Fe alone and with bleomycin + Fe in the presence or absence of radiation of varying periods of time and varying Mössbauer conditions.

6. Experimental designs

The cell lines are grown in nutrient media consisting of McCoy's 5A with 10% heat inactivated fetal bovine serum and 0.0223 M Hepes buffer. The Fe free media was Dulbecco's modified eagle medium formula #86-0083 with 1 gram/liter D-glucose, glutamine and sodium pyruvate added. Each well of the 96 well microtiter plate received, 2,000 cells in 150 μL of nutrient media. A 50 μL aliquot of drug in saline or saline alone was added to the top well of the column where appropriate and serially diluted down the column with four fold dilutions between wells. Initial concentrations of drugs before addition to the first well were 2 mg/ml.

Each 96 well plate has 12 rows of wells across and 8 columns of wells down the plate. Cells were added to all wells on July 17 and interventions were done to alternate columns. This provides an N of 6 for each intervention, with each intervention being done on a single plate. The IC 50 or IC 30 (that is the concentration of drug necessary to inhibit cell growth by 50% or 30% respectively) values were determined. For IC 50/30 calculations, the mean OD value of the 8 wells of the control column adjacent to the drug treated column was used. Except for the growth control plates, all plates were washed 2 times with saline, Fe free media added to the plate, drug or vehicle added to the appropriate columns of wells, radiation treated or not as per experimental protocol, saline washed 2 times, and normal Fe containing media added to the plate. A plate sealer was added to cover the wells and the plates were placed in the incubator until determination of *viable* cell number in each well occurred. Cell viability was

etermined by adding 50 μL of 1.0 mg/ml tetrazolium dye (XTT) containing 0.02 ım phenazine methosulfate (PMS). The XTT is reduced enzymatically by *viable ells* in the presence of PMS to an orange colored formazan that is water soluble. he amount of formazan produced is directly proportional to the number of ving cells present and to the time of incubation of the cells with the precursor ']. The amount of Formazan produced is quantitated by determining the ɔectrophotometric absorbance at 450 nm on a microtiter plate reader. A blank ɔntaining media and tetrazolium but without cells is read first and that value ıbtracted from each cell containing well. *Thus, O.D. readings reflect the live cell ımber per well.* All experiments were read on day six following treatment.

KPERIMENT 1 (A549 CELL LINE)

The following treatments were performed on alternating columns of the plates ɔecified.

late 1–Vehicle Treatment ($N = 6$), Drug only w/o Fe57 ($N = 6$)
late 2–Fe57 only w/o drug ($N = 6$), Drug-Fe57 only ($N = 6$)
late 3–Radiaton only ($N = 6$), Drug only w/o Fe57 + radiation ($N = 6$)
late 4–Fe57 w/o Drug + Radiation ($N = 6$), Drug-Fe57 + Radiation ($N = 6$).
radiation and drug treatments were begun on Day 1. Drugs were added 20 inutes prior to radiation. Nonirradiated plates were treated identically. Cells ere evaluated for viability using XTT on Day 6. The radiation source was the 25 mCI cobalt 57 source. The radiation was accomplished with ınstant acceleration of the source so that all resonances (between ±2 mm/sec) ere scanned. Results are given as the average =/− S.E.; For O.D. readings = 96 or 48, for IC50 values $N = 6$.

ESULTS OF EXPERIMENT 1 (A549 CELL LINE)

o radiation, no washes-growth control	O.D.	= 0.526 +/− 0.012
o radiation, saline washes	O.D.	= 0.410 +/− 0.011
no Fe media		
o radiation, bleo	IC 50	= 20 +/− 11
o radiation, Fe only	O.D.	= 0.457 +/− 0.019
o radiation, bleo + Fe	IC 50	= 41 +/− 12
hr. radiation, cells only	O.D.	= 0.452 +/− 0.016
hr. radiation, bleo.	IC 50	= 38 +/− 15
hr. radiation, Fe only	O.D.	= 0.331 +/− 0.011
hr. radiation, bleo + Fe	IC 50	= 10 +/− 3.

The A549 human lung cell line did appear to suffer cell loss due to the tensive plate washes. However, there were certainly adequate numbers of cells r experimental determinations. The non-irradiated plates had comparable OD lues and IC 50 values were not different for the bleo vs bleo + Fe treatments.

The irradiated plates had reasonable OD values for the controls and the bleo + Fe IC50 was less than the bleo. However, the difference did not reach statistical significance ($P > 0.1$). The other control, bleo + Fe non-radiated, IC 50 was significantly higher compared to the IC 50 of bleo + Fe of the irradiated cells ($P < 0.05$).

These data demonstrate a significant Mössbauer radiation effect and demonstrate that there was no effect due to radiation alone or in combination with iron or bleomycin.

EXPERIMENT 2 (B16 CELL LINE)

The protocol of Experiment 1 was repeated with the exceptions the following radiation levels were performed for the experiments receiving radiation.

Radiation levels

1- 1 hr constant acceleration (± 2.0 mm/sec limits)
2-15 min rad., constant velocity, +1.5 mm/sec
3-30 min rad., constant velocity, +1.5 mm/sec
4-30 min rad., constant velocity, +1.3 mm/sec

RESULTS OF EXPERIMENT 2 (B16 CELL LINE)

1 hr sweep radiation Fe only	O.D. = 0.158 + /− 0.012
1 hr sweep radiation bleo + Fe	IC 50 (mg/ml) = 213 ± 45
15 min rad.@ +1.5 mm/sec, cells only	O.D. = 0.189
15 min rad.@ +1.5 mm/sec, bleo	IC 50 = 27 + /− 9
15 min rad.@ +1.5 mm/sec, Fe only	O.D. = 0.294
15 min rad.@ +1.5 mm/sec, bleo + Fe	IC 50 = 81 + /− 17
30 min rad.@ +1.5 mm/sec, cells only	O.D. = 0.349
30 min rad.@ +1.5 mm/sec, bleo	IC 50 = 57 + /− 8
30 min rad.@ +1.5 mm/sec, cells only	O.D. = 0.326
30 min rad.@ +1.5 mm/sec, bleo	IC 50 = 64 + /− 9
30 min rad.@ +1.5 mm/sec, Fe only	O.D. = 0.372
30 min rad.@ +1.5 mm/sec, bleo + Fe	IC 50 = 46 + /− 12
30 min rad.@ +1.3 mm/sec, Fe only	O.D. = 0.201
30 min rad.@ +1.3 mm/sec, bleo + Fe	IC 50 = 14 + /− 2.5

These data demonstrate a dose response relationship for resonance radiation, ($P < 0.005$) for one hour of radiation at +1.3 mm/sec compared to one hour of radiation at constant acceleration. No radiation effect is demonstrated in combination with iron or bleomycin.

Finally, we state one of the fundamental assumptions of this work. This is that the fraction of recoil-free gamma absorption events is apparently not zero for Mössbauer active isotopes strongly bound to large molecules in a quasi-solution

state at or near ambient temperature. Precedence for this all-important observation comes from a study of the Mössbauer spectra of Iron-57 enriched whole bacteria [8]. Remarkable spectra are observed for *unfrozen solutions* of two types of bacteria. They consist of a Broad background extending over $\sim \pm 20$ mm/sec. However, superimposed on this is an intense Narrow line width central component (I_N/I_B varying from ± 0.10 to 0.30) for which Γ varies from ~ 1 to 2 mm/sec. The crucial nature of this observation in the present context is obvious.

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